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Protective effect of paeoniflorin against glutamate-induced neurotoxicity in PC12 cells via Bcl-2/Bax signal pathway

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Abstract

Paeoniflorin (PF), a monoterpene glycoside isolated from the aqueous extract of Radix Paeoniae Alba, is widely used in Traditional Chinese Medicine (TCM) for the treatment of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). In this study, we investigated the protective mechanism of PF on glutamate-induced neurotoxicity in cultured rat pheochromocytoma (PC12) cells. PC12 cells were cultured in vitro, cell viability was assessed by MTT assay, cell apoptosis as well as mitochondrial membrane potential (MMP) were detected by flow cytometric analysis and the expression profiles of apoptosis-related proteins including Bcl-2 and Bax were investigated by western blot. The results showed that PF could protect PC12 cells against glutamate-induced injury in a concentration-dependent manner and the mechanism of neuroprotective effect of PF was closely associated with up-regulation of Bcl-2 and down-regulation of Bax. It demonstrated that PF has neuroprotective effect on glutamate-induced apoptosis in PC12 cells via regulating mitochondrial membrane potential and Bcl-2/ Bax signal pathway.

Key words: *paeoniflorin, glutamate, PC12 cells, neuroprotective effect, signal pathway.*

Introduction

Traditional Chinese herbal medicine, with fewer side effects and better safety, has been widely investigated for drug development. *Radix Paeoniae alba*, the dry root of *Paeonia*, has been used for treating cerebral ischemia, epilepsy, and neurodegenerative disorders such as Alzheimer's disease [22] and Parkinson's disease [9]. Paeoniflorin (PF), a monoterpene glycoside isolated from the aqueous extract of *Radix Paeoniae alba*, has been identified as the main active ingredient responsible for the biological activities. Its beneficial effects can be found in improvement of learning and memory, analgesia, conscious-sedation and antioxidation [10,19]. Previous studies have demonstrated that PF might exert its neuroprotective effect by activating adenosine A1 receptor, ameliorating the function of the cholinergic nerve, regulating ion channel homeostasis, retarding oxidative stress and apoptosis of neurons and promoting nerve growth.

Glutamate, one of the 20-22 proteinogenic amino acids, is an important neurotransmitter that plays a key role in long-term potentiation and important for learning and memory in the central nervous system [4,16]. Under normal conditions, glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action poten-

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tial [8,18]. However, increased amounts of glutamate in the synaptic cleft can lead to neurotoxicity that has been well-established. It has been reported that plasma levels of glutamate in Alzheimer's disease patients were significantly higher than those in healthy controls and glutamate dysfunction drive to basal ganglia output nuclei is considered a likely contributor to the pathogenesis of Parkinson's disease [2].

Imbalance within the nerve cellslevels of glutamate in AD patients were significantly higher than those healthy controls. PC12 cell line is derived from a pheochromocytoma of rat adrenal medulla and can be induced to stop dividing and terminally differentiate when treated with high concentrations of glutamate [5,8,11]. This feature makes PC12 cells to be a useful model system for neuronal damage studies. Previous works reported that PF could exert a neuroprotective effect on glutamate-induced neurotoxicity in PC12 cells by inhibiting oxidative stress and Ca^{2+} overload [8]. In this study, we used different approaches to evaluate the neuroprotective effect of PF and investigated the potential mechanism.

Material and methods

Drugs and reagents

Paeoniflorin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). PC12 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Rhodamine123 (Rh123) was purchased from Sigma-Aldrich (St Louis, Mo, USA). DMEM culture medium and foetal bovine serum were purchased from Gibco (Grand Island, NY, USA). PI, RnaseA, dimethyl sulfoxide (DMSO) and ECL kit were purchased from Beyotime (Nantong, China). The information about the monoclonal antibodies used in this study shows as follows: Bax (PharMingen, CA, USA), Bcl-2 (Santa Cruz, CA, USA), β-actin (Santa Cruz, CA, USA), HRP conjugated goat anti rabbit secondary antibody and BeyoECL Plus (Beyotime, Nantong, China). All other chemicals and reagents were of analytical grade.

Cell culture and treatments

PC12 cells were cultured in flasks at 37°C under an atmosphere of 5% CO_2/95% air in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin. For the experiments, the cells were detached and re-seeded in plates. 24 h after seeding, the cells were treated with glutamate (30 mM) with the absence or presence of different concentrations of PF (0.1, 1.0 and 10 μM) for an additional 24 h.

Cell viability assay

Cell viability was measured by MTT assay as described in a reference [6]. PC12 cells (2×10^4 per well) were seeded in 96-well plates. After overnight incubation, PC12 cells were treated with glutamate (30 mM) or glutamate (30 mM) plus various concentrations of PF (0.1, 1.0 and 10 μM) for 24 h. Following incubation, 10 μl MTT (5 mg/ml) were added to each well. After 4 h at 37°C, the culture medium was removed and 100 μl DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with an ELISA reader (Model680, Bio-Rad, USA). Cell viability was expressed as a percentage of the value against the non-treated control group.

Measurement of cell apoptosis

DNA gel electrophoresis assay was used to examine the effect of cell apoptosis. PC12 cells treated with or without drugs for 24 h were harvested by centrifugation and washed twice with ice-cold PBS. The washed cells were fully re-suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% SDS) plus 0.2 μl Rnase A. After 1 h incubation at 50°C, proteinase K was added and incubated for another hour. DNA fragments in samples were separated on 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. Flow cytometric analysis was used to determine the apoptosis ratio caused by drugs. PC12 cells (2×10^5 per well) were seeded in 6-well plates and treated with or without drugs as mentioned before. Cells were harvested by centrifugation, fixed gently by adding 70% ethanol (in PBS) in 4°C overnight, before being resuspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase and 0.1% Trixon X-100 in a dark room. After 30 minutes at 37°C, the cells were analyzed by flow cytometry (Becton-Dickinson, CA, USA) with an argon ion laser at 488 nM. The data were collected by Cell Quest software (Becton-Dickinson, CA, USA).

Analysis of mitochondrial membrane potential (MMP)

For the measurement of mitochondrial membrane potential, PC12 cells (1×10^5 per well) treated with

drugs were incubated with 5 μM Rh123 for 30 min. After collection, excess rhodamine was removed by washing and the cells were incubated at 37°C for the indicated lengths of time. Then, cellular levels of Rh123 were measured by flow cytometry (Becton-Dickinson, CA, USA).

Relative expression of Bcl-2/Bax signal pathway

The expression profile of apoptosis-related proteins including Bcl-2 and Bax was examined by western blot using β-actin as the loading control. The total proteins were collected from PC12 cells treated with or without drugs for 24 h as described previously and the concentration of which was determined with the Bradford method. Then, samples were fractionated on SDS-PAGE gel and transferred onto PVDF membrane. The membrane was blocked for 1 h, immunoblotted with a specific primary antibody at 4°C overnight and then incubated for 1 h with a HRP-conjugated secondary antibody. Bound antibodies were detected by ECL kit and the density ratio of the protein to β-actin was calculated using the analysis tools.

Statistical analysis

Results are expressed as mean ± SD from at least three independent experiments. Two-group comparisons were performed using Student's *t*-test. Multiple-group comparisons were performed using one-way analysis of variance and Fisher's least significant difference (equal variances assumed) or Dunnett's T3-test (equal variances not assumed). Values of $p < 0.05$ were defined as statistically significant.

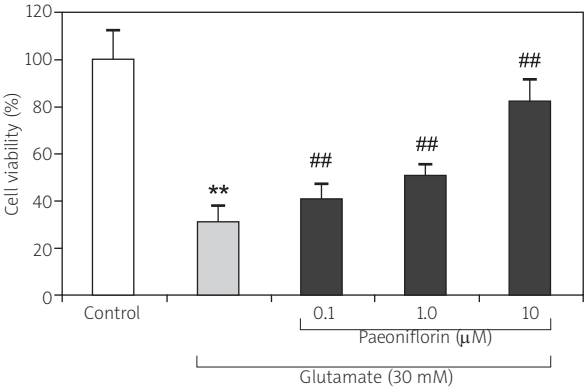

Fig. 1. MTT assays for the effect of PF in PC12 cells. The cell viability was significantly decreased after 24 h exposure to glutamate (30 mM), however, PF at a concentration of 0.1, 1 and 10 μM blocked this effect significantly. (**$p < 0.01$ vs. control group, ##$p < 0.01$ vs. glutamate group).

Results

Effect of paeoniflorin on glutamate-induced cell viability in PC12 cells

PC12 cells were incubated with or without drugs for 24 h and cell viability was determined by MTT assay. Upon treatment with glutamate (30 mM), cell viability was significantly reduced as compared with the control group. When treated with a combination of glutamate at 30 mM plus PF at different concentration (0.1, 1 and 10 μM), the cell viability was reduced to 27.12 ± 6.71%, as compared with the control group. Then, cell viability treated with glutamate plus different concentration of PF (0.1, 1 and 10 μM) was increased to 40.52 ± 4.74%, 50.67 ± 5.06% and 82.03 ± 8.78%, respectively (Fig. 1), which indicated that PF can inhibit glutamate-induced reduction of cell viability.

Effect of paeoniflorin on glutamate-induced apoptosis in PC12 cells

To examine the effects of the apoptotic program, DNA fragmentation was analyzed. A typical DNA ladder appeared when cells were treated with glutamate at 30 mM for 24 h. However, the DNA ladder disappeared when cells were treated with a combination of glutamate at 30 mM plus PF at different concentration (0.1, 1 μM) for 24 h (Fig. 2). To determine the apoptosis ratio caused by the drugs, apoptosis was analyzed by flow cytometry analysis. When glutamate was used alone, about 35.65% of cells were apoptotic at 24 h. However, the combination of glutamate at 30 mM and PF at different concentration (0.1, 1 and 10 μM) reduced the apoptosis rate to 18.34% ± 8.76, 9.27 ± 0.83% and 3.14 ± 0.29%, respectively ($P < 0.01$) (Fig. 3).

Effect of paeoniflorin on glutamate-induced MMP in PC12 cells

Lipophilic cationic dye Rh123 mainly gathered in the mitochondria and the intracellular intensity of Rh123 may reflect changes of mitochondrial membrane potential. After being treated with glutamate (30 mM) in DMEM medium for 24 h, the MMP of PC12 cells was reduced to 27.35 ± 2.74%, as compared with the con-

trol group. Then, MMP of cells treated with glutamate plus different concentration of PF (0.1, 1 and 10 μM) was increased to 42.35 ± 6.81%, 69.31 ± 5.94% and 78.26 ± 8.57%, respectively (Fig. 4), which indicated that PF can inhibit glutamate-induced reduction of MMP.

Effect of paeoniflorin on Bcl-2/Bax signal pathway

To determine whether apoptosis-related proteins including Bcl-2 and Bax participate in the process of apoptosis, cells were treated with or without different drugs and then the expression levels were examined by western blot. As shown in Figure 5, the expression level of Bcl-2 was significantly decreased when treated with 30 mM glutamate alone and significantly increased in a concentration-dependent manner when treated with a combination of glutamate at 30 mM plus PF at different concentration (0.1, 1, 10 μM) for 24 h as compared with the control group. However, the expression level of Bax was indeed contrary to that of Bcl-2. These results demonstrated that the protective effect of paeoniflorin on glutamate-induced apoptosis in PC12 cells was regulated through Bcl-2/Bax signal pathway.

Discussion

PF possesses wide pharmacological effects in nervous disorders such as Alzheimer's disease and Parkinson's disease [22]. In this study, we investigated the protective effect of PF on PC12 cells injury induced by glutamate. After treatment of PC12 cells with glutamate at 30 mM, a significant decrease in the cell viability and increase in the number of apoptotic cells appeared, confirming its neurotoxicity effect in PC12 cells. However, co-treatment with PF could increase the viability of cells exposed to glutamate alone, as well as a decrease in the percentage of apoptotic cells in a concentration-dependent manner. Apoptosis is now recognized as a normal feature in the development of the nervous system and may also play a role in neurodegenerative diseases, inappropriate cell death has been implicated in several neurodegenerative diseases [13]. Therefore, PF might be one of potential agents for treatment of these neurodegenerative diseases.

Mitochondria are the major source of energy in all eukaryotic cells, producing ATP through oxidative phosphorylation and the citric acid cycle. They regulate calcium homeostasis and modulate apoptosis through release of several cell death-inducing molecules [3,7].

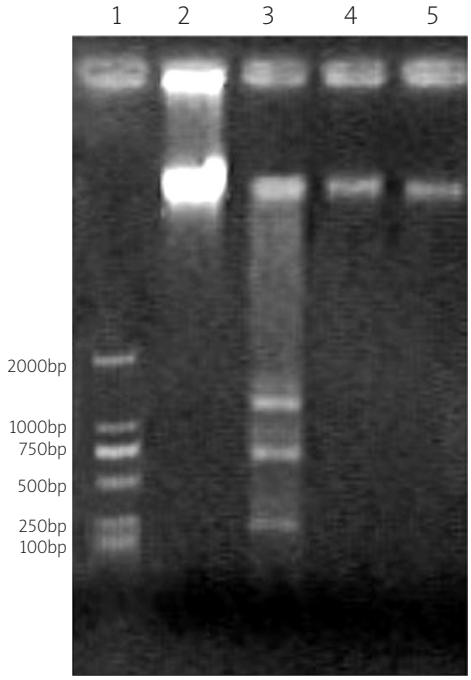

Fig. 2. The effect of PF on cell apoptosis by DNA fragmentation analysis. PC12 cells were exposed to drugs for 24 h and genomic DNA was extracted and separated by agarose gel electrophoresis. Lane 1: DNA marker; Lane 2: control; Lane 3: 24 h after glutamate treatment in the absence of PF; Lane 4-5: 24 h after glutamate treatment in the presence of PF (0.1, 1 μM).

The pattern of mitochondrial inheritance has a profound impact on development and reproductive performance [17]. Previous studies and our experiment showed that glutamate treatment caused a significant decrease in the level of MMP. However, we found that co-treatment of PF could inhibit reduction of MMP on PC12 cells, suggesting that the neuroprotective effect of PF may be related to the mitochondrial death pathway.

Moreover, we investigated the expression profiles of two apoptosis-related proteins including Bcl-2 and Bax [14]. Apoptosis is associated with the activation of a genetic program in which apoptosis effector genes promote cell death. It is regulated by the action of the Bcl-2 family of proteins, which includes anti- and pro-apoptotic members such as Bcl-2 and Bax [1,12,15,20,21]. It was reported that Bcl-2 binds to the mitochondrial membrane, competitive binding with Bax and forming Bcl-2/Bax heterodimer, which leads to closing mitochondrial permeability transition pore and preventing

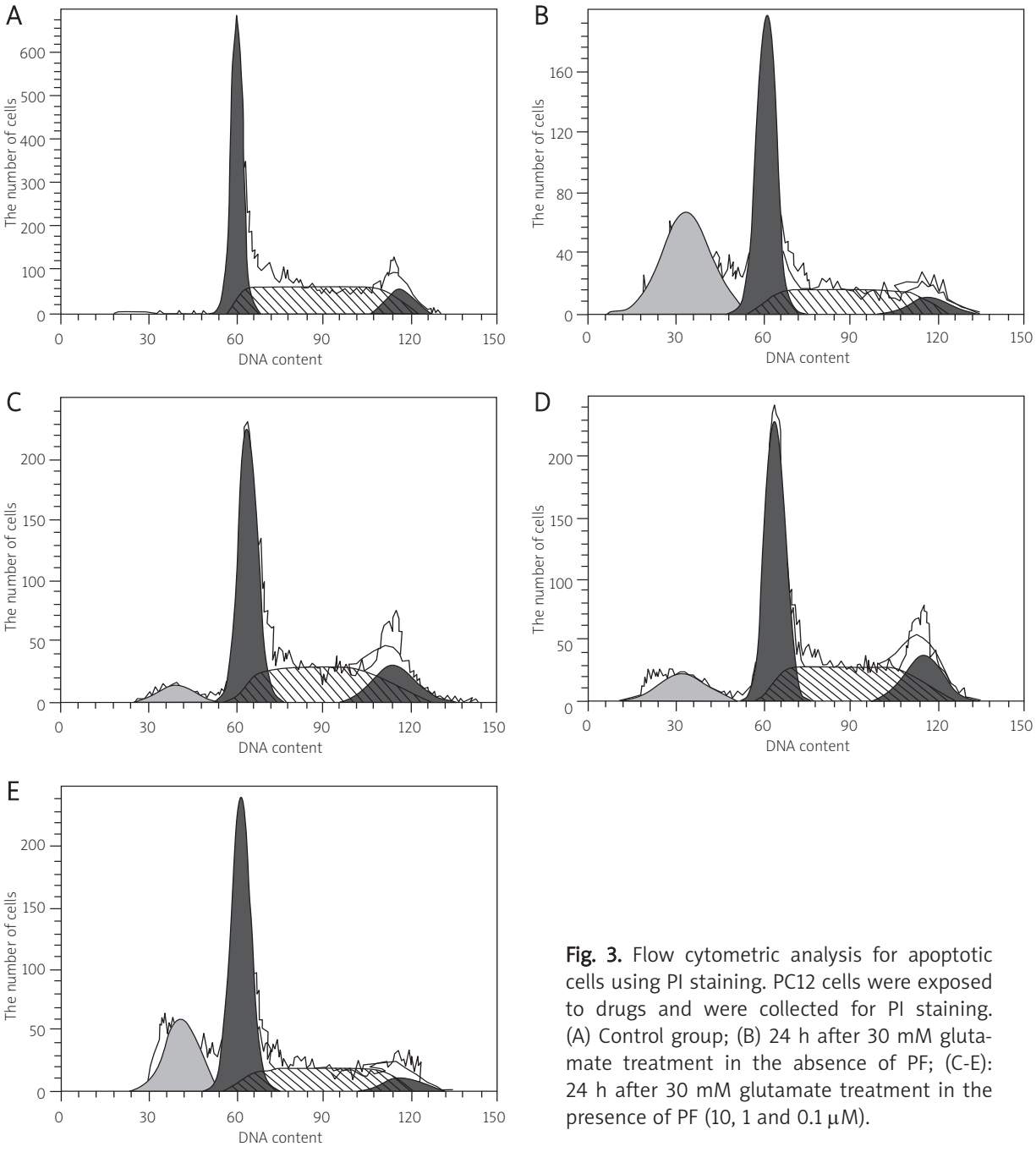

Fig. 3. Flow cytometric analysis for apoptotic cells using PI staining. PC12 cells were exposed to drugs and were collected for PI staining. (A) Control group; (B) 24 h after 30 mM glutamate treatment in the absence of PF; (C-E): 24 h after 30 mM glutamate treatment in the presence of PF (10, 1 and 0.1 μM).

the release of cytochrome C, thereby inhibiting apoptosis. As a proapoptotic molecule, Bax can be combined into a Bax-Bax homodimer to form the composition of the mitochondrial membrane permeable channels, through which cytochrome C can transfer from mitochondrial into cytoplasm, which activate the caspase-related apoptosis cascade, resulting in mitochondrial-dependent apoptosis. In the present study, the expression level of Bcl-2 was significantly decreased when treated with glutamate alone and significantly dose-dependent increased when treated with a combination of glutamate at 30 mM plus PF at different concentration (0.1, 1, 10 μM) for 24 h as compared with the control group. However, the expression level of Bax

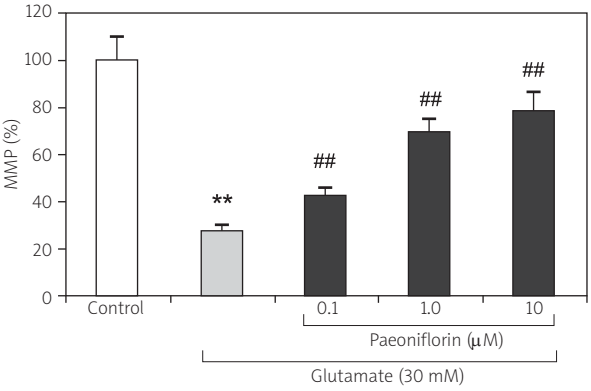

Fig. 4. The effect of PF on loss of MMP induced by glutamate in PC12 cells. (**$p < 0.01$ vs. control group, ##$p < 0.01$ vs. glutamate group).

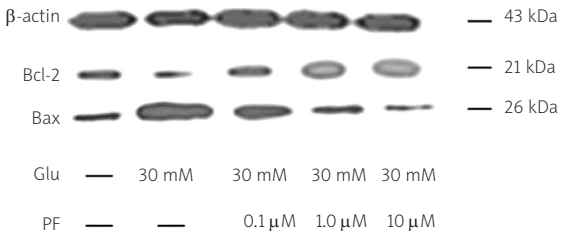

Fig. 5. The effect of PF on expression of Bcl-2 and Bax protein in PC12 cells by western blot. β-actin was used as the internal control. Lane 1: control; Lane 2: 24 h after 30 mM glutamate treatment in the absence of PF; Lane 3-5: 24 h after 30 mM glutamate treatment in the presence of PF (0.1, 1 and 10 μM).

was just the opposite. These results demonstrated that the protective effect of paeoniflorin on glutamate-induced apoptosis in PC12 cells was regulated through the Bcl-2/Bax signal pathway.

In summary, PF can remarkably inhibit glutamate-induced apoptosis in PC12 cells through regulating mitochondrial membrane potential and the Bcl-2/Bax signal pathway. These data suggest that PF has a protective effect on injured neurons and might be helpful in preventing neurotransmitter-related neurodegenerative disorders.

Acknowledgements

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