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Tenuigenin protects cultured hippocampal neurons against methylglyoxal-induced neurotoxicity

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Abstract

Methylglyoxal is a metabolite of glucose. Since serum methylglyoxal level is increased in diabetic patients, methylglyoxal is implicated in diabetic complications such as cognitive impairment. This study aimed to evaluate the effects of tenuigenin, an active component of roots of Polygala tenuifolia Willdenow, on methylglyoxal-induced cell injury in a primary culture of rat hippocampal neurons. MITT and Hoechst 33342 staining, together with flow cytometric analysis using annexin-V and propidium (PI) label, indicated that tenuigenin pretreatment attenuated methylglyoxal -induced apoptotic cell death in primary cultured hippocampal neurons, showing a dose-dependent pattern. Furthermore, 2, 7-dichlorodihydrofluorescein diacetate was used to detect the level of intracellular reactive oxygen species. Tenuigenin decreased the elevated reactive oxygen species induced by methylglyoxal. In addition, tenuigenin inhibited activation of caspase-3 and reversed down-regulation of the ratio of Bcl-2/Bax, both of which were induced by methylglyoxal stimulation. The results suggest that tenuigenin displays antiapoptotic and antioxidative activity in hippocampal neurons due to scavenging of intracellular reactive oxygen species, regulating Bcl-2 family and suppressing caspase-3 activity induced by methylglyoxal, which might explain at least in part the beneficial effects of tenuigenin against degenerative disorders involving diabetic cognitive impairment.

1. Introduction

Diabetes mellitus currently affects 250 million people worldwide, with 6 million new cases reported each year (Cole et al., 2007). Diabetes not only causes somatic complications but also may result in accelerated cognitive dysfunction. Cognitive decline is among the most common and feared conditions of old age, recognized as a risk factor for dementia. Many factors are thought to be involved in the pathomechanism of cognitive problems. Recent data from literature show that accumulation of toxic α-oxoaldehydes such as methylglyoxal may be one of the key determining factors.

Methylglyoxal is an endogenous toxic compound. Methylglyoxal accumulation is often seen under conditions of hyperglycemia, and impaired glucose metabolism (Haik et al., 1994). Essentially, glucose can react reversibly with protein amino groups, resulting in Schiff's base formation which, in turn, can rearrange to form an Amadori product. The Amadori product can subsequently degrade into dicarbonyl compounds (Skamarauskas et al., 1996), and methylglyoxal is one of the most important dicarbonyl compounds.

Some previously published studies have demonstrated that reactive methylglyoxal is capable of inducing apoptosis in hippocampal neurons through both mitochondrial and Fas-receptor pathways (Di Loreto et al., 2005), which could induce apoptosis through activating many intracellular signal transduction pathways (Min et al., 1999; Yamagishi et al., 2002). It is also known that methylglyoxal is a potent source of reactive oxygen species (Yim et al., 1995; Di Loreto et al., 2004), which is the main cause of oxidative stress, and the brain is more susceptible to oxidative damage than any other major organ because of its high oxygen consumption. Oxidative stress, advanced glycation end products and apoptosis are involved in the impairment of cognitive processes (Smith et al., 1994; Markesbery, 1997; Nagy and Esiri, 1997). Therefore it is possible to hypothesize that methylglyoxal cytotoxicity may be responsible for the relative impairment of cognitive functions.

The root of Polygala tenuifolia Willdenow, a traditional oriental medicine, has been used to improve memory and intelligence (Park et al., 2002; Shin et al., 2009) in traditional Chinese medicine for about 2000 years. Tenuigenin (Fig. 1) is an active component of roots of P. tenuifolia, and pharmacological data indicate that tenuigenin could suppress secretion of β-amyloid (Aβ) in SH-SYSY APP 695 cells by inhibiting beta-site APP-cleaving enzyme 1 (BACE1) or β-secretase (Jia et al., 2004), and showed protective effect against the cytotoxicity of some previously published studies have demonstrated that reactive methylglyoxal is capable of inducing apoptosis in hippocampal neurons through both mitochondrial and Fas-receptor pathways (Thornalley, 2005), which could induce apoptosis through activating many intracellular signal transduction pathways (Min et al., 1999; Yamagishi et al., 2002). It is also known that methylglyoxal is a potent source of reactive oxygen species (Yim et al., 1995; Di Loreto et al., 2004), which is the main cause of oxidative stress, and the brain is more susceptible to oxidative damage than any other major organ because of its high oxygen consumption. Oxidative stress, advanced glycation end products and apoptosis are involved in the impairment of cognitive processes (Smith et al., 1994; Markesbery, 1997; Nagy and Esiri, 1997). Therefore it is possible to hypothesize that methylglyoxal cytotoxicity may be responsible for the relative impairment of cognitive functions.

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of Aβ1-40 in primary cultured cortical neurons (Chen and Li, 2007). Thus in recent years, tenuigenin has been used in the traditional Chinese medicine treatment of Alzheimer’s disease.

In order to provide a new window into the pharmacological properties of tenuigenin, the present study was designed to investigate neuroprotection of tenuigenin against methylglyoxal-induced neuronal death in primary cultured hippocampal neurons of rats. Since cognitive dysfunction and dementia have been proven to be common complications of diabetes mellitus, we hope to expand the understanding of the potential therapeutic value of tenuigenin for diabetic cognitive dysfunction.

2. Materials and methods

2.1. Materials

Tenuigenin (purity >99%) was purchased from the National Institute of Pharmaceutical and Biological Products (Beijing, China). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), trypsin, poly-L-lysine, Neurobasal medium and B27 were purchased from Gibco (Grand Island, NY). Mouse anti-microtubule associated protein-2 (MAP-2), 3′-[4′, 5-dimethylthiazol-2-yl] - 2′, 5-diphenyl-tetrazolium bromide (MTT), and 2′, 7-dichlorofluorescein diacetate (DCFH-DA) were from Sigma-Aldrich (St. Louis, U.S.A.); 4′, 6-diamidino-2-phenylindole (DAPI) was from Dojindo (Kumamoto, Japan). Antibodies against Bcl-2, Bax, β-actin and IR Dye 800-conjugated goat anti-rabbit IgG were from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). Hoechst 33342, antibodies against cleaved caspase-3 and Annexin V/PI detection apoptotic kit were from Beyotime Institute of Biotechnology (Jiangsu, China). All the other chemicals used were purchased from Sigma, unless otherwise stated.

2.2. Cell culture and cell viability assay

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of newborn rat pups (obtained from Animal Breeding Center of Chinese Academy of Medical Sciences, China). All animal experiments were carried out in accordance with institutional guidelines and ethics. Every effort was made to minimize the number of animals used and their suffering. After treatment with 0.125% trypsin for 20 min at 37 °C in Ca2+ and Mg2+-free Hank’s balanced salt solution, the hippocampi were washed in DMEM/F12 with 10% FBS in order to stop trypsin activity, then the single-cell suspension was seeded in 48-well or 96-well plates coated with poly-L-lysine (0.1 mg/ml) at the density of 5 × 10^5/ml in a humidified atmosphere of 5% CO2 at 37 °C. After cells attached to the substrate, the medium was exchanged to neuronal culture medium (serum-free Neurobasal medium with 2% B27 supplement, 0.5 mM glutamine, 100 U/ml penicillin/100 U/ml streptomycin), followed by re-incubation for 7-8 days with half of the medium being changed every 3 days. Around 1 week later, neuronal networks formed, and the hippocampal neurons were treated with 0, 50, 100, 200 and 400 μM methylglyoxal for 24 h at 37 °C in a humidified incubator. Sister cultures were pre-treated with or without 1, 2, and 4 μg/ml tenuigenin prior to 100 μM methylglyoxal incubation for 24 h, and then MTT was added to the medium at a final concentration of 0.5 mg/ml and incubated at 37 °C for 4 h. The medium was removed carefully and dimethyl sulfoxide added to resolve the formazan dye crystals. The absorbance was measured by microplate reader at 540 nm. In the above procedures, treatments only with vehicle and only with methylglyoxal stimulation were considered as control and methylglyoxal groups, respectively.

2.3. Immunofluorescence staining

Hippocampal neurons were stained with an antibody against MAP-2, a marker for the cell body and neurites. Cultures were fixed with 4% paraformaldehyde for 15 min at 4 °C and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After blocking with 10% normal goat serum for 30 min, cells were incubated with monoclonal anti-MAP-2 antibody in blocking solution at a dilution of 1:800 overnight at 4 °C followed by fluorescent-conjugated secondary antibody. Cells were nuclear stained with DAPI (1 μg/ml, 15 min). Images were obtained using fluorescence microscope (IX-71, Olympus).

2.4. Hoechst 33342 staining

As a measure of apoptosis, cells were fixed in 4% paraformaldehyde, membranes were permeabilized with 0.1% Triton X-100 for 15 min, and cells were stained with the fluorescent DNA-binding dye Hoechst 33342 (1 mg/ml) dye for 10 min, followed by observation under a DMR fluorescence microscope (IX-71, Olympus). The hippocampal neurons with fragmented, condensed DNA or normal DNA were counted, respectively. The ratio of apoptotic neurons to total neurons was calculated.

2.5. Measurement of intracellular reactive oxygen species

Formation of reactive oxygen species was determined by use of fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses into cells where it is oxidized in the presence of reactive oxygen species into the fluorescent compound 2′, 7′-dichlorofluorescein (DCF). DCFH reacts with reactive oxygen species to form the fluorescent product DCF. Briefly, hippocampal neuron-enriched cultures were pretreated with different concentration of tenuigenin for 24 h prior to 100 μM methylglyoxal for 24 h. After treatment, the supernatant was removed and cells were washed with PBS. DCFH-DA was diluted in fresh DMEM/F12 at a final concentration of 10 μM and incubated with cells for 20 min at 37 °C in the dark. The cells were harvested and suspended in PBS. The fluorescence was read at 485 nm excitation and 530 nm emission with a fluorescence plate reader (Infinite, TECAN). The increasing production of reactive oxygen species was expressed as a percentage of control.

2.6. Flow cytometry with annexin V/PI staining

The hippocampal neurons that had been treated as above were harvested and resuspended in Phosphate Buffered Saline (PBS) buffer at a concentration of 1 × 10^6 cell/ml. After centrifuged at 1000 g for 5 min, 195 μl FITC-conjugated annexin V binding buffer and 5 μl of annexin V-FITC were added. Following incubation for 15 min at room temperature, 400 μl of annexin V-FITC were added. Following incubation for 15 min at room temperature, the cells were analyzed by flow cytometry (FACScan, Becton Dickinson, USA).
gentle vortex, the mixture was incubated for 15 min at room
temperature (20-25 °C) in the dark. After centrifuged at 1000 g for
5 min, 190 μl FITC-conjugated annexin V binding buffer and 10 μl
propidium iodide were added. Following gentle vortex, the sample
was analyzed using a dual-laser FACS Vantage SE flow cytometer
(Becton Dickinson, Mountain View, CA) within a 1 h period. The
percentages of apoptotic and necrotic cell for each sample were
estimated.

2.7. Western blot analysis

After treatment above mentioned, the hippocampal neurons were
subjected to Western blot analysis for cleaved caspase-3, Bcl-2 and
Bax protein expression. Cell proteins were extracted and quantified by
a BCA kit, followed by electrophoretic separation on SDS-PAGE. After
transferring to PVDF membranes, samples were allowed to react with
primary rabbit monoclonal antibodies against cleaved caspase-3
(1:1000), Bcl-2 (1:800) and Bax (1:800), and subsequently with IR
secondary rabbit monoclonal antibodies against cleaved caspase-3
(1:5000). The images were scanned with a scanner (Trellix), and the data of optical density
were analyzed using Image-J software. β-Actin was used as an internal
control.

2.8. Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical
differences between groups were analyzed by one-way analysis of
variance (ANOVA) followed by Turkey’s tests. Difference was
considered statistically significant at P<0.05.

3. Results

3.1. Effects of tenuigenin pretreatment on methylglyoxal-induced
decrease of cell viability in hippocampal neurons

MTT assay revealed the dose-dependent toxicity of methylglyoxal
on cultured hippocampal neurons. The median toxic concentration
(TC50) of methylglyoxal was 124 μM, which was calculated by logistic
regression of cell number on methylglyoxal concentration (logistic
regression coefficient r = 0.972 ± 0.005, Fig. 2A). In subsequent
experiments, an exposure to 100 μM methylglyoxal for 24 h was
used to induce cell insult. As illustrated in Fig. 2B, methylglyoxal
stimulation decreased the cell viability in hippocampal neurons to
49%, and tenuigenin at very low concentration (1 μg/ml) was not
effective for neuroprotection. Tenuigenin at 2 or 4 μg/ml, however,
significantly prevented cultured hippocampal neurons from methyl-
glyoxal-induced damage, and restored the cell survival to 62% and
75%, respectively, displaying dose-dependent protective effects. The
results of MTT assay suggested that tenuigenin at these concentra-
tions did not result in apparent cytotoxicity (data not shown).

Meanwhile, deterioration of hippocampal neurons was determined by
counting the number of neuron following immunofluorescence
staining through the Image-Pro Plus (IPP) software. Morphologically,
the changes of hippocampal neurons were shown in Fig. 2C. After
exposure to 100 μM methylglyoxal, hippocampal neurons exhibited a
49% decrease in the number of MAP-2-positive neurons and their
dendrites showed markedly retractable and tortuous appearances.
However, pretreatment with tenuigenin (1, 2, and 4 μg/ml) showed
protective effects: not only the amount of MAP-2-positive neurons
increased to 62%, 73% and 80%, respectively, but also the degenera-
tion of their dendrites was partially counteracted, which were
indicative of the neuroprotective effects of tenuigenin on neurons in
morphology (Fig. 2D).

3.2. Effects of tenuigenin pretreatment on methylglyoxal-induced
apoptosis of hippocampal neurons

Hoechst staining showed that after the neurotoxic insult of 100 μM
methylglyoxal for 24 h, chromatin condensation and nuclear
fragmentation were observed in hippocampal neurons. Pretreatment
with tenuigenin, however, blocked the apoptosis in terms of the
morphological appearance of hippocampal neurons (Fig. 3A). We
further found that 100 μM methylglyoxal produced apoptosis of 51% in
the total population of cultured hippocampal neurons, in comparison to the percentage of apoptotic neurons (11%) for control
group. Pre-incubation of tenuigenin (1, 2, and 4 μg/ml), however,
significantly reduced the percentage of methylglyoxal-induced
apoptotic neurons to 36%, 28% and 24%, respectively (Fig. 3B).

Similarly, cytometric analysis with apoptosis detection kit provided
a further protective evidence for tenuigenin against methylglyoxal.
As shown in Fig. 4A and B, the percentage of apoptotic hippocampal
neurons increased from 3% to 33% after challenging with 100 μM
methylglyoxal for 24 h. However, the percentage was significantly
decreased to 20%, 8% and 6% by pretreatment with tenuigenin (1, 2, and
4 μg/ml), respectively, while necrosis showed no significant
alternations.

3.3. Effects of tenuigenin pretreatment on expression of caspase-3, Bcl-2
and Bax in cultured hippocampal neurons after exposure to methylglyoxal

Western blot analysis showed that the expression level of cleaved
caspase-3 was significantly increased after exposure to methylglyoxal,
but declined in tenuigenin pretreatment groups (Fig. 5A and B). Bcl-2,
Bax, and Bax, as well as the ratio of Bcl-2/Bax, was significantly
increased after exposure to methylglyoxal. Conversely, the level of Bax, an important pro-apoptotic protein,
reduced markedly after exposure to methylglyoxal (24 h). As a
result, the ratio of Bcl-2/Bax decreased significantly. At the concentra-
tion range of 1–4 μg/ml tenuigenin enhancement of Bcl-2/Bax ratio
was achieved through an increase in expression of Bcl-2 and a
decrease in the expression of Bax (Fig. 5A and C).

3.4. Inhibitory effect of tenuigenin on reactive oxygen species formation

The action of tenuigenin on reactive oxygen species is paralleled with its effects on apoptosis. After exposure of hippocampal neuronal
cells to methylglyoxal (100 μM) for 24 h, the intracellular reactive oxygen species level increased to 176% of control, and decreased to
125% and 104% in the 2 and 4 μg/ml tenuigenin treatment group
respectively. Pretreatment with 1 μg/ml tenuigenin decreased the
intensity of fluorescence, but the difference was not statistically
significant (Fig. 6).

4. Discussion

In the present study, we investigated the mechanism of methyl-
glyoxal neurotoxicity and the effect of tenuigenin on methylglyoxal
neurotoxicity using primary cultures of rat hippocampal neurons, and
the results showed that by MTT assay, Hoechst 33342 staining, flow
cytometry analysis and DCFH-DA that pre-incubation with tenuigenin
extract protected cultured hippocampal neurons against methyl-
glyoxal toxicity in a dose-dependent manner.

To determine which type of neuronal death was induced by
methylglyoxal stimulation under our experimental conditions, we
conducted the measurements of cell viability and cell DNA
fragmentation, neuronal morphological examination as well as flow
cytometry analysis. In this study, exposure to methylglyoxal resulted in
the cell viability loss of hippocampal neurons in a dose-dependent
manner, identifying 100 μM methylglyoxal (corresponding to -50%
cell survival) as the best concentration to proceed with the following experiments. The morphological examinations indicated that exposure to methylglyoxal led to extensive apoptotic-like cell death in primary cultured rat hippocampal neurons. It was indicative that the direct neurotoxicity to hippocampal neurons triggered by methylglyoxal may be one of the central factors causing deterioration of hippocampal neurons and in turn contributed to the pathogenesis of neurodegeneration. These results are consistent with the previously reported findings that stimulation with a certain concentration of methylglyoxal within a delayed time period induces neuronal death in a prevailing form of apoptosis under in vitro conditions (McLellan et al., 1994).

Methylglyoxal is a metabolic byproduct of glycolysis, and under hyperglycaemic conditions, an increase in the concentration of methylglyoxal could lead to neuronal damage and contribute to neurodegeneration. This highlights the importance of controlling blood glucose levels to prevent neuronal damage and potentially delay the onset of neurodegenerative diseases.
methylglyoxal has been observed in human body fluids and tissues that seems to be responsible for diabetic complications (Haik et al., 1994; McLellan et al., 1994; Vander Jagt and Hunsaker, 2003). It is well known that the hippocampus plays a critical role in memory processing. Experimental results demonstrated that after 100 μM methylglyoxal treatment for 24 h, hippocampal neurons underwent extensive apoptotic like death may be associated with diabetes-mediated impairment of cognitive abilities.

Tenuigenin, a major active ingredient isolated from the plant Polygala tenuifolia Willdenow, has been reported to have a wide range of pharmacological properties (Shin et al., 2004). This study aimed to explore the neuroprotective effects of tenuigenin against methylglyoxal-induced cell damage in hippocampal neurons. However, pretreatment with different concentrations of tenuigenin decreased the cell viability loss induced by methylglyoxal, which was in parallel with the morphological analyses and Flow cytometry assay. These results suggest that tenuigenin pretreatment enhances the ability of hippocampal neurons to counteract methylglyoxal cytotoxicity.

Many molecules are involved in the apoptotic cascade, and the Caspase and Bcl-2 families are especially important among these molecules. Caspases are a family of cysteine proteases that are essential for apoptosis in cells, and thus have been termed "executioner" proteins for their roles in the cell apoptosis. Activation of caspase-3 is a hallmark of apoptotic cell death and precedes the changes in nuclear morphology (Almeida et al., 2005; Degterev et al., 2003). Bax and Bcl-2 are two important regulator of apoptosis in the Bcl-2 family, and alteration of the ratio of Bcl-2 to Bax is significant in determining whether apoptosis occurs (Yang and Korsmeyer, 1996; Kroemer, 1997). In the present study, exposure of cultured hippocampal neurons to methylglyoxal was shown to induce the elevation of cleaved caspase-3 expression; this suggests that caspase-3-like

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proteases are involved in the methylglyoxal induced apoptotic death of hippocampal neurons. Moreover, we also found that pretreatment with tenuigenin led to a significant decrease in caspase-3 activity compared to stimulation with methylglyoxal alone, suggesting the suppressive effect of tenuigenin on methylglyoxal-induced cell death. We also found that decreased Bcl-2 and increased Bax expression after exposure to methylglyoxal. The finding that tenuigenin decreased the expression of caspase-3 and increased the ratio of Bcl-2/Bax in methylglyoxal treated neurons suggests that tenuigenin interferes with the execution of the apoptotic program and favors the formation of Bcl-2-Bax heterodimers and then promote cell survival. Hence modulation of caspase-3 and Bcl-2/Bax ratio might be one of the major mechanisms whereby tenuigenin protects against hippocampal neuronal cell apoptosis induced by methylglyoxal.

Caspase-3 activation maybe only a part in methylglyoxal-mediated apoptosis, it was previously reported that methylglyoxal induced apoptosis via reactive oxygen species-mediated activation of JNK (Du et al., 2000, 2001; Ota et al., 2007), p38 (Fukunaga et al., 2004), ERK (Hsieh et al., 2007) or NF-κB (Hsieh et al., 2007; Kim et al., 2004). To acquire useful information on the mechanisms that responsible for the neuroprotective effect of tenuigenin, we further examine the changes in reactive oxygen species expression. The reactive oxygen species level was significantly increased by methylglyoxal treatment. The results showed that pretreatment with tenuigenin (1-4 μg/ml) dose-dependently attenuated methylglyoxal-induced reactive oxygen species production in hippocampal neuronal cells. Besides, we observed the generation of reactive oxygen species and apoptosis concurrently. Oxidative stress and apoptosis are closely linked physiological phenomena and are implicated in pathophysiology of some of the chronic diseases (Kannan and Jain, 2000). Literature has described Bcl-2 serving as an antioxidant, exerting a particular buffering effect on mitochondrial reactive oxygen species production, and to delay cell-cycle progression (Agostinis, 2009). Likewise, it was reported that reactive oxygen species expression of Bcl-2, but increased expression of Bax (Li et al., 2004), thereby contributing to the regulation of apoptosis (Simon et al., 2000).
Our results suggested that the protective effects of tenuigenin against methylglyoxal toxicity may be induced by directly inhibiting apoptosis and reactive oxygen species introduction, or indirectly attributed to an ability of the extract to reverse down-regulation of Bcl-2, which has antioxidative and antiapoptosis properties. Whether other pathways are involved requires further investigation.

In conclusion, tenuigenin displays antiapoptotic and antioxidative activity in hippocampal neurons due to the scavenging of intracellular reactive oxygen species and ameliorating hippocampal neuronal cells apoptosis induced by methylglyoxal. Thus, we believed that the antiapoptotic and antioxidative capacity of tenuigenin might provide at least in part clinical potential for preventing and/or treating neuronal damage and degenerative disorders involving diabetic cognitive problem.

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