Genistein Mitigates High Glucose-Induced Podocyte Adhesion Injury by Modulating Autophagy via Mtor Signal

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Abstract

Purpose: The study aimed to investigate the characteristics of autophagy on podocyte under high glucose (HG) conditions and further explore the effect of Genistein on podocyte autophagy/adhesion and the potential mechanism.

Materials and methods: CCK-8 was used to detect the viability of podocyte. The level of autophagy was mainly detected by western blot and immunofluorescence. The expression of autophagy related factors and podocyte adhesion markers, including LC3-II, p62, p-mTOR and integrin β 1-MF, were detected by immunofluorescence at 0, 6, 12, 24, 36, 48, 72h. The expression levels of proteins in the LC3-II, p62, p-mTOR/mTOR, integrin β 1-MF were further investigated by western blot. Wound healing test and cell migration assay were used to detect podocyte adhesion ability.

Results: The present study showed that HG-induced podocyte viability was reduced significantly for 6 h. Decreased integrin β 1-MF, LC3-II, increased p62 and abnormal activation of the mTOR signal was detected in podocyte under HG conditions. Genistein restored podocyte viability and up-regulated integrin β 1-MF, LC3-II expression, down-regulated p62, p-mTOR expression. Moreover, the HG-induced podocyte adhesion injury was abrogated by treatment with Genistein.

Conclusion: Our results demonstrated that podocyte adhesion injury in HG environment was related to the decrease of autophagy level. Genistein activated podocyte autophagy by inhibiting the mTOR signaling pathway, regulated the renewal expression of integrin β 1-MF, and finally reduced HG-induced podocyte adhesion injury.

1. Introduction

DKD is one of the most serious microvascular complications of diabetes which is the primary cause of ESRD, with progressive proteinuria as the hallmark caused by impaired glomerular filtration barrier (GFB) (1, 2). Currently, the renal protective drugs available in DKD have some side effects and limited efficacy(3). Therefore, the attempts of new drugs, especially those extracted from natural products, are necessary to provide a possibility for the development of successful therapeutic drugs in the future. Genistein is an isoflavone in soybean which have been shown to have numerous beneficial effects on diverse cells, such as anticancer, antioxidant, and anti-inflammatory activities(4, 5). The study intends to explore the effects of Genistein on podocyte under HG in vitro.

Podocytes are terminally differentiated epithelial cells which have no ability to proliferate in vivo, so various stimuli will cause irreversible damage, such as HG and mechanical force(6). Adhesion to the ECM is essential for cell survival, morphology and apoptosis. A large number of studies have demonstrated that the podocyte adhesion injury not only plays a critical role in the integrity of the GFB, but also is significantly associated with the production and development of albuminuria in patients with DKD(6–8). Therefore, exploring the mechanism of podocyte adhesion injury will provide a possible therapeutic strategy for the treatment of DKD. Podocyte adhere to the GBM principally via integrin β 1-MF (the form of

N-glycosylated mature / functional), which is abundantly expressed in the podocyte plasma membrane and a major receptor for GBM extracellular matrix components(7, 9). Integrin β 1-MF is mainly internalized to the perinuclear region of cells and then returned to the plasma membrane through RAB11-positive recycling endosomes, while a few internalized integrin β 1-MF is trafficking to the lysosomal degradation compartment(10–12). The degradation process is crucial to maintain the proper expression level of integrin β 1-MF on the cell surface, by which cell-GBM association is accurately regulated(13, 14). As the main degradative compartment, lysosomes are located at the end of autophagy pathway and play a vital role in the autophagic degradation of macromolecules and organelles(14).

Autophagy is a dynamic process in which lysosomes clear and degrade overexpressed or damaged proteins, mainly including the formation of autophagosomes, the emergence of autophagy lysosomes and substrate degradation(15). It has become a consensus that podocyte homeostasis is maintained by a relatively high level of basal autophagy in physiological conditions(7, 16). Recently, autophagy was found to be involved in the pathogenesis of podocyte adhesion injury(6, 17). The activation of autophagy enhances the stability of podocytes and helps to alleviate the progression of DKD, suggesting that autophagy is a potential therapeutic target against DKD(16, 18). However, the mechanism of autophagy in DKD remains to be studied and determined. The mTOR signal is widely considered one of the most critical autophagy regulators in eukaryotes, which is the intersection of multiple upstream signals, such as cell growth, proliferation, movement, survival and autophagy(19). The regulation of mTOR signal could be therapeutic mechanisms to improve the podocyte adhesion injury of DKD. Therefore, the study of the mTOR signal is helpful in elucidating the regulatory mechanism of autophagy and to explore the protective effect of Genistein on podocyte, providing a potential treatment for DKD.

2. Materials And Methods

2.1 Materials Collagen I (C8062), D-Mannitol(M8140), Fibronectin (from human plasma, F8180), Rapamycin (R8140) and BSA(A8020) were obtained from Solarbio.RPMI-1640 (22400-089), FBS (10100147C), penicillin-streptomycin (15070063), 0.25%Trypsin-EDTA solution (R001100), glucose (15023021), 1%Triton X-100(85111) and Tween-20(28320) were purchased from Gibco. Rabbit polyclonal anti-integrin β 1-MF (26918-1-AP), rabbit polyclonal anti-LC3(14600-1-AP), mouse monoclonal anti-p62/SQSTM1(66184-1-Ig), mouse monoclonal anti-phospho-mTOR(67778-1-Ig), β -actin monoclonal antibody(66009-1-Ig) were from Proteintech. Goat anti-mouse IgG (Dylight 488, E032210), goat anti-mouse IgG (Dylight 649, E032610), Goat anti-rabbit IgG (Dylight 488, E032220) were from EarthOX. Other laboratory consumables for cell culture and Genistein(G6649) were all from Sigma. Of note, all cell culture flasks and plates had been coated with type I collagen on the surface when the podocytes were inoculated.

2.2 Cell culture A conditionally immortalized human podocyte cell line was kindly provided by Prof. Moin Saleem (Bristol Royal Hospital for Children, Bristol, UK). Podocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin and streptomycin, and 10 U/ml recombinant mouse interferon- γ at 33°C with 5% CO₂ and 95% relative humidity for proliferation. After 2 to 3 days,

when the cells grew to approximately 70% -80% confluence, they were transferred to 37°C in RPMI 1640 medium to induce differentiation without interferon- γ for 10 to 14 days. Of note, the cell density should not exceed 80% - 90%, otherwise the cell activity will be affected. The differentiated podocytes were used between passages 7 and 10 for all subsequent experiments.

2.3 Cell treatment HG medium was made by supplementing RPMI 1640 medium (normal glucose concentration of 5.5 mmol/L; NG) with additional glucose for a final glucose concentration of 30.0 mmol/L. In addition, 19.5 mmol / L D-Mannitol was added to NG medium to maintain the hypertonic effect (HP) of HG concentration on cells. According to the solubility of Genistein, HG medium were treated with Genistein at various concentrations (2, 4, 6, and 8 $\mu\text{g/ml}$) to prepare the drug culture medium. Different mediums below were added to podocytes separately at 37°C for cck-8 test to select optimum drug concentration: NG medium, HP medium, HG medium, HG and 2 $\mu\text{g/ml}$ concentration Genistein medium, HG and 4 $\mu\text{g/ml}$ concentration Genistein medium, HG and 6 $\mu\text{g/ml}$ concentration Genistein medium, HG and 8 $\mu\text{g/ml}$ concentration Genistein medium. According to the results of CCK-8, podocytes were divided into NG group (NG medium), HG group (HG medium) and TM group (HG and 4 $\mu\text{g/ml}$ concentration Genistein medium) in subsequent experiments.

2.4 Podocyte viability assay Cell Counting Kit-8 (96992-100TESTS-F, sigma, USA) was used to detect podocyte viability following manufacturer's instructions. Briefly, the differentiated podocytes were seeded into 96-well plates (1.0×10^4 cells per well) and incubated at 37°C, 5% CO $_2$ overnight. We sequentially added 100 μl of corresponding medium to each well, and then cultured for 6, 12, 24, 36, 48 and 72 h respectively. CCK -8 solution was added in the amount of 10 μl per well, and then the cells were incubated for 2 h at 37°C and 5% CO $_2$. The absorbance values of each well were assessed in a microplate reader with 450 nm.

2.5 Immunofluorescence Podocytes were grown on glass coverslips in 24-well plates and subjected to various treatments, then were fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. After washing with phosphate-buffered saline (PBS) for three times, the cells were permeabilized with soaking buffer including 0.2% Triton X-100 for 20 min. Then cells were blocked with 5% BSA for 30 min at RT to block non-specific binding. Podocytes were incubated with the primary antibodies overnight at 4°(anti-integrin β 1-MF (1:300) anti-LC3-II (1:200), anti-p62(1:400), anti-p-mTOR (1:300)). Washed with PBS three times, followed by above 1 h of incubation with secondary antibodies at RT. Finally, after washing three times with PBS, the cells were stained with DAPI for 5 min to visualize the nuclear and observed under Immunofluorescence microscope (Axioscope 5, Carl Zeiss, Germany).

2.6 Western blot analysis Differentiated podocytes, with various experimental conditions, were washed twice with cold PBS. The washed podocytes were drained and extracted membrane protein from them with Membrane Extraction Kit (ThermoFisher,89842). Subsequently, protein (30 $\mu\text{g/lane}$) was separated by 8% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane, which was blocked with 5% fat-free milk in TBST (0.1% Tween-20) 1 h at room temperature and then incubated at 4°C overnight with the following primary antibodies: anti-integrin β 1 (1:1,000), anti-LC3-II (1:1,000), anti-p62(1:1,000), anti-p-

mTOR (1:1,000), anti-mTOR (1:1,000) and anti- β -actin (1:1,000). Following primary incubation, the membranes were washed twice with PBS, then were incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG for 1 h at RT. Subsequently, the immunoblots were washed three times with TBST (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween-20) and visualized in ECL Plus Western Blotting Detection Reagents (Beyotime Institute of Biotechnology). Results were quantified using Image J 1.52a software (Wayne Rasband national institutes of Health, USA) and with β -actin as the loading control.

2.7 Wound healing assay Cell migration was evaluated by wound healing test. Draw 5 lines on the bottom of the six well plate with a marker, and then inoculate an appropriate amount of differentiated and mature podocytes on the plate coated with type I collagen overnight. Scratched with 200 μ l pipette gun head, and then cleaned with sterile PBS twice. Finally, added 2ml medium of different groups into each well and cultured in a 37°C incubator. Photographs were taken at the specified time 0, 6, 12, 24, 36, 48 and 72 h, and analyzed using ImageJ 1.52a software (Wayne Rasband national institutes of Health, USA) image processing program. The percentage of cell migration area is calculated as follows.

2.8 Cell adhesion assay The differentiated podocytes were inoculated in type I collagen-coated 96-well plates overnight. Podocyte of different groups were cultured at 37°C for 6, 12, 24, 36 and 48 h respectively. Subsequently, discarded the culture medium, cells sticking to the bottom were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The dye was then washed three times with PBS and the cellular stain was dissolved in 33% acetic acid. The absorbance was quantified with a spectrophotometer at an optical density of 620 nm. The experiment was repeated three times to compare the percentage of podocyte adhesion (normalized to 1).

2.9 Statistical analysis Data was repeated at least three times independently and appeared as mean $\pm$ SD. Statistical analysis were processed by utilizing SPSS 24.0 software. Histogram and line chart were analyzed by GraphPad Prism 8.0 (GraphPad Software, USA) using Student's t test or One-way ANOVA analysis. $p < 0.05$ was regarded as statistically significant.

3. Results

3.1 Genistein Increased Hg-impaired Podocyte Viability

A CCK-8 kit was used to detect the viability of each group of podocytes. There was no significant difference in cell viability was between the podocytes with NG and HP medium, which indicated that hypertonic had a negligible effect on podocyte viability (Fig. 1. NG: 98.71 ± 0.1905 ; HP: 98.52 ± 0.4697 , $P > 0.05$). However, cell viability in the HG group was significantly abated compared with the NG group (Fig. 1, $P < 0.01$). Compared with the HG group, cell viability was significantly increased in the treatment of Genistein at all concentrations (Fig. 1; $P < 0.01$), which indicated that Genistein could increase HG-induced podocyte viability. In addition, the cell viability of the four concentrations of Genistein groups were different, the optimal drug concentration was 4 μ g/ml which was the only drug concentration retained in subsequent experiments (Fig. 1, $P < 0.001$). Comparing the cell viability curves of them, it indicated that

HG stimulation had an obvious negative effect in 6 h, while onset time of Genistein was about 12 h and reached the peak in 24 h.

3.2 Genistein Increased Podocyte Autophagy Under Hg Conditions

LC3-II and p62 were commonly used to estimate the level of autophagy. To clarify the role of autophagy in the reno-protective with HG effects of Genistein, the levels of LC3-II and p62 were detected at 0, 6, 12, 24, 36, 48 and 72 h by immunofluorescence co-localization analysis (Fig. 2A, B) and their expression levels at 24 h with obvious changes were quantified by western blot (Fig. 2C, D). The immunostaining analyses showed a reverse in autophagy levels with the prolongation of HG stimulation compared with NG group, and LC3-II expression significantly decreased in podocyte at 24 h of HG stimulation, whereas the expression of p62 was significantly raised after 24 h. In addition, the co-localization of LC3-II and p62 was observed in the cells and enhanced in the presence of HG after 24h (Fig. 2A). Compared with the HG group, the fluorescence intensities of LC3-II was stronger in the TM group, but p62 was weaker significantly, and the co-localization of LC3-II and p62 was decreased at 24h (Fig. 2B). Furthermore, western blot analyses showed that Genistein enhanced the protein levels of LC3-II expression and decreased the p62 expression compared with HG group at 24 h ($P < 0.001$) (Fig. 2C, D). Therefore, the results demonstrated that the protective effects of Genistein were associated with reversing insufficient autophagy in HG-induced podocyte.

3.3 Genistein Inhibited The Activation Of Mtor Signal

To investigate the potential mechanism of podocyte autophagy induced by Genistein with HG conditions, we utilized Rapamycin, a specific inhibitor of mTOR activation, to further verify the role of the mTOR signaling pathway in the inhibition of autophagy by Genistein. The results of immunofluorescence were represented in Fig. 3A-C below, the phosphorylation levels of mTOR were markedly increased in podocytes stimulated with HG, which was reduced with Genistein or Rapamycin treatment. Meanwhile, the results showed that Rapamycin counteracted the promoting effects of HG on mTOR expression, which was consistent with the effects of Genistein intervention. (Fig. 3D, E). Furthermore, the results of western blot also confirmed that Genistein and Rapamycin had the same inhibitory effects on mTOR phosphorylation induced by HG. Therefore, these results suggested that Genistein promoted HG-induced podocyte autophagy by down-regulating the mTOR signal.

3.4 Genistein affected the integrin β 1-MF expression of HG-induced podocyte

To determine the effects of Genistein on integrin β 1-MF in HG conditions, we detected the expression of integrin β 1-MF by immunofluorescence and Western blot. As shown in Fig. 4A, compared with NG group, the immunofluorescence intensity of integrin β 1-MF was mainly concentrated around the nucleus and rarely on the surface of plasma membrane in HG group. However, compared with the HG group, the

immunofluorescence intensity of TM group changed from perinuclear aggregation to uniform dispersion in membrane after 24 h, and the expression at 72 h was almost the same as that of the NG group (Fig. 4B). In addition, western blot results showed that the protein expression of cell membrane surface integrin β 1-MF was significantly decreased in the HG group from 12 to 72 h compared to that in the NG group ($P < 0.001$). By contrast, Genistein reversed the conditions, the expression of integrin β 1-MF on the surface of cell membrane was markedly increased in podocyte incubated with HG from 24 h (Fig. 4C, D).

3.5 Genistein alleviated the podocyte adhesion injury induced by HG conditions

The two experiments were performed at 0, 6, 12, 24, 36 and 48 h that showed a significant reduction in HG-induced podocyte adhesive capacity compared with NG group (Fig. 5). Wound healing test showed that the area of cell migration in NG and TM group was significantly larger than that in HG group at 48h (NG: 15.753 ± 0.475 ; HG: 38.337 ± 0.698 ; TM: 18.640 ± 0.834 , $p < 0.001$) (Fig. 5A-C). Cell adhesion assay demonstrated that compared with TM group, the adhesion rate of podocytes induced by HG all decreased significantly at each point in time. ($p < 0.001$) (Fig. 5D).

4. Discussion

In the current study, we focused on the protective effects of Genistein on podocytes induced by HG and the associated mechanisms. Genistein is a specific inhibitor of protein tyrosine kinase with heterocyclic bisphenol structure that has no cytotoxicity to normal tissues(5). Genistein plays an important role in the treatment of diseases by activating autophagy, especially in cancer(20, 21). Our results indicated that HG inhibited podocyte autophagy activity and adhesion ability, while Genistein effectively activated autophagy activity and enhanced integrin β 1-MF lysosomal dependent degradation recycling under HG conditions. As for the possible mechanisms of Genistein activating autophagy, our results showed that it reduced the phosphorylation of mTOR induced by HG. Collectively, the regulation of autophagy mediated integrin β 1-MF transport pathway was related to podocyte adhesion injury, Genistein activated basic podocyte autophagy by inhibiting mTOR signal.

It is known that autophagy serves as a protective mechanism for podocytes to maintain homeostasis(22). The autophagy flux is a very important index that evaluates the changes of autophagy activity(21). LC3-II is usually used as a specific marker of autophagy and degraded after the fusion of autophagy bodies and lysosomes (23). P62 is a multifunctional protein and itself is also a subject of autophagy degradation. Since the accumulation of p62 occurs when autophagy is inhibited, elevated p62 levels are generally considered to be as an important biomarker to assess autophagic flux(21, 24). Immunofluorescence co-localization intuitively showed that the accumulation of LC3-II and p62 increased concurrently, while Genistein significantly reduced the accumulation. Therefore, it is reasonable to speculate that Genistein could activate the autophagy lysosomal degradation pathway inhibited by HG. MTOR contains two complexes, mTORC1 and mTORC2, which is recognized as a key regulator of autophagy(25). The mTORC1 is generally considered to play a more important role in autophagy regulation. Activated mTORC1, acting as an autophagy blocker, phosphorylates Unc-51-like protein kinase

1(ULK-1) and preventing ULK1 activation by AMP-activated protein kinase (AMPK)(26). Inhibition of ULK1 complex could prevent formation of autophagic lysosomes(27). Therefore, the phosphorylation of mTORC1 could determine the degree of autophagy induction. In addition, some new studies have shown that mTORC1 could be located on the surface of lysosomal membrane(14, 25). And a large number of studies have demonstrated that mTORC1 is the main intersection of various pathways(26, 28, 29). The detection of mTOR signal may explain the mechanism of genistein activating autophagy.

The podocyte adhesion injury may occur in the early stage of DKD and play a key role in the progression of DKD(30). Integrin β 1-MF is the main receptor mediating podocytes adhesion to GBM, which is essential to maintain the adhesion ability of cells(31, 32). Of note, podocyte adhesion is a highly dynamic process, which depends on the continuous attachment and separation of integrin β 1-MF on the cell surface(7, 33). Therefore, the cycle of integrin β 1-MF maintains dynamic balance of adhesion spot synthesis and degradation, and endows podocytes with certain mobility and strong adhesion. Internalized integrin β 1-MF is continuously isolated and accumulated in the perinuclear region and reducing circulation to the plasma membrane in HG conditions. Furthermore, the lysosomal degradation pathway is blocked and the expression of surface integrin β 1-MF decreased continuously, resulting in adhesion injury. Podocytes maintain appropriately low mTOR levels and high autophagy activity levels which is important for controlling the stability of cell surface integrin β 1-MF levels(34). Some studies suggested that excessive autophagy activity will reduce the expression of integrins on the cell surface(14, 35). It is speculated that Genistein only restores the basic autophagy levels of podocytes, and then maintains the constant integrin β 1-MF on the cell surface.

In conclusion, our study investigates the underlying mechanisms by which Genistein reduced adhesive capacity damage in HG-induced podocytes, focusing on the involvement of autophagy and integrin β 1-MF. Genistein promotes to restore the lysosomal degradation and autophagy flux of HG-induced podocytes by inhibiting the phosphorylation of mTOR. The recovery of degradation process relieves the aggregation and isolation of integrin β 1-MF around the nucleus and promotes the expression on the cell surface, and then reduces the HG-induced cell adhesion injury. Although the in vitro model provides a convenience for exploring the effects and mechanisms of HG on podocyte autophagy and adhesion injury, we know that HG stimulation of podocytes may not fully reflect the pathological level or complete development of DN. Therefore, our next step is to verify the possible mechanism of Genistein in DKD through animal models.

Declarations

Ethical statement:

The manuscript has not been submitted elsewhere. No portion of the data has been or will be published elsewhere while the manuscript is under review by the Medicinal Chemistry Research. This manuscript has been read and approved by all authors. No conflict of interest was declared. All authors read and

approved the final manuscript. None of the authors has any potential financial conflict of interest related to this manuscript. Yu-Hang Lian on behalf of other co-authors.

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Figures

Figure 1

Effects of Genistein on HG-induced podocytes viability. The differentiated podocytes were treated with NG medium HP mediumHG medium and HG medium in the presence of 2, 4, 6 or 8 µg/ml Genistein for 0, 6, 12, 24, 36, 48 and 72 h respectively. The data represent the mean ± SD. ns($p \geq 0.05$), **($p < 0.01$), *** ($p < 0.001$).

Figure 2

Genistein upregulates autophagy in podocyte under HG conditions. (A, B) The levels of LC3-II and p62 in podocytes were examined by Immunofluorescence co-localization analysis at 0, 6, 12, 24, 36, 48 and 72 h. [Blue, nuclear staining (DAPI); green, LC3-II staining; red p62 staining; yellow, co-localization staining; Scale bar, 50 μ m] Fluorescence intensity and co-location were measured over time. (C, D) Western blotting

was further carried out for the protein expression of LC3-II and p62 at 24 h. The data are presented as the mean $\pm$ SD. $\square\square$ ($p < 0.01$), $\square\square\square$ ($p < 0.001$)

Figure 3

Effects of Genistein on mTOR signal related protein expression in podocyte under HG conditions. Based on the original grouping, podocytes treated with Rapamycin were added to verify the TM group. (A-C) The level of p-mTOR was examined by Immunofluorescence. [Blue, nuclear staining (DAPI); green, p-mTOR

staining; Scale bar, 50μm]. (D, E) Western blot analysis for mTOR and p-mTOR protein expression in each group of podocytes cultured for 24 h. Data are presented as the mean ± SD. Ns (p ≥0.05), *** (p < 0.001).

Figure 4

Effects of Genistein on integrin β1-MF under HG conditions. (A, B) Immunofluorescence microscopic images of integrin β1-MF in podocyte. [Blue, nuclear staining (DAPI); green,β1-MF staining]Scale bar, 50 μm]. It shows the changes of fluorescence intensity around nucleus (⊗) and cell surface (⊗) over time. (C,

D) Western blotting was used to detect the protein expression levels of integrin β 1-MF at 0, 6, 12, 24, 36, 48 and 72 h. The data represent the mean $\pm$ SD. ns ($p \geq 0.05$), $**$ ($p < 0.01$), $***$ ($p < 0.001$)

A. Wound healing

Figure 5

Podocyte adhesion injury was evaluated by wound healing assay and cell adhesion assay(A-C). Cells adhesion to collagen fibrinogen and migration were analyzed with spectrophotometry for 0, 6, 12, 24, 36 and 48 h. Scale bar, 50 μ m. All Values are expressed as the mean $\pm$ SD. $**$ ($p < 0.01$).