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Research report

# Dihydromyricetin ameliorates the oxidative stress response induced by methylglyoxal via the AMPK/GLUT4 signaling pathway in PC12 cells

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ABSTRACT

Dihydromyricetin (DMY), the major bioactive flavonoid ingredient extracted from the leaves of *Ampelopsis grossedentata* (Hand.-Mazz) W.T. Wang, displays multiple pharmacological activities, including oxidation resistance, antitumor properties and free radical scavenging capacities. However, the role of DMY in methylglyoxal (MG)-induced diabetes-associated cognitive decline and its underlying molecular mechanisms are unclear. The aim of the present study was to evaluate the effects of DMY on oxidative stress and glucose transport activity in a MG-induced PC12 cell line and to explore the related mechanisms. The effects of DMY on cell survival and apoptosis were examined, and the dysregulation of intracellular $Ca^{2+}$ was determined. Oxidative stress was evaluated by monitoring ROS production and the glutathione to glutathione disulfide ratio. The effects of DMY on glucose metabolism were investigated using a fluorescently labeled deoxyglucose analog and by measuring ATP and lactate production. Western blot analysis was performed to examine the protein levels of glyoxalase I (Glo-1), glucose transporter 4 (GLUT4), AMP-activated protein kinase (AMPKα) and phosphorylated AMPKα (p-AMPKα). The results revealed that DMY suppressed cellular oxidative stress in PC12 cells and balanced glucose metabolism. Additionally, DMY reduced GLUT4 translocation dysfunction and increased Glo-1 and p-AMPKα expression. We found that DMY protected PC12 cells against MG-induced apoptosis and glycometabolic disorders, at least in part by restraining the hyperactivation of p-AMPK activity and normalizing the translocation of GLUT4 from the intracellular compartment, resulting in a balance in glucose uptake. This result indicates that DMY may serve as a novel and effective candidate agent to treat diabetic encephalopathy by reducing the toxicity of MG.



## 1. Introduction

Diabetic encephalopathy is an emerging diabetic complication that is largely a consequence of changes within the central nervous system (CNS) (Kwon et al., 2013). The importance of diabetic encephalopathy is increasingly being recognized (An et al., 2012). Cerebrovascular changes, oxidative stress, increased levels of advanced glycation end products (AGEs) and impaired cerebral insulin signaling systems are considered as the underlying causes of diabetic encephalopathy (Feroz et al., 2012; Lin et al., 2009). Recent data have shown that the accumulation of toxic α-oxoaldehydes, such as methylglyoxal (MG), may be a key determinant of cognitive dysfunction (Di Loreto et al., 2008). Clinical data have also shown a significant increase in MG levels in the blood and tissues of diabetics (Eguchi et al., 2013; Liu et al., 2013).

MG is an endogenous toxic compound that is formed during the glycolytic pathway and is a by-product of several other metabolic pathways, including protein and fatty acid metabolism (Guo et al., 2013; Li et al., 2013; Wu et al., 2013). In normal mammalian cells, MG is efficiently detoxified via the glutathione-dependent glyoxalase pathway, which involves the enzymes glyoxalase 1(Glo-1) and glyoxalase 2 (Wang et al., 2013; Xu et al., 2013; Zeng et al., 2013). Under conditions of hyperglycemia and impaired glucose metabolism, MG often accumulates (Benhaddou-Andaloussi et al., 2011). Abundant MG levels induce dysfunction in mitochondrial respiration (Luiken et al., 2008) and increased production of reactive oxygen species (ROS) (Wijesekara et al., 2006). Glo-1 overexpression counteracts these effects (Li et al., 2008). Recent research has also confirmed that Glo-1 overexpression reduces the hyperglycemia-induced increase in the levels of AGEs and oxidative

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stress in diabetic rats (Song et al., 2010). Thus, Glo-1-mediated MG removal plays a leading role in the pathogenesis of diabetic complications, including diabetic encephalopathy. The exact molecular mechanisms of which MG induces cognitive dysfunction and several diabetic complications remain unclear. However, MG can both inactivate antioxidative enzymes and enhance intracellular oxidative stress (Ota et al., 2007). As described above, MG is a potent source of ROS (Lee et al., 2011; Shi et al., 2011), which a primary cause of oxidative stress. Furthermore, the brain is more susceptible to oxidative damage than any other major organ because of its high oxygen consumption. Evidence suggests that MG is capable of inducing apoptosis in hippocampal neurons via both the mitochondrial and Fas-receptor pathways (Schwenk et al., 2010). In addition to the above effects, MG may impair glucose metabolism and induce energy depletion in neurons (Li et al., 2004). In rat pheochromocytoma (PC12) cells exposed to 1 mmol/L MG, apoptosis is induced via the phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)/gamma-glutamyl cysteine ligase catalytic subunit (GCLc) redox signaling pathway (Feng et al., 2011). MG also elevates the GLUT4 levels on the surface of L6 myoblasts via both increased GLUT4 translocation from the intracellular compartment and reduced GLUT4 internalization, resulting in increased glucose uptake (Engelbrecht et al., 2014). AMP-activated protein kinase (AMPK), a multifunctional metabolic and energy sensor in the brain, is activated under conditions of cellular energy depletion (Nishino et al., 2004). MG neurotoxicity is also associated with energy depletion. Therefore, we speculated that MG is closely associated with AMPK activity. In addition, Gugliucci recently proposed that MG may cause a modification of AMPK that leads to the attenuated activation of this critical fuel sensor, which could produce metabolic dysregulation that is compatible with increased and perpetual liver insulin resistance (Gugliucci, 2009). Our data suggested that MG impaired GLUT4 trafficking and induced AMPK inactivation, which was associated with the induction of apoptotic cell death in PC12 cells.

*Ampelopsis grossedentata* (Hand.-Mazz) W.T. Wang grows wild in the southern region of China. Tea made from its stems and leaves is used to treat common colds, pyretic fever, pain, swelling of the pharynx and larynx and jaundice hepatitis and has been used for several hundred years by the Yao population in Guanxi and Hunan provinces (Jiu and Zhou, 1999; Murakami et al., 2004). Dihydromyricetin (DMY, also termed ampelopsin) is among the most common flavonoid compounds isolated from the tender stems and leaves of the plant species *A. grossedentata*, and the level of DMY can be greater than 27% in vine tea made from its stems and leaves (Guo et al., 2014) and greater than 40% in the cataphyll (Radhakrishnakartha et al., 2014). DMY displays many pharmacological activities, such as anti-inflammatory, cough relieving, antimicrobial, anti-hypertensive, antioxidative, hepatoprotective, and anti-carcinogenic effects (Daoud et al., 2014; Guo et al., 2014; Radhakrishnakartha et al., 2014; Zhu et al., 2014). Recently published data support the notion that DMY, as an antioxidant, protects PC12 and MT-4 cells against oxidative injury (Ye et al., 2008; Zou et al., 2014). The existing literature confirmed that DMY upregulates the level of phosphorylated AMPK (p-AMPK) in a dose- and time-dependent manner (Xia et al., 2014). Based on previous evidence, we explored for the first time whether DMY affects MG-induced damage to PC12 cells to guide the further investigation of its role in preventing diabetic encephalopathy.

## 2. Materials and methods

### 2.1. Materials

Dihydromyricetin was prepared from *A. grossedentata*, which was authenticated by Prof. Peigen Xiao. The incubation conditions for extracting DMY were 74% ethanol, a temperature of 65 °C, an incubation time of 94 min, and a 1:35 ratio of *A. grossedentata* to water (Zhao et al., 2014). The purity of DMY was demonstrated to be higher than 97% based on reversed-phase HPLC analysis. Infusions were prepared from 25 g of dried plant material according to the traditional method in China (5 g/100 mL $H_2O$) (Xu et al., 2012). The resulting aqueousextracts were lyophilized to obtain a dry residue, which served as the *A. grossedentata* total extract (AGTE). This dry residue was extracted using petroleum ether (TAPE), ethyl acetate (TAEA), *n*-butyl alcohol (TABU), and extracted water (TAW). The pheochromocytoma-derived cell line PC12 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Hyclone (Fisher, USA). MG and other reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated. Deionized water was used in all experiments. All other chemicals and reagents were of analytical grade. Compound C (AMPK inhibitor) (#ab120843) was purchased from Abcam. All other chemicals were purchased from Sigma–Aldrich Co. The following antibodies were used in the immunoblotting experiments at the indicated dilutions: mouse-derived anti-Glut-4 (1:200; Santa Cruz Biotechnology sc-53566, Santa Cruz, CA, USA); rabbit-derived anti-caspase-3 (#9662), anti-AMPKα (#2531), anti-GAPDH (1:1000; #5174) and anti-p-AMPKα (Thr-172) (#2532) (1:1000; Cell Signaling Technology, Danvers, MA, USA); anti-Glo-1 (1:1000; Epitomics #6778-1, USA); anti-β-actin (1:1000, CW0096A, CWBio); horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Santa Cruz Biotechnology sc-2004); and horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Santa Cruz Biotechnology sc-2768).

### 2.2. Cell culture

PC12 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C in humidified air containing 5% $CO_2$. Cells in the exponential phase of growth were used in all of the experiments. The PC12 cells were grown for 3 days and then divided into the different treatment groups.

### 2.3. Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to measure cell viability. The cells were seeded in 96-well plates ($1 \times 10^4$ cells/well) in 100 μL of DMEM. Twenty-four hours later, AGTE, TAPE, TAEA, TABU, TAW, MG or DMY was applied. The doses of DMY and MG were selected based on previous studies (de Arriba et al., 2006, 2007; Xia et al., 2014; Zhao et al., 2014). At end of the treatments, MTT (5 mg/mL in phosphate-buffered saline, PBS) was added to each well, and the plates were incubated for 4 h at 37 °C. Formazan dissolved in dimethyl sulfoxide (DMSO) was added to the wells, and the plates were agitated for 10–20 min on a shaker. The absorbance at 570 nm was measured using a microplate reader (BIO-TEK USA). Cell viability was expressed as the percentage of viable cells relative to that of the non-treated control cells.

### 2.4. TUNEL staining

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using the Fluorometric TUNEL System (Roche, Mannheim, Germany) according to the supplier's protocol. The cells were cultured in chamber slides and treated as described under "Drug treatment". At the end of treatment, the cells were fixed with 4% paraformaldehyde, washed with PBS, and permeabilized with 1% TritonX-100. After TUNEL staining, the slides