HGF Protected Against Diabetic Nephropathy via Autophagy-Lysosome Pathway in Podocyte by Modulating PI3K/Akt-GSK3 β -TFEB Axis

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Abstract

Background: Podocyte loss is a detrimental feature and major cause of proteinuria in diabetic nephropathy (DN). Our previous study revealed that hepatocyte growth factor (HGF) prevented high glucose-induced podocyte injury via enhancing autophagy. In the current study, we aimed to assess the role of HGF on podocyte homeostasis in DN and clarify its mechanisms further.

Methods: Streptozotocin (STZ)-induced DN mice were applied as a model to verify the impact of HGF on the prevention of type 1 diabetes. To study the mechanism involved, the autophagy flux and related signaling pathway upon HGF were examined and the relevance of lysosome acidification in cultured podocytes were investigated.

Results: Diabetic mice treated with HGF had markedly reduced ratio of kidney weight to body weight, urinary albumin excretion, podocyte loss and matrix expansion compared with that in the non-treated counterpart. Simultaneously, HGF-treated diabetic mice exhibited increased autophagy activity as indicated by the decreased accumulation of sequestosome 1 (SQSTM1/ p62) and increased microtubule-associated proteins 1 light chains 3 (LC3) II/LC3I ratio. These beneficial effects of HGF were blocked by HGF/c-Met inhibitor Crizotinib or phosphatidylinositide 3-kinases (PI3K) inhibitor LY294002. Moreover, HGF treatment obviously prevented inactivation of the protein kinase B (Akt)-glycogen synthase kinase 3 beta (GSK3 β)-transcription factor EB (TFEB) axis in high glucose-stimulated podocytes, which was associated with improved lysosome function and autophagy. Accordingly, adenovirus vector encoding constitutively active GSK3 β (Ad-GSK3 β -S9A) offset whereas small interfering RNA against GSK3 β (GSK3 β siRNA) recapitulated salutary effects of HGF on lysosome number and autophagy in podocytes.

Conclusions: These results suggested that HGF protected against diabetic nephropathy through restoring podocyte autophagy, which at least partially involved PI3K/Akt-GSK3 β -TFEB axis-mediated lysosomal function improvement.

Background

Diabetic nephropathy (DN) is a serious complication of diabetes and becomes the leading cause of end-stage renal disease worldwide. Depletion of podocytes, which are the critical constituent of the glomerular filtration barrier, initiates and drives the progression of proteinuria towards DN[1]. However, terminally differentiated podocytes have limited capacity of regeneration in response to injury. Autophagy is an intracellular self-repair mechanism that involves the degradation of defective proteins or organelles via lysosomes. Podocytes possess a relatively high basal level of autophagy, which is crucial for its stress adaptation[2]. Therefore, autophagy impairment has been proposed as a major contributor to the pathogenesis of podocytopathy and proteinuria.

Hepatocyte growth factor (HGF) is derived from some mesenchymal cells or tissues with multiple biological effects. Over the years, more and more animal experiments have confirmed that HGF could achieve significant prevention against and treatment contribution to DN[3-5], however, previous studies

have mainly focused on the antifibrotic and antioxidant effects of HGF in DN. What effects HGF has on podocyte injury, the important initiating factor of DN, remains largely unknown.

Glycogen synthase kinase 3 is a multifunctional serine/threonine protein kinase that regulates glycogen metabolism and plays a role in multiple intracellular signaling pathways. Emerging evidences have validated glycogen synthase kinase 3 beta (GSK3 β) mediates podocyte injury in various glomerular nephropathy including DN[6-8]. Consistently, genetic ablation or chemical inhibition of GSK3 β have been used to protect against podocyte injury, glomerulosclerosis and proteinuria[9, 10]. Our previous in vitro study has demonstrated that HGF could reduce high glucose (HG)-induced podocyte injury by restoring the autophagic flux[11]. In this study, we sought to further reveal the mechanism of HGF promoting autophagy of DN podocytes by regulating GSK3 β in vitro and in vivo.

Materials And Methods

Ethics statement and patients

Permissions on using human kidney biopsy sections and performing animal experiment (No. 20170223-039) for research purposes were approved by the Ethics Committee of Shanghai Medical College, Fudan University, China. All procedures were carried out according to the approved guidelines. Renal specimens were obtained from diabetic nephropathy (n=10) patients, and patients with minimal change nephrotic syndrome/minimal change disease (n=6) were used as control. The definite diagnosis was made based on WHO histologic classification of glomerular diseases (1982 and 1995).

Antibodies and reagents

Antibodies against NPHS2/Podocin (ab181143), SQSTM1/p62 (ab109012), Akt (ab32505), GSK3 β (ab32391), GAPDH (ab181602), rabbit IgG H&L (Alexa Fluor 488) (ab150073) and goat IgG H&L (Alexa Fluor 647) (ab150131) were purchased from Abcam (Abcam, Cambridge, MA, USA); antibodies against LC3B (#3868), PI3Kinase (#2429), phospho-Akt (Ser473) (#4060) and phospho-GSK3 β (Ser9) (#9323) were from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA); antibodies against Synaptopodin (sc-21537), HA-probe (sc-7392), mouse IgG-HRP (sc-2004) and rabbit IgG-HRP (sc-2005) were from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA), and anti-TFEB (3372-1-AP) was from ProteinTech (ProteinTech Group, Rosemont, IL, USA). Streptozotocin (STZ) (S0130), Crizotinib (PZ0191) and LY294002 (L9908) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Recombinant Human HGF (rh-HGF) was from PeproTech (100-39, PeproTech, Cranbury, NJ, USA).

Animal studies

The 9-week-old male DBA/2J mice were purchased from SLAC Laboratory (Shanghai SLAC Laboratory Animal, Shanghai, China) and maintained in accordance with Institutional Animal Care and Use Committee procedures of Fudan University. Diabetes was induced by intraperitoneal injection of STZ (100 mg/kg body weight for 3 consecutive days) and after 2 weeks, mice with a non-fasting blood glucose of

more than 15 mmol/l (280 mg/dl) were considered as diabetic. Diabetic mice were then allocated randomly into DN, DN+HGF, DN+HGF+Crizotinib and DN+HGF+LY294002 groups. Rh-HGF (150 µg/kg, i.v.) was given every other day and Crizotinib (20 mg/kg, i.g.) and LY294002 (6 mg/kg, i.p.) or equivalent vehicle were given daily for 4 weeks. All mice were maintained for 12 weeks and the blood glucose level and body weight were monitored throughout the course of treatment. At the end of the study, 24h-urine was collected in the metabolic cages and mice were then killed under chloral hydrate anesthesia to collect blood samples and kidney tissues. Total protein, creatinine and albumin in urine were detected by standard diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Transmission electron microscopy

Kidney tissues from mice were fixed in 2.5% glutaraldehyde overnight and processed for standard scanning at the Electron Microscopy Core Laboratory, School of Basic Medical Sciences, Fudan University. Five glomeruli were randomly selected from each mouse and 3-5 electron micrographs were taken. The glomerular basement membrane (GBM) thickness and the number of foot processes were calculated as described[12].

Histology and immunohistochemistry analysis

The paraformaldehyde-fixed and paraffin-embedded sections of kidney were stained with hematoxylin-eosin (H&E) and periodic acid–Schiff (PAS) and glomerulosclerotic index was assessed on PAS staining as described previously[13]. For immunohistochemistry staining, the sections were dewaxed and hydrated. The endogenous peroxidase was removed using 3% H₂O₂ and the antigen was retrieved in citrate buffer. After that, nonspecific binding sites were blocked with 5% normal goat serum and the sections were incubated with primary antibody at 4 °C overnight (NPHS2/Podocin: 1:500), and secondary antibody in 37 °C for 45 min. The expression was detected by diaminobenzidine and hematoxylin was used as the nuclear counterstain. Images were captured by Nikon camera system (Nikon, Tokyo, Japan) and immunoreactivity was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining of tissue sections

The kidney sections were dewaxed and hydrated. The antigen was retrieved in citrate buffer. The sections were incubated with primary antibodies at the following dilutions: LC3B (1:100), SQSTM1/p62 (1:100), Synaptopodin (1:100), p-GSK3β (1:100) overnight at 4 °C, and then incubated with Alexa Fluor 488- and Alexa Fluor 647-conjugated secondary antibodies at 37 °C for 1 h. Nuclei were counterstained with DAPI (1:1000, Sigma-Aldrich, St. Louis, MO, USA). Images were captured by Vectra Automated Imaging System (Perkin Elmer, Waltham, MA, USA).

Glomerulus isolation and western blotting analysis

The procedure of glomerulus isolation from mice is similar to the isolation from rat described previously[14]. Briefly, the kidney cortex from mice were minced and passed through a system of sieves (BD Falcon, San Diego, CA, USA) with decreasing pore diameters (100, 70, and 40 μ m) to obtain suspension of glomeruli in PBS. The glomeruli were homogenized in the RIPA lysis buffer (Sangon Biotech, Shanghai, China) containing protease inhibitor cocktail (Selleck, Houston, TX, USA) and vortexed every 10 min for 5 times on ice, which was followed by centrifugation. The protein lysate was measured by BCA protein assay reagent kit (Thermo Piece, Waltham, MA, USA) and used for western blot as described[11]. The primary antibodies were used at the indicated dilution: 1:1000 (LC3B, p-GSK3 β , p-Akt, PI3K), 1:2000 (Akt, GSK3 β), 1:10000 (p62, GADPH). The bands were quantified by densitometry measurement using Gel-pro software (Media Cybernetics, Bethesda, MD, USA).

Cell culture and treatment

Conditionally immortalized podocytes MPC-5 were kindly provided by Professor Peter Mundel (Albert Einstein College of Medicine, New York, NY, USA) and cultured as previously reported in the literature[15]. All experiments were performed with differentiated podocytes, free of mycoplasma infection. HG culture medium was made by supplementing normal glucose RPMI 1640 medium (Thermo Gibco, Waltham, MA, USA) containing 5.5 mmol/l D-glucose with additional D-glucose (Sinopharm, Shanghai, China) for a final D-glucose concentration at 30 mmol/l. Podocytes cultured in normal or high glucose were stimulated with or without 25 ng/ml HGF in the absence or presence of 2.5 μ mol/l SU11274 (S1080, Selleck, Houston, TX, USA) for 24 h. SU11274 was added 1 h before HGF supplementation. After treatment, podocytes grown on 60mm culture dishes (Corning, New York, NY, USA) were harvested and the lysate measurement and western blotting were the same as above.

Transfection of adenovirus and small interfering RNAs

The plasmids which contained wild-type (GSK3 β -WT), constitutively active (GSK3 β -S9A) and catalytically inactive (GSK3 β -K85A) forms of GSK3 β were purchased from Addgene (14753-14755, Addgene, Watertown, MA, USA) and cloned into adenovirus vector (Hanbio Technology, Shanghai, China). At 80-90% confluence, differentiated podocytes were transfected with control GFP vector or GSK3 β adenovirus for 24h. SiRNAs targeting GSK3 β were synthesized by GenePharma (GenePharma, Shanghai, China) and transfected using Lipofectamine RNAiMAX Transfection Reagent (13778, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Immunofluorescence staining of podocytes

Podocytes on the glass sides were fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing twice with PBS, cells were permeabilized with 0.1% Triton (Sinopharm, Shanghai, China) for 5min, incubated with primary antibody (TFEB: 1:50) at 4 °C overnight and secondary antibody at room temperature for 1 h, and counterstained with DAPI. Images were taken using Zeiss Axioplan2 Imaging System (Zeiss, Oberkochen, Germany).

Lysosome staining and flow cytometric analysis

The acidification of lysosomes was determined with Lysosome Staining Kit (E607506, Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. Cultured podocytes were incubated with LysoBrite Red for 2 h. The cells were then washed with PBS to remove any excess lysosomal marker, and the live cell images were taken using the EVOS fluorescence microscope (Life technologies, Grand Island, NY, USA). For fluorescence quantification, the podocytes were digested by trypsin (Thermo Gibco, Waltham, MA, USA) and resuspended with PBS. The fluorescent intensity value of LysoBrite Red was quantified by flow cytometry using BD LSRFortessa (BD Biosciences, San Diego, CA, USA). The MFI (mean fluorescent intensity) of the fluorescence was calculated and presented as a histogram photograph by FlowJo software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

The experiments were all independently repeated at least three times. Results were expressed as means $\pm$ SEM. Differences between two and more groups were performed by means of the unpaired t test and ANOVA, respectively. Statistical analysis was conducted using the GraphPad Prism software (GraphPad, La Jolla, CA, USA). $P < 0.05$ was considered to indicate statistically significant difference.

Results

HGF alleviated podocyte injury and improved kidney function in STZ-induced

DN mice

To validate the effect of HGF on podocytes in DN, we first injected rh-HGF into Streptozotocin (STZ)-induced diabetic mice. As shown in Supplementary figure, all the STZ groups had hyperglycemia and lower body weight compared with the control counterparts. Firstly, we showed decreased expression of NPHS2/Podocin, a podocyte-specific transmembrane protein, in renal biopsies from DN patients by immunohistochemical detection, suggesting the occurrence of podocyte injury (Fig. 1a). Secondly, we found lower expression of this marker in STZ-induced DN mice than in the control group. Moreover, HGF treatment significantly prevented the loss of podocytes, which could be abrogated by Crizotinib (Criz), a specific inhibitor of HGF receptor. Consistently, matrix expansion and glycogen accumulation assessed by periodic acid–Schiff (PAS) staining were shown in the mesangial region of nontreated and HGF+Criz-treated DN mice but not in HGF-treated diabetic mice and nondiabetic control mice (Fig. 1b).

Transmission electron microscopy (TEM) analysis further revealed thicker glomerular basement membrane (GBM) and much more abnormal changes of podocytes such as foot process effacement and vacuolization in DN or HGF+Criz treated mice (Fig. 1c). As an index of renal function, urinary albumin to creatinine ratio (UACR) was measured and expectedly higher levels of UACR was observed in DN or HGF+Criz treated mice relative to normal and HGF-treated mice (Fig. 1d). The ratio of kidney to body weight, another parameter indicating renal hypertrophy, was also increased in DN mice but markedly decreased in HGF-treated DN mice (Fig. 1e).

HGF restored podocyte autophagy in STZ-induced DN mice

Autophagy deficiency is a well-recognized contributor to podocyte injury and proteinuria in DN[16]. We examined if autophagy regulation would be one of the mechanisms by which HGF reduces renal damage. Consistent with other studies, double staining of immunofluorescence with autophagy related proteins and Synaptopodin (Synapt), one of the podocyte cytoskeleton proteins, located fewer dots of LC3 (Fig. 2a) and more accumulated SQSTM1/p62 (Fig. 2b) in podocytes from DN patients compared with the control subjects, indicating impaired autophagy flux. Similar results were also obtained in STZ-induced DN mice tissue (Fig. 2c,d). Protein of renal cortex was collected, and western blotting analysis displayed decreased ratio of LC3II/LC3I and increased p62 expression (Fig. 2e). Of note, we found that HGF significantly recovered the level of LC3 and relatively suppressed p62 expression, which could be almost completely abolished by HGF inhibitor Crizotinib (Fig. 2c-e). These results indicated that restoring podocyte autophagy was the relevant mechanism underlying the salutary effects of HGF in DN described above.

HGF-restored podocyte autophagy in STZ-induced DN mice involved Akt-GSK3 β signaling pathway

As a vital component of glycogen metabolism, GSK3 β has been confirmed as a downstream target of phosphoinositide 3-kinase (PI3K) /Akt pathway[17]. Some studies proposed that GSK3 β activity is inversely correlated with autophagy[18-20], and our previous research also verified HGF promoted autophagic flux by inhibition of high glucose-reduced Ser9 phosphorylation of GSK3 β , an indicator of GSK3 β activation[11]. In this study, we logically hypothesized and examined whether HGF played the role through the Akt-GSK3 β signaling pathway in vivo. Immunoblotting (Fig. 3a) or double immunofluorescence staining (Fig. 3b) showed decreased ratio of p-Akt(ser473) /Akt and p-GSK3 β (ser9) /GSK3 β in DN mice group compared with control group. HGF treatment nearly normalized the phosphorylation of Akt and GSK3 β to the level in control group, which was efficiently blocked by HGF inhibitor Crizotinib or PI3K/Akt inhibitor LY294002 (LY). Taken together, these results suggested that HGF enhanced podocyte autophagy via PI3K/Akt-GSK3 β pathway.

HGF improved lysosome function in HG-stimulated mouse podocytes

Lysosomes-mediated intracellular degradation is the major compartment in autophagy activation. Dysfunctional lysosomes participate in podocytes damage, leading to glomerular disease and proteinuria[16]. Thus, we further examined whether correcting lysosome dysfunction could account for HGF-enhanced autophagy flux in HG-stimulated cultured podocytes. Lysosome staining reagent was used to label and track the acidic intracellular compartments (lysosomes) in live cells, and the fluorescence intensity increases with the decrease of pH. As shown in Figure 4a and 4b, live cell imaging and quantitative analysis by flow cytometry displayed declined fluorescence intensity in HG-stimulated mouse podocytes in comparison to the normal glucose (NG) group, indicating weakened lysosomal acidification. However, HGF co-treatment partially recovered lysosomal acidification and HGF inhibitor SU11274 antagonized this effect. Transcription factor EB (TFEB), as the master regulators of lysosome biogenesis, is dephosphorylated followed by translocation to the nucleus to activate the transcription of

genes responsible for coordinated lysosomal expression and regulation[21]. High glucose sequestered more TFEB granules in cytoplasm while pre-HGF treatment caused more TFEB granules entry into the nucleus as evidenced by distribution of fluorescence intensity. HGF inhibitor SU11274 completely blocked the effect of HGF on nuclear translocation of TFEB (Fig. 4c). Together, these results implied that HGF played an important role in maintaining lysosome function in HG-stimulated podocytes.

Overexpression of GSK3 β abrogated the effect of HGF on lysosome function in HG-stimulated mouse podocytes

Several studies have revealed negative effect of GSK3 β on autophagy may be relevant with lysosomes[19, 22, 23]. Based on the findings described above, we speculated that GSK3 β activity regulation may be attributed to the improved lysosome function by HGF. Podocytes were transfected with adenovirus vector encoding the constitutively active GSK3 β (Ad-S9A), dominant-negative GSK3 β (Ad-K85A), or wild-type GSK3 β (Ad-WT) plasmid and then subjected to HG+HGF treatment. The virus carrying GFP vector was used as the control transfection treatment, and successful adenoviral transfection of GSK3 β was corroborated by the immunoblotting for hemagglutinin (HA, the tag of GSK3 β adenovirus vector) or increased GSK3 β protein expression (Fig. 5a). Lysosome red fluorescence intensity greatly reduced in Ad-GSK3 β -S9A but not in Ad-GSK3 β -WT and Ad-GSK3 β -K85A group (Fig. 5b,c). In addition, the nuclear translocation of TFEB was also depressed in GSK3 β transfection groups, with the strongest cytosolic TFEB expression being observed in Ad-GSK3 β -S9A group (Fig. 5d).

Knockdown of GSK3 β partially recapitulated the effect of HGF on lysosome function in HG-stimulated mouse podocytes

To further verify the role of GSK3 β , podocytes were transfected by small interfering RNA against GSK3 β (GSK3 β siRNA) before exposure to HG or HG+HGF (Fig. 6a). As expected, knocking down GSK3 β increased lysosome fluorescence intensity (Fig. 6b,c), as well as promoted TFEB translocation (Fig. 6d). Moreover, HGF treatment in the Ctrl siRNA group certainly enhanced the lysosome acidification and function, however, there was no significant difference between HG+GSK3 β siRNA group and HG+HGF+GSK3 β siRNA group regarding lysosome fluorescence intensity and TFEB translocation (Fig. 6b-d), indicating the silencing of GSK3 β replaced the inhibitory effect of HGF on GSK3 β and thus highlighting the importance of targeting GSK3 β for DN treatment. All these data in Figure 5 and 6 illustrated that GSK3 β activity modified by Ser9 phosphorylation status was essential for improved lysosome function and autophagy by HGF in HG-stimulated podocytes.

Discussion

As the essential mechanism of maintaining podocytes homeostasis, autophagy is suppressed by excess nutrition or energy in diabetes mellitus state with the results of making podocytes more prone to dysfunction and apoptosis[16]. Consistently, we observed podocyte injury, glomerulosclerosis and proteinuria in STZ-induced type 1 DN mouse model, with decreased LC3 (LC3II/LC3I) and increased SQSTM1/p62 expression indicative of impaired autophagy flux. Although there was no difference in

blood glucose, HGF treatment had significantly improved in all aforementioned histological and functional parameters of kidney in DN model, accompanied by restored autophagy, proving that the protective effect of HGF against DN may be achieved by normalizing autophagy level in podocytes without affecting hyperglycemia. This in vivo data is concordant with our previous in vitro study reporting the favorable effects of HGF on high glucose-treated podocyte[11]. Furthermore, in line with our data, Peng KY et al. demonstrated that HGF had a role in the amelioration of diabetic vascular complications via autophagic clearance of advanced glycation end (AGE) products[24]. Since numerous clinical trials revealed that patients subjected to intensive glycemic control gained little improvement on preservation of renal function compared with patients receiving less aggressive glucose control, the treatment strategy independent of glucose lowering is promising like sodium-glucose cotransporter (SGLT) inhibitors.

As an important regulator of glycogen metabolism, renal expression of total and activated GSK3 β was elevated in diabetic patients and mice[25]. Mechanistically, enhanced GSK3 β activity was associated with podocyte apoptosis in DN[8]. In our study, expression of p-Akt (ser473) and p-GSK3 β (Ser9) in podocytes in DN mice were reduced, which indicated lowered Akt activity and elevated GSK3 β activity, respectively. However, these changes in Akt-GSK3 β phosphorylation status were remarkably diminished in podocytes of HGF-treated DN mice. Given that GSK3 β has been recognized as a downstream signaling molecule of the HGF/c-Met-Akt pathway, we reasoned that HGF could benefit podocytes through the PI3K/Akt-GSK3 β signaling pathway in diabetic milieu. As a multifunctional kinase, GSK3 β also plays a role in the regulation of autophagy, and the therapeutic effect of GSK3 β inhibitors is linked to activated/enhanced autophagy in various models [18, 26-28]. In this study, HGF treatment lessened GSK3 β activity in parallel with restored autophagy flux in podocyte of diabetic mice, which could be blocked by co-treatment of HGF or PI3K/Akt inhibitors. Therefore, consistent with our previous study in vitro, we confirmed that HGF could promote podocyte autophagy by suppression of GSK3 β activity in vivo.

Then, we raised the question of how GSK3 β affects autophagy. Azoulay-Alfaguter I et al. found GSK3 reduced lysosomal acidification (where GSK3 β has a stronger effect than GSK3 α) in breast cancer cells[19]. Avrahami L et al. and Parr C et al. confirmed the inhibition of GSK3 promoted lysosomal biogenesis and autophagic degradation, which reduced the accumulation of β -amyloid in Alzheimer's disease mouse model[22, 29]. Therefore, we speculated lysosome function might be influenced by the change of GSK3 β during HGF treatment in DN. As we expected, HGF partially recovered the defective lysosomal acidification in immortalized mouse podocytes with HG stimulation. However, overexpression of GSK3 β weakened the restoration of lysosome acidification by HGF. Moreover, the extent of weakening was correlated to the activity pattern of overexpressed GSK3 β with constitutively active GSK3 β being the strongest and dominant dead GSK3 β the weakest. When GSK3 β is interfered in the opposite direction, that is GSK3 β silence by siRNA, the effect of HGF on lysosome acidification was recapitulated as demonstrated by the finding that there was no difference in lysosome acidification between HG+siGSK3 β and HG+HGF group. Furthermore, lysosomal acidification level in HG+HGF+siGSK3 β group was comparable with that in HG+siGSK3 β group, suggesting that GSK3 β is a pivotal downstream target of HGF to influence lysosome function. We also found GSK3 β expression in HG+HGF group was numerically reduced as compared with HG group but it did not reach statistical significance.

TFEB is one of the major transcription factors related to autophagy. Studies have reported reduced TFEB phosphorylation by GSK3 inhibitors caused more TFEB nuclear translocation and promoted lysosomal function[20, 29, 30]. Therefore, we also explored the localization of TFEB in this study.

Immunofluorescence staining demonstrated HG made more TFEB sequestered in cytoplasm, which was mostly abolished by HGF. This HGF-promoted TFEB nuclear translocation under HG conditions was blocked by HGF inhibitor SU11274 or partly abrogated by overexpression GSK3 β , especially constitutively active GSK3 β -S9A. Upon GSK3 β silenced by siRNA, TFEB nuclear translocation was enhanced, albeit to a lesser extent in HG group in comparison to HG+HGF group, pointing at mechanisms other than GSK3 β -dependent signaling pathway whereby HGF could influence TFEB location. Considering that TFEB entry into nucleus is instrumental to lysosome function, we proposed HGF improved lysosome function and then accelerated autophagic flux in podocytes exposed to HG stimulation via modulation of GSK3 β -TFEB axis. In accordance with our findings, Zhao X et al. confirmed TFEB inhibition mediated the AGE-stimulated autophagy impairment in cultured podocytes and db/db mice[31]. As a multifunctional transcription factor, TFEB can be regulated by multiple serine/threonine kinases, driving both expression of autophagy and lysosomal genes to coordinate the autophagy-lysosome degradation system[21]. Overexpression of TFEB promoted the elimination of storage factors in some accumulation or metabolic diseases[32-34]. It warrants further study in future that TFEB may be a promising therapeutic target both in DN and for favorable effects of HGF.

Conclusions

In summary, we show in this study that HGF protects against DN through restoring podocyte autophagy. The mechanism is partly due to improved lysosome function that correlates with enhanced TFEB nuclear translocation and GSK3 β activity inhibition. Since PI3K/Akt is a well-recognized signaling pathway of HGF as well as in the pathogenesis of DN, we suggest that PI3K-GSK3 β -TFEB axis could be a potential therapeutic target beyond accounting for the salutary effects of HGF in DN treatment.

Abbreviations

DN, Diabetic nephropathy; GBM, Glomerular base membrane; GSK3 β , Glycogen synthase kinase 3 beta; HG, High glucose; HGF, Hepatocyte growth factor; LC3, Microtubule-associated proteins 1 light chains 3, NG, Normal glucose; PI3K, Phosphatidylinositide 3-kinases; SQSTM1/p62, Sequestosome 1; STZ, Streptozotocin; TFEB, Transcription factor EB; UACR, Urinary albumin to creatinine ratio

Declaration

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Shanghai Medical College, Fudan University, China (No. 20170223-039).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that there is no potential conflict of interest.

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Authors' contributions

BH and HL were responsible for the study design, acquisition of data, interpretation of results and preparation and revision of the manuscript. YKL, XL, CYZ, ZHZ and QC contributed to the acquisition and analysis of data. NZ and HL contributed to the study design, interpretation of data and manuscript preparation. All authors were involved in drafting and editing the article, and all authors gave their approval for the final manuscript to be published. HL is the guarantors of this work. All authors read and approved the final manuscript.

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References

References

- [1] Wolf G, Chen S, Ziyadeh FN (2005) From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *DIABETES* 54: 1626-1634
- [2] Hartleben B, Godel M, Meyer-Schwesinger C, et al. (2010) Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J CLIN INVEST* 120: 1084-1096
- [3] Dai C, Yang J, Bastacky S, Xia J, Li Y, Liu Y (2004) Intravenous administration of hepatocyte growth factor gene ameliorates diabetic nephropathy in mice. *J AM SOC NEPHROL* 15: 2637-2647

- [4] Li H, Jiang T, Lin Y, Zhao Z, Zhang N (2006) HGF protects rat mesangial cells from high-glucose-mediated oxidative stress. *AM J NEPHROL* 26: 519-530
- [5] Flaquer M, Franquesa M, Vidal A, et al. (2012) Hepatocyte growth factor gene therapy enhances infiltration of macrophages and may induce kidney repair in db/db mice as a model of diabetes. *DIABETOLOGIA* 55: 2059-2068
- [6] Xu W, Ge Y, Liu Z, Gong R (2015) Glycogen synthase kinase 3beta orchestrates microtubule remodeling in compensatory glomerular adaptation to podocyte depletion. *J BIOL CHEM* 290: 1348-1363
- [7] Bao H, Ge Y, Peng A, Gong R (2015) Fine-tuning of NFkappaB by glycogen synthase kinase 3beta directs the fate of glomerular podocytes upon injury. *KIDNEY INT* 87: 1176-1190
- [8] Paeng J, Chang JH, Lee SH, et al. (2014) Enhanced glycogen synthase kinase-3beta activity mediates podocyte apoptosis under diabetic conditions. *APOPTOSIS* 19: 1678-1690
- [9] Zhou S, Wang P, Qiao Y, et al. (2016) Genetic and Pharmacologic Targeting of Glycogen Synthase Kinase 3beta Reinforces the Nrf2 Antioxidant Defense against Podocytopathy. *J AM SOC NEPHROL* 27: 2289-2308
- [10] Li C, Ge Y, Dworkin L, Peng A, Gong R (2016) The beta isoform of GSK3 mediates podocyte autonomous injury in proteinuric glomerulopathy. *J PATHOL* 239: 23-35
- [11] Zhang C, Hou B, Yu S, Chen Q, Zhang N, Li H (2016) HGF alleviates high glucose-induced injury in podocytes by GSK3beta inhibition and autophagy restoration. *Biochim Biophys Acta* 1863: 2690-2699
- [12] Kenichiro K, Hitonari N, Yuki K, et al. (2011) Defective glycosylation of α -dystroglycan contributes to podocyte flattening. *KIDNEY INT* 79: 311-316
- [13] Thallas-Bonke V, Thorpe SR, Coughlan MT, et al. (2008) Inhibition of NADPH Oxidase Prevents Advanced Glycation End Product-Mediated Damage in Diabetic Nephropathy Through a Protein Kinase C-Dependent Pathway. *DIABETES* 57: 460-469
- [14] Wu L, Feng Z, Cui S, et al. (2013) Rapamycin upregulates autophagy by inhibiting the mTOR-ULK1 pathway, resulting in reduced podocyte injury. *PLOS ONE* 8: e63799
- [15] Shankland SJ, Pippin JW, Reiser J, Mundel P (2007) Podocytes in culture: past, present, and future. *KIDNEY INT* 72: 26-36
- [16] Tagawa A, Yasuda M, Kume S, et al. (2016) Impaired Podocyte Autophagy Exacerbates Proteinuria in Diabetic Nephropathy. *DIABETES* 65: 755-767
- [17] Beurel E, Grieco SF, Joep RS (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* 148: 114-131

- [18] Ren F, Zhang L, Zhang X, et al. (2016) Inhibition of glycogen synthase kinase 3 β promotes autophagy to protect mice from acute liver failure mediated by peroxisome proliferator-activated receptor α . *CELL DEATH DIS* 7: e2151
- [19] Azoulay-Alfaguter I, Elya R, Avrahami L, Katz A, Eldar-Finkelman H (2015) Combined regulation of mTORC1 and lysosomal acidification by GSK-3 suppresses autophagy and contributes to cancer cell growth. *ONCOGENE* 34: 4613-4623
- [20] Marchand B, Arsenault D, Raymond-Fleury A, Boisvert FM, Boucher MJ (2015) Glycogen synthase kinase-3 (GSK3) inhibition induces prosurvival autophagic signals in human pancreatic cancer cells. *J BIOL CHEM* 290: 5592-5605
- [21] Settembre C, Di Malta C, Polito VA, et al. (2011) TFEB links autophagy to lysosomal biogenesis. *SCIENCE* 332: 1429-1433
- [22] Avrahami L, Farfara D, Shaham-Kol M, Vassar R, Frenkel D, Eldar-Finkelman H (2013) Inhibition of glycogen synthase kinase-3 ameliorates beta-amyloid pathology and restores lysosomal acidification and mammalian target of rapamycin activity in the Alzheimer disease mouse model: in vivo and in vitro studies. *J BIOL CHEM* 288: 1295-1306
- [23] Marchand B, Arsenault D, Raymond-Fleury A, Boisvert FM, Boucher MJ (2015) Glycogen synthase kinase-3 (GSK3) inhibition induces prosurvival autophagic signals in human pancreatic cancer cells. *J BIOL CHEM* 290: 5592-5605
- [24] Peng KY, Horng LY, Sung HC, Huang HC, Wu RT (2011) Hepatocyte growth factor has a role in the amelioration of diabetic vascular complications via autophagic clearance of advanced glycation end products: Dispo85E, an HGF inducer, as a potential botanical drug. *METABOLISM* 60: 888-892
- [25] Liang X, Wang P, Chen B, et al. (2020) Glycogen synthase kinase 3 β hyperactivity in urinary exfoliated cells predicts progression of diabetic kidney disease. *Kidney Int* 97: 175-192
- [26] de Munck E, Palomo V, Muñoz-Sáez E, et al. (2016) Small GSK-3 Inhibitor Shows Efficacy in a Motor Neuron Disease Murine Model Modulating Autophagy. *PLOS ONE* 11: e162723
- [27] Shimada K, Motoi Y, Ishiguro K, et al. (2012) Long-term oral lithium treatment attenuates motor disturbance in tauopathy model mice: implications of autophagy promotion. *NEUROBIOL DIS* 46: 101-108
- [28] Wei H, Qu H, Wang H, et al. (2017) Corrigendum to "1, 25-Dihydroxyvitamin-D3 prevents the development of diabetic cardiomyopathy in type 1 diabetic rats by enhancing autophagy via inhibiting the beta-catenin/TCF4/GSK-3 β /mTOR pathway" [*J. Steroid. Biochem. Mol. Biol.* 168 (2017) 71-90]. *J Steroid Biochem Mol Biol* 174: 312-313

- [29] Parr C, Carzaniga R, Gentleman SM, Van Leuven F, Walter J, Sastre M (2012) Glycogen Synthase Kinase 3 Inhibition Promotes Lysosomal Biogenesis and Autophagic Degradation of the Amyloid-Precursor Protein. *MOL CELL BIOL* 32: 4410-4418
- [30] Li L, Friedrichsen HJ, Andrews S, et al. (2018) A TFEB nuclear export signal integrates amino acid supply and glucose availability. *NAT COMMUN* 9: 2685
- [31] Zhao X, Chen Y, Tan X, et al. (2018) Advanced glycation end-products suppress autophagic flux in podocytes by activating mammalian target of rapamycin and inhibiting nuclear translocation of transcription factor EB. *The Journal of Pathology*
- [32] Settembre C, De Cegli R, Mansueto G, et al. (2013) TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *NAT CELL BIOL* 15: 647-658
- [33] Pastore N, Blomenkamp K, Annunziata F, et al. (2013) Gene transfer of master autophagy regulator TFEB results in clearance of toxic protein and correction of hepatic disease in alpha-1-anti-trypsin deficiency. *EMBO MOL MED* 5: 397-412
- [34] Rega LR, Polishchuk E, Montefusco S, et al. (2016) Activation of the transcription factor EB rescues lysosomal abnormalities in cystinotic kidney cells. *KIDNEY INT* 89: 862-873

Figures

Figure 1

HGF protected against podocyte injury and improved kidney function. (a) Representative images of immunohistochemical staining and quantification of NPHS2/Podocin in renal biopsies from control subjects (n=6) and DN patients (n=10). The percentage of Podocin stained area per glomerulus were calculated from 1-3 images of each case. Data were represented as means $\pm$ SEM, ****P<0.0001 for Control (Ctrl) vs DN. (Scale bar=40μm) (b) Representative of Immunohistochemical and H&E and PAS

staining of mice renal tissues. The percentage of Podocin stained area and the glomerulosclerotic index were calculated from 5-10 randomly images of each mouse (n=3). Data were represented as means $\pm$ SEM, *P<0.05, ***P<0.001 for Ctrl vs DN, **P<0.01, *P<0.05 for DN vs DN+HGF, **P<0.01, *P<0.05 for DN+HGF vs DN+HGF+Criz. (Scale bar=20 μ m) (c) Representative TEM image of podocytes morphological changes in different groups of mice. GBM thickness and the number of foot processes/ μ m GBM were calculated from 3 images of each mouse (n=5). Data were represented as means $\pm$ SEM, ****P<0.0001, ***P<0.001 for Ctrl vs DN, ****P<0.0001, ***P<0.001 for DN vs DN+HGF, ***P<0.001 for DN+HGF vs DN+HGF+Criz. (Scale bar=2 μ m) (d) UACR of each group after 12 weeks (n=3 or 5). Data were represented as means $\pm$ SEM, **P<0.01 for Ctrl vs DN, *P<0.05 for DN vs DN+HGF, **P<0.01 for DN+HGF vs DN+HGF+Criz. (e) Kidney weight to body weight ratio of each group (n=4 or 5). Data were represented as means $\pm$ SEM, *P<0.05 for Ctrl vs DN, ****P<0.0001 for DN vs DN+HGF, *P<0.05 for DN+HGF vs DN+HGF+Criz.

Figure 2

HGF normalized podocyte autophagy. (a,b) Representative images of immunofluorescence double staining of autophagy related proteins LC3 (a) (green) or SQSTM1/p62 (b) (green) in control and DN renal biopsies. Synaptopodin (red) was used as a podocyte marker and the LC3-positive (yellow) dots indicated decreased number of autophagosomes in podocytes of DN patients. (Scale bar=40μm) (c,d) Immunofluorescence double staining images of LC3 (c) (green) or p62 (d) (green) with Synaptopodin

(red) in different treatment mice groups. Shown were representative images with similar results. (Scale bar=20μm) (e) Western blot analysis of LC3 and p62 expression from glomeruli protein of mice. GAPDH served as a loading control and the statistic based on the densitometric quantification of bands. Data were represented as means ± SEM from three independent experiments, **P<0.01 for Ctrl vs DN and DN vs DN+HGF, **P<0.01, *P<0.05 for DN+HGF vs DN+HGF+Criz.

Figure 3

Akt-GSK3 β signaling pathway involved in HGF-restored autophagy. (a) Western blot analysis of PI3K/Akt-GSK3 β pathway expression from glomeruli protein of mice. Mice glomeruli were isolated as detailed in Materials and Methods. GAPDH served as a loading control and the statistic based on the densitometric quantification of bands for the ratio of p-Akt/Akt and p-GSK3 β /GSK3 β . Data were represented as means $\pm$ SEM from three independent experiments, **P<0.01 for Ctrl vs DN, **P<0.01, *P<0.05 for DN vs DN+HGF, *P<0.05 for DN+HGF vs DN+HGF+Criz, *P<0.05, **P<0.01 for DN+HGF vs DN+HGF+LY. (b) Immunofluorescence double staining images of p-GSK (green) with Synaptopodin (red) in different mice groups. Shown were representative images with similar results. (Scale bar=20 μ m)

Figure 4

HGF improved lysosome function in HG-treated podocytes. (a) Images of lysosome staining (red dots) in cultured podocytes. Shown were representative images with similar results. (Original magnification×400) (b) Histogram and mean fluorescent intensity of lysosome staining signals quantified by flow cytometry. Data were represented as means $\pm$ SEM from at least three independent experiments, **** P <0.0001 for NG vs HG, * P <0.05 for HG vs HG+HGF, **** P <0.0001 for HG+HGF vs HG+HGF+SU11274. (c) Images of

immunofluorescence staining of TFEB (green) location in podocytes. Shown were representative images with similar results. (Original magnification×400)

Figure 5

Overexpression constitutively active GSK3β abrogated HGF-improved lysosome function. (a) Western blot analysis of GSK3β and HA expression in transfected podocytes. GAPDH served as a loading control and shown were representative results from three experiments. (b) Representative images of lysosome

staining in live podocytes. (Original magnification×200) (c) Mean fluorescent intensity quantified by flow cytometry. Data were represented as means ± SEM from at least three independent experiments, ****P<0.0001 for HG+HGF vs HG+HGF+Ad-GSK3β-S9A, ns, no significance for HG+HGF vs HG+HGF+Ad-GSK3β-K85A, *P<0.05 for HG+HGF vs HG+HGF+Ad-GSK3β-WT. (d) Images of immunofluorescence staining of TFEB location in podocytes. Shown were representative images with similar results. (Original magnification×400)

Figure 6

Knockdown of GSK3 β recapitulated HGF-improved lysosome function. (a) Western blot analysis of GSK3 β expression in transfected podocytes. GAPDH served as a loading control and the statistic based on the densitometric quantification of bands. Data were represented as means $\pm$ SEM from three independent experiments, *P<0.05 for HG vs HG+siGSK3 β and HG+HGF vs HG+HGF+siGSK3 β (b) Representative images of lysosome staining in live podocytes. (Original magnification $\times$ 200) (c) Mean fluorescent intensity quantified by flow cytometry. Data were represented as means $\pm$ SEM from at least three independent experiments, **P<0.01 for HG vs HG+siGSK3 β , ns, no significance for HG+HGF vs HG+HGF+siGSK3 β , ***P<0.0001 for HG vs HG+HGF, *P<0.01 for HG+ siGSK3 β vs HG+HGF+siGSK3 β . (d) Images of immunofluorescence staining of TFEB location in podocytes. Shown were representative images with similar results. (Original magnification $\times$ 400)

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