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 $A\beta$ has been recognized as a hallmark in AD. Therefore, the effective agents targeting $\beta$-amyloid ($A\beta$)-induced mitochondrial dysfunction may be useful for the treatment or prevention of AD. In the present study, the neuroprotective effect of paeoniflorin (PF), one monoterpenoid 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, $\beta$-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 $\beta$-amyloid ($A\beta$) peptides [7,13]. Studies of postmortem brains of AD patients and transgenic mouse models of AD suggest that $A\beta$ exerts neurotoxicity by promoting oxidative stress that is believed to directly affect the mitochondrial function [4,17]. Then, mitochondrial dysfunction induced by $A\beta$ has been recognized as a prominent and early event in AD [16,19]. Therefore, the effective agents targeting $A\beta$-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 monoterpenoid 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 $\beta$-amyloid in the animal model and

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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 Aβ25-35-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). Aβ25-35, 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% CO2/95% 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 Aβ25-35 (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.
results showed that the percentage of apoptotic cells induced by Aβ25-35 (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 Aβ25-35 (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 Aβ25-35-induced apoptosis in PC12 cells.

Effect of paeoniflorin on Aβ25-35-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 Aβ25-35 (25 μM) for 24 h markedly decreased Rh123 staining, indicating a drop in MMP which is related to mitochondrial dysfunction, while PF significantly improved Aβ25-35-induced impairments of MMP in a dose-dependent manner.

Effect of paeoniflorin on Aβ25-35-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, Aβ25-35 (25 μM) significantly increased the release of cytochrome c from mitochondria to cytosol. However, PF pretreatment could

Results

Effect of paeoniflorin on Aβ25-35-induced cell injury

Cell viability of PC12 cells treated with the indicated drugs was evaluated with MTT assay. As shown in Figure 2, Aβ25-35 (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 Aβ25-35-induced cell apoptosis

Apoptosis of PC12 cells treated with the indicated drugs was assessed by annexin V-PI dual-staining assay. The ratio of annexin V-positive cells (apoptotic) was determined by flow cytometry. As shown in Figure 3, the percentage of annexin V-positive cells 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 Aβ25-35 (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 Aβ25-35-induced apoptosis in PC12 cells.


Fig. 2. Effect of PF on Aβ25-35-induced cytotoxicity in PC12 cells. Determination of the viability of PC12 cells by the MTT assay after treatment with Aβ25-35 (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. Aβ25-35 alone.
inhibit the release of cytochrome c in a dose-dependent manner.

**Effect of paenoflorin on Aβ25-35-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 Aβ25-35 (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
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 Aβ25-35-induced mitochondrial dysfunction in PC12 cells.

Previous studies suggested that Aβ25-35-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 Aβ25-35 (25 μM)-induced loss of cell viability in PC12 cells by MTT assay. Then, the protective effect of PF against Aβ25-35-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 Aβ25-35 in PC12 cells. Furthermore, the molecular mechanism of the neuroprotective effect of PF on Aβ25-35-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 Aβ25-35-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 Aβ25-35 in PC12 cells. The subsequent experiments revealed that pretreatment of PF could attenuate all
of these biochemical changes which are tightly associated with Aβ25-35-induced apoptosis.

In conclusion, our results confirmed for the first time the neuroprotective effect of PF on Aβ25-35-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.

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Disclosure

Authors report no conflict of interest.

References