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Ginsenosides attenuate methylglyoxal-induced impairment of insulin signaling and subsequent apoptosis in primary astrocytes

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ABSTRACT

Diabetes mellitus (DM), which is characterized by chronic hyperglycemia, is known to increase the risk of neurodegeneration. In type 2 diabetes, hyperglycemia could cause insulin resistance and neurodegeneration in various cells including neurons and astrocytes. Hyperglycemia is also known to result in the formation of advanced glycation end-products (AGE) Methylglyoxal (MG) is one of the most reactive AGE precursors in which its abnormal accumulation is usually found in diabetic patients and induces neuronal cell death in central nervous system. Ginseng is a herb that has been widely used to treat various diseases in traditional Chinese medicine. Ginsenosides, the pharmacologically active component isolated from ginseng, have been shown to have cryoprotective effects in different neural cells.

In the present study we investigated the effects of MG in disturbing insulin signaling and leading to further cellular apoptosis in rat primary astrocytes. Furthermore, the protective effects of different subtypes of ginsenosides were studied. From the results, impairment of insulin signaling was found in astrocytes under MG treatment. Moreover, cleavage of caspase and Poly ADP ribose polymerase (PARP) was observed in line with insulin signaling disruption, showing the neurotoxic effects of MG towards astrocytes. The effects of ginsenosides in MG treated astrocytes were also investigated. After treatment, ginsenosides Rd and R-Rh2 were shown to ameliorate the cell viability of MG-treated astrocytes. In addition, Rd and R-Rh2 could improve insulin signaling and inhibit apoptosis, indicating that Rd, R-Rh2 and related compounds may have therapeutic potential in treating diabetes-induced neurodegeneration.

1. Introduction

Diabetes Mellitus (DM) is a chronic metabolic disorder due to the failure of pancreas to produce sufficient insulin or the body could not utilize the secreted insulin effectively. It is characterized by hyperglycemia and distinctive complications in various tissues. Recently, it has been shown that diabetic patients have a higher risk of developing neurodegenerative diseases such as Alzheimer disease (AD) or Parkinson's disease and with significant deterioration of cognitive functions including psychomotor efficiency, intelligence, attention and information processing in diabetic patients (Strachan et al., 2011; Xu et al., 2011). However, the underlying mechanisms of diabetes-related neurodegeneration remain largely unknown. Nowadays, increasing evidences show that astrocytes may play pivotal roles in the progression of neurodegenerative disease (Allaman et al., 2011). In particular, astrocytes could be activated under pathophysiological conditions and they are involved in various diabetes-related complications including neuropathy and neurodegeneration (Coleman et al., 2004; Maragakis and Rothstein, 2006; Gandhi et al., 2010).

Astrocytes are one of the major cell types found in human central nervous system (CNS) and they are classically considered as structural cells which hold neurons together. It is also known that

Abbreviations: AD, Alzheimer disease; AGE, advanced glycation end-product; CNS, central nervous system; DM, Diabetes Mellitus; EDTA, Ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IR, insulin receptor; IRS, insulin receptor substrate; MG, methylglyoxal; PARP, Poly ADP ribose polymerase; PBS, phosphate buffer saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ROS, reactive oxygen species.

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astrocytes participate in many housekeeping functions such as stabilizing the extracellular environment, regulating cerebral blood flow, maintaining synaptic functions and even supporting neuronal survival (Maragakis and Rothstein, 2006). For instance, astrocytes are responsible for recycling glutamate via glutamate transporter from the synaptic cleft into itself (Danbolt, 2001). Dysfunction of astrocytes could result in increased glutamate concentration and excitotoxicity (Maragakis and Rothstein, 2004). Furthermore, astrocytes could protect neurons from different stress insults such as reactive oxygen species (ROS) and oxidative stress by its high antioxidative capacity and the release of neurotrophic factors such as brain derived neurotrophic factor and nerve grow factor (Desagher et al., 1996; Lindsay, 1979; Chen et al., 2005). However, upon neurodegeneration, dysfunction of astrocytes is observed which accompanies with neuronal cell death in brain (Maragakis and Rothstein, 2006; Forman et al., 2005). Not only these abnormal astrocytes give less support to the neurons, they also release proinflammatory mediators to cause neuronal cell death (Berliocchi et al., 2007; Bordon, 2013). Until recently, one of the possible mechanisms that link dysfunction of astrocytes and diabetes-related neurodegeneration is the formation of methylglyoxal (MG) and related advanced glycation end product (AGE) in diabetic patients (Kuhla et al., 2005; Srikanth et al., 2013; Belanger et al., 2011).

In human body, MG is formed as a result of normal glucose metabolism (Turk, 2010). It is an AGE precursor that can foster the formation of AGE and these AGE in turn could interact with free amino groups, lipids and peptides in blood (Turk, 2010; Vlassara and Palace, 2002). MG is one of the major representative alpha-oxoaldehyde compounds and it is inevitably produced from glucose, fatty acid and protein metabolism (Kalapos, 1999). Within the intracellular environment, the glycolytic pathway is the most important endogenous source of MG via fragmentation of glyceraldehydes-3-phosphate (Belanger et al., 2011; Kalapos, 2008). Since the brain has very high demand of energy requirement in order to maintain neural cells function, neural cells are more likely to be exposed to MG compare with other cell types. In previous reports, MG could induce cellular degeneration by induction of ROS and oxidative stress (Desai et al., 2010; Heimfarth et al., 2013). Also, depleted ATP production and mitochondria dysfunction were found in MG-treated neuronal cells (de Arriba et al., 2007), therefore suggesting the role of MG in energy production disturbance and degeneration. Such MG burden associated with stress stimulus may cause neurodegeneration in CNS.

Panax ginseng is a well-known herb that has been widely used in traditional Chinese medicine to treat diseases such as type 2 diabetes, inflammation, cancer and hyperlipidemia (Cho et al., 2006; Chan et al., 2012; Wang et al., 2007). Active components of ginseng, namely ginsenosides, have been isolated and investigated. Ginsenosides are recognized as the bioactive components in ginseng and they are reported to have a wide spectrum of physiological and pharmacological effects such as modulating cardiovascular and neurological functions, protecting against chemically-induced tissues damage and delaying aging (Sengupta et al., 2004; Leung et al., 2007; Ramesh et al., 2012). In addition, their protective effects have been demonstrated in various diabetic models (Jang et al., 2003; Liu et al., 2012; Shapiro and Gong, 2002). Since abnormal MG production was found in diabetic patient, we therefore investigated whether ginsenosides could protect astrocytes from MG-associated stress. Furthermore, possible mechanisms of neuroprotective effects of ginsenosides in astrocytes were studied. From our results, we were able to demonstrate in *in vitro* primary astrocytic cultures that MG-induced astrocytic dysfunction was associated with the disturbance of insulin signaling and activation of apoptotic pathway. In addition, ginsenosides Rd and R-Rh2 could counteract the effects of MG by attenuating insulin signaling and inhibiting subsequent apoptosis. These results therefore demonstrated the beneficial effects of ginsenosides against MG-induced astrocytic dysfunction.

2. Materials and methods

2.1. Animal

Post natal day 1 Sprague Dawley rats were obtained from University of Hong Kong. The handling of rats and all procedures were in accordance with the Animals (Control of Experiments) Ordinance, Hong Kong, China, and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University, and conformed to the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals employed and the extent of their suffering in the experiments.

2.2. Chemicals

Methylglyoxal, insulin, MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Anti-glial fibrillary acidic protein (GFAP) antibody was purchased from Dako (Copenhagen, Denmark). Other primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma (St. Louis, MO, USA). Ginsenosides Rd ($C_{48}H_{82}O_{18}$) and R-Rh2 ($C_{36}H_{62}O_8$) with approximately 98% purity were obtained from Fleton (Chengdu, China). The purities of all ginsenosides are verified by high-performance liquid chromatography and thin-layer chromatography.

2.3. Preparation of primary rat astrocytic cultures

Primary astrocytes were prepared and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/neomycin (PSN) antibiotics (Invitrogen, Carlsbad, CA, USA) according to previous protocol with a few modifications (Lee et al., 2012; De Marinis et al., 2013). Briefly, brain tissues without hindbrain were isolated from postnatal day 1 Sprague–Dawley rats. It was minced and dissociated in trypsin solution for 15 min at 37°°C. The cultures were allowed to seed overnight and mechanical shaking (200 rpm) was applied to dissociate neurons and microglia. Finally, astrocytes were yielded and grown for 3 weeks which were developed into homogenous cultures.

2.4. Methylglyoxal and pharmacological treatment

Astrocytes were seeded on 96 well plates (for MTT assay) and 6 well plates (for Western Blotting) for 24 h with $5*10^3$ and $2*10^5$ of cells respectively. Cell cultures were then switched to DMEM low glucose medium supplemented with 1% FBS for 24 h. To examine the effects of MG in astrocytic cultures, cells were exposed to different concentrations of MG at increasing concentrations from 200 μM to 2000 μM for 24 h or longer. In order to investigate the protective effects of insulin and active ginsenosides compound, 1 μM of insulin and different concentrations of ginsenosides were incubated with MG in cultures for 24 h respectively and proceeded to MTT assay and Western blotting experiments.

2.5. MTT assay for cell viability

MTT assay was performed to determine cell viability after different treatments. 0.5 mg/ml MTT solution was added into each well and incubated at 37°°C for 4 h. Formazan salt formed in live cells was dissolved by dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm by micro-plate reader (Bio-Rad, Hercules, CA, USA).

2.6. Western blotting

Cells were washed with phosphate buffer saline (PBS) and total cell lysates were obtained by scrapping cells with lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with protease inhibitor. The protein supernatants were extracted after 14000 g centrifugation. These protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). It was incubated with different primary antibodies at 4° C for overnight followed by horseradish peroxidase (HRP)-conjugated secondary goat anti mouse/rabbit antibodies (Sigma, St. Louis, USA) for 1 h. Protein bandings were visualized with enhanced chemiluminescence reagents (Invitrogen, Carlsbad, CA, USA). Semi-quantifications of the signals were performed by densitometric analysis of the Image J software.