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Hispidulin alleviates high-glucose-induced podocyte injury by regulating protective autophagy

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ABSTRACT

Objectives: Diabetic nephropathy (DN) is one of the most common complications in patients with diabetes, and the discovery of novel targeted therapeutic approaches for DN treatment still faces severe challenges. In the current study, we aimed to discover a novel natural product for potential DN treatment and determine its molecular mechanisms.

Materials and methods: Methylthiazoltetrazolium (MTT) assay was employed to evaluate cell viability. Transmission electron microscopy, GFP-LC3 fluorescence fusion plasmid, and Annexin V/PI apoptosis assay were carried out to determine cellular autophagy and apoptosis. Moreover, quantitative proteomics and bioinformatics analysis, Western blotting, and RNA interference were performed to investigate potential molecular mechanisms.

Results: Hispidulin displayed protective capacity on the high-glucose-induced podocyte injury models by activating autophagy and inhibiting apoptosis. The mechanism for hispidulin-induced autophagy was associated to Pim1 inhibition and the regulation of Pim1-p21-mTOR signaling axis. Moreover, quantitative proteomics and bioinformatics analysis revealed that the hispidulin-regulated Pim1 inhibition was associated to RAB18, NRas, PARK7, and FIS1.

Conclusions: These results indicate that hispidulin induces autophagy and inhibits apoptosis induced by high glucose in murine podocytes. This study will illuminate future developments in DN-targeted therapy.

1. Introduction

With the extension of life expectancy and changing lifestyles, the incidence of diabetes mellitus (DM) has rapidly risen in China and worldwide [1]. In 2013, the prevalence of DM among adults worldwide was about 8.8%. Moreover, China has the highest number of patients with diabetes, and the prevalence of DM among Chinese adults is about 10.9% [2]. Furthermore, the total number of Chinese adult DM patients has exceeded 100 million. Without intervention, DM patients in China are expected to exceed 150 million in 2030 [3]. About one-third of patients with diabetes have diabetic nephropathy (DN), which is the main cause of end-stage renal disease [4–6]. No major symptoms occur at the beginning of DN; however, as DN progresses, the condition deteriorates and causes the kidneys to become less effective at removing toxins and drugs from the body [7]. The main factors of occurrence and development of DN include chronic hyperglycemia, reactive oxygen species (ROS), inflammatory cytokines, and advanced glycation end products (AGEs), and so on. These factors trigger a number of signaling pathways, such as protein kinase B (Akt), thioredoxin interacting protein (TXNIP), adenosine monophosphate-activated protein kinase (AMPK), and mammal target of rapamycin (mTOR), including glomerular basement membrane (GBM) thickening, mesangial expansion, impaired glomerular filtration function, and the accumulation of extracellular matrix and proteins such as fibronectin (FN) [4–7]. The complex pathogenesis of DN further complicates the search for targeted therapeutic agents. Common therapies for DN

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include medicines against hyperglycemia and hypertension, changing dietary habits, and renal replacement therapy [8]. In the early stages, angiotensin-converting enzyme (ACE) inhibitors, such as captopril and lisinopril, can be used to reduce the amount of lost protein and control cardiovascular complications. Moreover, angiotensin II receptor (ARB) blockers, such as losartan potassium and candesartan, can be used to lower blood pressure and protect the kidneys. Other treatment options include diuretics and beta blockers [9–11].

Podocytes are terminally differentiated glomerular epithelial cell that are among the main constituents of the renal filtration barrier. Injury and loss of podocytes lead to proteinuria, which is a hallmark and predictive indicator of DN [12]. Due to the limited capacity of cell proliferation and replacement of podocytes, the mechanism of self-renewal, represented by autophagy, is crucial to maintaining homeostasis [13]. Autophagy is a process that relies on the lysosomal pathway to degrade cytoplasmic proteins and organelles for the degradation and recovery of cellular proteins and removal of damaged organelles [14–17]. Podocytes have high levels of basal autophagy, and the podocyte-specific depletion of the Atg5 gene leads to glomerulopathy in aging mice, whose oxidative and ubiquitinated protein accumulation and podocyte endoplasmic reticulum stress eventually lead to loss of podocytes, increased proteinuria, and glomerulosclerosis [18]. In addition, the induction of proteinuria in mice with podocyte-specific deletion of the Atg5 gene induced by puromycin or doxorubicin resulted in more severe proteinuria, loss of podocytes, and glomerulosclerosis compared with control mice [19]. Knockout of autophagy increases the susceptibility to the development of glomerular diseases, and autophagy manifests a pressure-adaptive response of podocytes that have cytoprotective effects on glomerular diseases [20–22].

Chinese medicine describes natural products that have traditionally been used as complementary therapeutic agents for diabetes due to their effectiveness, low cost, and structural diversity [23]. Moreover, medicinal plant extracts and their constituent phytochemicals have an improved effect on diabetes and its complications. The use of flavonoids and polyphenolic compounds from traditional medicinal plants has been shown to reduce DN; such phytochemicals include silybin from *Silybum Marianum* [24], curcumin from *Curcuma longa* [25], luteolin from *Plantago asiatica* [26–28] and berberine from *Berberis vulgaris* [29]. These results show that the characteristics of these flavonoids or polyphenols can effectively improve renal deficits and pathological metabolic changes by controlling various signaling pathways. In addition to specific molecular mechanisms, medicinal plants are rich in antioxidants and provide nephron protection by reducing oxidative stress, thereby ultimately controlling diabetes and its complications [30].

In this study, we found that hispidulin, a flavonoid extracted from *Plantago asiatica*, alleviates high-glucose-induced podocyte apoptosis. Further mechanism studies suggested that hispidulin induces podocyte-protective autophagy by inhibiting pim1-p21-mTOR signaling axis. iTRAQ-based proteomics analysis was performed to identify the differentially expressed proteins regulated by hispidulin. Bioinformatics analysis suggested that autophagy and mTOR pathways were significantly enriched. Subsequently, the expression levels of autophagy- and apoptosis- related proteins were determined to explore the molecular mechanisms of hispidulin. Thus, we identified natural pim1 inhibitor hispidulin as a novel autophagy activator, and this sheds new light on diabetic nephropathy therapy regarding Pim1-p21-mTOR signaling axis.

2. Materials and methods

2.1. Cell culture and reagents

Immortalized murine podocyte MPC-5 cell lines were obtained from the National infrastructure of cell line resource (Wuhan, China). The proliferation and differentiation of MPC-5 podocytes were cultured and stimulated as previously reported [31]. In brief, cells were cultured in permissive conditions at 33 °C and 5% CO_2 and RPMI 1640 medium supplemented with 10% FBS (fetal bovine serum, Invitrogen, Carlsbad, CA, USA), which contained 10 U/ml of recombinant mouse interferon-γ (Invitrogen, Carlsbad, CA, USA) for proliferation. Subsequent experiments were conducted with differentiated cells. The cells were incubated with 5.6 mM glucose as normal glucose control (NC) or 50 mM glucose as the high glucose (HG) at 37 °C for 24 h. The other regents were of analytical grade used without additional modifications.

2.2. Cellular proliferation assay

The cellular proliferation assay was performed by using MTT assay kit (Keygen, Nanjing, China). The differentiated podocytes (5×10^4 cells per well) were seeded into 96-well plates for 12 h with different glucose concentrations, and then treated by hispidulin for another 24 h. Subsequently, the media in each well were replaced with 20 μL MTT. After culturing for another 4 h, the resulting formazan was dissolved by 150 μL DMSO and assessed for absorbance at 550 nm. Afterward, cell viability was calculated as the mean percentage of the OD value of experiments and control groups.

2.3. Apoptosis and caspase3 activity assay

Cellular apoptosis was detected with a FITC-conjugated AnnexinV-PI (propidium iodide) dual-staining Kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Briefly, the differentiated MPC5 podocytes (5×10^4 cells per well) were cultured in a 24-well plate with different glucose concentrations and then treated with hispidulin for another 24 h. The cells were stained with FITC-AnnexinV/PI dual staining kit under dark conditions for 15 min. The apoptotic cell rates were determined by a flow cytometer (BD FACSAria II, BD Biosciences, CA, USA). The activity of caspase3 were evaluated by using a caspase3 assay kit (Sigma, MO, USA) following the manufacturer's protocol. The differentiated podocytes were treated as stated above and then harvested and lysated. After centrifugation and quantification of protein concentrations by using the Bradford method, the produced *p*-nitroaniline (pNA) levels were determined spectrophotometrically at 405 nm using a microplate reader.

2.4. iTRAQ proteomics and bioinformatics analysis

The quantitative proteomics analysis based on iTRAQ method were performed in MPC-5 cells. In brief, MPC-5 cells were lysated and labelled with iTRAQ labelling reagents. After UPLC-MS-MS analysis, the protein identification and relative quantification were performed by using ProteinPilot™ Software 4.2 (AB SCIEX, Shanghai, China) and Paragon™ algorithm for peptide identification. The cutoff value for the differentially expressed proteins was adjusted to $p < 0.05$ and the fold change was set to > 2.0 or < 0.5. GO (gene ontology). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment and cluster analysis were performed using the Metascape webtool with default settings.

2.5. RNA interference western blot analysis

The podocytes were transfected by Pim1 siRNAs or negative control siRNA (Ribobio, Guangzhou, China) for 24 h and incubated with the conditions stated above. The different groups of podocytes were harvested, rinsed with cold PBS thrice, and lysed in the RIPA buffer (Abcam, Cambridge, USA) for 30 min. The protein concentrations were analyzed by using micro-BCA kit (Pierce, Rockford, IL, USA) and then quantified. Cell lysates (30 μg/lane) were separated by using 6%–24% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Pierce, Rochford, IL, USA). The 5% fat-free dry milk or 5% bovine