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ORIGINAL ARTICLE

Protective effect of selaginellin on glutamate-induced cytotoxicity and apoptosis in differentiated PC12 cells

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Abstract L-glutamate plays a key role in neuronal cell death associated with many neurodegenerative conditions such as cerebral ischemia, hypoxia, Alzheimer's, Huntington's, and Parkinson's diseases. Selaginellin, a component extracted from *Saussurea pulvinata* (Hook.et Grev.) Maximo, was assessed for its ability to protect rat pheochromocytoma (PC12) cells against oxidative toxicity induced by glutamate. The differentiated PC12 cells were pretreated with various concentrations (10^{-7}, 3×10^{-7}, or 10^{-6} M) of selaginellin for 1 h prior to exposure to L-glutamate. Selaginellin was shown to protect PC12 cells against glutamate toxicity, as determined by characteristic morphological features, lactate dehydrogenase release and cell viability, and apoptosis as evaluated by Hoechst 33342 staining assay and caspase-3 activity. In addition, the increase in levels of reactive oxygen species and decrease in *klotho* gene expression induced by glutamate were significantly reversed by selaginellin. Our study suggests that selaginellin has a neuroprotective effect against L-glutamate-induced neurotoxicity through mechanisms related to anti-oxidation and anti-apoptosis via scavenging reactive oxygen species and up-regulating the expression of *klotho* gene.

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Introduction

L-glutamate, an excitatory amino acid, has been identified as the principal transmitter mediating excitatory synaptic responses and neuronal development via glutamate receptor activation in the central nervous system (CNS; Jin et al. 2002; Molnar and Isaac 2002). However, excessive excitatory transmission can be transformed into an implement of neuronal destruction resulting in CNS disorder, such as cerebral ischemia, hypoxia, alcoholism, autoimmune encephalomyelitis, Alzheimer's, Huntington's, and Parkinson's diseases (Doble 1999; van Os et al. 2006; Camins et al. 2008; Flatscher-Bader et al. 2008; Mattson 2008; Benveniste 2009; Shijie et al. 2009). Glutamate can induce cell death by two different pathways: excitotoxicity and oxytosis (Choi 1988; Murphy et al. 1989; Penugonda et al. 2005; Herrera et al. 2007). The excitotoxicity of glutamate is mediated through the activation of several types of excitatory amino acid receptors, and subsequently massive influx of extracellular Ca^{2+} (Froissard and Duval 1994; Urushitani et al. 2001; Bleich et al. 2003). The oxytosis of glutamate is mediated by competitive inhibition of cystine uptake, which leads to a marked decrease in cellular glutathion resulting in the devoid of anti-oxidant defense against oxidative stress and the activation of calcium-dependent enzymes (Murphy et al. 1990; Pereira and Oliveira 1997; Penugonda et al. 2006; Zablocka and Janusz 2008). The exponential increase of both oxidative stress and intracellular Ca^{2+} levels causes a massive neuronal cell apoptosis and necrosis (Tan et al. 2001).

Selaginella species have been used as traditional Chinese medicine for a long history. Studies have shown that the crude extract of *selaginella* species possess a variety of pharmacological activities, including enhancing immune function, reducing the levels of blood pressure and serum glucose (Miao et al. 1996), and strong anti-oxidation (Chen et al. 2005; Sah et al. 2005). Selaginellin is a component extracted from *Saussurea pulvinata* (Hook.et Grev.) Maximo (Fig. 1). Selaginellin contains hydroxy group, we therefore presumed that it would have the ability of binding free radicals. Our pilot study did show that selaginellin inhibited the lower density lipoprotein oxidation induced by 2,2′-azobis(2-amidinopropane) dihydrochloride and $CuSO_4$ (unpublished data).

In the present study, we therefore studied the protective effect of selaginellin on glutamate-induced cytotoxicity and apoptosis in differentiated PC12 cells. Because klotho, an anti-aging protein with anti-oxidative property has been shown to have anti-apoptotic function in a variety of cell types (Ikushima et al. 2006; Tang et al. 2009), we also investigated whether the anti-apoptotic effect of selaginellin on PC12 cells is related to regulation of *klotho* gene expression.

Materials and methods

Materials

PC12 cells (ATCC, CRL-1721) were obtained from the Academia Sinica (Shanghai, China). Selaginellin (purity was 99.4% by high-performance liquid chromatography and the structure was elucidated on the basis of spectral evidences) was extracted from *Saussurea pulvinata* (Hook. et Grev.) Maximo. (School of Pharmaceutical Sciences, Central South University, China). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] kit was purchased from Promega Chemical Co. (Madison, WI, USA). RPMI 1640 medium, fetal bovine serum, and horse serum were provided from Gibco BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen Corp. (Carlsbad, CA, USA). First Strand cDNA Synthesis Kit was a product of MBI Fermentas Inc. (Vilnius, Lithuania). Lactate dehydrogenase (LDH) assay kit was obtained from Jiancheng Institute of Biological Engineering (Nanjing, China). Hoechst 33342, caspase-3 activity assay kits, and reactive oxygen species (ROS) detection kit were purchased from Beyotime Biotechnology (Jiangsu, China).

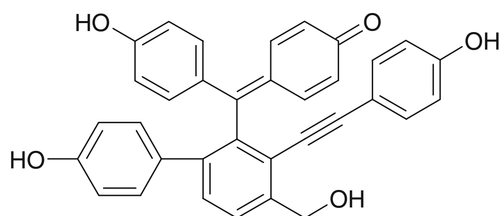

Fig. 1 Chemical structure of selaginellin

Cell culture and treatment

PC12 cells were cultured in RPMI 1640 medium supplemented with 5% (*v/v*) fetal bovine serum, 10% (*v/v*) horse serum, 100 U/ml penicillin (Gibco BRL Life Technologies), and 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged into poly-L-lysine-coated six-well culture plates at 2×10^5 cells/well and differentiated by treating with 100 ng/ml nerve growth factor for 1 week as described previously (Greene and Tischler 1976; Penugonda et al. 2005). After cultured with the serum-free medium for 24 h, the differentiated PC12 cells were pretreated with various concentrations (10^{-7}, 3×10^{-7} or 10^{-6} M) of selaginellin for 1 h prior to exposure to L-glutamate. All measurements were performed 24 h after the cells were exposed to L-glutamate (final concentration, 0, 0.01, 0.1, 1, 5, 10, and 20 mM). Control cells were not treated with selaginellin and L-glutamate. L-glutamate was dissolved in RPMI 1640 medium. Selaginellin was dissolved in DMSO. The final concentration of DMSO was less than 0.1% (*v/v*). The concentrations of selaginellin selected were according to our preliminary study. Each independent experiment was carried out at least three times.

Observation of morphologic changes

PC12 cells were seeded in a six-well culture plate at 2×10^5 cells/well and cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cellular morphology was observed by using an inverted microscope (Nikon, Japan).

LDH release assay

PC12 cells were seeded in a 96-well culture plate at 1×10^4 cells/well and LDH leakage in the conditioned medium, an indicator of cellular injury, was detected with LDH assay kit. According to the manufacturer's instructions, the reaction was initiated by mixing 40 μl of the conditioned medium with pyruvate, and then reacted with 2,4-dinitrophenylhydrazine. After reaction, the absorbance of the each sample was read at 440 nm. Data were normalized to the LDH activity released from control cells.

Determination of cell viability

PC12 cells were seeded in a 96-well culture plate at 1×10^4 cells/well and cell viability was measured by MTS assay.

After treatment of the cells with glutamate (0.01, 0.1, 1, 5, 10, and 20 mM) for 24 h, 20 μl of MTS solution was added into each well in 100 μl of culture medium and cells were incubated at 37°C for 4 h in a humidified atmosphere containing 5% CO_2. Each well was then added 25 μl of 10% sodium dodecyl sulfate to stop the reaction and sodium dodecyl sulfate-treated plate was stored in a humidified chamber at room temperature for up to 18 h protected from light. Absorbance at 490 nm was measured using a 96-well plate reader (Beckman, USA).

Apoptosis analysis

Hoechst 33342 staining assay PC12 cells were seeded in the 12-well plates at 1×10^5 cells/well and treated with 0.1 mg/ml Hoechst 33342 for 10 min at 37°C in dark. Hoechst-stained nuclei were observed by using a fluorescence microscope (Olympus, Japan) at 521 nm of emission wavelength. A total of 200 cells from five random high power fields were counted. The percent of apoptosis was expressed as ratio of apoptotic cells to total cells.

Measurement of caspase-3 activity PC12 cells were seeded in the 12-well plates at 1×10^5 cells/well and the activity of caspase-3 like protease in the lysate was measured using a colorimetric caspase-3 assay (Jiang et al. 2006). The caspase-3 activity was expressed by value of OD_{405}.

Measurement of intracellular ROS

The intracellular ROS production was measured by the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate to fluorescent dichlorofluorescein. Culture medium was removed and cells seeded in six-well plates at 2×10^5 cells/well were washed with phosphate-buffered saline three times. 2′,7′-dichlorofluorescein diacetate, diluted to a final concentration of 10 μM with RPMI1640, was added and incubated in the dark at 37°C for 20 min. Then the relative levels of fluorescence were quantified in fluorospectro-photometer (F4000, Japan) at an excitation wavelength at 488 nm and an emission wavelength at 525 nm.

Quantitative real-time PCR analyses

Total RNA was extracted from PC12 cells using the TRIzol Reagent (Invitrogen) and aliquote of 1 μg RNA from each sample was reverse-transcribed using reverse transcription system (Promega, Madison, WI, USA).

Quantitative analysis of mRNA expression was performed by using the ABI 7300 real-time PCR system with the Power SYBR Green PCR Master Mix kit (Applied Biosystems, Warrington, UK). Generated cDNA was amplified by quantitative real-time PCR with specific forward and reverse primers for *klotho* (sense: 5′-CGAAG GATGACCGACTATCACA-3′; anti-sense: 5′-CCAGCC TAGCACGAAGTCAAG-3) and *GAPDH* (sense: 5′-TGGCCTCCAAGGAGTAAGAAAC-3′; anti-sense: 5′-GGCCTCTCTCTTGCTCTCAGTATC-3′). A 25-μL reaction mixture containing 1 μL cDNA template, 12.5 μL SYBR Master mix, and 0.40 μL of each primer (10 mM) was amplified using the following thermal parameters: denaturing at 95°C for 10 min and followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. All amplification reaction for each sample was carried out in triplicates and the averages of the threshold cycles were used to interpolate curves using 7300 System SDS Software. Results were expressed as the percentage of *klotho* mRNA normalized to *GAPDH* mRNA.

Statistical analysis

Data were expressed as mean ± SEM and were analyzed by ANOVA followed by the Student–Newman–Keuls test. A P value less than 0.05 was considered to be statistically significant.

Results

Differentiation of PC12 cells

In keeping with previous study (Greene and Tischler 1976; Penugonda et al. 2005), treatment of PC12 cells with 100 ng/ml nerve growth factor for 1 week successfully induced differentiation as shown by round cell bodies with fine dendritic networks (Fig. 2).

Cytotoxicity of L-glutamate

MTS assay showed that the cell viability was inhibited by glutamate in a concentration-dependent manner (Fig. 3). Glutamate at 10 mM led to about 45% inhibition of cell viability. We therefore chose 10 mM of glutamate for the subsequent experiments.

Effects of selaginellin on L-glutamate-induced cell injury

Under a phase-contrast microscopy, we found that normal differentiated PC12 cells showed round cell bodies with fine dendritic networks, and the cell edges were intact and clear (Fig. 4a). In contrast, incubation of cells with 10 mM of L-glutamate for 24 h induced shrinkage of the cell bodies, disappearance in cell reticular formation, and

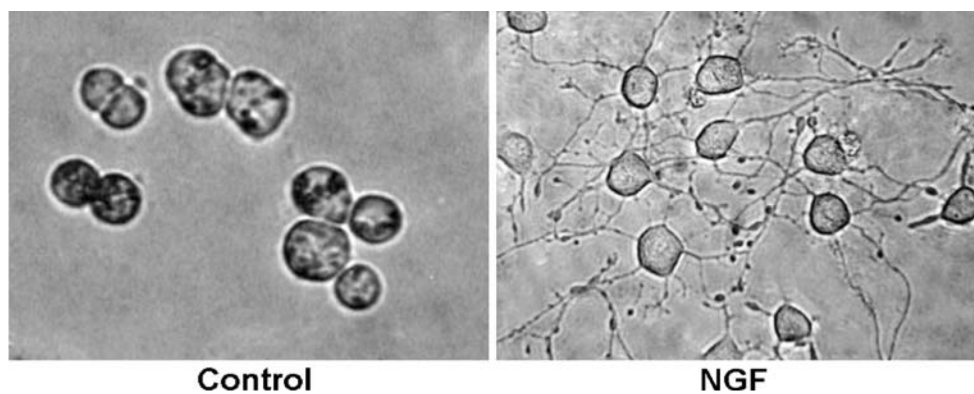

Fig. 2 Differentiation of PC12 cells induced by nerve growth factor (*NGF*). Cells were cultured with NGF 100 ng/ml for 1 week. Differentiated cells showed round cell bodies with fine dendritic networks like neurite outgrowth which resembled neuronal cells. Photographs were taken with an inverted microscope at ×400 magnification. The results are representative of three independent experiments

disruption of the dendritic networks. Pretreatment with selaginellin dramatically alleviated morphological manifestations of cell damage in a concentration-dependent manner.

In keeping with morphological manifestations of cell damage, glutamate also significantly increased the release of LDH and decreased the cell viability (Fig. 4b and c). These effects of glutamate were reversed by selaginellin in a concentration-dependent manner. DMSO, 0.1%, alone had no effect on the release of LDH and the cell viability in PC12 cells. Selaginellin itself had no effect on the viability of PC12 cells in absent of glutamate (data not shown).

Effect of selaginellin on apoptosis of PC12 Cells

Hoechst 33342 staining assay showed that treatment with glutamate (10 mM) for 24 h significantly increased the ratio of cells with a profile of cell shrinkage, chromatin condensation, and fragmented fluorescent nuclei (Fig. 5a and b). The caspase-3 activity, a marker of apoptosis, was also found to be increased in glutamate-treated cells (Fig. 5c). The number of Hoechst 33342-positive cells and the activity of caspase 3 induced by glutamate were significantly reduced by selaginellin in a concentration-dependent manner. DMSO, 0.1%, alone had no effect on apoptosis of PC12 cells.

Effects of selaginellin on L-glutamate-induced ROS accumulation

It has been documented that glutamate-induced cell injury involves oxidative stress (Kume et al. 2004; Li et al. 2007). Our pilot study also found that selaginellin had anti-oxidant effect (unpublished data). We therefore measured intracellular ROS production in PC12 cells. Glutamate increased intracellular ROS generation as indicated by the increase in 2′,7′-dichlorofluorescein fluorescence (Fig. 6). Selaginellin significantly reduced the increase in ROS generation induced by glutamate in a concentration-dependent manner. DMSO, 0.1%, alone had no effect on ROS generation in PC12 cells.

Effects of selaginellin on downregulation of *klotho* mRNA expression induced by glutamate

It has been shown that *klotho* gene has anti-apoptotic function in a variety of cell types (Ikushima et al. 2006; Tang et al. 2009). To explore the role of *klotho* gene in glutamate-induced cell apoptosis, the expression of *klotho* mRNA was determined. The results showed that incubation of PC12 cells with glutamate (10 mM) for 24 h led to a significant decrease in *klotho* mRNA expression. In contrast, the decreased mRNA expression of *klotho* was reversed by pretreatment with selaginellin in a concentration-dependent manner (Fig. 7). DMSO, 0.1%, alone had no effect on *klotho* mRNA expression in PC12 cells.

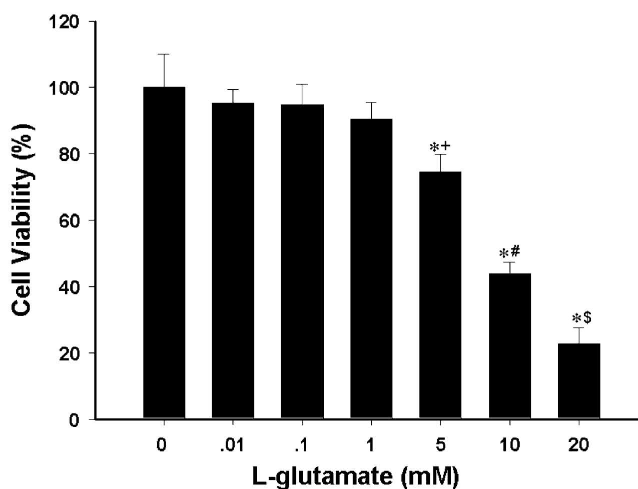

Fig. 3 Effect of glutamate on viability of PC12 cells. MTS assay showed that glutamate decreased cell viability in a concentration-dependent manner. Data are summarized from three independent experiments. *$P<0.05$, compared with control in the absence of glutamate; $^{+}P<0.05$, compared with glutamine (1 mM); $^{\#}P<0.05$, compared with glutamine (5 mM); $^{\$}P<0.05$, compared with glutamine (10 mM)

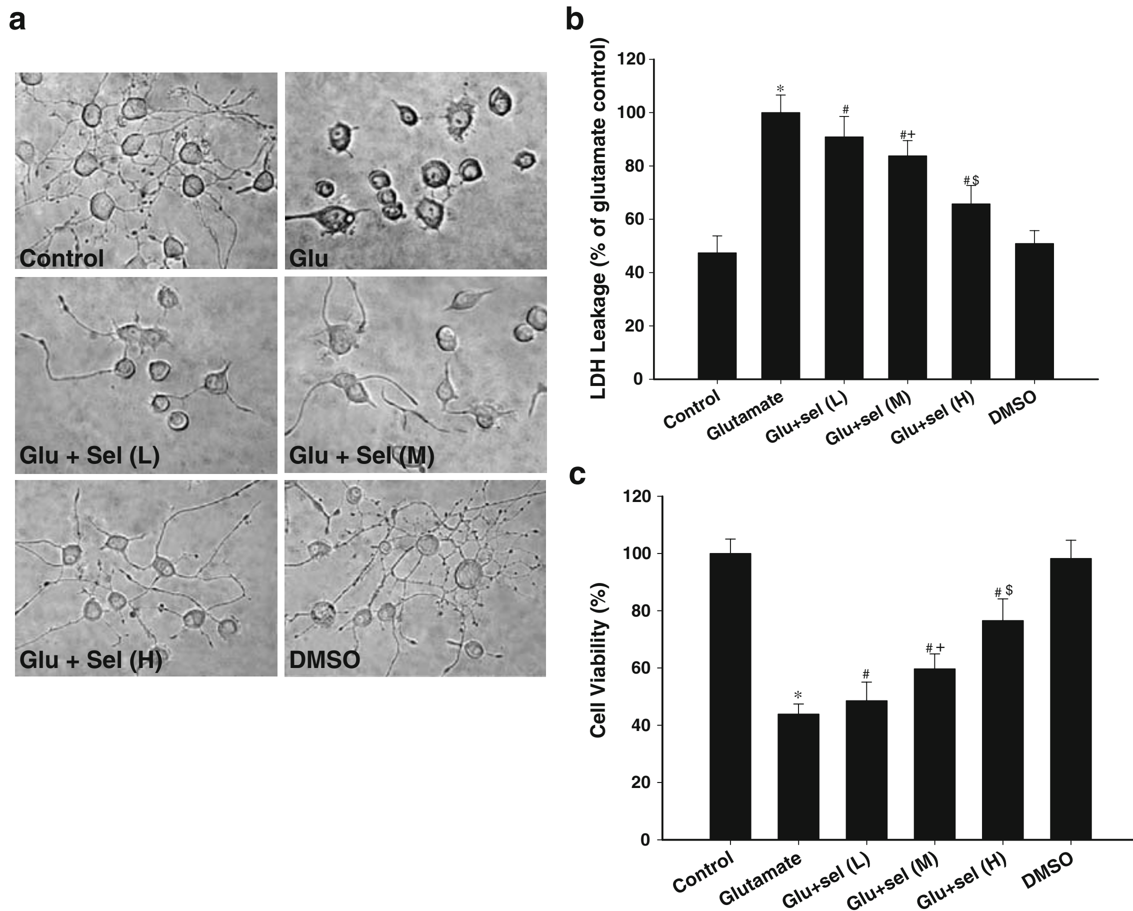

Fig. 4 Effect of selaginellin on cell injury induced by L-glutamate. **a** Changes in morphology. **b** LDH leakage. **c** Cell viability. Incubation of cells with 10 mM of L-glutamate for 24 h induced shrinkage of the cell bodies, disappearance in cell reticular formation, and disruption of the dendritic networks concomitantly with the increased LDH leakage and the decreased cell viability. Selaginellin reversed all these changes in a concentration-dependent manner. Data are summarized from four independent experiments. $^{*}P<0.05$, compared with control; $^{\#}P<0.05$, compared with glutamate; $^{+}P<0.05$, compared with Glu + sel (L); $^{\$}P<0.05$, compared with Glu + sel (M). Glu:L-glutamate; Sel (L): selaginellin (10^{-7} M); Sel (M): selaginellin (3×10^{-7} M); Sel (H): selaginellin (10^{-6} M)

Discussion

In the present study, we investigated the protective effects of selaginellin on glutamate-induced injury and apoptosis in differentiated PC12 cells. The results demonstrated that pretreatment with selaginellin inhibited glutamate-induced cell injury and apoptosis in a concentration-dependent manner which was related to inhibition of intracellular ROS generation and up-regulation of *klotho* mRNA expression.

Glutamate, a major excitatory neurotransmitter, plays an important role in synaptic transmission, formation of neuronal circuit, and neuronal development in the nervous system (Walton and Dodd 2007). Elevated level of glutamate due to excess release or/and uptake disorder have been shown to induce extensive neuronal damage and cell loss in brain tissue. Glutamate cytotoxicity has been associated with the activation of glutamate receptors and non-receptor-mediated oxidative glutamate toxicity. It has been shown that cell death via excessive activation of glutamate receptors generally occurs within a shorter time span following exposure of lower concentration of glutamate (Bonfoco et al. 1995; Cheung et al. 1998; Seyfried et al. 2000).

Differentiated PC12 cell, a sympathetic nerve cell line derived from rat pheochromocytoma, has been widely used for neurological studies (Schubert et al. 1992; Froissard and Duval 1994). Although PC12 cells express *N*-methyl-D-aspartate (NMDA) receptors, cell death exhibited by exposure high concentration of glutamate for a longer time

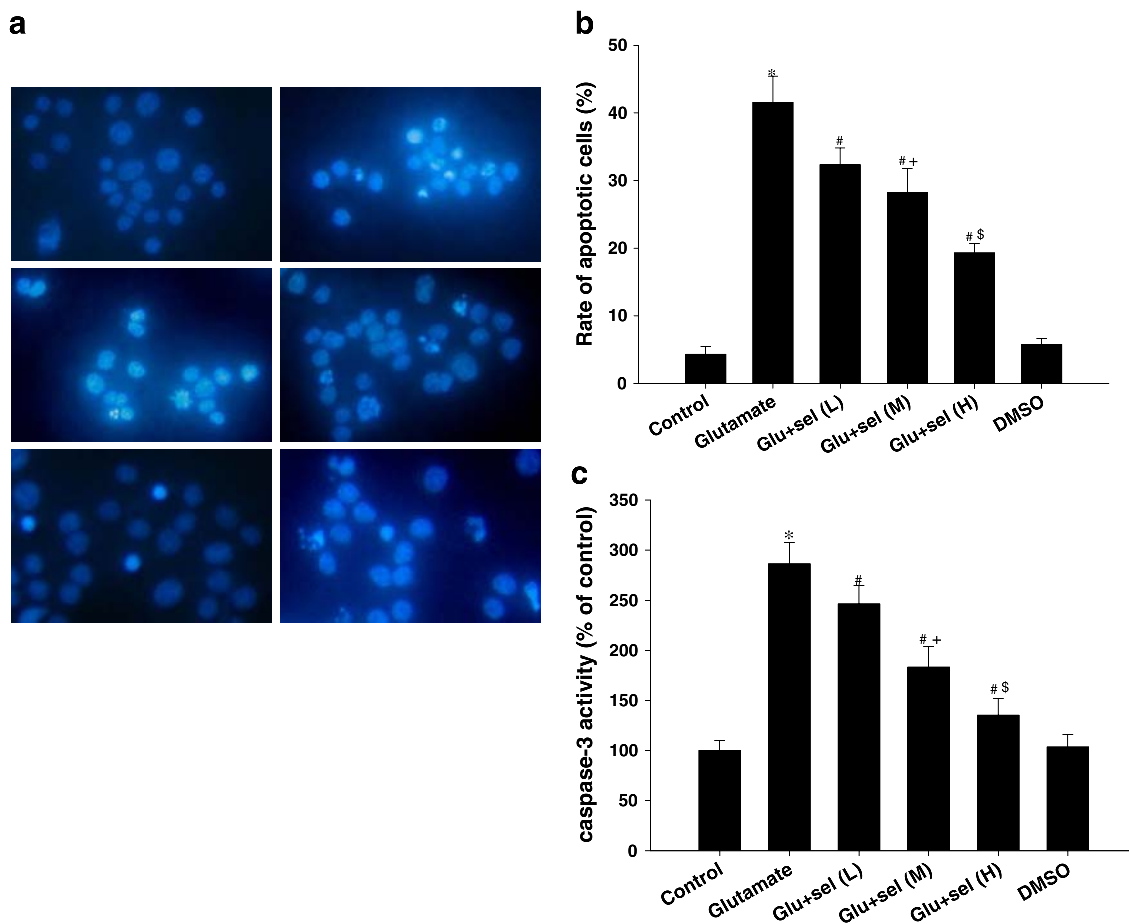

Fig. 5 Effect of selaginellin on apoptosis of PC12 cells induced by glutamate. **a** Fluorescence photomicrographs of PC12 cells with Hoechst 33342 staining. **b** Summarized data of Hoechst 33342-positive cells. **c** Caspase-3 activity. The number of Hoechst 33342-positive cells and the activity of caspase 3 induced by glutamate were significantly reduced by selaginellin in a concentration-dependent manner. Data are summarized from four independent experiments. *P<0.05, compared with control; $^{\#}P$<0.05, compared with glutamate; ^{+}P<0.05, compared with Glu + sel (L); $^{\$}P$<0.05, compared with Glu+sel (M). Glu:glutamate; Sel (L): selaginellin (10^{-7} M); Sel (M): selaginellin (3×10^{-7} M); Sel (H): selaginellin (10^{-6} M)

span does not solely relate to NMDA receptors (Schubert et al. 1992; Froissard and Duval 1994; Seyfried et al. 2000). In addition, 6,7-dinitroquinoxaline-2,3-dione, a known antagonist of non-NMDA receptors, is able to significantly decrease the glutamate-induced cell death (Froissard and Duval 1994). In the present study, we found that treatment of differentiated PC12 cells with L-glutamate (10 mM) for 24 h induced cell injury and apoptosis via promoting intracellular ROS generation. Taken together, we presumed that the cell death induced by glutamate may be not mediated by NMDA receptors, but mainly by reactive oxygen intermediates.

Glutamate-induced mismatched redox equilibrium has been widely accepted as the leading mechanism of neuronal disorder (Coyle and Puttfarcken 1993; Kume et al. 2004; Parfenova et al. 2006). Oxidative stress occurs when the production of oxygen free radicals increases and overrides the ability of the endogenous anti-oxidant system to scavenge free radicals (Chong et al. 2005). It has been well documented that anti-oxidants superoxide dismutase, catalase, and ubiquinone protect nerve cell injury from glutamate toxicity (Schwartz et al. 1998; Sandhu et al. 2003; Wang et al. 2003). Ezaki et al. have also demonstrated that Vitamin E prevents glutamate-induced neuronal cell death (Ezaki et al. 2005). Godkar et al have reported *Celastrus paniculatus* seed oil via anti-oxidation attenuates glutamate-induced injury in embryonic rat forebrain neuronal cells (Godkar et al. 2006). The results of the present study demonstrated that pretreatment of PC12 cells with selaginellin dramatically protected against L-glutamate-

induced cell injury and apoptosis via inhibition of intracellular ROS generation. Our pilot study showed that *selaginellin* had the ability to remove free radicals induced by 2,2′-azobis(2-amidinopropane) dihydrochloride and $CuSO_4$. These findings suggest that selaginellin via its anti-oxidant property protects against glutamate-induced oxidative stress injury. However, further investigation deserves to be done to rule out whether selaginellin affects the NMDA receptors.

The *klotho* gene expressed predominantly in the kidney and brain functions as an aging-suppressor gene that extends life span. The abnormal regulation of *klotho* gene exhibits multiple disorders resembling human premature-aging syndrome, including a shortened lifespan, infertility, arteriosclerosis, mitral annular calcification, skin atrophy, osteoporosis, and emphysema (Kuro-o et al. 1997). It has been reported that purified recombinant Klotho protein acts as a humoral factor to reduce H_2O_2-induced apoptosis in endothelial cells (Ikushima et al. 2006). Klotho protein also reduces apoptosis in the kidney subjected to ischemia-reperfusion injury (Sugiura et al. 2005). *Klotho* gene, also as a primary factors, contributes to aging changes in brain white matter of the rhesus monkey (Duce et al. 2008). Another report showed that a-tocopherol, an anti-oxidant, decreases the number of apoptotic cells in the *klotho* mutant mouse hippocampus (Nagai et al. 2003). Therefore, Klotho protein may be involved in the regulation of anti-oxidative defense (Nagai et al. 2003). In our present study, we firstly demonstrated that L-glutamate down-regulated the expression of *klotho* mRNA gene in differentiated PC12 cells concomitantly with cell injury and apoptosis, and that selaginellin protected against L-glutamate-induced cell injury and apoptosis by up-regulation expression of *klotho* mRNA gene.

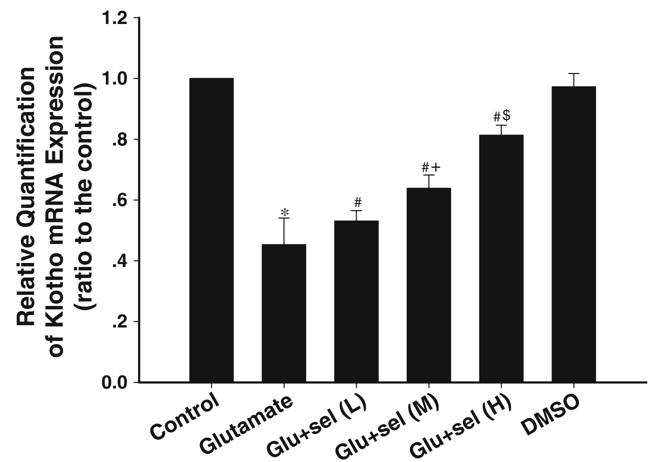

Fig. 7 Effects of selaginellin on downregulation of *klotho* mRNA expression induced by glutamate. *Klotho* mRNA expression was analyzed by real-time PCR. Selaginellin upregulated the decrease in mRNA expression of *klotho* induced by glutamate in a concentration-dependent manner. Data are summarized from six independent experiments. $^*P<0.05$, compared with control; $^{\#}P<0.05$, compared with glutamate; $^{+}P<0.05$, compared with Glu + sel (L); $^{\$}P<0.05$, compared with Glu + sel (M). Glu:glutamate; Sel (L): selaginellin (10^{-7} M); Sel (M): selaginellin (3×10^{-7} M); Sel (H): selaginellin (10^{-6} M)

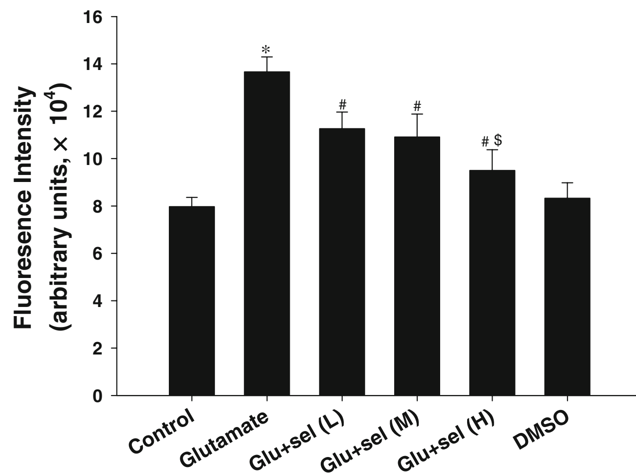

Fig. 6 Effects of selaginellin on glutamate-induced intracellular accumulation of ROS. Selaginellin significantly reduced the increase in ROS generation induced by glutamate as indicated by 2′,7′-dichlorofluorescein fluorescence. Data are summarized from three independent experiments. $^*P<0.05$, compared with control; $^{\#}P<0.05$, compared with glutamate; $^{\$}P<0.05$, compared with Glu+sel (M). Glu: glutamate; Sel (L): selaginellin (10^{-7} M); Sel (M): selaginellin (3×10^{-7} M); Sel (H): selaginellin (10^{-6} M)

Substantial evidence has demonstrated that the anti-apoptotic property of *klotho* gene is associated with suppression of oxidative stress (Saito et al. 2006; Kuro-o 2008). *Klotho* suppresses insulin/IGF-1 signaling, which negatively regulates Fork-head box O (FOXO; Yamamoto et al. 2005). The FOXO family is closely related to anti-apoptosis. In the nucleus, FOXO can upregulate anti-oxidant enzymes, including catalase and mitochondrial manganese superoxide dismutase, which remove ROS and reduce oxidative stress (Kampkotter et al. 2008; Olmos et al. 2009). Besides the direct anti-oxidation property, whether selaginellin has indirect anti-oxidation via up-regulation expression of *klotho* mRNA gene needs to be further investigated.

In summary, the present study demonstrated that selaginellin protected against L-glutamate cell injury and apoptosis in differentiated PC12 cells through regulation of ROS/*klotho* gene pathway. Selaginellin may be a potential neuroprotective compound, and the further study is needed to define its effect in in vivo experiments with CNS disorder.

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