Emodin Ameliorates High Glucose-Induced Podocyte Apoptosis via Regulating AMPK/mTOR-Mediated Autophagy Signaling Pathway

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ABSTRACT Objective: To investigate the effect of emodin on high glucose (HG)-induced podocyte apoptosis and whether the potential anti-apoptotic mechanism of emodin is related to induction of adenosine-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR)-mediated autophagy in podocytes (MPC5 cells) in vitro. Methods: MPC5 cells were treated with different concentrations of HG (2.5, 5, 10, 20, 40, 80 and 160 mmol/L), emodin (2, 4, 8 µmol/L), or HG (40 mmol/L) and emodin (4 µmol/L) with or without rapamycin (Rap, 100 nmol/L) and compound C (10 µmol/L). The viability and apoptosis of MPC5 cells were detected using cell counting kit-8 (CCK-8) assay and flow cytometry analysis, respectively. The expression levels of cleaved caspase-3, autophagy marker light chain 3 (LC3) I/II, and AMPK/mTOR signaling pathway-related proteins were determined by Western blot. The changes of morphology and RFP-LC3 fluorescence were observed under microscopy. Results: HG at 20, 40, 80 and 160 mmol/L dose-dependently induced cell apoptosis in MPC5 cells, whereas emodin (4 µmol/L) significantly ameliorated HG-induced cell apoptosis and caspase-3 cleavage (P<0.01). Emodin (4 µmol/L) significantly increased LC3-II protein expression levels and induced RFP-LC3-containing punctate structures in MPC5 cells (P<0.01). Furthermore, the protective effects of emodin were mimicked by rapamycin (100 nmol/L). Moreover, emodin increased the phosphorylation of AMPK and suppressed the phosphorylation of mTOR. The AMPK inhibitor compound C (10 µmol/L) reversed emodin-induced autophagy activation. Conclusion: Emodin ameliorated HG-induced apoptosis of MPC5 cells in vitro that involved induction of autophagy through the AMPK/mTOR signaling pathway, which might provide a potential therapeutic option for diabetic nephropathy.

KEYWORDS emodin, diabetic nephropathy, autophagy, podocyte apoptosis, adenosine-monophosphate-activated protein kinase/mammalian target of rapamycin signaling pathways

Diabetic nephropathy (DN) is a diabetes-induced microvascular complication which has become the primary cause of end-stage renal failure. Progressive proteinuria is a significant clinical feature of DN caused by an impaired glomerular filtration barrier. Podocytes are highly differentiated glomerular epithelial cells, which constitute glomerular filtration barrier together with glomerular basement membrane (GBM) and vascular endothelial cells. Glomerular podocyte injury and apoptosis play an important role in the progression of DN, especially in the formation of proteinuria and glomerulosclerosis.

Autophagy is an important cellular process that involved in the maintenance of cell renewal and homeostasis through the degradation of lysosomal proteins and the removal of damaged structures or overexpressed proteins. Autophagy pathway can be activated under the stress conditions of nutrition deficiency, ischemia and hypoxia, oxidative stress, etc.
Research has shown that the basic level of autophagy in podocytes is significantly higher than that in other intrinsic glomerular cells, and high level of autophagy is necessary to maintain the normal physiological function of podocytes. The high level of autophagy activity of podocytes is conducive to the degradation or removal of damaged proteins and aging organelles for maintaining cell homeostasis. Apoptosis is a programmed method of gene regulation and biological autonomy for maintaining a constant number of cells. Studies have demonstrated that autophagy is closely related with apoptosis in the development and progression of DN. Apoptosis of podocytes is present in early stage of DN, and autophagy activity is significantly increased when the podocytes are damaged. Therefore, exploring the relationship between podocyte autophagy and apoptosis may provide an important therapeutic strategy for drug treatment of DN.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active anthraquinone constituent that extracted from the rhizome of rhubarb Rheum officinale Baill. It has been shown that emodin possesses various pharmacological properties, including anti-bacterial, anti-inflammation, immunosuppressive, antiproliferation, anticancer and antioxidant activities, etc. Rhubarb preparations have been widely used in clinical treatment of DN. Previous studies have shown that the treatment mechanism of emodin in DN may be related to the inhibition of cell proliferation and inflammatory response. Recent study has shown that emodin can improve the damage of DN by regulating autophagy signaling pathway. The pathogenesis of DN is related to nutrition sensitive pathways such as AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), autophagy is positively regulated by AMPK, but negatively regulated by mTOR. AMPK activation leads to the phosphorylation and activation of tuberous sclerosis complex 1/2 (TSC1/2) complex, which can indirectly inhibit the activity of mTOR by suppressing the activity of Rheb enzyme. It can also directly phosphorylate a subunit of mTORC1, raptor, to inhibit mTOR and enhance autophagy. It has been reported that emodin is an effective AMPK activator, and can also regulate mTOR pathway. Our in vivo study shows that emodin ameliorates podocyte injury in DN rats by regulating AMPK/mTOR-mediated autophagy signaling pathway. To further verify the protective effect of emodin on podocytes of DN, we conducted experiments in vitro and the effects and molecular mechanisms of emodin on podocyte injury induced by high glucose was investigated.

**METHODS**

**Reagents**

Emodin (Cat. No. E7881), rapamycin (Rap, CAS#: 53123-88-9) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Dorsomorphin (Compound C, Cat. No. S7840) was purchased from Selleck Chemicals LLC (Houston, Texas, USA). Recombination interferon-γ (IFN-γ) was purchased from Pepro Tech Inc (Rocky Hill, New Jersey, USA). D-glucose (HG) was purchased from MedChemExpress (New Jersey, USA). Emodin was dissolved in DMSO to a concentration of 10 mmol/L for use.

**Cell Culture**

The conditioned immortalized mouse podocytes (MPC5) cells were kindly provided by Prof. YUAN Jun, Department of Nephrology, the Affiliated Hospital of Hubei University of Chinese Medicine, which were obtained from Professor Peter Mundel Laboratory (Mount Sinai Medical Center, New York, USA). Undifferentiated podocytes were cultured in RPMI 1640 medium (Hyclone, Thermo Fisher, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) containing 10 U/mL IFN-γ, 100 U/mL penicillin G and 100 μg/mL streptomycin (Gibco USA) at 33 ℃ under an atmosphere of 5% CO₂, and induced to differentiate supplemented with 10% FBS without IFN-γ at 37 ℃ and 5% CO₂ for 10–14 days in RPMI-1640 medium. The differentiated podocytes were used in subsequent experiments.

**Evaluation of Viable Cells**

The number of viable cells was assessed by trypan blue exclusion. MPC5 cells were incubated in medium containing normal glucose (5.5 mmol/L) and different concentrations of HG (20, 40, 80, 160 mmol/L), or 40 mmol/L HG with emodin (2, 4, 8 μmol/L) for 48 h and were subjected to phase-contrast microscopy (magnification, ×200), then the viable cells were collected and resuspended in the trypan blue solution (0.4%), finally the number of viable cells were counted under a light microscope with a hemacytometer. At least 3 independent experiments were conducted.

**Cell Proliferation Assay**

The cell counting kit-8 (CCK-8, C0037, Beyotime
Biotechnology, Shanghai, China) was used to detect cell viability according to the manufacturer's instructions. The differentiated MPC5 cells (5 x 10^3 cells per well) were seeded into 96-well plates and incubated with 5% CO_2 at 37 °C. After the cells proliferated to 70%–80% fusion in the plate, subsequently, the cells were divided into several groups and treated with different concentrations of HG (2.5, 5, 10, 20, 40, 80 and 160 mmol/L) medium respectively. After 48 h treatment, CCK-8 and serum-free RPMI 1640 medium were mixed at a ratio of 1:10, and then the cells were incubated for 2 h. The absorbance values of each well were measured at 450 nm using a microplate reader (SpectraMax i3x, Molecular Devices, Shanghai, China), the relative ratio was used to reflect the proliferation rate.

Flow Cytometric Analysis of Apoptosis

The apoptosis of MPC5 cells was assessed by flow cytometry (Becton Dickinson, USA) using the Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, KGA108, Nanjing, China) according to the manufacturer's protocol. Cells were incubated in medium containing normal glucose (5.5 mmol/L) and 40 mmol/L HG with or without 100 nmol/L Rap for 48 h, and then MPC5 cells were collected and resuspended in binding buffer. Subsequently, cells were incubated with Annexin V-FITC (5 μL) and PI (5 μL) for 15 min. The percentage of Annexin V-FITC and PI-stained cells was calculated using Accuri C6 software (Becton Dickinson, USA).

Western Blot Analysis
Podocytes were collected and lysed with RIPA lysis buffer (Beyotime, Hainan, China). Samples were obtained via centrifugation at 13,000 x g and 4 °C for 5 min. The supernatants were boiled at 100 °C for 5 min in loading buffer. Lysate protein concentrations were determined by bicinchoninic acid (BCA) protein concentration assay kit (Beyotime Biotechnology, P0012, Shanghai, China). Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then was electrophoretically transferred to polyvinylidene fluoride membrane (Millipore, Bedford, USA). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, and then incubated overnight at 4 °C with primary antibody as follows: anti-rat AMPK (Cat. No. ab80039), anti-rat p-AMPK (Cat. No. ab23875), anti-rat β-actin (Cat. No. ab227387) antibodies were purchased from Abcam Ltd, HKSP, New Territories, HK, China; anti-rat LC3 I/II (Cat. No. 12741), anti-rat mTOR (Cat. No. 2983), anti-rat p-mTOR (Cat. No. 5536), anti-rat cleaved caspase-3 (Cat. No. 9661) antibodies were obtained from Cell Signaling Technology Company, Beverly, MA, USA; anti-rat caspase-3 (Cat. No. BM4620) antibodies were purchased from Boster Biological Technology Co., Ltd., Wuhan, China; Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins and anti-mouse immunoglobulins (KPL Company, USA) were used as the secondary antibody. The membranes were coated using HRP-labeled chemiluminescent substrates (Millipore, Bedford, USA), eventually exposed, and fixed in the dark box. This procedure was carried out 3 times. The results were quantified using Image-Pro Plus 6.0 software (Media Cybernetic, Washington, USA), which were contrasted with densitometric signal of β -actin, respectively, and the ratios were expressed as the relative protein contents.

Detection of Autophagosomes by Transient Transfection

MPC5 cells were transfected with the pmRFP-LC3 plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. pmRFP-LC3 (Cat. No. 21075) plasmid was obtained from Addgene (Massachusetts, USA). MPC5 cells transfected with pmRFP-LC3 were incubated with or without 4 μmol/L emodin and 10 μmol/L compound C for 1 h and formation of autophagosomes was observed under a fluorescent microscope.

Statistical Analysis
The data were analyzed using statistical software SPSS 24.0 (SPSS Inc., Chicago, Illinois, USA). Significant differences were evaluated using one-way ANOVA with Bonferroni post-hoc test (GraphPad Prism 6.0, La Jolla, CA, USA). A P-value less than 0.05 was statistically significant.

RESULTS

HG Induces Apoptosis in Podocytes
Results of CCK-8 analysis revealed that the proliferation rate of cells was gradually decreased when HG concentration is higher than 5 mmol/L (Figure 1A). Additionally, HG resulted in concentration-dependent cell apoptosis, and cells treated with 40 mmol/L HG showed obviously apoptosis (P<0.01, Figure 1B).
Western blot analysis showed that after 12-h treatment with 40 mmol/L HG, the expression of cleaved caspase-3 increased ($P<0.01$, Figure 1C). After 48 h of treatment, the results of flow cytometry revealed that the proportion of apoptotic cells increased after 40 mmol/L HG treatment (11.4% early apoptotic cells and 1.69% late apoptotic cells; $P<0.01$, Figure 1D).

Effect of Emodin on Podocyte Apoptosis Induced by HG

The results indicate that 40 mmol/L HG-treated cells for 48 h exhibited abundant cellular apoptosis that was markedly attenuated by 4 $\mu$mol/L emodin treatment ($P<0.01$, Figure 2A). Under phase-contrast microscopy, 40 mmol/L HG treatment decreased the number of viable cells, which was obviously reversed by 4 $\mu$mol/L emodin (Figure 2B). Additionally, Western blot analysis revealed that 40 mmol/L HG treatment in MPC5 cells for 48 h significantly increased cleaved caspase 3 levels, whereas 4 $\mu$mol/L emodin significantly suppressed the expression of cleaved caspase-3 ($P<0.01$, Figure 2C).

Emodin Induces Autophagic Activity in MPC5 Cells

As shown in Figure 3A, when MPC5 cells were exposed to 40 mmol/L HG at 0, 1, 2, 3, 6, 12 h, LC3-Ⅱ markedly increased and reached a maximum level at 1 h ($P<0.01$). Similarly, MPC5 cells were treated with 4 $\mu$mol/L emodin, the level of LC3-Ⅱ was also increased at 1 h ($P<0.01$, Figure 3B). Under the fluorescence microscope, when MPC5 cells were treated with 4 $\mu$mol/L emodin for 1 h, the bright fluorescent particles increased significantly, whereas cells without emodin treatment showed a diffuse distribution of red fluorescence (Figure 3C), which indicated an increase in the formation of autophagosomes.

Autophagy Protects Podocytes from Apoptosis Induced by HG

Western blot analysis showed that autophagic
activity was significantly induced by 100 nmol/L Rap treatment at 0, 1, 2, 3, 6 h, especially at 1 h and 6 h (P<0.01, Figure 4A). Subsequently, cells were exposed to 40 mmol/L HG with or without 100 nmol/L Rap for 48 h and were subjected to Annexin V and PI assays. D: Cells were treated with 40 mmol/L HG with or without 4 μmol/L emodin and 100 nmol/L Rap for 48 h and were subjected to Western blot analysis of caspase-3 and cleaved caspase-3 levels.

Emodin also can suppress caspase-3 cleavage caused by 40 mmol/L HG (P<0.01, Figure 4D), supporting the cytoprotective effect of emodin and Rap.

**Emodin Induces Autophagy by Regulating the AMPK/mTOR Signaling Pathways**

When MPC5 cells were exposed to 4 μmol/L emodin at 0, 1, 2, 3, 6 h, the phosphorylation of AMPK was significantly increased, especially at 1 h, while the phosphorylation of mTOR was markedly suppressed, which also most obviously at 1 h (P<0.01, Figure 5A). As shown in Figure 5B, when MPC5 cells were exposed to 4 μmol/L emodin for 1 h, pmRFP-tagged LC3-transfected cells exhibited increased punctate

**Figure 4. Autophagy Protects Podocytes from Apoptosis Induced by High Glucose**

Notes: A: MPC5 cells were treated with 100 nmol/L Rap at different time points and were subjected to Western blot analysis of LC3- I and LC3- II protein levels. B: MPC5 cells were treated with 4 μmol/L emodin for 0–6 h and were subjected to Western blot analysis of LC3- I and LC3- II protein levels. C: Fluorescent microscopic analysis of MPC5 cells transfected with pmRFP fluorescent-tagged LC3 plasmid and exposed to 4 μmol/L emodin for 1 h. The red color indicates autophagosomes. \*P<0.01
Chin J Integr Med 2023 Sep;29(9):801-808

have suggested that HG could induce apoptosis in glomerular podocytes,\(^{(22,23)}\) in this experiment, apoptosis of podocyte and expression of pro-apoptotic protein cleaved caspase-3 increased significantly with 40 mmol/L HG. After emodin intervention, all these indice were significantly reversed, which indicates that emodin has protective effect on podocyte injury.

Autophagy participates in organelle metabolism and bioenergy supply through degrades long-lived proteins and organelles, which can maintain the stability of the cell environment.\(^{(24)}\) Under normal physiological conditions, the basic level of autophagy exists in almost all cells and plays an important role in cell growth, proliferation, and death. Studies have reported that in the pathophysiological process of the kidney, autophagy is closely related to the intrinsic cells of the kidney, such as podocytes and renal tubular epithelial cells.\(^{(25,26)}\) There are also studies reported that autophagy has appeared in diabetic kidney injury,\(^{(27)}\) renal ischemia-reperfusion injury,\(^{(28)}\) and toxic kidney injury,\(^{(29)}\) indicating that autophagy may be involved in a variety of kidney diseases. Under normal conditions, podocytes maintain a certain level of autophagy; in our experimental results, a few autophagosomes were found in podocytes cultured with basic concentration of HG (5.5 mmol/L). LC-3, known as microtubule associated protein light

structures, while these number of punctate structures was significantly decreased when 10 μ mol/L compound C was added (P<0.01). Western blot showed that 4 μ mol/L emodin treated cells for 48 h significantly increased the ratio of LC3-II/LC3-I, while the ratio was significantly decreased when 10 μ mol/L compound C was added (P<0.01, Figure 5C).

**DISCUSSION**

DN has now gradually become a major cause of end stage renal disease. However, the completely effective treatment is still limited. Increasingly studies show that Chinese medicine treatment can delay the progression of DN through variety mechanisms,\(^{(21)}\) including antioxidation, anti-inflammation, immune regulation, anti-fibrosis, etc. In the present investigation, emodin, a bioactive substance found in rhubarb, which increased autophagy and suppressed HG-induced podocyte apoptosis, might exert protective effects via inhibiting podocyte apoptosis and promoting cell autophagy in DN.

Podocytes were highly differentiated glomerular epithelial cells and located on the surface of GBM, which play a key role in maintaining the structure and function of the glomerular filtration barrier. The loss and impairment of podocytes is a major cause of nephrotic proteinuria and glomerular sclerosis, which lead to the initiation and progression of DN.\(^{(4)}\) Previous studies have suggested that HG could induce apoptosis in glomerular podocytes,\(^{(22,23)}\) in this experiment, apoptosis of podocyte and expression of pro-apoptotic protein cleaved caspase-3 increased significantly with 40 mmol/L HG. After emodin intervention, all these indice were significantly reversed, which indicates that emodin has protective effect on podocyte injury.

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emodin against HG-induced podocyte apoptosis, that involved induction of autophagy through the AMPK/mTOR pathway. This study confirmed that emodin ameliorates HG-induced podocyte apoptosis and provides additional evidence in support of the clinical usage of emodin in the treatment of DN.

Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
Liu H, Zhang YM and Chen WD conceived and designed the experiment. Liu H, Chen WD and Yang WQ performed the experiment. Liu H, Hu TT and Wang HL analyzed the data. Liu H and Hu YL wrote the manuscript. All authors read and approved the final manuscript for publication.

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Autophagy is regulated by two main nutrient-sensing pathways, i.e., mTOR and AMPK. Rapamycin, the activator of autophagy, which has been reported to activate autophagy by inhibiting mTOR signaling pathway, was used to verify the protective effect of autophagy on HG-induced podocyte apoptosis. mTOR is a target protein of rapamycin, which can regulate cell growth and autophagy. In the condition of adequate nutrition or without stress, mTOR is activated and autophagy is inhibited; however, mTOR activity is inhibited and autophagy pathway is activated when the cells in a stress state or starvation environment under nutritional deficiency. Under stress conditions, rapamycin can specifically bind to mTOR and inhibit the protein kinase activity of mTOR, thus inducing autophagy. In this study, Rap was used to interfere with MPC5 cells. It was found that rapamycin treatment could increase the ratio of LC3-II/LC3-I. Our previous studies have shown that emodin can regulate mTOR pathway, in the present experiment, the results showed that the ratio of LC3-II/LC3-I also increased significantly, which indicated that emodin can induce autophagy analogue to rapamycin. Emodin treatment also increased the autophagy fluorescence granules, which further confirmed that emodin can induce autophagy.

AMPK pathway is one of the upstream pathways of mTOR. Activation of AMPK can inhibit mTOR and enhance autophagy. It has been reported that emodin is an effective AMPK activator, our results showed that the expression of p-mTOR protein was significantly down-regulated and the expression of p-AMPK was up-regulated with the prolongation of emodin intervention time, which most obvious at 1 h. Therefore, emodin may induce autophagy in MPC5 cells by regulating AMPK/mTOR signaling pathway. Compound C is a well-known AMPK inhibitor, when cells were treated with emodin alone or in combination with compound C, autophagy fluorescence granules increased by emodin was obviously suppressed when added compound C. Similarly, Western blot results showed that emodin can increase the ratio of LC3-II/LC3-I, which reversed by compound C. It was further confirmed that emodin might regulate AMPK/mTOR signaling pathway.

In conclusion, experimental results indicated that emodin could induce autophagy in HG-treated MPC5 cells and revealed the underlying mechanism of emodin against HG-induced podocyte apoptosis, that involved induction of autophagy through the AMPK/mTOR pathway. This study confirmed that emodin ameliorates HG-induced podocyte apoptosis and provides additional evidence in support of the clinical usage of emodin in the treatment of DN.

ch 3, is synthesized in cells and located in the cytoplasm. In the process of autophagy, LC-3 type-I is modified by ubiquitin-like system, covalently combined with phosphatidylethanolamine, and located on the autophagosome membrane to form LC-3 type-II. The inversion of the relative expression ratio of type-I and type-II can be used to indicate the activity of autophagy. In this experiment, the results showed that the ratio of LC3-II/LC3-I was significantly increased at 1 and 6 h after HG and emodin treatment, indicating that autophagy activity was enhanced. The results indicated that autophagy has a self-stabilizing effect and plays a protective role in podocyte damage, and emodin may reduce HG-induced podocyte apoptosis by enhancing autophagy.

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(Accepted July 11, 2022; First Online October 11, 2022)

Edited by TIAN Lin