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Emodin protects hyperglycemia-induced injury in PC-12 cells by up-regulation of miR-9

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ABSTRACT

Background: Diabetic foot is a severe complication of diabetes mellitus, mainly caused by diabetic peripheral neuropathy. The objective of this study was to investigate the function of emodin (a neuroprotective agent reported previously) in diabetic peripheral neuropathy.
Methods: A neuron-like cell line PC-12 was subjected with high level glucose, before which emodin was applied to treat cells. The expression of miR-9 in cell was overexpressed or suppressed by miRNA transfection. Thereafter, cell viability, apoptosis and autophagy were assessed, respectively.
Results: High glucose exhibited cytotoxicity in PC-12 cells. Emodin protected PC-12 cells against high glucose-induced apoptosis and viability impairment. These observations were coupled with the down-regulations of p21, p16, Bax, cleaved caspase-3 and -9, and the up-regulations of CyclinD1 and Bcl-2. Additionally, high glucose-induced autophagy was alleviated by emodin, as Beclin-1 was down-regulated, p62 was up-regulated, and the conversion of LC3-I to LC3-II was decreased. miR-9 was highly expressed in response to emodin treatment. More interestingly, the protective actions of emodin on high glucose-induced injury were reversed by miR-9 suppression. Also, the activation of PI3K/AKT signaling and deactivation of NF-κB signaling induced by emodin were recovered by miR-9 suppression.
Conclusion: Emodin protected PC-12 cells against high glucose-induced apoptosis and autophagy. The neuroprotective activities might be realized by up-regulation of miR-9, and modulation of PI3K/AKT and NF-κB signaling pathways.

1. Introduction

Diabetic foot is one of the common and severe complications of diabetes mellitus, with a high incidence (approximately 25%) during lifetime (Singh et al., 2005). To date, the treatment of diabetic foot is still challenging. Patients with diabetic foot require treatment with systemic antibiotics, but antibiotic resistance has become a major problem (Amjad et al., 2017). Majority of the patients have to have their extremities amputated to prevent further infection, and amputation reduces the quality of life significantly. The pathogenesis of diabetic foot is complex and remains unclear, but diabetic peripheral neuropathy has been recognized as a major cause of diabetic foot (Obrosova, 2009). Perpetually high blood serum glucose appears to lead to damage nerves, and then leading to gradual loss of protective sensation in both the skin and foot joints (Bodman and Dulebohn, 2017). Prevention of peripheral neuropathy is a promising way to prevent the happening of diabetic foot.

Emodin, 1,3,8-trihydroxy-6-methyl-9,10-anthraquinone ($C_{15}H_{10}O_5$, molecular weight 270.23), is a natural anthraquinone derivative extracted from the bark of Rhamnus, the rhizome of the rhubard, and Semen Cassiae (a seed of *Cassia obtusifolia*). It is well-accepted that emodin possesses multiple biological activities, including anti-inflammatory (Chen et al., 2016a), anti-bacterial (Li et al., 2016), anti-oxidant (Chen et al., 2017), anti-fibrosis (Ma et al., 2017a), anti-aromatase (Molee et al., 2018) and anti-tumor (Ma et al., 2017b) activities. Additionally, emodin has neuroprotective effects after brain injury, which might resulted from decreasing glutamate excitotoxicity (Gu et al., 2005), and increasing the activation of activin A pathway (Guo et al., 2013). Because of the protective effect of emodin on the nervous system, emodin has been proposed as an effective therapy for nervous system diseases, such as Alzheimer's disease (Tao et al., 2014), muscle atrophy (Chen et al., 2016b), and neuropathic pain (Gao et al.,

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2011). Therefore, we are interested in investigating whether emodin can also exhibit protective effect on diabetes-induced nerve damage.

microRNAs (miRNAs), a class of short, non-coding RNAs, are key regulators involved in multiple biological processes, including cell proliferation, apoptosis, autophagy, differentiation, and cell cycle. It is highly believed that miRNAs are contributed in the pathogenesis of diabetic foot, and miRNAs are evidenced as promisingly therapeutic targets (Ramirez et al., 2017; Zhu et al., 2017). High-throughput human miRNA array analytical results showed that, miR-9 was down-regulated in placental tissues derived from gestational diabetes mellitus (Li et al., 2015). However, the serum level of miR-9 was increased in the progression of diabetic nephropathy (Xiao et al., 2017), and in the spinal dorsal horn neurons of rats with painful diabetic neuropathy (Liu et al., 2017). miR-9 is abundant in neuronal cells and pancreatic islets, and functions in executing exocytosis of insulin, which of course is one of the fundamental canons of diabetes (Krupanidhi et al., 2009).

In the present study, PC-12 cells, a cell line which can differentiated into neuron-like cells under the induction of mouse nerve growth factor, were subjected to high level glucose. The effects of emodin on PC-12 cell damage caused by high glucose were tested, and the underling mechanisms of the action were explored by focusing on the regulatory role of emodin in miR-9 expression. The findings of this study will reveal the potential role of emodin in the prevention of diabetic peripheral neuropathy, which was unstudied before.

2. Materials and methods

2.1. Cell culture

Rat adrenal pheochromocytoma cell line PC-12 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) with 10% heat-inactivated horse serum (Gibco) and 5% heat-inactivated fetal bovine serum (Gibco). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. Medium was changed every two days. Subculture was achieved by trypsin/EDTA digestion, when cells grown to about 80–90% confluence.

2.2. Study design

Cells were divided into three groups and were treated as follows: 1) HG group, in which cells were treated by 25 mM glucose for 24 h; 2) NG group, a control group in which cells were treated by 5 mM glucose and 20 mM mannitol; 3) HG + E group, in which cells were treated with 10 μM emodin for 24 h before HG treatment.

Emodin purchased from Sigma-Aldrich was dissolved in DMSO and made up with the medium so that the final concentration of the DMSO was less than 0.1%.

2.3. Oligonucleotide transfection

PC-12 cells were transfected with mimic or inhibitor specific for rno-miR-9 (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A scrambled oligonucleotide was used as a negative control (NC). Briefly, PC-12 cells were planted in the 24-well plates with a density of 2×10^5 cells/well. After adherence, transfection was performed under antibiotics-free condition for 48 h. The final concentration of miR-9 mimic (miR-9 group), miR-9 inhibitor (anti-miR-9 group), and NC was 50, 200, and 100 nM, respectively. The sequences of the oligonucleotides were as follows: miR-9 mimic, sense 5′-UCUUUGGUUAUCUAGCUGUAUGA-3′, anti-sense 5′-AUACAGCUAGAUAACCAAAGAUU-3’; miR-9 inhibitor, 5′-UCAUACAGCUAGAUAACCAAAGA-3’.

2.4. CCK-8 assay

Changes in cell viability were detected by the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kyushu, Japan). The miR-transfected PC-12 cells were planted in 96-well plates and cultured for 12 h in complete medium. The cells were then treated with NG, HG, or HG + E as indicated. After washing with PBS for twice, 10 μl CCK-8 was added into each well, and the plates were incubated at 37 °C for 2 h. The absorbance of each well was detected by a Microplate Reader (Bio-Rad, Hercules, CA, USA) under 450 nm.

2.5. Assessment of cell apoptosis

Cell apoptosis was analyzed by the Annexin V-FITC and PI double-stain using FITC-annexin V/PI detection kit (Beijing Biosea Biotechnology, Beijing, China). The miR-transfected cells were seeded in 6-well plates with a density of 5×10^5 cells/well. After the treatment of NG, HG, or HG + E, cells were collected and resuspended in 200 μl Binding Buffer containing 10 μl FITC-annexin V and 5 μl PI. The samples were incubated in the dark over ice for 30 min, after which 300 μl PBS was added. Apoptotic cells (FITC-positive and PI negative) were distinguish by a FACS can (Beckman Coulter, Fullerton, CA, USA), and the percentage of the apoptotic cells was analyzed by FlowJo software (Tree Star, San Carlos, California, USA).

2.6. qRT-PCR

RNA was isolated from PC-12 cells by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA (50 ng) was converted to first-strand cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), and qRT-PCR was performed by the FastStart Universal SYBR Green Master (Roche). To quantify the mature miR-9 expression in PC-12 cells, Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used. Two housekeeping genes β-actin and U6 represented as internal controls. Relative gene expressions were calculated using the classic $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Western blot

Cellular proteins were extracted by using M-PER Protein Extraction Reagent (Pierce, Appleton, WI, USA) supplemented with PMSF (Sigma-Aldrich). The purity and concentration of the whole-cell extracts were tested by the BCA™ Protein Assay Kit (Pierce). 0.1 mg protein was resolved over SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5% non-fat dry milk for 1 h at room temperature, after which the membranes were incubated in primary antibodies at 4 °C overnight. The primary antibodies used in this analysis was as follows: anti-p21 (orb255850), anti-p16 (orb228200), anti-CyclinD1 (orb77308), anti-caspase-3 (orb10237), anti-Beclin-1 (orb74657), anti-p-PI3K (orb106105), anti-AKT (orb29949, Biorbyt, San Francisco, CA, USA), anti-p62 (#39749), anti-Bcl-2 (#3498), anti-Bax (#2774), anti-caspase-9 (#9508), anti-LC3B (#2775), anti-PI3K (#4255), anti-p-AKT (#4060), anti-IκBα (#9242), anti-β-actin (#4970, Cell Signaling Technology, Danvers, MA, USA), anti-p-IκBα (ab133462), anti-p65 (ab16502), anti-p-p65 (ab86299, Abcam, Cambridge, MA). The membranes were then incubated with the secondary antibodies for 1 h at room temperature. After rinsing, the positive signals were visualized by enhanced chemiluminescence method. Intensity of the blots was tested by Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

2.8. Statistics

All experiments were performed in triplicate and repeated 3 times. Data were presented as mean ± SD. The difference between two or