Orientin Protects Podocytes from High Glucose Induced Apoptosis through Mitophagy

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Diabetic nephropathy (DN) is one of the serious complications of diabetes mellitus. Orientin, a major bioactive constituent of Fenugreek, has been reported to possess antihyperglycemic properties. However, its effects on DN remain unclear. Therefore, we explored the protective effect of orientin on podocytes. Here, we assessed cell viability and toxicity, level of autophagy, mitochondrial morphological changes, and podocyte apoptosis. The results indicated that high glucose (HG) induced podocyte apoptosis as well as mitochondrial injury can be partially blocked by orientin. The results showed that orientin could repair autophagy disorder induced by HG, while 3-methyladenine (3-MA) reversed the protection of orientin. Our study demonstrated the possibility of treating DN with orientin.

Keywords: orientin, autophagy, mitochondria, podocytes, diabetic nephropathy.

Introduction

One of the main microvascular complications of diabetes mellitus is diabetic nephropathy (DN), which is also one of the important causes of the end-stage renal disease. [1] Although the pathogenesis of DN is multifactorial, increased numbers of diabetic patients and animal models have reported the structural and functional abnormalities of podocytes associated with diabetic renal disease and progression of renal injury. Podocytes, the epithelial cells in the visceral layer of renal microcystis, adhere to the outside of glomerular basement membrane (GBM). Together with vascular endothelial cells and GBM, they constitute the glomerular blood filtration barrier. As podocytes have limited ability to repair and regenerate,[2] the extent of podocyte injury is considered a major prognostic determinant in DN.

The current treatment of DN is limited. For example, patients with DN need to strictly control blood sugar, reduce protein intake, and are treated with drugs such as angiotensin II type 1 (AT1) receptor antagonists, angiotensin II-converting enzyme inhibitors, and others.[3] However, there is a lack of effective therapeutic drugs to protect the podocytes.

It has been reported that traditional medicinal plants may play an important role in the cure of DN and its complications.[4] Trigonella foenum-graecum also, known as ‘Fenugreek’ in Chinese, has been considered to be a promising supplement for the treatment of DN.[5] Orientin (Figure 1), a C-glycosyl flavonoid, is the main bioactive component of fenugreek,[6] which has multiple bioactivities and therapeutic effects, such as anti-inflammatory, antidia-

![Figure 1. The chemical structure of orientin.](image-url)
betes, antioxidation and autophagy induction.\[^{7-10}\] Therefore, it is of great significance to better understand the protective effect of orientin on podocytes for the new drug development for the cure of DN.

Emerging evidence has indicated that impaired autophagic activity is associated with injury of podocytes. Podocytes have a high level of basal autophagy, which is crucial for preserving homeostasis in podocytes.\[^{11-13}\] Significantly, it has been found that podocyte autophagy has a synergistic protective effect on glomeruli induced by diabetes.\[^{14}\] In addition, impaired podocyte autophagy aggravates proteinuria in the animal model of type 2 diabetic nephropathy.\[^{15}\] Furthermore, Dong et al.\[^{16}\] revealed that HG leads to increased autophagy in podocytes at an early stage, which is one of the survival mechanisms in the model of type 1 diabetic nephropathy under these conditions.

The purpose of this study is to verify the hypothesis that orientin attenuates the podocyte damage induced by HG and to investigate its relative mechanism.

**Results and Discussion**

_Orientin Recovered HG-Induced MPC-5 Cell Decrease by Reducing Apoptosis_

Apoptosis assay is one of the important methods to detect whether orientin can reduce the apoptosis of HG-induced in MPC-5 cells, and we observed that HG led to increased apoptosis of podocytes, but orientin (120 μM) reversed these results (Figure 2A and 2B). Then, CCK-8 assays were used to detect the cytotoxic effects of orientin on MPC-5 cells.

**Figure 2.** Orientin protects podocyte from HG-induced cell death. (A, B) Podocytes were exposed to HG and treated with orientin (120 μM) for 48 h. (C) Podocytes were exposed to orientin (20, 40, 80, 160, 320, and 640 μM) for 48 h. Cell viability was assessed by CCK-8 assay and expressed relative to untreated control cells. (D) MPC-5 cells were exposed to HG and treated with orientin (40, 80, and 120 μM) for 48 h. Cell viability was assessed by CCK-8 assay and expressed relative to untreated control cells. Apoptosis was evaluated by Annexin V staining. Data were expressed as the mean ± SEM from independent experiments. Comparisons between multiple groups were analyzed by one-way ANOVA followed by the Turkey post-test. \(^*p<0.05\) and \(^{**}p<0.01\), relative to the control group; \(^{#}p<0.05\) and \(^{##}p<0.01\), relative to the HG group.
effect of orientin and its influence on reducing apoptosis of MPC-5 cells induced by HG. In order to achieve this goal, we treated MPC-5 cells with different concentrations of orientin (20 μM to 640 μM). We can observe that orientin of 40–120 μM has no significant toxic effect on MPC-5 cells. However, when treated with 160 μM orientin, cell survival was significantly reduced (Figure 2C). To evaluate whether orientin protected podocytes from cell injury induced by HG after 48 h of incubation, the cell viability was tested by CCK-8 Assay Kit. As shown in Figure 2D, compared with the control group, the cell viability of HG group was significantly reduced, while orientin (40–120 μM) was reversed in a dose-dependent manner. Based on these findings, orientin concentrations of 120 μM were used in the subsequent experiments.

**Orientin Restored the Autophagy Suppressed by HG**

To investigate the formation of LC3 induced by orientin (120 μM), MPC-5 cells were transfected with GFP-LC3 plasmid. Compared with the control group, the number of GFP-LC3 puncta was decreased sharply in cells treated with HG. However, orientin reversed this phenomenon. Furthermore, autophagy inhibitor 3-MA reversed the increase of autophagy induced by orientin. Western blot demonstrated that the expression of LC3-II/LC3-I was reduced by HG, but the expression of p62 was increased (Figure 3A and 3B). It is acknowledged that LC3 is one of the important biomarkers of autophagy formation, and p62 is one of the biomarkers of autophagy degradation. According to these results, we knew that HG reduces the autophagy, while orientin restores it. In order to reveal whether orientin’s protection of MPC-5 cells is related to autophagy, we used 3-MA, an autophagy inhibitor. In treatment group, we found that orientin increased the LC3 and depressed the p62 expression induced by HG. However, when 3-MA was added, the result was opposite. These results suggest that the protective effect of orientin on HG-induced apoptosis might be related to the enhancement of autophagy (Figure 3C–3E).

**Orientin Protects Podocytes by Repairing Mitochondrial Transmembrane Potential**

Using JC-10 staining method to detect mitochondrial membrane potential, we observed that HG significantly increased the number of cells in depolarized mitochondria (green fluorescence), which can be reversed by orientin (120 μM) treatment (Figure 4A and 4B).

**Orientin Might Protect Mitochondria by Restoring Autophagy**

The protective effect of orientin on the mitochondria and its relationship with autophagy was analyzed through immunofluorescence staining. Compared with the control group, the percentage of swelling, fragmentation, and roundness of mitochondria in HG group was higher, but orientin group showed alleviated HG-induced mitochondrial injury. Interestingly, the treatment of 3-MA eliminated the protective effect of orientin on mitochondria (Figure 5A). These results indicated that when autophagy was increased, the mitochondrial injury was alleviated. In MPC-5 cells, TOM 20 was distributed on the outer membrane of mitochondria colocalized with GFP-LC3 (Figure 5B). The confocal images also localized the LC3 on the outer membrane of the mitochondria, and orientin can restore the colocalization reduced by HG, indicating the role of orientin to protect mitochondria by strengthening the autophagy of the MPC-5 cells.

DN is one of the complications of diabetes mellitus leading to end-stage renal disease. Autophagy and mitophagy have been proved to be the key to maintain the homeostasis of podocytes by reducing apoptosis, which was changed under the condition of diabetes. Convincing pieces of evidence suggested that orientin can induce autophagy in the brain and cardiomyocytes. However, its effect on autophagy induction of podocytes is not clear. In our study, we confirmed that orientin significantly reduces the apoptosis rate of MPC-5 cells induced by HG by activating autophagy. Significantly, our results indicated that orientin protects podocytes by protecting mitochondria. Therefore, this study revealed a new possible strategy to protect podocytes against apoptosis in DN.

*Trigonella foenum-graecum* (fenugreek) seed is a well-known traditional medicinal herb and possesses antidiabetic, hepatoprotective, antihyperlipidemic, antiobesity, anticancer, antioxidant, anti-inflammatory, and miscellaneous pharmacological effects. Orientin, one of the isobal bioactive compounds of Fenugreek, is often used in various bioactivity studies due to its extensive beneficial properties, such as antioxidant, anti-inflammation, cardioprotective, neuroprotective, antidepressant-like, anti-adipogenesis, and antinociceptive effects. In a word, orientin is one of the important bioactive components of fenug-
greek, and the fenugreek has been proved to have a therapeutic effect on diabetes and DN,\textsuperscript{30,31} and orientin itself also has many functions such as anti-inflammatory and antioxidation.\textsuperscript{29} Meanwhile, the oxidative stress pathway in the pathogenesis of diabetes and DN is very important. To our knowledge, there are few studies on orientin in DN and podocytes. Therefore, in the present

Figure 3. Orientin is an autophagy activator. (A, B) Confocal images of LC3 puncta in MPC-5 cells expressing GFP-LC3 that were treated with HG, orientin (120 μM), or 3-MA. Bar: 25 μm. 100 to 150 cells from each group were counted, and three independent experiments were performed. (C–E) Western blot was used to investigate the protein expression of LC3 and p62. Data were expressed as the mean ± SEM of independent experiments. Comparisons between multiple groups were analyzed by one-way ANOVA followed by the Turkey post-test. *p < 0.05.
Figure 4. Orientin protects mitochondrial membrane integrity. (A, B) JC-10 staining was used to investigate mitochondrial depolarization. Cells with depolarized mitochondria were detected by green fluorescence and quantified as the percentage of the total cell, bar = 50 μm (Data were expressed as the mean ± SEM from independent experiments. Comparisons between multiple groups were analyzed by one-way ANOVA followed by the Turkey post-test. **p < 0.01, relative to the HG group).
Figure 5. Orientin protects mitochondria by enhancing autophagy of podocytes. (A) Images of mitochondria and LC3 in podocytes of HG group, orientin (120 μM) group and 3-MA group obtained through immunofluorescence confocal microscopy. (B) LC3 is located on the outer membrane of the mitochondria of podocytes. Regions in the white boxes are analyzed. Control group mean co-localization coefficient: 0.58 ± 0.03, HG group mean co-localization coefficient: 0.13 ± 0.06, orientin group mean co-localization coefficient: 0.65 ± 0.05, 3-MA group mean co-localization coefficient: 0.22 ± 0.03. Scale bars are 5 μm. Co-localization coefficients are the combined results of 2–3 independent experiments (n = 28–30 total fields/group).
study, we have made a concerted effort to reveal the role of orientin on podocytes.

The current study showed that orientin has the protective effect on podocytes injury induced by HG. In MPC-5 cells, we found that orientin attenuated the podocyte apoptosis induced by HG. Flow-cytometry analysis showed that HG treatment could cause early apoptosis of podocytes, while orientin treatment could reverse this result. Together, these results suggest that orientin protects podocytes by attenuating apoptosis induced by mercury. However, it requires in-depth studies to unravel its exact mechanism.

Autophagy is a process in which autophagy lysosomes are formed by phagocytosis of cytoplasmic proteins or organelles and their inclusion into vesicles and fusion with lysosomes to degrade the contents of the inclusions, so as to realize the metabolic needs of cells themselves and the renewal of some organelles. Autophagy has proven to be one of the most important mechanisms to protect the kidney. In the present study, we found that HG disturbed the LC3 expression of podocytes that were reversed by orientin, indicating the possible protective role of autophagy. Subsequently, we have confirmed its effect on autophagy using 3-MA, an autophagy inhibitor, which showed that the increased expression of LC3 caused by orientin in HG group was reversed by the 3-MA.

Mitochondria are double-membrane-bound organelles that are generally considered to be ‘powerhouse units’ of the cell. Apart from the heart, the kidney is thought to be the densest place in every tissue. Some studies have shown that diabetic nephropathy is characterized by decreased mitochondrial content and function, at the same time, with the change of mitochondrial membrane potential. Herein, we used JC-10 Assay Kit to test mitochondrial membrane depolarization in the high glucose treatment group which was found to be partially blocked by orientin.

Mitochondria are highly dynamic organelles that play a significant role in keeping homeostasis. They are a class of highly shape-changing organelles which continuously undergo fusion and fission. In physiological conditions, the shape of the mitochondria is normal, in the form of a rod or an ellipse, but their shape becomes swollen and ruptured to round under stress including various kidney diseases. The selective scavenging effect of autophagy on mitochondria is closely related to the biological activity of mitochondria. It can maintain the stability of mitochondria and meet the metabolic needs of the body. Here we have shown that orientin can reduce the ratio of the swelling and fragmentation of mitochondria caused by HG by enhancing autophagy. Furthermore, we also observed that orientin could increase the localization of LC3 and TOM20 though enhancement of autophagy of MPC-5 cells.

These results revealed that orientin has mitochondrial protection and anti-apoptosis effects, which might be related to the restore of autophagy in HG-induced podocyte injury.

Despite the significant findings, there are still some deficiencies of this study that need to be addressed. First, the specific mechanism through which orientin reduced apoptosis of podocytes through autophagy is yet to be explored. Secondly, the complex mechanism between autophagy and mitochondria in podocytes of DN has not been studied. Third, the mechanism through which orientin enhanced the localization has not been studied in depth.

Conclusions

In conclusion, our study examined the protective effects of orientin on MPC5 cells and the potential molecular mechanisms. We found that orientin significantly induced cell anti-apoptosis and autophagy via protecting mitochondria of podocytes. Our study revealed that orientin restored cell proliferation in HG-induced MPC-5 cells through decreased apoptosis. Furthermore, we demonstrated that autophagy had a vital role in anti-apoptosis induced by HG. Significantly, we found that orientin had protective effects on mitochondrial membrane integrity induced by HG, which might be related to autophagy.

This study indicates that orientin is a novel drug candidate for DN that has the benefits and clinical application for reversing the toxicity of high glucose to the kidney.

Experimental Section

Chemicals and Reagents

Purified orientin (>98%) was purchased from the Beijing Putian Tongchuang Biotechnology Co., Ltd. (Beijing, China). Stock solution at 100 mM was made in dimethyl sulfoxide (DMSO) and stored at −80°C. Cell Counting Kit-8 (CCK-8) was purchased from Med Chem Express (New Jersey, America). JC-10 Kit was acquired from Sinobest Biotechnology Co., Ltd. (Shanghai, China). BCA Protein Kit Assay was obtained from Beyotime Institute of Biotechnology (Nanjing, Jiangsu,
China). Anti-rabbit IgG/HRP, β-actin, LC3II/I, and p62 antibodies were supplied by Cell Signaling Technology (Beverly, MA, USA).

**Cell Culture and Treatment**

Conditionally immortalized mouse podocyte cell lines (MPC-5) were acquired from Beijing Zhongke Quality Inspection and Technology Co., Ltd. (Beijing, China), and cells were cultured in DMEM (5.5 mM glucose) containing 10% FBS. Cells were cultured and maintained in DMEM (low glucose) medium containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin plus 100 μg/ml streptomycin at permissive temperature of 37°C and 5% CO₂.

MPC-5 cells were plated into 24-well plates. All the subsequent experimental assays were performed under the following conditions: control group (5.5 mM glucose), HG group (30 mM glucose), orientin group (30 mM glucose with 120 μM orientin), 3-MA group (30 mM glucose with 1 mg/ml 3-MA).

**Apoptosis Assay**

Podocytes were plated in 12-well plates (5×10⁵ cells per well) and cultured for 6 h, then treated with orientin (120 μM) for 48 h. Apoptosis rate was assessed by Annexin V-FITC/PI Apoptosis Kit (CST, Shanghai, China).

**CCK-8 Assay**

MPC-5 cells were inoculated in 96-well plates at a density of 2000 cells/well for 3 h followed by addition of HG and orientin and incubated for 48 h as mentioned above. We used CCK-8 Assay Kit to evaluate cell viability. Then, the absorbance was measured at the wavelength of 450 nm.

**Western Blot Analysis**

In order to lyse MPC-5 cells, we added RIPA lysis buffer (Sinobest, China) containing 1 mM PMSF (Sinobest, China) into the cells according to the instructions and incubated it on ice for 30 min. Equal amounts of proteins (15 μg) on 15% SDS-PAGE gel and transferred it to PVDF membrane. The following primary antibodies were incubated overnight at 4°C: β-actin (1:5000), LC3II/I (1:1000), p62 (1:1000) antibodies.

**Mitochondrial Membrane Potential Assay**

Mitochondrial membrane potential was assessed by JC 10 Kit. Observation with fluorescence microscope, the depolarization of mitochondria showed green fluorescence, while the normal mitochondria showed red fluorescence.

**Immunofluorescence Staining**

MPC-5 cells were transfected with GFP-LC3 plasmid by Lipofectamine 3000 (Invitrogen). The cells were then treated with HG, 3-MA or orthin, incubated for 48 h, then incubated overnight at 4°C with an anti-Tom20 (1:200) primary antibody.

**Statistical Analysis**

We use SPSS 19.0 software (IBM, Armonk, New York, USA) for data analysis.

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**Author Contributions Statement**

Z.-L. K. conceived the concept, designed the experiment, carried out the experiment and wrote the article. K. C. did the experiment. Z.-L. K., J.-W. C. and Y.-Y. W. carried out the experiments and contributed to the data analysis. J.-X. H. facilitated the data analysis. Y. C., Y.-Y. W., and X. W. contributed to the revision of the article. W.-S. L. analyzed the results.
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