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 (Anjali 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 (C15H10O5, molecular weight 270.23), is a natural anthraquinone derivative extracted from the bark of Rhamnus, the rhizome of the rhubarb, 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., 2018).
highly believed that miRNAs are contributed in the pathogenesis of proliferation, apoptosis, autophagy, and progression of diabetic nephropathy (Xiao et al., 2017), and in the spinal dorsal horn neurons of rats with painful diabetic neuropathy (Liu 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 underlying 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% CO2. Medium was changed every other day. 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 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'-UCUUUGGUUAUCUCAGCUGUAUGA-3'; anti-sense 5'-UAUCACGUAGUAACCAAGAUU-3'; miR-9 inhibitor, 5'-UCAUAUCACGUAGUAACCAAG-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-staining 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 distinguished 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 were represented as internal controls. Relative gene expressions were calculated using the classic 2^-ΔΔCT 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-Bcl-2 (#3498), anti-Bax (#2774), anti-caspase-9 (#9508), anti-LC3 (orb37277), anti-p62 (orb10237), anti-p-AKT (#4060), anti-p-IκBα (#4970), Cell Signaling Technology, Danvers, MA, USA), anti-caspase-9 (#9508), anti-LC3B (#2775), anti-PI3K (#4255), anti-p-AKT (#4060), anti-Beclin-1 (#4970), Cell Signaling Technology, Danvers, MA, USA), anti-p-IκBα (ab133462), anti-p-p65, anti-p-p65 (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
more groups was analyzed by the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) using the one-way analysis of variance (ANOVA) followed by Duncan post-hoc test. Results with p < 0.05 were considered as significant differences.

3. Results

3.1. Emodin protects PC-12 cells against high glucose-induced cell apoptosis

PC-12 cells were subjected with various doses of emodin for 24 h to test the cytotoxicity of emodin in PC-12 cells. CCK-8 analytical results showed that cell viability was significantly reduced by 15 and 20 μM of emodin (p < 0.05, Fig. 1A). No cytotoxicity was observed in doses below 10 μM, thus 10 μM was selected as an emodin treating condition for use in the following experiments. Data in Fig. 1B showed that, viability of PC-12 cells was significantly lower in HG group than in NG group, while the inhibition of viability was recovered by addition of emodin (p < 0.05). Fig. 1C and D showed that, the mRNA and protein levels of p21 and p16 were up-regulated in HG group than in NG group, and emodin notably abolished these up-regulations (p < 0.05). The opposite tendency was observed in the expression of CyclinD1, as the mRNA and protein levels of CyclinD1 were down-regulated by HG and up-regulated by addition of emodin (p < 0.05). Additionally, HG induced a significant apoptosis in PC-12 cells (p < 0.05), and emodin alleviated HG-induced apoptosis significantly (p < 0.05, Fig. 1E). Western blotting results in Fig. 1F showed that, Bcl-2 was down-regulated, Bax was up-regulated and caspase-3 and -9 were cleaved in HG group, while these protein alterations were partially abolished by...
emodin treatment (Fig. 1F).

3.2. Emodin protects PC-12 cells against high glucose-induced autophagy

The protein expressions of autophagy markers were assessed by Western blot analysis, to see the impacts of emodin on high glucose-induced autophagy. As shown in Fig. 2A and B, significant up-regulations of LC-II and Beclin-1, and a significant down-regulation of p62 were found in HG group than those in NG group (p < 0.05). However, these protein alterations induced by HG were alleviated by addition of emodin (p < 0.05). Protein expression of LC-I was unaffected either by HG or by HG + E.

3.3. miR-9 is up-regulated by emodin

By performing qRT-PCR, we found that the RNA level expression of miR-9 was significantly increased in response to 10 μM of emodin (p < 0.05, Fig. 3). Slightly increase of miR-9 was observed in 5 μM group than in untreated group (the control), but did not reach the significant difference.

3.4. Emodin protects PC-12 cells against high glucose-induced apoptosis via up-regulation of miR-9

Next, the expression of miR-9 in PC-12 cells was overexpressed or suppressed by miRNA transfection to reveal whether miR-9 was involved in the protective activities of emodin in cell. As shown in Fig. 4A, the expression of miR-9 was significantly increased by miR-9 mimic transfection, while was significantly reduced by inhibitor transfection (both p < 0.05). Fig. 4B–D showed that miR-9 overexpression significantly increased cell viability, down-regulated the expressions of p21 and p16, and up-regulated the expressions of CyclinD1 (p < 0.05). Of contrast, miR-9 suppression significantly reduced cell viability, up-regulated p21 and p16, and down-regulated CyclinD1 even in the presence of emodin (p < 0.05). Fig. 4E and F showed that miR-9 overexpression repressed cell apoptosis, as the apoptotic cell rate was decreased (p < 0.05), Bcl-2 protein expression was up-regulated, and Bax protein and the cleaved forms of caspase-3 and -9 were down-regulated. As expected, the protective effects of emodin on high glucose-induced apoptosis were markedly attenuated by miR-9 suppression.

3.5. Emodin protects PC-12 cells against high glucose-induced autophagy via up-regulation of miR-9

The involvement of miR-9 expression in the protective effects of emodin on high glucose-induced autophagy was also explored. As shown in Fig. 5A and B, miR-9 overexpression down-regulated the protein expressions of LC-II and Beclin-1, and up-regulated the protein expression of p62 (p < 0.05). The protective effects of emodin on high glucose-induced autophagy were attenuated by miR-9 suppression, as the protein expressions of LC-II and Beclin-1 were significantly up-regulated, and the protein expression of p62 was significantly down-regulated by miR-9 inhibitor transfection (p < 0.05). LC-I expression was unaffected by any experimental treatment.

3.6. Emodin protects PC-12 cells via modulation of PI3K/AKT and NF-κB pathways

To further reveal the underlying mechanisms of which emodin protected PC-12 cells against high glucose-induced cell damage, the expression changes of core proteins in PI3K/AKT and NF-κB pathways were tested. Western blotting results showed that the phosphorylation of PI3K and AKT was significantly suppressed by HG, and promoted by emodin (p < 0.05, Fig. 6A). The phosphorylation of PI3K and AKT induced by emodin was further promoted by miR-9 overexpression, while was repressed by miR-9 suppression (p < 0.05). Of contrast, HG induced significant phosphorylation of IκBα and p65, and emodin reduced the phosphorylation (p < 0.05, Fig. 6B). The suppression of IκBα and p65 phosphorylation induced by emodin was further suppressed by miR-9 overexpression, while it was reversed by miR-9 suppression (p < 0.05).
Increasing attention is being given to the use of Chinese medicine for the prevention and treatment of diabetic foot. Tuo-Li-Xiao-Du-San, a traditional Chinese medicine formula, has been reported to improve diabetes-impaired wound healing (Zhang et al., 2016a). Notoginsenoside Ft1, a saponin from Panax notoginseng, may accelerate diabetic wound healing by promoting fibroblast proliferation, enhancing angiogenesis, and attenuating inflammatory response (Zhang et al., 2016b). In the present study, our data suggest that emodin protects PC-12 cells against high glucose-induced damage by inhibition apoptosis and autophagy. The protective actions may be realized by up-regulation of miR-9 expression, and modulation of PI3K/AKT and NF-κB signaling pathways. These data bring emodin as an effective therapeutic agent for preventing diabetic peripheral neuropathy.

Perpetually high blood serum glucose appears to damage small blood vessels which compromise oxygen and nutrients to the nerves (Bodman and Dulebohn, 2017). Early recognition of diabetic peripheral neuropathy will prevent diabetic foot and possible limb loss (Vas and Edmonds, 2016). High glucose contributes significantly to the pathogenesis of nervous system malfunction (Smith and Singleton, 2012). Apoptosis is induced by high glucose, which in turn has toxic effects on nerve tissues (Kaeidi et al., 2013). This was also confirmed in this study, that high glucose exhibited cytotoxicity in PC-12 cells, as viability was decreased, and apoptosis was induced by high glucose. These observations were coupled with the up-regulations of p21 and p16 (two inhibitors of cyclin-dependent kinase), down-regulation of CyclinD1, decrease of Bcl-2 to Bax ratio, and cleavages of caspase-3 and -9. Additionally, we observed that autophagy was induced by high glucose, as Beclin-1 was up-regulated, p62 was down-regulated, and the conversion of LC3-I to LC3-II was increased. Similar findings were reported by Li et al., suggesting that high glucose augmented autophagy level (Li et al., 2017). Recently, the emerging role of autophagy in diabetic neuropathy has gained a lot of attention, but whether autophagy in diabetic neuropathy is a hero or culprit is still complicated. More efforts are required to reveal the significant role of neuropathy in the
Emodin has been previously demonstrated as a neuroprotective agent (Gu et al., 2005; Guo et al., 2013), which has potential for prevention and treatment of nervous system diseases (Tao et al., 2014; Chen et al., 2016a,b; Gao et al., 2011). Herein, we for the first time demonstrated that emodin preconditioning significantly attenuated high glucose-induced apoptosis and autophagy, implying the protective activities of emodin on diabetic peripheral neuropathy.

Recently, a growing number of studies have begun to recognize the importance of miRNAs in the therapeutic activities of emodin. But, the research on the role of emodin in the regulation of miRNAs remains scanty. In vitro and in vivo investigations demonstrated that miR-30a-5p was a target gene of emodin, which helped emodin attenuated pancreatic acinar cell injury (Xiang et al., 2017). miR-126 was proved as another target of emodin, that emodin prevented intimal thickening via modulation of miR-126 in balloon-injured carotid artery rats (Hua et al., 2015). In the current study, our data evidenced that emodin protected PC-12 cells against high glucose-induced damage via up-regulation of miR-9, indicating the neuroprotective effect of emodin possibly realized by increasing miR-9 expression.

The pathogenesis of diabetic peripheral neuropathy is still unknown, but high glucose has been shown to impair PI3K/AKT signaling, and thereby inducing apoptosis and neuron loss (Anitha et al., 2006; Cai and Helke, 2003). In addition to PI3K/AKT, NF-κB is another signaling pathway participates in the regulation of sensory neurons’ function and survival in diabetes (Fernyhough et al., 2005). It has been reported that NF-κB signaling can be activated by high glucose (Busik et al., 2008). More interestingly, high glucose repressed PI3K/AKT signaling, inducing the activation of NF-κB, which in turn triggered the caspase-3 activity that facilitated human endothelial cell apoptosis (Sheu et al., 2005). In consistence with these previous studies, our results showed that PI3K/AKT signaling was deactivated while NF-κB signaling was activated in response to high glucose. We additionally demonstrated that emodin alleviated high glucose-induced deactivation of PI3K/AKT signaling and activation of NF-κB signaling possibly via up-regulation of miR-9.

Fig. 5. Involvement of miR-9 in the effects of emodin on high glucose-induced autophagy in PC-12 cells. A. Protein levels of autophagy-related factors in PC-12 cells in the NG, HG, HG + E, HG + E + NC, HG + E + miR-9, HG + E + anti-miR-9 groups. *, p < 0.05 compared to the indicated group. Data are reported as mean ± SD.

Fig. 6. Effects of emodin and miR-9 expression on the activation of PI3K/AKT and NF-κB pathways. A. Protein levels of PI3K and AKT. B. Protein levels of IκBα and p65 in PC-12 cells in NG, HG, HG + E, HG + E + NC, HG + E + miR-9, HG + E + anti-miR-9 groups. *, p < 0.05 compared to the indicated group. Data are reported as mean ± SD.
Taken together, our findings provided evidence that 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. Emodin may be an effective agent for preventing diabetic peripheral neuropathy and diabetic foot.

Conflicts of interest
The authors declare that they have no competing interests.

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References

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