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Paeoniflorin attenuates $A\beta_{25-35}$-induced neurotoxicity in PC12 cells by preventing mitochondrial dysfunction

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Abstract

The pathogenic mechanism of neurodegenerative brain disorder such as Alzheimer's disease (AD) has been still far from clearly understood. Previous research has identified that mitochondrial dysfunction induced by Aβ has been recognized as a hallmark in AD. Therefore, the effective agents targeting β-amyloid (Aβ)-induced mitochondrial dysfunction may be useful for the treatment or prevention of AD. In the present study, the neuroprotective effect of paeoniflorin (PF), one monoterpene glycoside isolated from the Chinese herb Radix Paeoniae alba, on $A\beta_{25-35}$-induced toxicity in PC12 cells was investigated for the first time. The results showed that PF could attenuate or restore the cell injury induced by $A\beta_{25-35}$ in PC12 cells through preventing mitochondrial dysfunction, including decreased mitochondrial membrane potential, increased cytochrome c release as well as activity of caspase-3 and caspase-9. Therefore, our data provide the evidence that PF could protect PC12 cells against $A\beta_{25-35}$-induced neurotoxicity and might be a potentially therapeutic approach for AD in the future.

Key words: *paeoniflorin, Alzheimer's disease, β-amyloid, mitochondrial dysfunction, PC12 cells.*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder which approximately affects 14 million people worldwide [2,6,9]. One major pathological feature of AD is the deposition of amyloid plaques in the cerebral cortex, which are mainly composed of β-amyloid (Aβ) peptides [7,13]. Studies of postmortem brains of AD patients and transgenic mouse models of AD suggest that Aβ exerts neurotoxicity by promoting oxidative stress that is believed to directly affect the mitochondrial function [4,17]. Then, mitochondrial dysfunction induced by Aβ has been recognized as a prominent and early event in AD [16,19]. Therefore, the effective agents targeting Aβ-induced mitochondrial dysfunction may be useful for the treatment or prevention of AD.

Oriental herbal medicine, with fewer side effects and better safety, has been widely investigated for drug development [3]. Paeoniflorin (PF), a monoterpene glycoside isolated from the aqueous extract of the Chinese herb *Radix Paeoniae alba*, was reported to exert wide pharmacological effects in the nervous system (Fig. 1) [1,10-12,21]. Previous studies have identified that PF could attenuate the neurotoxicity induced by β-amyloid in the animal model and

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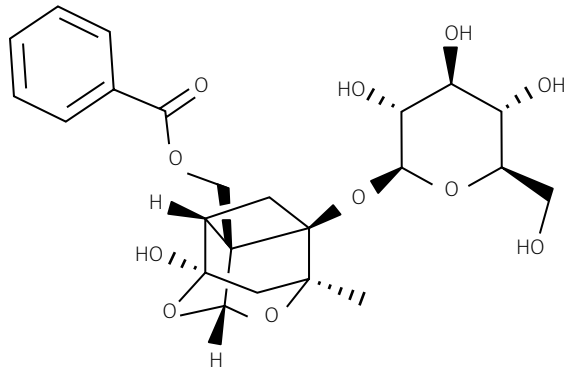

Fig. 1. Chemical structure of paeoniflorin.

might exert beneficial action for the treatment of AD [25]. However, the molecular mechanisms by which PF exerts its neuroprotective effect against β-amyloid-induced toxicity are still unclear.

In this study, we aimed to elucidate the protective effect of PF on $A\beta_{25-35}$-induced cytotoxicity in PC12 cells. Furthermore, the molecular mechanisms by which PF acted in models of neuron injury was also analyzed and this analysis focused on the mitochondrial pathway.

Material and methods

Materials and chemicals

Paeoniflorin (purity ≥ 98%, MW: 480.46) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All cell culture reagents were purchased from Gibco (Grand Island, NY, USA). $A\beta_{25-35}$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Annexin V-FITC and PI double staining kit were purchased from PharMingen (San Diego, CA, USA). Dimethyl sulfoxide (DMSO), ribonuclease A (Rnase A), polyvinylidene fluoride (PVDF) membranes and enhanced chemiluminescence (ECL) detection kit were purchased from Beyotime (Nantong, China). Antibody against cytochrome c was obtained from Santa Cruz Biotechnology (CA, USA). Caspase-3 and caspase-9 fluorometric assay kits were obtained from BioVision (SF, USA). All other chemicals and reagents were of analytical grade.

Cell culture and treatment

The rat pheochromocytoma (PC12) cell line was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PC12 cells were cultured in flasks at 37°C under an atmosphere of 5% CO_2/95% air in RPMI-1640 medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. For the experiments, the cells were detached and re-seeded in plates. After seeding, cells were pretreated with or without various concentrations of PF for 24 h, and then $A\beta_{25-35}$ (25 μM) was added to the medium for an additional 24 h.

MTT assay for cell viability

Cell viability was measured by MTT assay as described previously [17]. Briefly, after treated with the indicated drugs, 10 μL of MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Then, the culture medium was removed and 100 μL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with an ELISA reader (Model 680, Bio-Rad, USA). Cell viability was expressed as a percentage of the value against the non-treated control group.

Measurement of cell apoptosis

Apoptosis of PC12 cells was examined by flow cytometry (Becton Dickinson FACS Calibur, Franklin Lakes, USA). After treated with the indicated drugs, cells were washed twice with ice-cold PBS and resuspended in 300 μL of binding buffer (Annexin V-FITC kit) containing 10 μL of Annexin V-FITC stock and 10 μL of PI. After incubation for 15 min at room temperature in the dark, the samples were analyzed by flow cytometry for the evaluation of cell apoptosis.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured by uptake of lipophilic cation Rh123. Cells were treated with the indicated drugs and incubated with 5 μM of Rh123 at 37°C for 30 min. Then, the cells were washed twice and resuspended in PBS. The cellular levels of Rh123 were analyzed by flow cytometry (Becton-Dickinson, CA, USA).

Measurement of cytochrome c release

For measurement of cytochrome c release, the cytosol and mitochondrial fractions were prepared as described previously [24]. The protein concentration of samples was determined with Bradford method

[8]. Then, the samples (50 µg) were applied to 10% SDS polyacrylamide gel and transblotted onto PVDF membranes. After blocking with 5% BSA in Tris-buffer saline (TBST) for 1 h, membranes were incubated with the primary antibody against cytochrome c overnight and followed by secondary antibody incubation for 1 h at room temperature. Protein bands were visualized by ECL detection kit.

Measurement of caspase-3 and caspase-9 activity

Fluorometric assay was used to detect the cleavage of substrate to caspase-3 or caspase-9. Cells were collected and lysed in buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 100 mM NaCl, 2 mM ethylene diamine tetraacetic acid (EDTA), 0.1% 3 [(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 10% sucrose and 5 mM dithiothreitol (DTT). Aliquots of 6 mg of crude cell lysate were incubated with caspase-3 substrate DEVD-AFC or caspase-9 substrate LEHD-AFC at 37°C for 30 min. The activity was quantified by a spectrofluorometer with an excitation wavelength at 400 nm and an emission wavelength at 505 nm.

Statistical analysis

Biostatistical analyses were conducted with SPSS 16.0 software. All experiments were done in triplicates and the results were indicative of three independent studies. Data were presented as the mean ± SEM. Difference was considered statistically significant when $p < 0.05$.

Results

Effect of paeoniflorin on $A\beta_{25-35}$-induced cell injury

Cell viability of PC12 cells treated with the indicated drugs was evaluated with MTT assay. As shown in Figure 2, $A\beta_{25-35}$ (25 µM) exhibited a remarkably inhibitory effect on the growth of PC12 cells. However, the cytotoxic effects were attenuated by the pretreatment with PF in a dose-dependent manner.

Effect of paeoniflorin on $A\beta_{25-35}$-induced cell apoptosis

Apoptosis of PC12 cells treated with the indicated drugs was assessed by annexin V-PI dual-staining assay. The results showed that the percentage of apoptotic cells induced by $A\beta_{25-35}$ (25 µM) increased from 1.58 ± 1.52% to 42.51 ± 6.53% as compared to the control. While with the pretreatment of 2, 10 and 50 µM of CNTF, cell apoptosis induced by $A\beta_{25-35}$ (25 µM) decreased to 32.72 ± 4.48%, 22.23 ± 3.07% and 10.35 ± 3.27%, respectively (Fig. 3). These results indicated that PF could suppress $A\beta_{25-35}$-induced apoptosis in PC12 cells.

Effect of paeoniflorin on $A\beta_{25-35}$-induced loss of mitochondrial membrane potential

The change of MMP was evaluated by the fluorescence probe Rh123. As shown in Figure 4, cells exposed to $A\beta_{25-35}$ (25 µM) for 24 h markedly decreased Rh123 staining, indicating a drop in MMP which is related to mitochondrial dysfunction, while PF significantly improved $A\beta_{25-35}$-induced impairments of MMP in a dose-dependent manner.

Effect of paeoniflorin on $A\beta_{25-35}$-induced cytochrome c release

The reduction in MMP could induce a release of cytochrome c from the mitochondria to cytosol. As shown in Figure 5, $A\beta_{25-35}$ (25 µM) significantly increased the release of cytochrome c from mitochondria to cytosol. However, PF pretreatment could

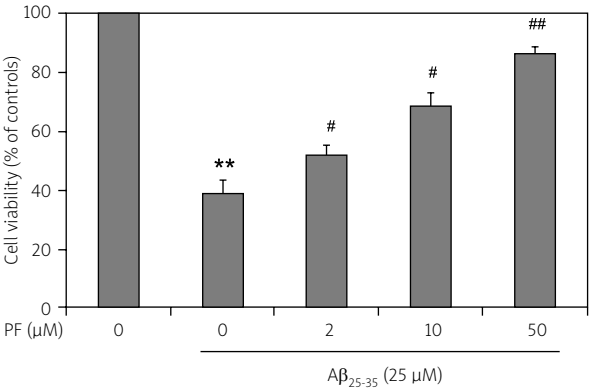

Fig. 2. Effect of PF on $A\beta_{25-35}$-induced cytotoxicity in PC12 cells. Determination of the viability of PC12 cells by the MTT assay after treatment with $A\beta_{25-35}$ (25 µM) in the absence or presence of the indicated concentrations of PF. The results are shown as mean ± SEM of three experiments and each included triplicate sets. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. $A\beta_{25-35}$ alone.

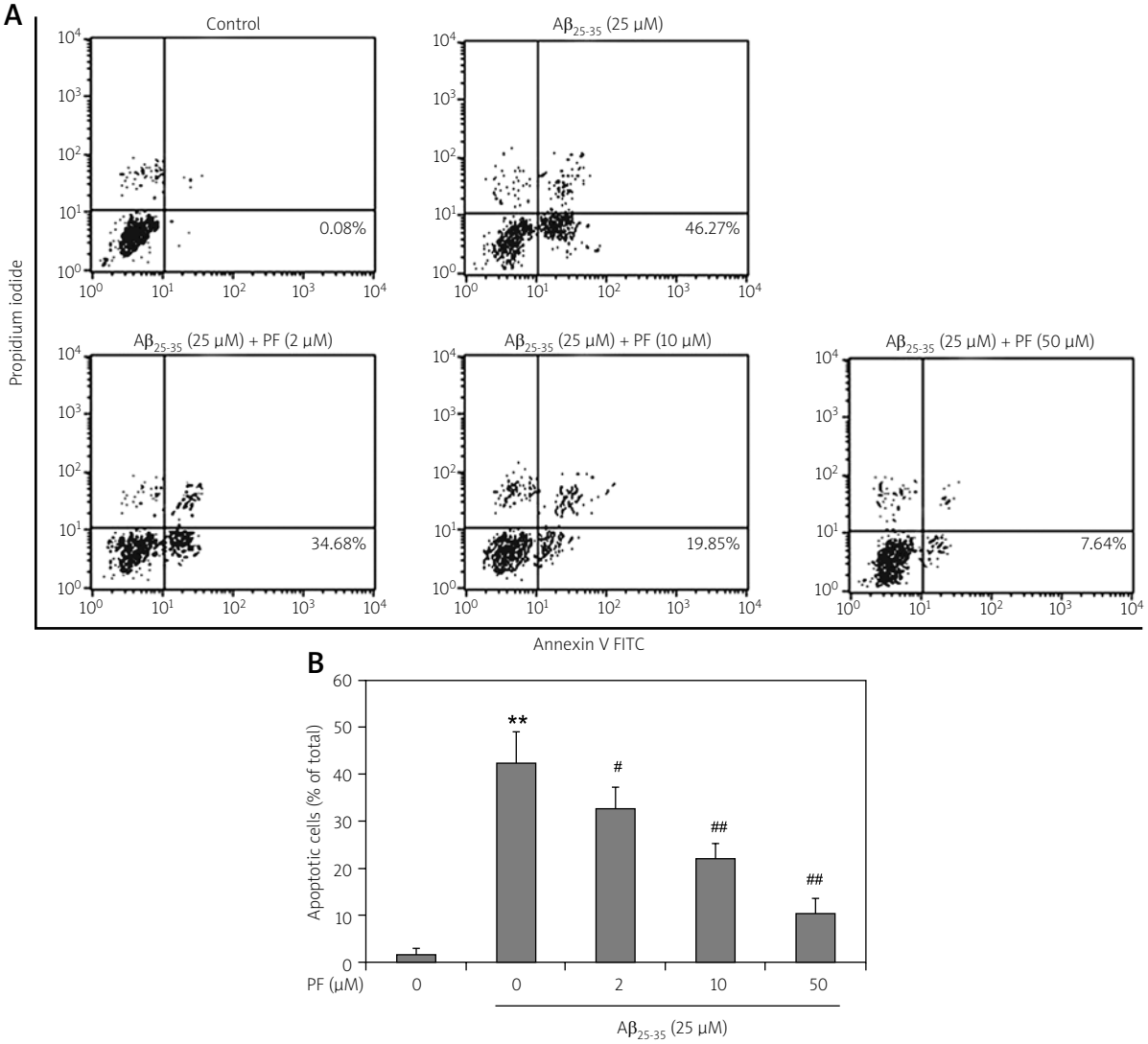

Fig. 3. Effect of PF on $A\beta_{25-35}$-induced apoptosis in PC12 cells. Determination of the apoptosis of PC12 cells by the annexin V-PI dual-staining assay after treatment with $A\beta_{25-35}$ (25 μM) in the absence or presence of the indicated concentrations of PF. **A)** Flow cytometry analysis of cell apoptosis. The dual parametric dot plots combining annexin V-FITC and PI fluorescence show the viable cell population (lower left quadrant, annexin V^-PI^-), the early apoptotic cells (lower right quadrant, annexin V^+PI^-) and the necrotic cells (upper right quadrant, annexin V^+PI^+). **B)** The percentage distribution of apoptotic cells. The results were shown as mean ± SEM of three experiments and each included triplicate sets. $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. $A\beta_{25-35}$ alone.

inhibit the release of cytochrome c in a dose-dependent manner.

Effect of paeoniflorin on $A\beta_{25-35}$-induced caspase-3 and caspase-9 activation

The release of cytochrome c could activate caspase-9, and then activate effector caspase-3. The activation status of caspase-3 and caspase-9 was further investigated when cells were treated with the indicated drugs. As shown in Figure 6, the activity of caspase-3 and caspase-9 significantly increased following $A\beta_{25-35}$ (25 μM) treatment for 24 h and which were dose-dependently reversed when cells were pretreated with PF (Fig. 6).

Discussion

More and more scientific research has identified that mitochondrial dysfunction is a hallmark of

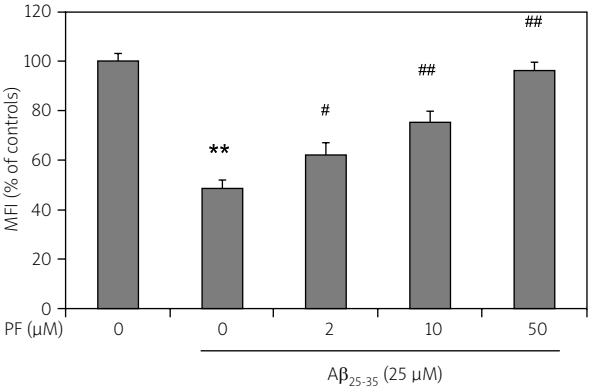

Fig. 4. Effect of PF on $A\beta_{25-35}$-induced loss of MMP in PC12 cells. Mean relative fluorescent density (MFI) of Rh123 was calculated after treatment with $A\beta_{25-35}$ (25 μM) in the absence or presence of the indicated concentrations of PF. The results are shown as mean ± SEM of three experiments and each included triplicate sets. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. $A\beta_{25-35}$ alone.

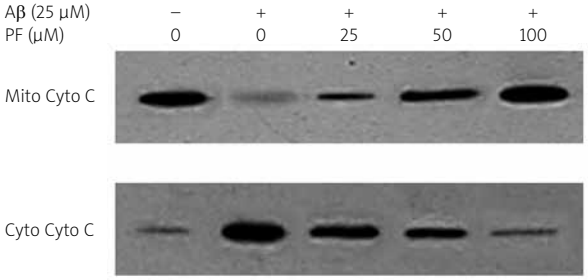

Fig. 5. Effect of PF on $A\beta_{25-35}$-induced cytochrome c release in PC12 cells. The expression level of cytochrome c in mitochondria and cytosol was assessed by western blot after treatment with $A\beta_{25-35}$ (25 μM) in the absence or presence of the indicated concentrations of PF. The results are shown as mean ± SEM of three experiments and each included triplicate sets.

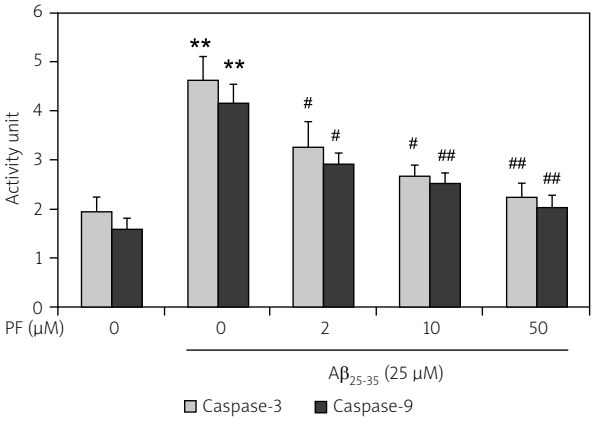

Fig. 6. Effect of PF on $A\beta_{25-35}$-induced caspase-3 and caspase-9 activation in PC12 cells. The activity of caspase-3 and caspase-9 was evaluated by fluorometric assay after treatment with $A\beta_{25-35}$ (25 μM) in the absence or presence of the indicated concentrations of PF. The results are shown as mean ± SEM of three experiments and each included triplicate sets. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. $A\beta_{25-35}$ alone.

Aβ-induced neuronal toxicity in AD [14,18]. Therefore, any substances that can decrease mitochondrial dysfunction may be useful for the treatment or prevention of AD. Paeoniflorin, one of components of the aqueous extract of the Chinese herb *Radix Paeoniae alba*, has recently been reported to be an active neuroprotective agent in animal models of neurodegenerative diseases [25]. To further understand the biological function of PF on the AD *in vitro* model, the present study focused on the molecular effect of PF on $A\beta_{25-35}$-induced mitochondrial dysfunction in PC12 cells.

Previous studies suggested that $A\beta_{25-35}$-induced cytotoxicity in PC12 cells was recognized as a typical model of Alzheimer's disease [5,15]. In this study, we confirmed for the first time that pretreatment with PF could markedly attenuate $A\beta_{25-35}$ (25 μM)-induced loss of cell viability in PC12 cells by MTT assay. Then, the protective effect of PF against $A\beta_{25-35}$-induced cell apoptosis was evaluated by annexin V-PI dual-staining assay. The results showed that PF pretreatment significantly reduced the percentage of apoptotic cells induced by $A\beta_{25-35}$ in PC12 cells. Furthermore, the molecular mechanism of the neuroprotective effect of PF on $A\beta_{25-35}$-induced cell apoptosis in PC12 cells was investigated.

One classification of neuronal apoptosis is based on compelling evidence that mitochondrial changes are pivotal in the cell death decision in many cases [20,22]. Our results showed that mitochondrial dysfunction is involved in $A\beta_{25-35}$-induced apoptosis in PC12 cells which includes opening of pores in cell membrane, release of cytochrome c and activation of caspases. Then, we investigated whether PF can regulate mitochondrial dysfunction induced by $A\beta_{25-35}$ in PC12 cells. The subsequent experiments revealed that pretreatment of PF could attenuate all

of these biochemical changes which are tightly associated with $A\beta_{25-35}$-induced apoptosis.

In conclusion, our results confirmed for the first time the neuroprotective effect of PF on $A\beta_{25-35}$-induced cell injury in PC12 cells by preventing mitochondrial dysfunction. The potency of PF presented here provides a rational reason for exploring its clinical efficiency.

Acknowledgements

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Disclosure

Authors report no conflict of interest.

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