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Tenuigenin protects cultured hippocampal neurons against methylglyoxal-induced neurotoxicity

Yu-Jing Chen [a,b,1], Xiao-Bo Huang [a,1], Zong-Xin Li [a], Lin-Lin Yin [b], Wen-Qiang Chen [a], Lin Li [b,*]

[a] Department of Traditional Chinese Medicine, Xuanwu Hospital of Capital Medical University, Beijing 100053, PR China
[b] Department of Pharmacology, Xuanwu Hospital of Capital Medical University, Beijing 100053, PR China

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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. MTT 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., 2008). The methylglyoxal-protein reaction has also been shown to produce advanced glycation end products (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 related 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-SY5Y 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

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* Corresponding author. No. 45, Chang-Chun Street, Beijing 100053, PR China. Tel.: +86 10 83198886; fax: +86 10 83198855.
E-mail addresses: cyj0504@sina.com.cn (Y.-J. Chen), linli97@hotmail.com (L. Li).

[1] Both authors contribute equally to this work.

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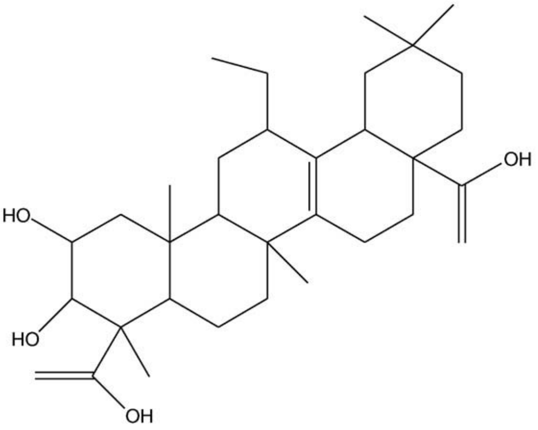

Fig. 1. Chemical structure of tenuigenin.

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 damage 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 Ca^{2+} and Mg^{2+}-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% CO_2 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 (DCFH). 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 mentioned 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

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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 VantageSE 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 Dye 800-conjugated goat anti-rabbit IgG (1:5000). The images were scanned with Mustek 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 (S.D.). 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 (TC_{50}) of methylglyoxal was 124 μM, which was calculated by logistic regression of cell number on methylglyoxal concentration (logistic regression coefficient $r=0.972\pm0.005$, 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 methylglyoxal-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 concentrations did not result in apparent cytotoxicity (data not shown). Meanwhile, deterioration of hippocampal neurons was determined by counting the number of neuron following immunofluorence 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 retractile 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 degeneration 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 of 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 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 reduced 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 exposed to methylglyoxal, but declined in tenuigenin pretreatment groups (Fig. 5A and B). Bcl-2, a key protein contributing to maintain cell survival, was present at a relatively high level in the normal hippocampal neuronal cells and decreased after exposure to 100 μM methylglyoxal for 24 h. On the other hand, the level of Bax, an important pro-apoptotic protein, increased markedly after exposure to methylglyoxal for 24 h. As a result, the ratio of Bcl-2/Bax decreased significantly. At the concentration 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 μg/ml 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 methylglyoxal 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 methylglyoxal toxicity in a dose-dependent manner.

To determine which type of neuronal death was induced by methylglyoxal stimulation under our experimental conditions, we carried out 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%

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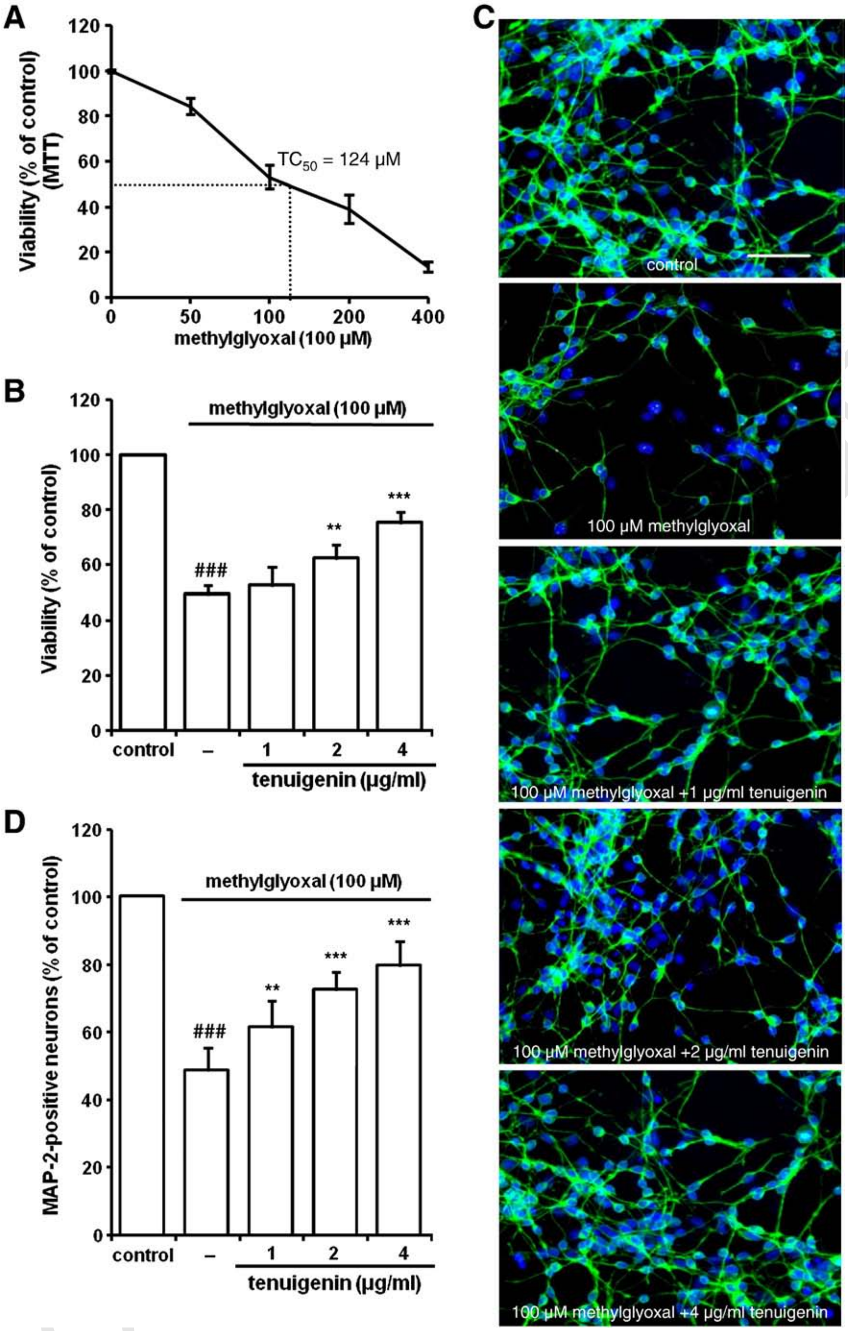

Fig. 2. Effect of tenuigenin against methylglyoxal-induced neurotoxicity to hippocampal neural cells. (A) Effect of methylglyoxal on hippocampal neural cells. Dose-response curve and toxic concentration (TC_{50}) value calculated after 24 h of methylglyoxal incubation in 8- 10 DIV neuronal cultures. Values are mean ± S.D. of three different cultures. (B) Tenuigenin protects cultured hippocampal neurons against methylglyoxal-induced cell death. Tenuigenin exerted a protective effect in a concentration- dependent manner. Tenuigenin was applied to the hippocampal neurons at 1, 2 and 4 µg/ml. After 24 h, the cultures were then incubated with methylglyoxal (100 µM) for another 24 h in the presence or absence of tenuigenin, followed by MTT assay. Results are presented as % of control cell survival (100%). (C) Effect of tenuigenin on methylglyoxal-induced morphological alternations in hippocampal neural cells. The cells immunofluorence-stained with MAP-2 primary antibody as described in experimental procedures. MAP-2 positive cells (green) represented neurons, cell nuclei (blue) were stained with DAPI. Scale bar = 50 µm. (D) After cell treatment, the number of MAP-2-positive hippocampal neurons was counted through Image-Pro Plus (IPP) software. Pretreatment with tenuigenin attenuated the neurotoxicity induced by methylglyoxal and inhibited the degeneration of neurons to some extent. Data are expressed as mean ± S.D. of three independent experiments. ### $P<.001$ vs. control. ** $P<0.01$ and *** $P<0.001$ vs. methylglyoxal stimulation alone.

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

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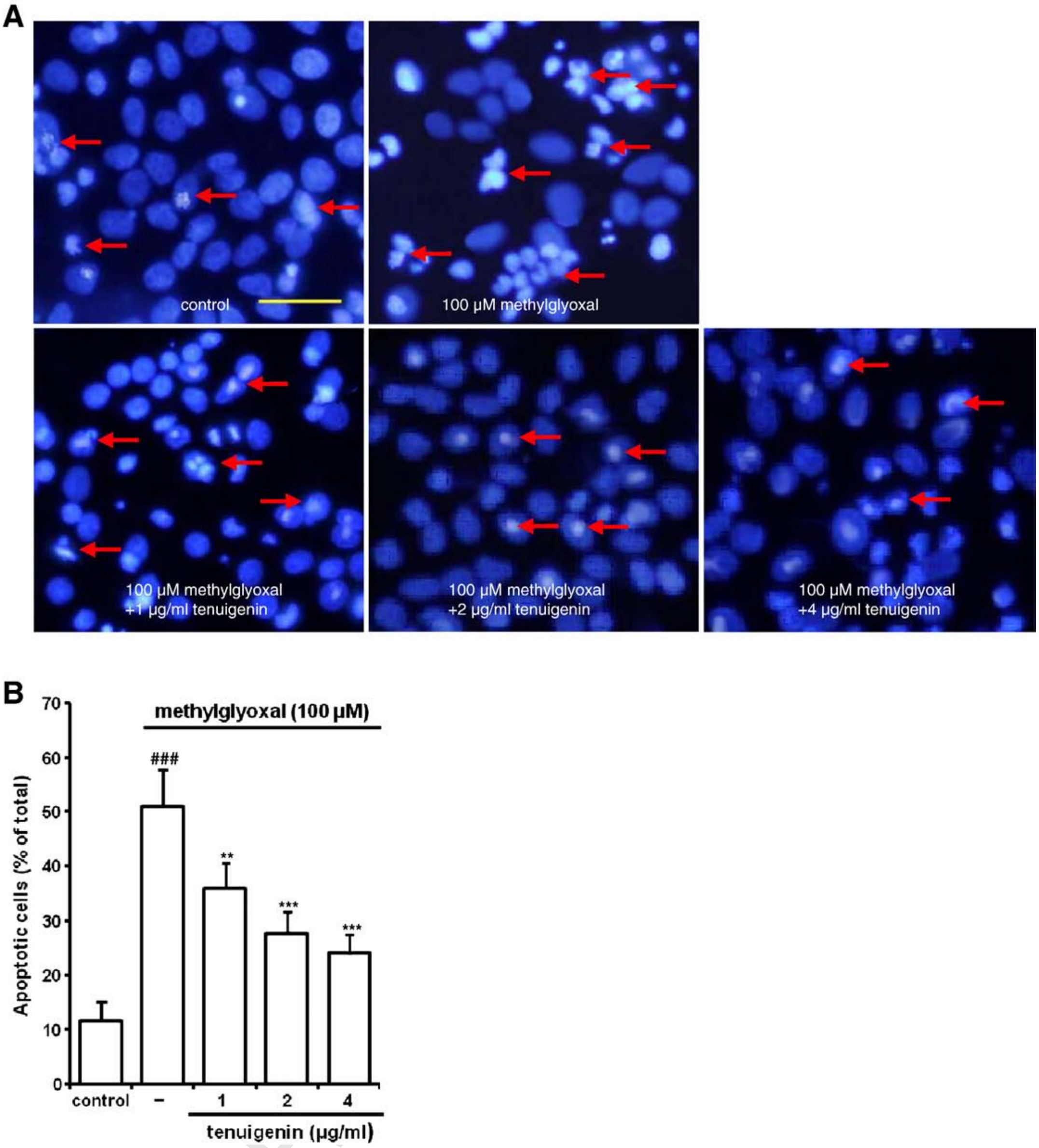

Fig. 3. Inhibitory effect of tenuigenin on methylglyoxal-induced apoptosis of hippocampal neurons (Hoechst 33342 staining). Cells were preincubated for 24 h in the presence or absence of tenuigenin, then exposed for 24 h to methylglyoxal. (A) Hoechst 33342 staining was performed to visualize the extent of programmed cell death. Condensed or fragmented nuclei were considered as apoptotic cells. Arrows indicated condensed nuclei. Scale bar = 20 μm. (B) Quantification of hippocampal neurons apoptosis after exposure to methylglyoxal in the presence or absence of tenuigenin. ### $P<0.001$ vs. control, ** $P<0.01$ and *** $P<0.001$ vs. methylglyoxal alone. All data were expressed as mean ± S.D. of three independent experiments.

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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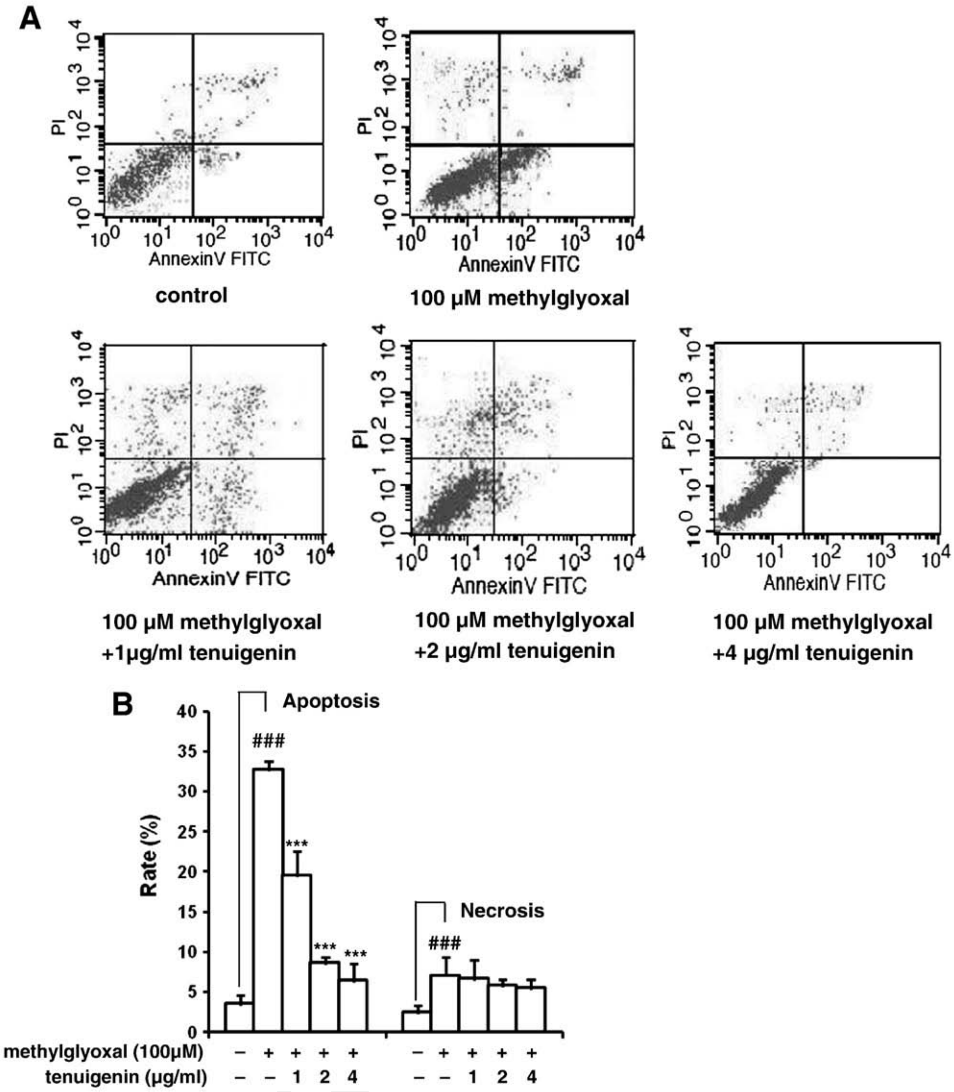

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Fig. 4. Inhibitory effect of tenuigenin on methylglyoxal-induced apoptosis of hippocampal neurons (Flow cytometric analyses). Cells were preincubated for 24 h in the presence or absence of tenuigenin, and then exposed for 24 h to methylglyoxal. Cells that stain positive for annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both annexin V-FITC and PI are in the end stages of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both annexin V-FITC and PI are alive and not undergoing measurable apoptosis. Tenuigenin pretreatment significantly suppressed methylglyoxal-induced cell apoptosis, while necrosis showed no significant alternations. (A) Apoptosis determined by staining with annexin-V + PI. (B) The percentage of apoptotic or necrotic hippocampal neurons in total hippocampal neurons, ### $P<0.001$ vs. control. *** $P<0.001$ vs. methylglyoxal stimulation alone. All data were expressed as mean ± S.D. of three independent experiments.

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 that reactive oxygen species suppressed expression of Bcl-2, but increased expression of Bax (Li et al., 2004), thereby contributing to the regulation of apoptosis (Simon et al., 2000).

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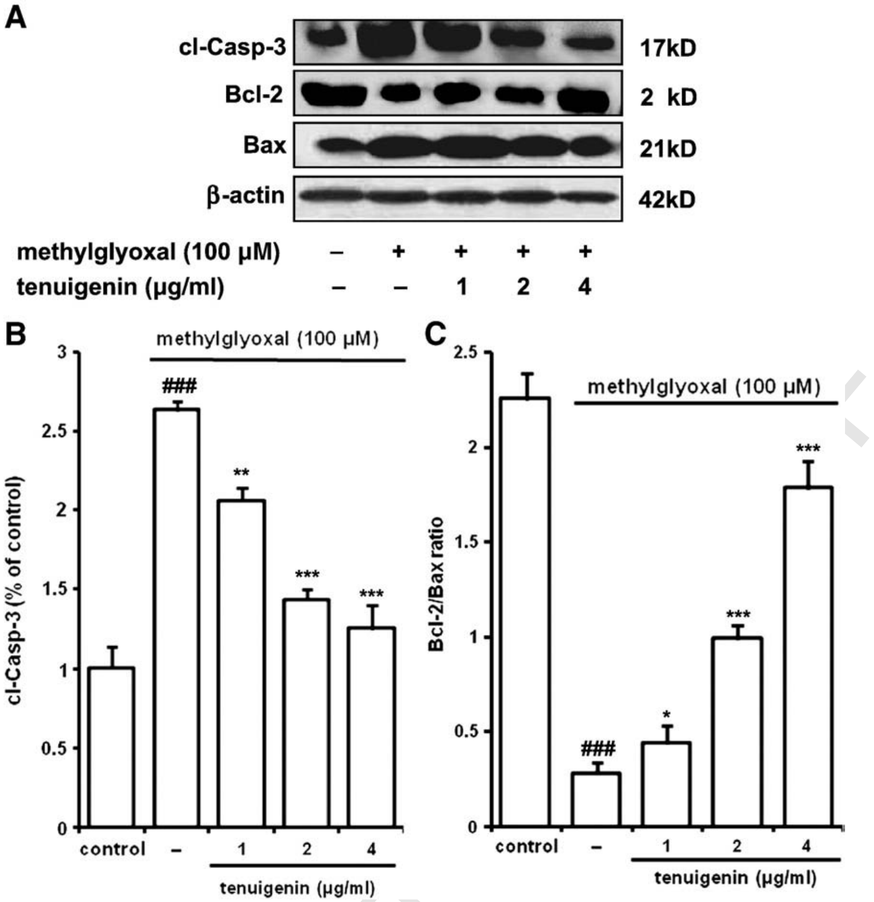

Fig. 5. Effect of tenuigenin on expression of caspase-3, Bcl-2 and Bax in cultured hippocampal neurons after exposure to methylglyoxal (western blotting analysis). (A) Representative image of immunoblots for cleaved caspase-3, Bax and Bcl-2. (B) Level of cleaved caspase-3 (cl-Casp-3). (C) Ratio of values of Bcl-2/Bax. Cells were preincubated for 24 h in the presence or absence of tenuigenin, and then exposed for 24 h to methylglyoxal. Densitometric analysis is mean ± S.D. of three independent experiments. ### $P<0.001$ vs. control. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. methylglyoxal stimulation alone. β-Actin as internal standard.

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.

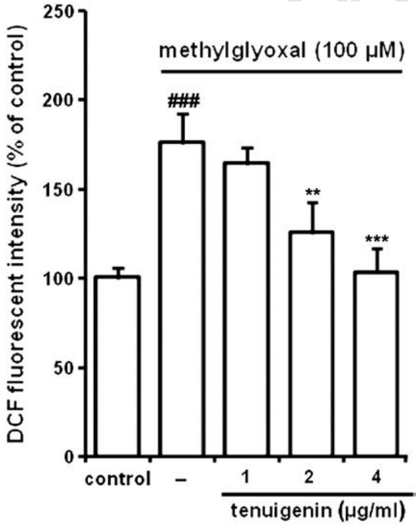

Fig. 6. Inhibitory effect of tenuigenin on methylglyoxal-induced production of intracellular reactive oxygen species. Neural hippocampal cells were pretreated for 24 h with vehicle or indicated concentrations of tenuigenin prior to stimulation with 100 μM methylglyoxal. The levels of intracellular reactive oxygen species were determined by DCFH-DA as described in Material and Methods. The results are the mean ± S.D. of three independent experiments. ### $P<0.001$ vs. control. ** $P<0.01$ and *** $P<0.001$ vs. methylglyoxal stimulation alone.

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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