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Full Length Research Paper

Antioxidant and neuroprotective effect of PSE-1 against oxidative stress-induced cytotoxicity in N18-RE-105 cells

Hyun-Jung Kim[1], Eun-Ah Lee[1], Bo-Young Seo[2], Ae-Ran Choi[1], Seong-Hee Moon[1], Sung-Ku Lee[1], Eunju Park[2], Hae-Ryong Park[1], Jum-Soon Kang[3] and Yong-Il Hwang[1]*

[1]Department of Food Science and Biotechnology, Kyungnam University, Changwon 631-701, Korea.
[2]Department of Food and Nutrition, Kyungnam University, Changwon 631-701, Korea.
[3]Department of Horticultural Bioscience, Pusan National University, Miryang, 627-706, Korea.

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Neurodegenerative conditions such as the Alzheimer and Parkinson diseases, are associated with the production of reactive oxygen species and resultant oxidative stress. Glutamate is the major excitatory neurotransmitter of the central nervous system and may induce cytotoxicity through persistent activation of glutamate receptors and through oxidative stress mechanisms. On the basis of this information, we established a screening system using N18-RE-105 cells to identify therapeutic agents that can protect cells from glutamate toxicity. During the course of our screening program, we recently isolated an active compound, 8,13-dihydroxy-9,11-octadecadienoic acid (PSE-1), from peanut sprouts, which prevents glutamate-induced cell death. The chemical structure of PSE-1 was identified using spectroscopic methods and by comparison with the value in the literature. The antioxidant and neuroprotective effects of PSE-1 were evaluated using the oxygen radical absorbance capacity assay, Comet assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide reduction assay, the lactate dehydrogenase release assay, a morphological assay and Hoechst 33342 staining. The results of the assays demonstrate that PSE-1 has neuroprotective effects and that PSE-1 could be a new potential chemotherapeutic agent against neuronal diseases.

Key words: Glutamate, PSE-1, peanut sprout, antioxidant, neuroprotective, N18-RE-105 cells.

INTRODUCTION

With life expectancies increasing around the world, populations are aging and neurodegenerative diseases have become a global issue. There is an increasing amount of experimental evidence that oxidative stress is a causal, or at least an ancillary, factor in the neuro-pathological mechanism of several adult neurode-generative disorders, as well as in stroke, trauma and seizures (Gołembiowska and Dziubina, 2011; Leyen et al., 2005; Gardner et al., 1997). Oxidative stress-induced cell apoptosis which is involved in the pathogenesis of neurodegenerative disorders, such as stroke, Alzheimer disease and Parkinson disease, (Behl, 1999; Canals et al., 2003; Olanow and Tatton, 1999) through the production of reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, which mediate oxidative stress, readily damage biological molecules and ultimately lead to apoptotic or necrotic cell death (Hou et al., 2003; Fridovich, 1983; Zhang et al., 2007).

Oxidative stress injury is a leading mechanism of glutamate neurotoxicity. Glutamate is the major excitatory neurotransmitter in the central nervous system, playing an important role in neuronal development, synaptic plasticity, learning and memory processes under physiological conditions (Murphy et al., 1990). Glutamate is stored in synaptic vesicles after active transport by vesicular glutamate transporters and when exocytosed, it

*Corresponding author. E-mail: yihwang@kyungnam.ac.kr. Tel: +82-55 249 2685. Fax: +82 0505 999 2171.

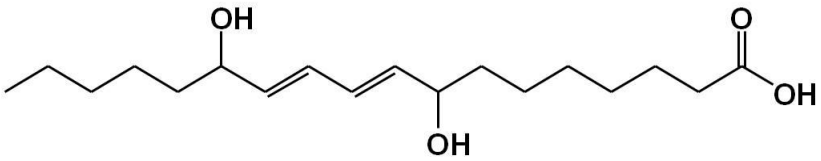

Figure 1. Structure of PSE-1.

binds to a variety of glutamate receptors, leading to glutamatergic neurotransmission (Murphy et al., 1898; Stanciu et al., 2000; Murphy et al., 1898; Fei et al., 2006). However, large amounts of glutamate release intersynaptic spaces which can cause neuronal cell death and neurodegeneration via excitotoxicity processes. Glutamate can induce cell death by two different pathways (Monaghan et al., 1989): excitotoxicity and oxytosis. Excitotoxicity is triggered by the over-activation of glutamate ionotropic receptors and the consequent massive influx of extracellular calcium (Ca^{2+}) (Hwang et al., 2002; Colwell and Levine, 1999), whereas oxytosis is triggered by the blockade of the cystine/glutamate antiporter, which causes the progressive depletion of the cellular antioxidant, glutathione. It is well established that glutamate-induced cell death is related to oxidative stress and the potential activation of the mitogen-activated protein kinase pathway (Tortarolo et al., 2003; Greenwood and Connolly, 2007).

Oxidative stress has long been involved in the etiology and progression of many neurodegenerative diseases. Currently, there is no effective cure for neurodegenerative diseases. Many synthetic chemicals such as phenolic compounds are powerful radical scavengers, but they commonly cause severe side effects (Yoon et al., 2007; Lee et al., 2008). Therefore, research about the neurodegenerative disease focus more on modulating or emulating the protective effects that regulate oxidative stress, with the aim of developing an ideal drugs or genetic therapies (Rao and Balachandran, 2002; Sayre et al., 2008). Thus, we established a screening system to discover therapeutic agents that can protect cells from glutamate-induced cytotoxicity. Using this system, we isolated an active compound, 8,13-dihydroxy-9,11-octadecadienoic acid (PSE-1), from peanut sprouts, which can protect N18-RE-105 cells against glutamate-induced cytotoxicity. The purpose of this study was to assess the neuroprotective effects of PSE-1 against glutamate-induced oxidative stress on neurons, which has not been previously assessed.

MATERIALS AND METHODS

The peanut sprouts were obtained from the Busan National University horticultural bioscience (Busan, Korea). PSE-1, isolated from peanut sprouts, was prepared as a stock solution in 20 mM dimethyl sulfoxide and stored at -20°C. Histopaque 1077, glutamate and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were provided by Sigma chemical company (St. Louis, MO). Lactate dehydrogenase (LDH) release assay kit was purchased by Wako pure chemical industries, Ltd. (Osaka, Japan). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS) and HAT supplement were obtained from Gibco-BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Purification of PSE-1 from peanut sprouts

The potential neuroprotective compound PSE-1 was isolated and purified from peanut sprouts as follows: dried peanut sprouts powder (1 kg) was percolated overnight with methanol (10 L), and then the extract was concentrated under a vacuum to eliminate the methanol. The resulting aqueous solution was extracted with diethyl ether. The organic layer was concentrated, applied to a silica gel column and eluted with chloroform-methanol (100:1 to 1:1 [v/v]). Finally, pure PSE-1 was obtained by reverse-phase column chromatography and eluted with 70% methanol to give a dark-yellow matter (9.8 mg). Using liquid chromatography and mass spectrometry, the molecular formula of PSE-1 was found to be $C_{18}H_{32}O_4$. The structure of PSE-1 was determined from its physicochemical properties and with various nuclear magnetic resonance spectroscopic methods. PSE-1 was determined by examining previously published studies and found to have an 8,13-dihydroxy-9,11-octadecadienoic acid skeleton (Hamberg, 1983) as an aliphatic compound, which had previously been reported as a prototypical compound (Figure 1).

Oxygen radical absorbance activity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay was carried out on a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm and emission wavelength: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with either 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, 20 mM) as a peroxyl radical generator in peroxyl radical-scavenging capacity ($ORAC_{ROO.}$) assay. Trolox (1 µM) was used as a control standard and prepared fresh daily. The analyser was programmed to record the fluorescence every 2 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. $ORAC_{ROO}$ was expressed as micromoles of Trolox equivalents (TE). One ORAC unit is equivalent to the net protection area provided by 1 µM Trolox.

Preparation of human leukocytes

Blood samples were obtained from two healthy male volunteers. 5

ml of fresh whole blood was added to 5 ml of phosphorous buffered saline (PBS) and layered onto 5 ml of Histopaque 1077. After centrifugation for 30 min at 400 × g at room temperature, the leukocytes were collected from just above the boundary with the Histopaque 1077 and washed with 5 ml PBS. Finally, they were freshly used for Comet assay.

Treatment of human lymphocytes with PSE-1

Leukocytes (2×10^4 cell/ml) were incubated with various concentration of PSE-1 (0, 1, 5, 10 and 50 μM) for 30 min at 37°C in a dark incubator and then resuspended in PBS with 200 μM H_2O_2 for 5 min on ice. After each treatment, samples were centrifuged at 1450 rpm for 5 min and washed with PBS. All the experiments were repeated twice with lymphocytes from each of the two donors on the separate days.

Determination of DNA damage (Comet assay)

The cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 75 μl of 0.5% LMA, and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4°C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for deoxyribonucleic acid (DNA) unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C and then treated with ethanol for another 5 min before staining with 50 μl of ethidium bromide (20 μg/ml). Measurements were made by image analysis (kinetic imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides). Cell viability, as measured by trypan blue exclusion, was above 95% for all treatments.

Cell culture and treatments

Hybridoma N18-RE-105 cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). The cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS), 1 × HAT supplement, penicillin (100 U/ml), streptomycin (100 μg/ml), and 3.7 mg/ml $NaHCO_3$ in a humidified incubator set at 37°C and 5% CO_2. PSE-1 was added to the cell culture medium such that the DMSO made up less than 0.5% of the total volume of the culture.

MTT reduction assay for cell viability

Cell viability was measured with blue formazan that was metabolized from colorless tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide) (MTT) by mitochondrial dehydrogenase, which are active only in live cells. N18-RE-105 cells were preincubated in 96-well plates at a density of 5×10^4 cells/ml for 24 h. Cells with various concentrations of PSE-1 were treated with glutamate of 20 mM for 24 h. After incubation, MTT reagent (5 mg/ml) was added to each of the wells and the plate was incubated for an additional about 1 h at 37°C. The intracellular formazan product was dissolved in 100 μl of dimethyl sulfoxide (DMSO). For MTT assay, the optical density (OD) of each well was then measured at 540 nm wavelength using an ELISA reader (Model 680, BioRad, Hercules, CA, USA). OD values from untreated control cells were designated 100% as a standard.

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). An LDH release assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). N18-RE-105 cells were seeded in 96-well plates (5×10^4 cells/ml), cultured overnight in the presence of glutamate and treated with various concentrations of PSE-1 extracts for 24 h, as described. The LDH assay reaction was initiated in a 96 well plate by mixing 50 μl of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μl. A colorimetric assay was performed, in which the amount of formazan salt was proportional to the level of LDH activity in the sample. The intensity of the resultant red color measured at 540 nm was therefore proportional to LDH activity.

Morphological analysis

To observe the effects of PSE-1 on glutamate-induced morphological alteration, N18-RE-105 cells in DMEM containing 10% FSB and 5% HS were seeded into 6-well plates (5×10^4 cells/well) and incubated with various concentrations of PSE-1. After incubation for 30 min, cells were treated with 20 mM glutamate for 24 h. The cellular morphology was photographed under a phase-contrast microscope (Nikon, Tochigi, Japan). The photographs were taken at a magnification × 100.

Hoechst 33342 staining assay

The fluorescent dye hoechst 33342 was used to detect DNA condensation, nuclear fragmentation, and characteristic features of apoptotic cells. N18-RE-105 cells (5×10^4 cells/well in 6-well plate) were seeded for 24 h. Cells were treated with various concentration of PSE-1, and then incubated at 37°C with 30 min. The cells were treated with 20 mM glutamate for 24 h with or without pretreatment with PSE-1, and then washed with PBS. The cells were fixed in PBS containing 10% formaldehyde for 4 h at room temperature. After twice rinsed with PBS, the cells were stained with hoechst 33342 for 30 min at room temperature. Subsequently, the cells were observed under a fluorescence microscope (Nikon).

Statistical analysis

All data are expressed as the means of three determinations ± SE and was analyzed using the SPSS package for Windows (Version 11.5). The mean values of the Trolox equivalents and DNA damage (tail intensity) from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's test. *P*-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Antioxidant activity of PSE-1

Oxygen radical absorbance capacity (ORAC) has been recently accepted as a standard method to analyze the

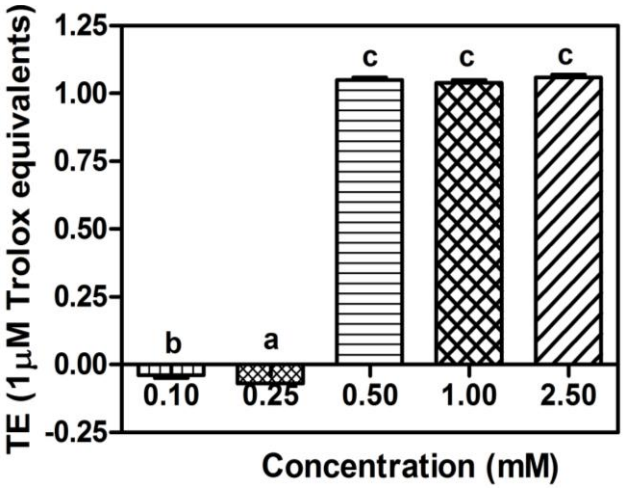

Figure 2. The effect of peroxyl radical scavenging activities (Trolox equivalent, M) of PSE-1 different concentrations *in vitro* system. The oxygen radical absorbance capacity (ORAC) value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 M. The area under the curve for the sample is compared to the area under the curve for Trolox, and the anti-oxidative value is expressed in micromoles of Trolox equivalent per liter. The results represent the mean ± SE. of values obtained from three measurements. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's multiple range test.

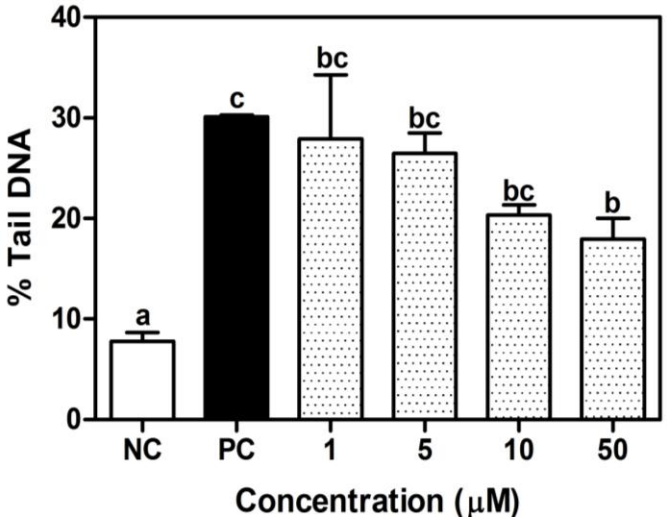

Figure 3. The effect of supplementation *in vitro* with different concentrations of PSE-1 on 200 μM H_2O_2 induced DNA damage in human leukocytes. NC, 1% DMSO (without oxidative stimulus) treated negative control; PC, 200 μM H_2O_2 treated positive control. The results represent the mean ± SE. of values obtained from three measurements. Different corresponding letters indicate significant differences at $p < 0.05$ by Duncan's multiple range test.

antioxidant potential of active substances of plants (Kurihara et al., 2004). This method involves the completion of free radical activities as a means of quantization and combines the extent of inhibition and length of inhibition time of free radical action by antioxidants into a single quantity (Cao et al., 1997). The antioxidant capacity of PSE-1 measured using the ORAC assay is shown in Figure 2. The peroxyl radical-scavenging activities were 1.04, 1.06 and 1.05 μM Trolox equivalents at a PSE-1 concentration of 0.5, 1 and 2.5 mM. The PSE-1 reducing potentials and metal chelating activity is identical with that of the 1 μM Trolox activity. Next, the genomic damage was also assessed using, the Comet assay. The genomic damage evaluated by the Comet assay is directly related to fragment size, single-strand breaks and alkali-labile sites (Collins et al., 1996). H_2O_2 a primary precursor for the generation of hydroxyl radicals, can have profoundly deleterious effects on cells through base modifications and strand breakage in the cell nucleus and genomic DNA (Deutsch, 1998).

The genotoxic effects of H_2O_2 and the protective ability of PSE-1 were assessed in normal human leukocytes by Comet assay (Figure 3). The presence of PSE-1 prevented H_2O_2 induced DNA damage dose dependently in human leukocytes. The maximum inhibition of H_2O_2-induced DNA damage was 44%, which occurred at the PSE-1 concentration of 50 μM, which significantly reduced the maximum inhibition of H_2O_2-induced DNA damage in the human leukocytes compared the H_2O_2-treated positive control. The possible mechanism by which PSE-1 inhibited oxidative DNA damage in human leukocytes can be ascribed to the keto group contained in the chemical structure of PSE-1. Hirayama et al. (1994) suggested that the singlet oxygen scavenging potency could be due to the existence of conjugated keto groups and that isolated C = C double bonds seem to have an additional effect on quenching the oxygen scavenging effects of H_2O_2 (Hirayama et al., 1994).

PSE-1 protection of N18-RE-105 cells against glutamate-induced cytotoxicity

The main effect of glutamate observed in N18-RE-105 cells seems to be cytotoxicity. In the N18-RE-105 cells, the toxic effects of glutamate are thought to be the inhibition of the glutamate-cystine antiporter and consequent depletion of cellular cystine and its metabolic product glutathione. Glutathione, an important free radical scavenger, is required to protect cells from injury and its depletion could cause oxidative stress and induce cell death either by apoptosis or necrosis, especially in organs such as the brain. To determine the effects of PSE-1 on neuronal protection, the MTT reduction and cytoplasmic LDH release assays were used. As shown in Figure 4A, the N18-RE-105 cells treated for 24 h with 20 mM glutamate had a reduction icell viability of 55.1%

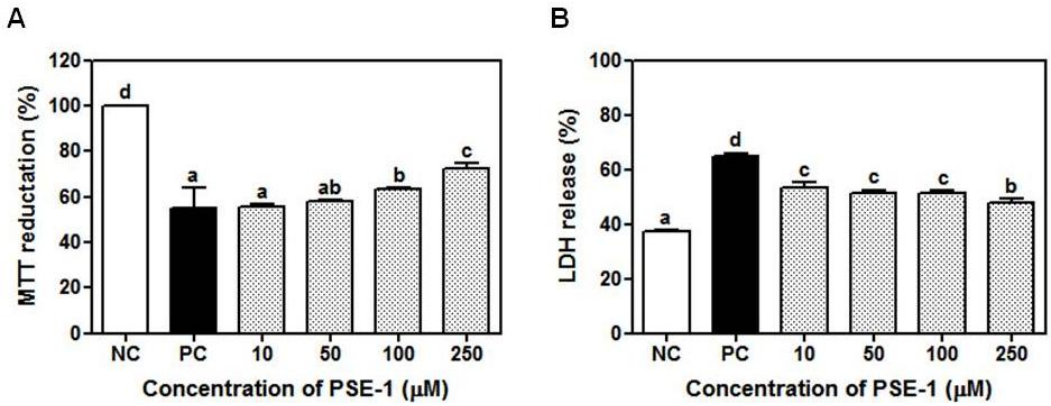

Figure 4. Effect of PSE-1 on glutamate-induced cytotoxicity in N18-RE-105 cells. (A) MTT reduction assay. (B) LDH release assay. Values are mean with standard error of triplicate experiments. Cells were pretreated with the indicated concentration of PSE-1 for 30 min, and then further treated with 20 mM glutamate for 24 h. The MTT reduction rate was the difference in absorbance values between the treated and control wells divided by the control absorbance. The LDH release rate was presented as percentage of control values. As the results of LDH release assay, data were normalized to the activity of LDH released from vehicle-treated cells (100%) and expressed as percentage of the control (obtained from separate plating).

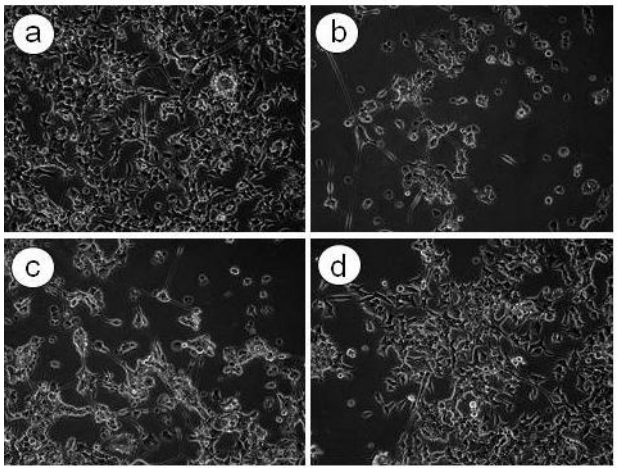

Figure 5. Effects of PSE-1 on glutamate-induced morphologic changes in N18-RE-105. (a) Control; (b) N18-RE-105 cells exposed to 20 mM of glutamate for 24 h; (c) N18-RE-105 cells treated with 100 μM PSE-1 for 30 min before exposure to 20 mM of glutamate for 24 h; (d) N18-RE-105 cells treated with 250 μM PSE-1 for 30 min before exposure to 20 mM of glutamate for 24 h. Photographs were taken with a phase-contrast microscope at magnification × 100 and show that glutamate treatment decreased the number of viable cells and induced shrinkage and aggregation of cell bodies, whereas PSE-1 pretreatment attenuated the effects of glutamate treatment.

compared with the controls. However, after exposure to various concentrations of PSE-1 (10, 50, 100, 250 μM) and then to 20 mM glutamate for 24 h, the viability of the glutamate-stressed N18-RE-105 cells recovered from 57.6% to 72.6% when compared with the viability of the controls. To further investigate the protective effects of PSE-1, we performed the LDH release assay as another indicator of cell toxicity. The N18-RE-105 cells were exposed to various concentrations of PSE-1 (10, 50, 100, 250 μM) and 20 mM glutamate for 24 h.

LDH release was significantly inhibited to 53.2, 51.5, 51.3, 47.6% of the control values, respectively, at each of the PSE-1 concentrations (Figure 4B). We characterized the effects of PSE-1 on the cell viability of the glutamate-stressed N18-RE-105 cells, by incubating the cells with PSE-1 and 20 mM glutamate. Morphological alterations in the cells were verified using a phase-contrast microscope. As shown in Figure 5, the control (untreated) cells had round cell bodies and fine dendritic networks, which were clearly visible under the phase-contrast microscope. However, after 24 h of exposure to 20 mM glutamate, many cell bodies showed cytoplasmic shrinkage and aggregation, either detached from each other or floating in the medium. In contrast, the N18-RE-105 cells that were treated with PSE-1 were protected against glutamate-induced cytotoxicity. These results indicate that PSE-1 offered almost complete protective action against glutamate-induced cell death and the reinstated viability level was reinstated as well as that of the control. Considering all our results, we conclude that PSE-1 was effective for neuroprotection and viability of N18-RE-105 cells.

Effects of PSE-1 on apoptosis induced by glutamate

To determine whether cell protection by PSE-1 was as a result of the inhibition of apoptosis, the N18-RE-105 cells were treated with glutamate and various concentrations

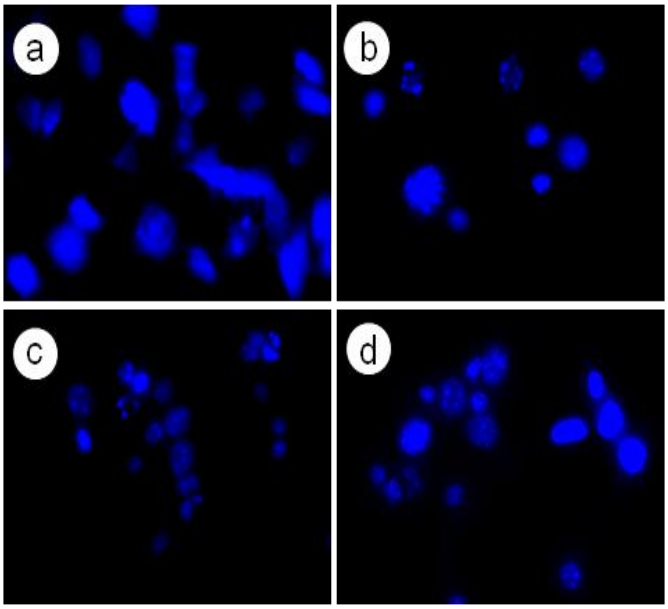

Figure 6. Inhibition of glutamate-induced apoptosis in N18-RE-105 cells by PSE-1. Cells were preincubated with PSE-1 at the indicated concentrations for 30 min. The cells were then stimulated with 20 mM of glutamate for 24 h and then used for cell staining with Hoechst 33342. (a) Control; (b) 20 mM of glutamate; (c) 20 mM glutamate + PSE-1 100 μM; (d) 20 mM glutamate + PSE-1 250 μM. The nuclear morphology of cells was examined by flourescence microscopy (magnification × 400).

of PSE-1. We found that the cells stained with Hoechst 33342 revealed chromatin condensation and apoptotic body formation when examined with a fluorescence microscope. Hoechst 33342 staining also revealed that the glutamate-treated cells exhibited highly condensed and fragmented nuclei morphologies and nuclei shrinkage, which are typical characteristics of apoptosis. In contrast, pretreatment with 100 or 250 μM PSE-1 prevented these glutamate-induced morphological alterations of the nuclei, and the number of cells with nuclear condensation and fragmentation was significantly decreased in the PSE-1-treated group compared with the glutamate control group (Figure 6). From these results, we concluded that PSE-1 has neuroprotective effects by preventing cell death and apoptosis in the N18-RE-105 cells.

Although additional research is needed to delineate the relative contribution of these pathways, our results suggest that PSE-1 may be a potential candidate for novel therapeutic agents for neuronal diseases associated with oxidative stress. In conclusion, PSE-1 was an effective antioxidant and was shown to protect N18-RE-105 cells by strongly suppressing cytotoxicity and apoptosis caused by glutamate-induced oxidative stress. Thus, we propose that PSE-1 may be used in treatment agents that could enable effective curing of the neurodegenerative diseases and prevent it from worsening.

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