



DIHYDROMYRICETIN ATTENUATES HIGH GLUCOSE-INDUCED CASPASE 3 EXPRESSION, ROS PRODUCTION AND INCREASES AMPK PHOSPHONYLATION IN PC12 CELLS

Shuo Wang^{1&}, Lin Wang^{1&}, Haijian Li^{1&}, Shumei Wang^{1&}, Zhenzhen Li¹, Bao Li^{2*}, Chunzhen Zhao^{1*}

¹ *Laboratory of Applied Pharmacology, Weifang Medical University, Weifang 261053, China*

² *Department of urology, Affiliated Hospital of Weifang Medical College*

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Co-first authors[&]: These authors contributed equally to the work.

Corresponding author[&]: Chunzhen Zhao, Li Bao

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ABSTRACT:

Dihydromyricetin (DMY) has a protective effect on neural function under central nervous system dysfunction conditions. There is growing interest concerning the beneficial effects of DMY on treating diabetic neuropathy (DN). This study was carried to detect protective effects of DMY on high glucose (HG)-induced cell damage and related mechanisms. The effect of DMY on cell survival was detected by MTT assay. Caspase-3 and phosphorylated AMP-activated protein kinase (AMPK) was evaluated by Western blotting. The effects of DMY and AMPK agonist AICAR on ROS production was determined. Our results showed that DMY treatment protect against HG-induced cell damage. DMY treatment significantly reduced the expression of caspase-3 and phosphorylated AMPK. ROS production was inhibited by DMY or AMPK agonist AICAR treatment. These studies demonstrate that DMY may inhibit ROS production, caspase-3 expression through AMPK pathway.

Keywords: dihydromyricetin, caspase, oxidative stress

Introduction

Diabetes mellitus is a major cause of high morbidity and mortality in the world. Diabetic neuropathy (DN) is one of the most common diabetes-related complications leading to cognitive impairment, motor and sensory dysfunction^[1]. The importance of DN is being recognized increasingly. Oxidative stress, impaired cerebral insulin signaling systems are considered as being closely related to DN development^[2]. Many evidence have reported that overproduction of reactive oxygen species (ROS) contributed to neural cell apoptosis and inhibition of oxidation may block the progression of DN^[3].

Dihydromyricetin (DMY), a flavonoid compound, was isolated from the leaves of *Ampelopsis grossedentata*^[4]. It has been reported to possess multiple pharmacological activities including anti-inflammatory^[5], anti-oxidative^[6], anticancer^[7] and hepatoprotective effects^[8]. Recent data supported dihydromyricetin attenuated methylglyoxal induced- oxidative stress in PC12 cells^[9].

Dihydromyricetin treatment can improve A β induced cognitive impairment and reverse progressive neuropathology in a mouse model of Alzheimer's disease^[10]. However, the effects of DMY on hyperglycemia neural injury and related mechanisms have not been clearly studied.

The aim of the present study was to investigate the protective effects of DMY on high glucose-induced cell damage in cultured PC12 cells. We further examined whether DMY can affect high glucose-induced caspase-3 expression, ROS production and AMPK phosphorylation.

Materials and Methods

Reagents

Dihydromyricetin (purity \geq 98%) was purchased from Sigma-Aldrich. ROS production was measured by using a ROS assay kit (Nanjing Jiancheng Bioengineering Institute, China). Antibodies against phospho-AMPK, cleaved caspase 3 was purchased from Boster Bio Tech (Wuhan, China). Antibodies

against GAPDH were obtained from Beyotime, Nanjing, China.

Cell Culture

PC12 cells were cultured in DMEM medium containing 10% fetal bovine serum under conditions of 37 °C in humidified air containing 5% CO₂. The cultured medium was changed every 48 hours and the cells in the exponential phase of growth were used in all experiments.

Cell viability detection

Cell viability was evaluated by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) detection assay. PC12 cells were seeded in 96-well plates. Cells were pretreated with different conditions of Dihydromyricetin (10, 20, 40 μM) for 1 h and treated with high glucose with concentration of 100 mM for 48 h. MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 h at incubator of 37 °C. The medium was removed and dimethyl sulfoxide (DMSO) was added to the wells. The absorbance was measured at 570 nm using multiscan spectrum. Cell viability was expressed normalized to percentage of non-treated control cells.

Intracellular ROS detection assay

Intracellular ROS production was evaluated by 2,7-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe detection assay. PC12 cells were seeded in 24-well plates and pretreated with DMY 20 μM for 1 h or AICAR 10 nM and exposed to 100 mM of HG for 48 h. The medium was removed and incubated with 20 μmol/L of DCFH-DA for 30 min at 37 °C, then cells were washed with PBS to remove DCFH-DA. Cells were collected and analyzed immediately using a microplate reader.

Western Blot Analysis

PC12 cells were harvested and washed with ice-cold PBS, and the cellular lysates were prepared. Protein concentrations were determined by BCA assay. The protein samples were subjected to electrophoresis on 10-12% SDS polyacrylamide gels and transferred onto a nitrocellulose membrane. Then the membrane was blocked with 7.5% nonfat milk, and incubated with anti-cleaved caspase 3, anti-phospho-AMPK and anti-GAPDH antibodies overnight.

Then the blots were incubated with anti-rabbit IRDye700DX[®]-conjugated antibody or anti-mouse IRDye800DX[®]-conjugated antibody. The signals were scanned by an Odyssey infrared imaging system. Protein bands were quantitatively evaluated by Quantity One[®] analysis software. The ratio to confidence reference items was calculated.

Statistical Analysis

All data were expressed as mean ± SD. The significance of differences among three or more groups was performed using One-way ANOVA followed by Bonferroni's post hoc test (SPSS 15.0 for Windows, SPSS inc., USA). *P* < 0.05 was considered statistically significant.

Results

DMY attenuated HG-induced PC12 cell damage

To investigate protective effect of DMY in high glucose-induced neural cell damage model, PC12 cells were pretreated with DMY with different concentrations of 10, 20, 40 μM and exposed to high glucose with concentrations of 100 mM for 48 h. Then cell viability was detected by MTT detection assay. HG induced cell damage significantly compared to the control group. DMY with concentrations of 10, 20, 40 μM can increase cell survival significantly as compared to HG group (Fig.1, *P* < 0.01).

DMY decreased HG-induced caspase-3 expression in PC12 cells

Apoptosis related protein caspase 3 was detected by Western blotting and protective effect of DMY on caspase-3 expression was evaluated. As shown in Fig.2, compared to control group, cleaved caspase-3 protein expression increased significantly. 20 μM of DMY can significantly reduced caspase-3 expression in PC12 cells compared to HG group (Fig.2A and 2B, *P* < 0.05).

Effect of DMY on HG-induced intracellular ROS production and AMPK activation

Many studies have reported that ROS contributed to HG-induced PC12 cell injury. Here we struggled to demonstrate whether DMY would affect the elevated level of ROS and related mechanisms in HG-treated PC12 cells. Our results showed that DMY significantly reduced HG-induced ROS production in PC12 cells. Also we found DMY can recover HG-induced inhibition of AMPK

phosphorylation (Fig.2, $P < 0.05$). As compared with HG group, AMPK agonist AICAR 10 nM can significantly reduced HG-induced ROS level in PC12 cells (Fig.3, $P < 0.01$).

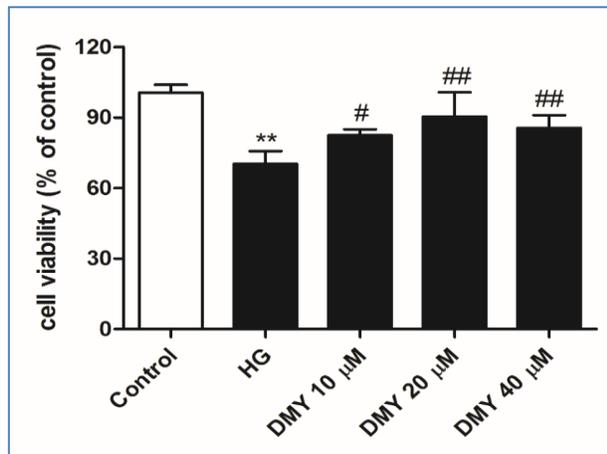


Fig.1: Effect of DMY in PC12 cells after HG exposure. Cell viability was evaluated by MTT detection assay. PC12 cells are pretreated with DMY for 1 h and then exposed to HG 100 mM for 48 h. Data are expressed as mean \pm SD. ** $P < 0.01$ compared to control group. ## $P < 0.01$, # $P < 0.05$ compared to HG group.

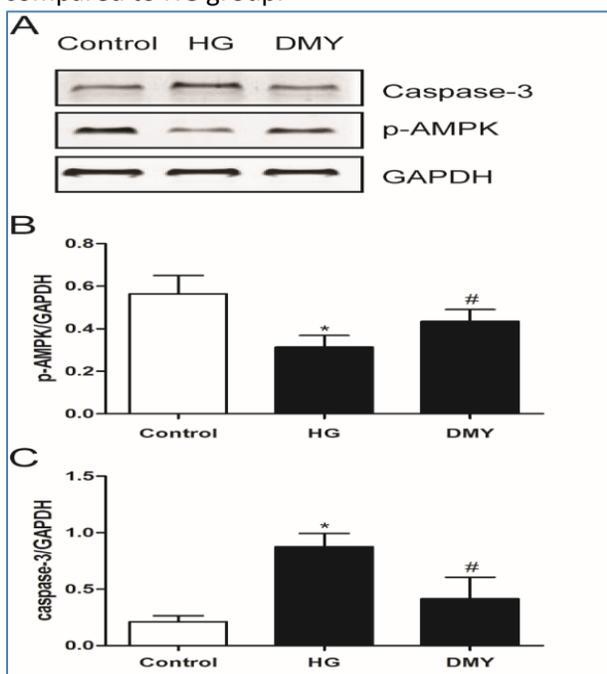


Fig.2: Effect of DMY on caspase-3 expression and AMPK phosphorylation in PC12 cells. PC12 cells are pretreated with DMY 20 μ M for 1 h and then exposed to HG for 48 h (A). The density of caspase 3 and p-AMPK was determined by Western blotting and the ratio to GAPDH was calculated. * $P < 0.05$

compared to control group, # $P < 0.05$ compared to HG group.

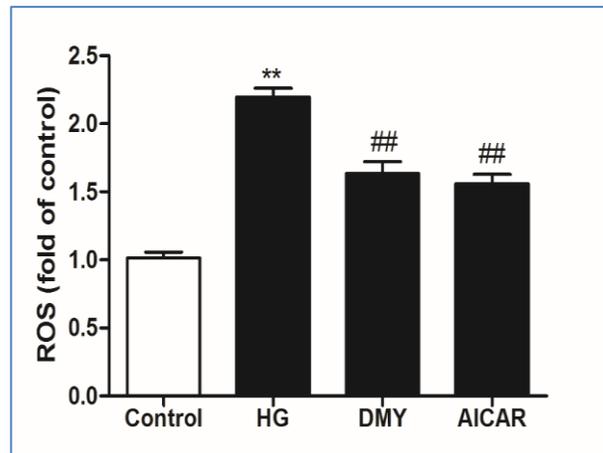


Fig.3: Effect of DMY and AMPK agonist on ROS production in PC12 cells. PC12 cells are pretreated with DMY 20 μ M or AMPK agonist AICAR 10 nM for 1 h and then exposed to HG for 48 h. Intracellular ROS generation in PC12 cells was determined using a detection assay. Values are expressed as ratio of control group and reported as mean \pm SD. ** $P < 0.01$ compared to control group. ## $P < 0.01$ compared to HG group.

Discussion

In the present study, we reported that DMY pretreatment protected against HG-induced cell damage, and reduced the expression of caspase-3 and phosphorylated AMPK. DMY reduced ROS production possibly through AMPK pathway.

The flavonoids are a large a large class of powerful antioxidative compounds that are mainly found in fruits, tea and other plant-derived materials^[11]. Many studies have demonstrated the effects of flavonoids and their metabolites on cancer, diabetes mellitus and Alzheimer's disease^[12]. Dihydromyricetin has been showed a potential neuroprotective effect against oxidative stress-induced and amyloid-induced neuronal cell injury^[13]. As a flavonoid compound, isolated from traditional southern Chinese herb Ampelopsis grossedentata, DMY possessed neuroprotective effects against oxidative stress in neurodegenerative diseases^[14]. It was reported that DMY significantly reduced oxidative stress and inhibited microglial inflammation in transgenic mouse model of AD^[15]. Moreover, DMY also decreased oxidative stress induced cell injury in a concentration-dependent manner^[16]. Consistent

with previous studies, our results showed DMY protected PC12 neural cells against HG-induced cell cytotoxicity.

It's well known that imbalance of Bax/Bcl-2 leads to the release of cytochrome C, caspase-3 activation and subsequent cell apoptosis in neural cells^[17]. Caspase-3 plays a pivotal role as a final stage of apoptosis^[18]. It was reported that increased caspase-3 prompted apoptosis in PC12 cells^[19]. Thus, our study found that DMY pretreatment is able to suppress the activation of caspase-3 in PC12 cells.

Previous studies showed that hyperglycemia-induced intracellular ROS production and inhibition of AMPK phosphorylation^[20]. We observed that the influences of DMY on inhibition of ROS production and activation of AMPK phosphorylation. Since AMPK signaling pathway is a crucial pathway involved in cell apoptosis. AMPK agonist also have similar protective effect with DMY treatment in HG-induced ROS level. It indicated that DMY might reduce cell damage and ROS production through AMPK pathway. Our study supported the AMPK signaling pathway involved in the HG-induced PC12 cell apoptosis and cytotoxicity.

In conclusion, our data highlight the activation of AMPK signaling pathway as a potential mechanism by which DMY protects PC12 cells from HG-induced ROS production and cell apoptosis. These results may suggest that DMY therapy could be employed as a promising agent in prevention therapy for diabetic neuropathy.

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