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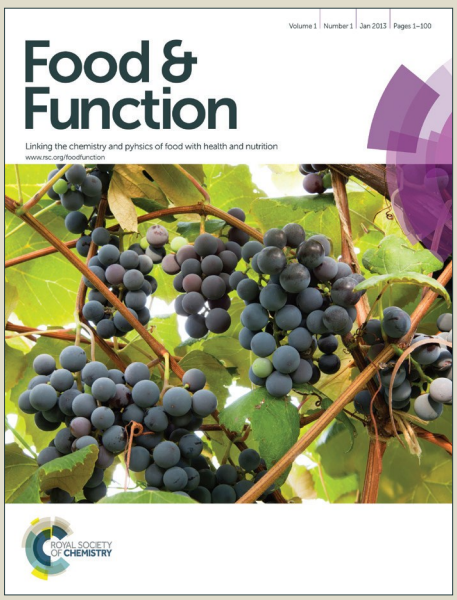

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ARTICLE

Effects of piceatannol and pterostilbene against β-amyloid-induced apoptosis on the PI3K/Akt/Bad signaling pathway in PC12 cells

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Neuron apoptosis induced by β-amyloid (Aβ) is an important precipitating factor in the pathogenesis of Alzheimer's disease (AD). In the present study, effects of piceatannol (PT) and pterostilbene (PS) against Aβ-induced apoptosis in PC12 cells were evaluated. PT and PS both showed observable anti-apoptosis activity. Increased cell viability, decreased apoptosis rate and declining intracellular ROS were observed after PT and PS treatment. For signaling pathway, PT significantly promoted phosphorylation of Akt and Bad, further suppressed Bcl-2/Bax expression and inhibited cleavage of caspase-9, caspase-3 and PARP. PS promoted phosphorylation of Akt without affecting the other factors. The experimental results, for the first time, unambiguously suggested that PT showed a comprehensive protective effect against Aβ-induced apoptosis in PC12 cells via a novel PI3K/Akt/Bad signaling pathway and downstream mitochondria-mediated and caspase-dependent signaling pathway. Unlike PT, PS inhibited apoptosis against Aβ through a different PI3K/Akt signaling pathway in which the downstream targets need further investigated. The results also provide the basis for dietary intervention involved in the prevention and adjunctive therapy of AD.

Keywords: Piceatannol, Pterostilbene, Alzheimer's disease, β-amyloid, PC12 cells, Akt, Bad, Apoptosis

1 Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease that causes a decline in memory and recognition. AD is characterized by two typical neuropathological hallmarks, neurofibrillary tangle deposition and senile plaque accumulation, which eventually lead to progressive degeneration and loss of neurons in the brain.[1] Although the definite cause of the disease remains unclear, recent studies have confirmed that β-amyloid (Aβ) accumulation in the brain has a crucial role in the development of AD.[2] Aβ aggregation increases cellular oxidative stress, induces neuron inflammation and promotes neuron apoptosis.[3-5] Therefore, many therapeutic efforts on AD have focused on reducing the toxicity of Aβ and inhibiting Aβ-induced neuronal apoptosis.

Compelling evidence indicates that Aβ accumulation may induce oxidative stress and promote the release of pro-apoptosis factors to contribute to neuron apoptosis in AD.[6, 7] PI3K/Akt is a set of vital kinases that play a pivotal role in the regulation of genes associated with apoptosis, and its activation is one manner through which anti-apoptosis drugs counteract Aβ neurotoxicity.[8] Active AKT accelerates the phosphorylation of multiple downstream targets, such as GSK-3β and mTOR, to promote cell growth and survival.[9, 10] In addition, PI3K/Akt is intimately related with Bcl-2 family proteins which are crucial apoptosis-associated factors around mitochondria. When stimulated, Akt leads to the phosphorylation of the pro-apoptosis protein Bad and inhibit the translocation of Bad from the cytoplasm to the mitochondria, resulting in the suppression of Aβ-induced mitochondrial dysfunction.[11] Mitochondrial dysfunction is an important event that leads to stimulate multiple downstream caspases. These active caspases regulate other protein substrates, such as DNase and ribose polymerase, to finally trigger the apoptosis process.[12] Therefore, the PI3K/Akt/Bad signaling pathway may be a vital target in the treatment or prevention of AD.

Currently, several methods to attack Aβ have been tested. Numerous synthetic Aβ-targeting drugs, such as An-1792, Flurizan, and Dimebon, have fallen by the wayside due to a lack of efficacy or side effects.[13] Therefore, researchers turned their attention to natural active compounds. In recent reports, many bioactive natural products with antioxidant activity such as flavonoids,[14] terpenes[15] and stilbenes,[16] have been shown to be potential therapeutics for AD, and these studies supported the notion that oxidative stress responses can be an attractive target for treatment with antioxidative drugs. Among these drugs, stilbenes have attracted tremendous attention for their extremely clear antioxidant activity and neuroprotective properties.

Stilbenes are a series of food-derived bioactive natural products primarily represented by resveratrol (RES) and its derivatives found in grapes and wines, such as piceatannol (PT) and pterostilbene (PS) (Fig. 1).[17] In recent studies, stilbenes were shown to have neuroprotective activity. Twenty Stilbenes including RES, PT and PS were reported to inhibit the β-amyloid peptide formation in vitro

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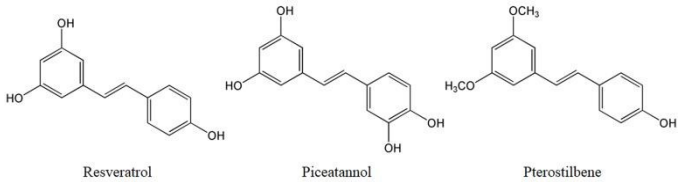

Figure 1. Chemical structures of three stilbenes: resveratrol, piceatannol and pterostilbene.

and to show possible therapeutic activity for AD.[18] Among all the stilbenes, RES received the most widespread attention. RES has been proved to regulate neurological disorders, including Huntington's disease and strokes[19, 20] as well as to protect neurons against Aβ from dying and apoptosis, which are considered significant pathological symptoms of AD.[21, 22]. However, other stilbenes, such as PS and PT, were reported with more eminent neuroprotective activity than RES. One study has indicated that PS, rather than RES, is a potent neuromodulator of aging and AD.[23] The other three studies have confirmed that PT was more prominent than RES in the neuroprotection against 4-hydroxynonenal, hydrogen peroxide, peroxynitrite and Aβ, however, the underlying mechanism especially the signaling pathway remains largely incomplete.[24-26] Therefore, the neuroprotective activity of PT and PS deserve more attention, and the underlying mechanism needs further investigated. The present study is aimed to investigate the neuroprotective effects and potential AD-therapeutic value of PT and PS. In our research, the anti-apoptosis activity of PT and PS against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells was investigated, and their underlying molecular mechanisms and signaling pathways were also targeted. The results also provide the basis for dietary intervention involved in the prevention and adjunctive therapy of AD.

2 Materials and methods

2.1 Reagents and chemicals

PT (≥ 99%) and PS (≥ 99%) were obtained from J&K Scientific Inc. (Beijing, China). $A\beta_{25-35}$ (≥ 99%) was purchased from Chinese Peptide Inc. (Beijing, China). RPMI 1640 medium, 0.25% trypsin solution, and penicillin/streptomycin solution were obtained from GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from Hyclone Laboratory, Inc. (Logan, UT, USA). MTT was obtained from AMRESCO Inc. (Solon, OH, USA). The Annexin-V-Fluos Staining Kit was purchased from Roche (Basel, CH). DCFH-DA and rabbit antibodies against β-actin were obtained from Solarbio Technology Inc. (Beijing, China). The following rabbit antibodies were purchased from Cell Signaling Technology Inc. (Boston, MA, USA): phospho-Bad (Ser-136), phospho-Akt (Ser-473), Akt, Bax, Bcl-2, caspase-8, cleaved caspase-3, cleaved caspase-9 and cleaved PARP.

2.2 Cell culture and Peptide Preparation

PC12 cells were purchased from Tongpai Biotechnology Inc. (Shanghai, China); PC12 cells were grown in RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were seeded in 6-well or 96-well culture plates in serum-free medium for 12 h prior to drug treatment. The $A\beta_{25-35}$ peptide was dissolved in PBS solution at 1 mM and incubated for 14 days at 37 °C for aggregation; the solution was stored at -20 °C until use.

2.3 Cell Viability Assay

The quantitative colorimetric MTT assay was used to determine cell viability. The MTT assay is based on the ability of living cells to transform MTT to formazan. PC12 cells were seeded into 96-well plates at a density of 1.0×10^5 cells/well. After drug treatment, 100 µL of MTT solution at a final concentration of 1 mg/mL was added, and the cells were incubated for 4 h at 37 °C. Then, the dark formazan crystals formed in living cells were dissolved with 50% isopropanol, 40% deionized distilled water and 10% Triton-X 100 in 200 µL of co-solvent buffer for 6 h at 37 °C. Finally, the absorbance at 570 nm of each well was measured with a microplate reader (Tecan, Männedorf, CH). Cell viability was expressed as a percentage of the untreated controls.

2.4 Intracellular ROS Assay

The level of intracellular ROS was detected with DCFH-DA. DCFH-DA reacts with ROS in cells and is converted into a fluorescent product, DCF, which can be detected by flow cytometry. In brief, PC12 cells were seeded into 6-well plates at 3.0×10^5 cells/well. After drug treatment, the cells were incubated with 5 µM DCFH-DA for 30 min at 37 °C, followed by three washes with PBS. The fluorescence of DCF was detected with flow cytometry (BD, San Jose, CA, USA) at Ex = 488 nm and Em = 605 nm. ROS production was expressed as the fold change compared to untreated controls.

2.5 Apoptosis Assay

Apoptosis was detected with the Annexin-V-Fluos Staining Kit, which consists of Annexin-V and propidium iodide (PI). PC12 cells in an early stage of apoptosis were stained only with Annexin-V, cells in late apoptosis were observed with PI and Annexin staining, and dead cells were stained only with PI. Therefore, the number of apoptosis cells was measured with flow cytometry. In brief, PC12 cells were seeded into 6-well plates at 3.0×10^5 cells/well. After drug treatment, the cells were washed twice with PBS, harvested, and centrifuged at 600 g for 5 min. Then, the cells were incubated in 100 µL of Hepes buffer with 10 µL of Annexin-V and 10 µL of PI for 15 min at room temperature. Annexin staining was measured with a flow cytometer (BD, San Jose, CA, USA) at Ex = 488 nm and Em =518 nm, and the PI staining was measured at Ex = 488 nm and Em =605 nm.

2.6 Western Blot Analysis

PC12 cells were seeded into 6-well plates at 3.0×10^5 cells/well. After drug treatment, the cells were washed twice with PBS, harvested, and centrifuged at 600 g for 5 min. The cells were collected with 100 µL of lysis buffer on ice for 30 min, followed by ultrasonic degradation 10 times at 30 W with an Ultrasonic Processor (Scientz, Ningbo, China). The supernatant was obtained by centrifugation at 12,000 g for 30 min. The protein concentrations were measured with the BCA protein assay kit. Equal protein solutions were mixed with 4× loading buffer and heated at 100 °C

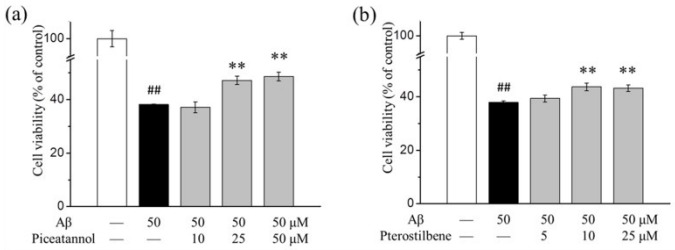

Figure 2. Effects of piceatannol (PT) and pterostilbene (PS) on cell viability in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25-35}$ for 24 h. **(a)** Pre-treatment with PT; **(b)** pre-treatment with PS. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25-35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25-35}$ treatment.

for 6 min. Proteins were separated on an 8% or 10% SDS-polyacrylamide gel, and then transferred onto a PVDF membrane for 100 min at 200 mA in a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk and 0.1% Tween-20 in TBST buffer for 1 h at room temperature. Then, the membrane was incubated overnight with primary antibodies including p-Bad, p-Akt, Akt, Bax, Bcl-2, caspase-8 c-caspase-3, c-caspase-9, c-PARP and β-actin diluted 1:1000 at 4 °C. After five washes with TBST containing 0.1% Tween-20 for 8 min each, the membrane was incubated with HRP-conjugated secondary antibodies diluted 1:5000 for 1 h at room temperature. After another five washes with TBST for 8 min, the membrane was visualized using western blotting luminal reagents.

2.7 Statistical analysis.

Data of each experiment showed the mean ± SD (n = 3). Data analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). A one-way ANOVA was used for the comparison of multiple groups. A t-test was used for the comparison of two groups.

3 Results

3.1 Effects of PT and PS on cell viability in $A\beta_{25-35}$-induced PC12 cells

Losing neurons in the brain is a significant pathological characteristic of AD, and it has been confirmed that neurons death is a key consequence and representation of Aβ-induced neurotoxicity.[27] To determine the effects of PT and PS on cell death, PC12 cells were pretreated with PT and PS for 1 h, damage was induced by 50 μM $A\beta_{25-35}$ for 24 h, and cell viability was measured by MTT assay. Increasing concentrations of PT (10, 25, and 50 μM) or PS (5, 10, and 25 μM) alone did not show cytotoxicity in PC12 cells (data not shown). As shown in Figure 2, $A\beta_{25-35}$ significantly ($p < 0.01$) decreased the cell viability (38.0%) compared with the control without $A\beta_{25-35}$ treatment. Cells pretreated with PT (25, 50 μM) exhibited a significantly increased level of cell viability (47.2%,

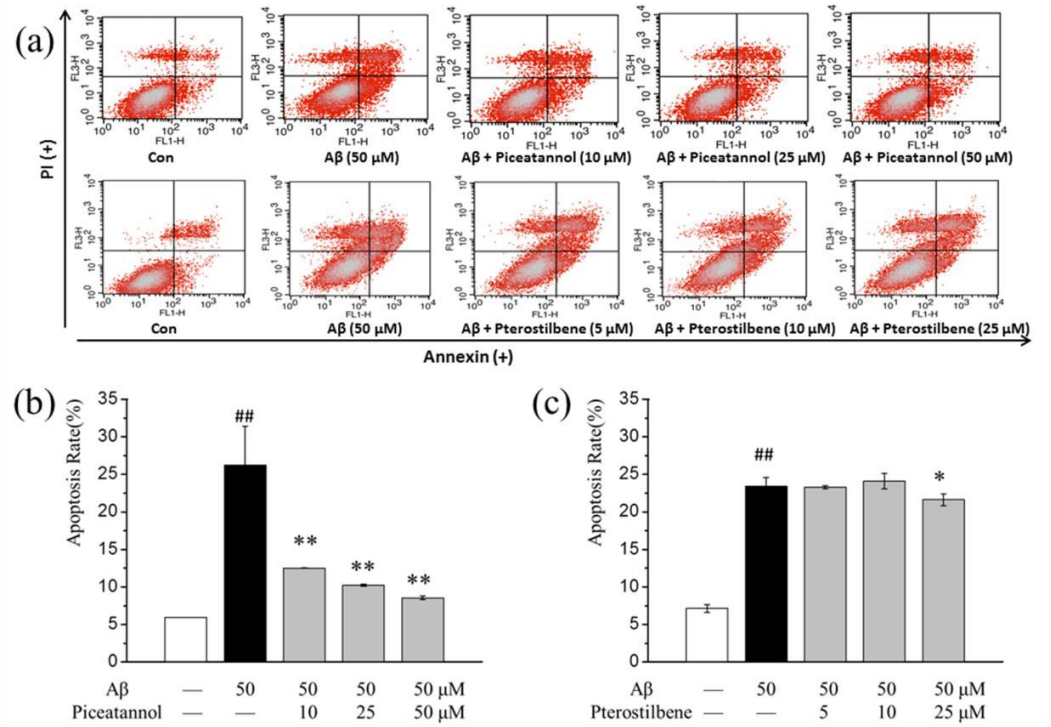

Figure 3. Effects of piceatannol (PT) and pterostilbene (PS) on cell apoptosis in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25-35}$ for 24 h. Annexin-V/propidium iodide (PI) double staining was used to examine cell apoptosis. **(a)** Annexin-V/PI double staining in PC12 cells. The apoptosis rate was calculated and presented in **(b)** Pre-treatment with PT or **(c)** pre-treatment with PS. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25-35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25-35}$ treatment.

48.8%) in comparison to the $A\beta_{25-35}$ treatment group ($p < 0.01$). On the other hand, pre-treatment with PS (10, 25 μM) also increased cell viability (43.2%, 43.7%), although to a lesser extent than PT.

3.2 Effects of PT and PS on apoptosis in $A\beta_{25-35}$-induced PC12 cells

Cell apoptosis induced by various exogenous disadvantageous factors is the primary manner of neuron death in the brains of AD patients.[28, 29]. In addition, Aβ accumulation results in apoptosis initiation in various neurons, including cortical and hippocampal neurons.[30] To determine the potential neuroprotective ability of PT and PS, apoptosis in PC12 cells exposed to different treatments were measured with Annexin V and PI double staining, followed by flow cytometry. As shown in Figure 3, $A\beta_{25-35}$ treatment significantly ($p < 0.01$) increased the level of apoptosis cells, which is consistent with previous studies.[31] PT (10, 25, and 50 μM) dramatically ($p < 0.01$) reversed the above effects in a dose-dependent manner (12.5%, 10.2%, 8.6%) in comparison to $A\beta_{25-35}$ group (26.1%). This result is also analogous to a previous study in which PT (5, 10, and 20 μM) dose-independently decreased the apoptosis level in PC12 cells.[24] On the other hand, PS at 5 and 10 μM did not affect the apoptosis rate (23.3%, 23.6%) in comparison to $A\beta_{25-35}$ group (23.4%). Only PS at 25 μM slightly ($p < 0.05$) decreased the level of apoptosis cells (21.6%). The combined results on cell viability imply that PT has significant potential neuroprotective ability against $A\beta_{25-35}$, and the neuroprotection by PS was much weaker.

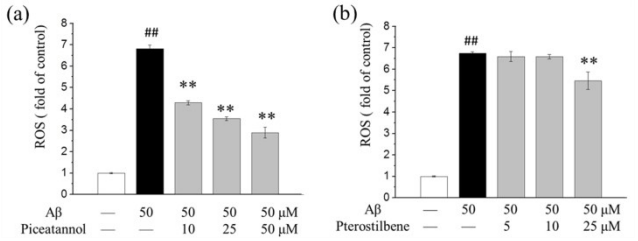

Figure 4. Effects of piceatannol (PT) and pterostilbene (PS) on ROS in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25-35}$ for 24 h. **(a)** Pre-treatment with PT; **(b)** pre-treatment with PS. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25-35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25-35}$ treatment.

To further confirm this result, we next investigated some related intracellular factors.

3.4 Effects of PT and PS on intracellular ROS in $A\beta_{25-35}$-induced PC12 cells

ROS is an important intracellular response factor related to apoptosis. Aβ-induced cytotoxicity is accompanied by an increase in intracellular ROS, redundant ROS would cause direct oxidative damage to lipids, proteins and DNA in cells or act as signal factor to stimulate multiple downstream gene targets related to apoptosis.[6] In the present study, intracellular ROS in PC12 cells were detected. As shown in Figure 4, $A\beta_{25-35}$

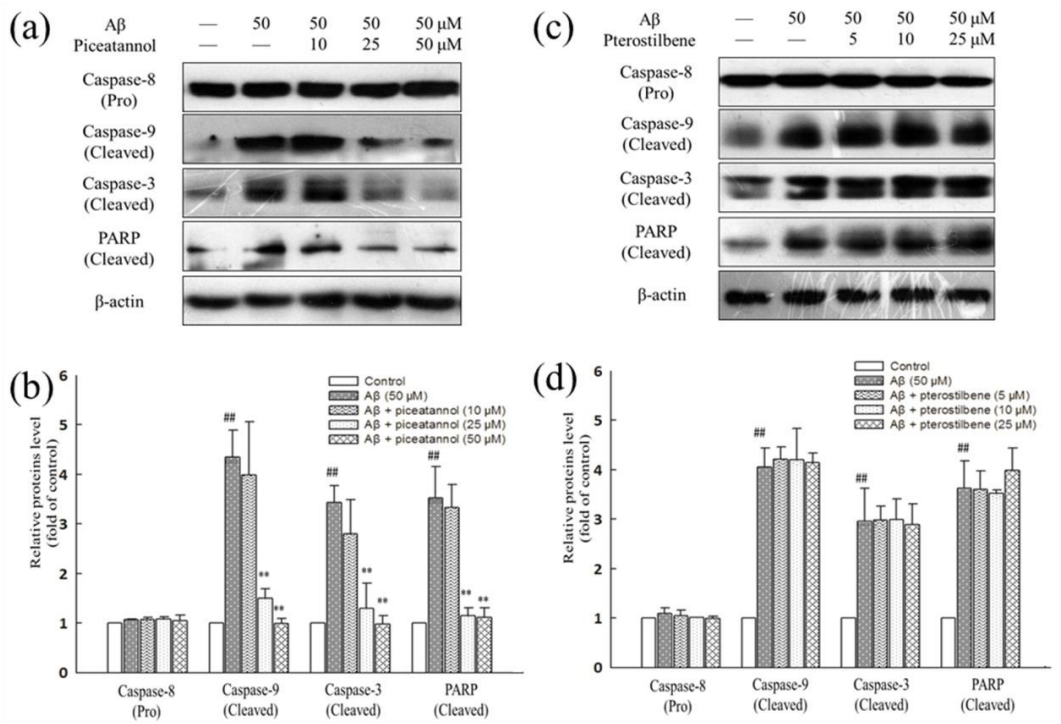

Figure 5. Effects of piceatannol (PT) and pterostilbene (PS) on the activation of multiple caspases in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25-35}$ for 24 h. Western blotting was used to analyse the expression or cleavage of caspase-8, -9, and -3 and PARP in **(a)** the group with PT pre-treatment or **(c)** the group with PS pre-treatment. The relative proteins level was calculated and presented in **(b)** the group with PT pre-treatment or **(d)** the group with PS pre-treatment. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25-35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25-35}$ treatment.

significantly ($p < 0.01$) increased the intracellular ROS (681.1%). PT (10, 25, and 50 μM) pre-treatment significantly ($p < 0.01$) caused a dose-dependent decrease (429.1%, 354.4%, and 288.9%) in the level of ROS in comparison to the $A\beta_{25-35}$ treatment group. On the other hand, PS at 25 μM also decreased intracellular ROS (546.5%, $p < 0.01$), although the effect was weaker than PT. In addition, the variation in the ROS level (Fig. 4) correlated very closely with that of the apoptosis level (Fig. 3) in PC12 cells, suggesting that PT and PS protect PC12 cells from Aβ-induced apoptosis via receding intracellular ROS, exactly.

3.4 Effects of PT and PS on the activation of multiple caspases in $A\beta_{25-35}$-induced PC12 cells

To further investigate the underlying signaling pathway of the neuroprotective effects of PT and PS, key signal factors related to apoptosis were measured with western blot. The complex cascade of caspase activation is specific to apoptosis, and caspases-3, -8,-9 and PARP have been shown to play vital roles.[12] As illustrated in Figure 5, $A\beta_{25-35}$ alone activated caspase-9/-3 cleavage and PARP cleavage, the levels of cleaved caspase-9/-3 and cleaved PARP were significantly elevated ($p < 0.01$). PT significantly suppressed caspase-9/-3 activation and PARP cleavage in a dose-dependent manner ($p < 0.01$). No obvious effect was detected in the PS pre-treated group. In addition, the level of caspase-8 remained constant in all treatment groups. Caspase-8 is considered a significant signal molecule to initiate apoptosis in the death receptor-mediated signaling pathway,[32] while caspase-9 and -3 are crucial in the mitochondria-mediated apoptosis signaling pathway.[33] The results indicated that PT inhibited Aβ-induced apoptosis by inactivation of caspase-9/-3 in the mitochondria-mediated signaling pathway rather than caspase-8 in the death receptor-mediated signaling pathway.

3.5 Effects of PT and PS on the expression of Bcl-2 family proteins in $A\beta_{25-35}$-induced PC12 cells

To further confirm the mitochondria-mediated signaling pathway in PT and PS anti-apoptosis activity, we focused on the Bcl-2 family proteins, which are critical regulatory factors in mitochondria-mediated apoptosis. Bcl-2 family proteins are divided into two broad categories: pro-apoptosis proteins (Bax, Bad, Bak, etc.) and anti-apoptosis proteins (Bcl-2, Bcl-xL, etc.).[34] Previous study has revealed that either single or double phosphorylated Ser-112 or Ser-136 of Bad would inhibit its pro-apoptosis function.[35, 36] In the present experiment, Bcl-2, Bax and phosphorylated Ser-136 of Bad were measured by western blot analysis. As shown in Figure 6, $A\beta_{25-35}$ treatment significantly suppressed Bcl-2/Bax expression and inhibited Bad phosphorylation in PC12 cells ($p < 0.01$), indicating obvious pro-apoptosis activity. PT markedly reversed the above effects in a dose-dependent manner ($p < 0.01$) and showed a significant anti-apoptosis consequence. Our study, for the first time, indicated that PT inhibits $A\beta_{25-35}$-induced apoptosis by promoting Bad phosphorylation. On the other hand, PS did not exhibit such effect, the level of Bcl-2, Bax and p-Bad did not change in comparison to the $A\beta_{25-35}$-stimulated control group.

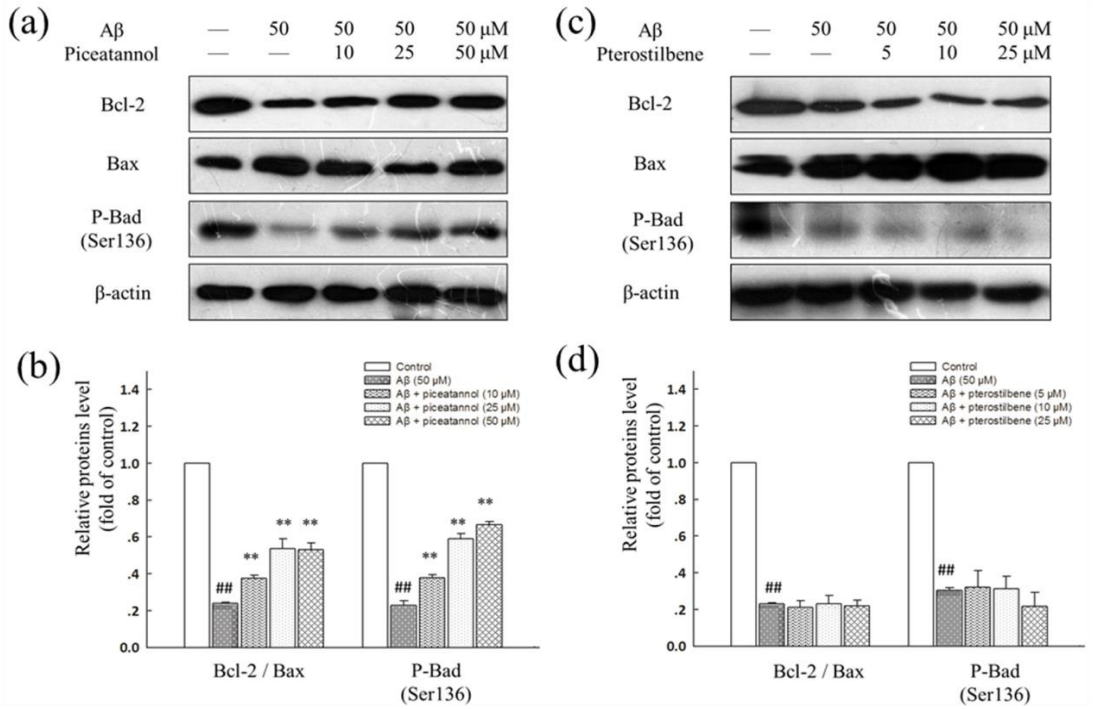

Figure 6. Effects of piceatannol (PT) and pterostilbene (PS) on Bcl-2 family proteins in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25-35}$ for 24 h. Western blotting was used to analyse the expression of Bax, Bcl-2 and p-Bad in **(a)** the group with PT pre-treatment or **(c)** the group with PS pre-treatment. The relative proteins level was calculated and presented in **(b)** the group with PT pre-treatment or **(d)** the group with PS pre-treatment. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25-35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25-35}$ treatment.

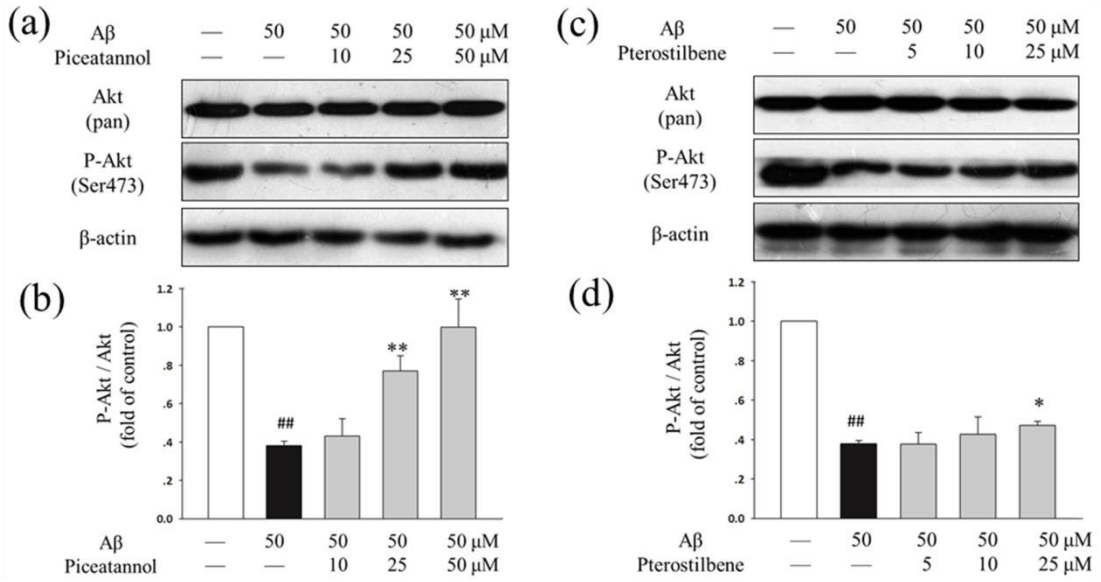

Figure 7. Effects of piceatannol (PT) and pterostilbene (PS) on Akt phosphorylation in $A\beta_{25\text{-}35}$-induced PC12 cells. Cells were pretreated with drugs at the indicated concentrations for 1 h and then exposed to 50 μM $A\beta_{25\text{-}35}$ for 24 h. Western blotting was used to analyse Akt phosphorylation **(a)** in the group with PT pre-treatment or **(c)** in the group with PS pre-treatment. The relative proteins level was calculated and presented in **(b)** the group with PT pre-treatment or **(d)** the group with PS pre-treatment. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25\text{–}35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25\text{–}35}$ treatment.

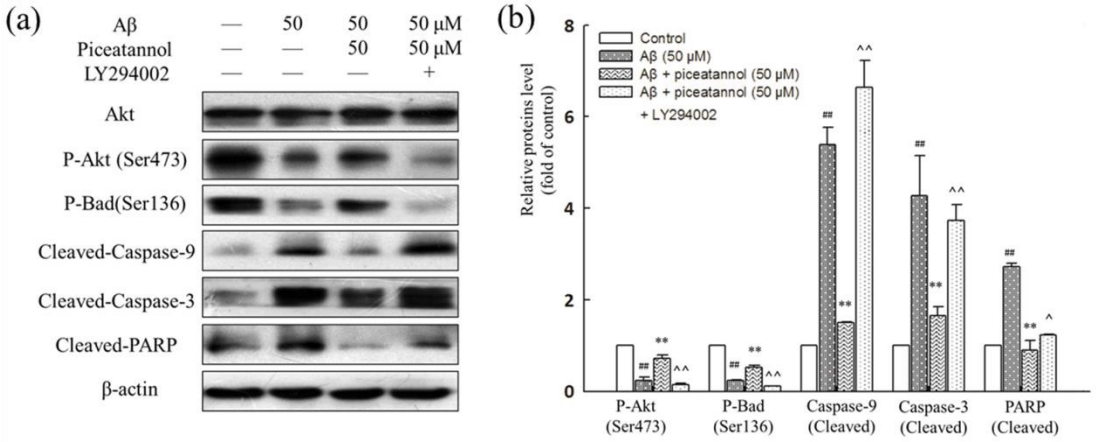

Figure 8. Effects of LY294002 (inhibitor of Akt phosphorylation) in the PC12 cells pretreated with piceatannol (PT). Cells were pretreated with LY294002 for 1 h, and then treated with PT at indicated concentrations for 1 h, followed by exposed to 50 μM $A\beta_{25\text{-}35}$ for 24 h. **(a)** Western blotting was used to analyze Akt phosphorylation, Bad phosphorylation, caspase-9/-3 cleavage and PARP cleavage. **(b)** The relative proteins level was calculated and presented. Each bar represents the mean ± SD from three independent experiments. (##) $p < 0.01$ and (#) $p < 0.05$ compared with the control without $A\beta_{25\text{–}35}$ treatment. (**) $p < 0.01$ and (*) $p < 0.05$ compared with the group with only $A\beta_{25\text{–}35}$ treatment.(^^) $p < 0.01$ and (^) $p < 0.05$ compared with the group with PT and $A\beta_{25\text{–}35}$ treatment

3.6 Effects of PT and PS on Akt phosphorylation in $A\beta_{25\text{-}35}$-induced PC12 cells.

Phosphorylated Bad is an important target of Akt kinase. Akt Ser-473 phosphorylation targets Bad Ser-136 phosphorylation to further regulate cell apoptosis.[36] To investigate the effect of PT and PS on Akt phosphorylation and whether PT-induced Bad ser-136 phosphorylation could be actually due to activation of Akt, Akt phosphorylation on Ser-473 was measured by western blot analysis. As shown in Figure 7, PT led to a dose-dependent increase in Akt phosphorylation compared to the group with only $A\beta_{25\text{-}35}$ treatment ($p < 0.01$), which was exactly consistent with Bad ser-136 phosphorylation. Meanwhile, PS (25 μM) promoted Akt phosphorylation on Ser-473 ($p < 0.05$), while the lower concentrations (5, 10 μM) did not make any difference on Akt phosphorylation. The results suggest that both PT and PS activate PI3K/Akt signaling pathway, in which the downstream targets were different.

To confirm the pivotal role of Akt phosphorylation in the whole anti-apoptosis signaling pathway of PT, we measured Akt phosphorylation, Bad phosphorylation, caspase-9/-3 cleavage and PARP cleavage in PC12 cells which were pretreated with an Akt pharmacological inhibitor, LY294002. As shown in Figure 8, Akt phosphorylation was significantly blocked by its inhibitor

LY294002 ($p < 0.01$). Bad phosphorylation induced by PT was also prominently inhibited ($p < 0.01$). In agreement with Akt and Bad inactivation, the cleavage of caspase-3/-9 and PARP were dramatically increased ($p < 0.05$). In conclusion, the results strongly suggest that Akt phosphorylation in the suppression of apoptosis induced by PT is associated with Bad phosphorylation, and Akt phosphorylation plays a crucial role in the entire anti-apoptosis pathway.

4 Discussion

The cytotoxic effect of Aβ against neurons has been regarded as a major factor in AD onset. Aβ accumulation and deposition in the brain would eventually lead to the loss of neurons via apoptosis.[28] Therefore, the inhibition of neuron apoptosis is thought to be a promising therapeutic approach in AD. However, due to a lack of efficacy and the side effects of synthetic drugs, bioactive natural products attracted much attention as predominant and promising therapeutic agents for AD.[13] Many bioactive natural products have been reported to have anti-apoptosis activity and potential therapeutic effect in AD. Stilbenes, a series of important bioactive natural products, are a group of phytoalexins found in *Vitis* species to resist disease.[17] Several studies have found that stilbenes binded to mutually exclusive sites on the Aβ plaques with ligand–protein interaction, so stilbenes were useful as a probe in detection of Aβ plaques.[37, 38] In addition, twenty stilbenes have been confirmed to demonstrate an inhibition of Aβ aggregation process.[18] Several stilbenes such as RES[21] and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS),[39] were efficient in protecting neurons from neurotoxicity induced-apoptosis. Therefore, stilbenes would be predominant and potential in diagnosis and therapeutic of AD. Among the stilbenes, due to the greater neuroprotective activity than RES, PS and PT deserve more attention.

4.1 PT suppresses $A\beta_{25-35}$-induced apoptosis via novel PI3K/Akt/Bad signaling pathway as well as downstream mitochondria-mediated and caspase-dependent signaling pathway.

PT (3,5,4',3'-*trans*-trihydroxystilbene, as shown in Figure.1) is a naturally occurring hydroxyl derivatives of resveratrol widely found in the *Vitis* species. The scientific research team of Lee has carried out a series of experiments on the anti-apoptosis activity of PT, and demonstrated that PT protected 4-hydroxynonenal-, hydrogen peroxide-, peroxynitrite- and Aβ-induced neuron apoptosis through the suppression of ROS.[24-26] In the research of PT against Aβ-induced neurotoxicity,[26] the apoptosis features, such as cell viability, intracellular ROS and cleavage of PARP and caspase-3, were investigated, suggesting that PT suppresses apoptosis by blocking accumulation of ROS. However, the underlying mechanism, especially the signaling pathway remains unclear. In the present study, we confirmed that PT efficiently increased the cell viability (Fig.2a), decreased the apoptosis rate (Fig.3b), and suppressed the accumulation of ROS (Fig.4a) in $A\beta_{25-35}$-treated PC12 cells. In addition, the underlying mechanism was targeted. We suggest the neuroprotection appears to correlate with the block of multiple caspases cleavage (Fig.5a, 5b), inhibition of Bcl-2/Bax (Fig.6a, 6b), promotion of Bad phosphorylation (Fig.6a, 6b) and Akt phosphorylation (Fig.7a, 7b).

The caspase cascade is the best-known and widest studied apoptosis factors. Among at least 14 known caspases, those involved in apoptosis can be further subdivided into initiator caspases (caspase-2, -8, -9 and -10) and effector caspases (caspase-3, -6 and -7).[40] Our study confirmed that Aβ leads to a caspase-dependent apoptosis. $A\beta_{25-35}$ caused the activation of caspase-9 and -3, as well as the cleavage of its downstream target PARP (Fig.5), which is an important poly ADP-ribose polymerase to sustain cellular homeostasis. And these effects were strongly reversed by PT pre-treatment (Fig.5a, 5b), suggesting PT exerts its anti-apoptosis ability through the inactivation of caspase-dependent pathway, which keeps consistent in the previous study.[26] However, not all apoptosis processes are dependent on caspase activation. For instance, Curcumol induced apoptosis via caspases-independent mitochondrial pathway in human lung adenocarcinoma ASTC-a-1 cells. The inhibitor of caspases did not reduce the curcumol-induced apoptosis.[41] In addition, we found that PT did not affect the caspase-8 (Fig.5a, 5b), which is an important factor involved in death receptor-mediated pathway, suggesting that mitochondria-mediated pathway which include caspase-9 and -3 might be potential upstream pathway. It is worth mentioning that Kim.*et al*[24] indicated that PT attenuated hydrogen-peroxide- and peroxynitrite-induced apoptosis of PC12 cells through the inhibition of both caspase-8 and caspase-3. In the present study, PT dramatically increased Bcl-2/Bax expression (Fig.6a, 6b). Bcl-2/Bax was an important set of protein related to apoptosis on mitochondria. Bax is a pro-apoptosis protein regulated by its binding partner Bcl-2 with anti-apoptosis activity. Bax promotion causes Bcl-2 suppression, which would further results in an increase in mitochondrial permeability and the release of cytochrome c, which subsequently leads to the activation of caspase-9.[42] Therefore, combined with the results of caspases mentioned above, these results provided further evidence that PT inhibits $A\beta_{25-35}$-induced apoptosis via the caspase-dependent and mitochondria-mediated signaling pathway.

Bad is a member of the pro-apoptosis bcl-2 family proteins. The pro-apoptosis activity of Bad is determined by its phosphorylation status.[43] The phosphorylation of Bad at Ser-112 or Ser-136 results in loss of its pro-apoptosis activity.[36, 44] unphosphorylated Bad can translocate to mitochondrial form cytoplasm and subsequently interact with Bcl-xl, an anti-apoptosis protein of Bcl-2 family, induced mitochondrial dysfunction and cell apoptosis.[11] Therefore, the suppression of Bad phosphorylation was a promising target of therapeutic agents to cancer cells.[45] Our study provides evidence that $A\beta_{25-35}$ treatment inhibits the phosphorylation of Bad at Ser-136 (Fig.6), which was reported by only one previous study.[11] PT pre-treatment significantly promoted Bad phosphorylation (Fig.6b). In addition, phosphorylation of Bad at Ser-136 was confirmed to be related to Akt phosphorylative activation (Fig.7b). Akt activation resulted in a significant phosphorylation of Bad and promoted cell survival. Our study first suggests that PT represses apoptosis in PC12 cells by promoting Akt phosphorylation and Bad phosphorylation via a PI3K/Akt/Bad signaling pathway. Meanwhile, PI3K/Akt pathway was reported to be involved in PT suppressing breast cancer cell invasion in which the downstream factor was

mTOR rather than Bad.[46] Furthermore, Akt phosphorylation and Bad phosphorylation were significantly inhibited, the cleavage of caspase-9, caspase-3 and PARP were dramatically promoted by LY294002, a pharmacological inhibitor of Akt (Fig.8b), suggesting the Akt phosphorylation is vital in the entire anti-apoptosis pathway. Therefore, the results strongly support a novel mechanism of PT against Aβ-induced apoptosis via the promotion of Akt phosphorylation and Bad phosphorylation as well as the suppression of the downstream mitochondria-mediated signaling pathway and caspase-dependent pathway. In addition, the PI3K/Akt/Bad signaling pathway plays a critical role (Fig. 9).

4.2 PS exhibits weaker anti-apoptosis ability than PT against $A\beta_{25\text{-}35}$ through inhibition of ROS accumulation, promotion of Akt phosphorylation, and a different downstream signaling pathway with PT

PS (3,5-Dimethoxy-4'-hydroxy-*trans*-stilbene, as shown in Figure.1) is a resveratrol derivative of methoxy. Recent study showed that PS, rather than RES, suppresses cellular stress and inflammation in aging and AD animal models.[23] In addition, PS was reported with a neuroprotective ability against hydrogen peroxide-induced neurotoxicity in SY5Y cells[47] and PC12 cells.[48] Apart from this, no report regarding the anti-apoptosis property of PS against Aβ has been found so far. PS is often considered with higher bioavailability because it is more easily transported into the cell and more resistant to degradation and elimination.[49] However, our study first shows that PS possesses somewhat weaker anti-apoptosis activity than PT against Aβ in PC12 cells. In the present study, PS (25 μM) increased the cell viability (Fig.2b), decreased the apoptosis rate (Fig.3c), inhibited the accumulation of ROS (Fig.4b), however, the effect was much weaker when compared with PT. In addition, PS exerts the anti-apoptosis activity via a different signaling pathway with PT. PS (25 μM) promoted the Akt phosphorylation (Fig.7d), however, did not affect the other downstream factors including Bad, Bcl-2, Bax, caspase-3, caspase-9, PARP (Fig.5b, 6b, 7b).

In the present study, PS suppressed the Aβ-induced apoptosis via PI3K/Akt signaling pathway, however, the downstream signaling pathway was different with that of PS. One potential explanation account for our results is the chemical structure differences between these two compounds, just like the result in previous study that PS, not RES inhibited the cellular stress and inflammation.[23] Meanwhile, the Akt phosphorylation caused by PS might target some downstream factors other than Bad, such as GSK-3β[9] and mTOR,[10] which would also promote cell survival. In addition, PS might also target other signaling pathways. Recent study suggested that PS suppressed neurotoxicity through both PI3K/Akt and MAPK/ERK signaling pathway in vitro.[47] Another study indicated that PS alleviated injury through inhibition of NF-κB p65 and JNK phosphorylation which were associated with inflammatory signaling pathway in aging and AD animal models.[23] Therefore, further studies regarding the signaling pathway of PS ant-apoptosis ability are needed.

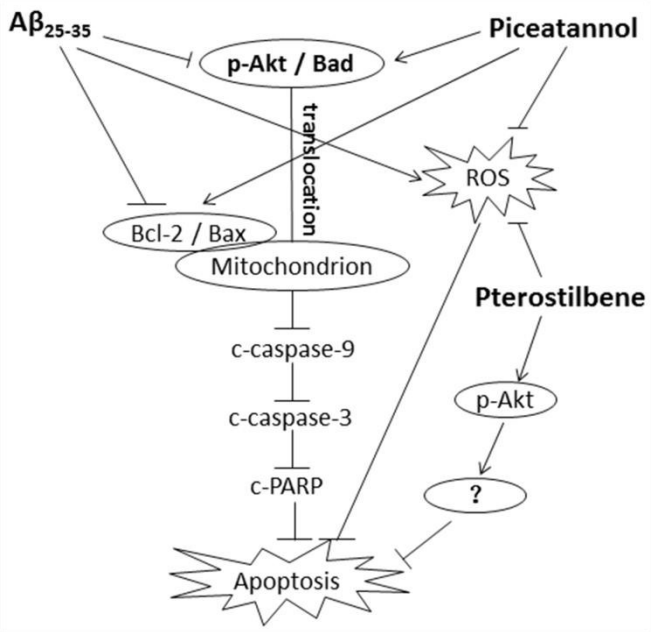

Figure 9. Signaling pathways underlying the mechanism of piceatannol (PT) and petrostilbene (PS) against $A\beta_{25\text{-}35}$-induced apoptosis in PC12 cells. $A\beta_{25\text{-}35}$ treatment led to promotion intracellular ROS, suppressed Bcl-2/Bax expression and Akt, Bad phosphorylation, further induced the cleavage of capase-9, capase-3 and PARP, eventually resulted in apoptosis in PC12 cells. Pre-treatment with PT was capable of reversing all the $A\beta_{25\text{-}35}$-induced effects mentioned above, further suppress apoptosis. Pre-treatment with PS inhibit apoptosis by decreasing ROS and promote Akt phosphorylation, and the underlying mechanism needs further investigated.

Our study found that $A\beta_{25\text{-}35}$ increased the ROS level (Fig.4) in PC12 cells, and the suppression of ROS accumulation is an important mechanism involved in PS inhibiting $A\beta_{25\text{-}35}$-induced apoptosis. Many studies have confirmed that $A\beta_{25\text{-}35}$ cytotoxicity is accompanied by ROS accumulation.[7, 25, 50]. $A\beta_{25\text{-}35}$ inhibited the catalase, superoxide dismutase and glutathione peroxidase, which further resulted in the increasing of ROS including hydrogen peroxide and hydroxyl radical.[51] High levels of ROS can cause direct oxidative injury to cellular molecules, including lipids, proteins and DNA, following by cellular structural damage, cellular function disorder and cell apoptosis.[6] On the other hand, redundant ROS can also act as signal factor to stimulate downstream factors. For instance: ROS initiated the expression of transcription factors related to apoptosis, such as multiple caspases, to further induce cell apoptosis;[52] ROS induced the expression of pro-inflammatory cytokines such as TNF-α and IκB to result in an inflammation response, which would further induce cell apoptosis;[53] Or Even ROS triggered the expression of hypoxic homeostasis transcription factors, such as HIF-1α, Cav-β3, and STAT3, and further induced cell death.[54] In the present study, PT and PS both suppressed cell apoptosis via the inhibition of ROS production, however, with a different mechanism. The result might be related to the different role of ROS in PC12 cells. The decreased ROS after PT and PS treatment may regulate different downstream gene expression or PS just relieve direct oxidative injury of ROS to promote survival. The hypothesis needs further identification.

5 Conclusions

In conclusion，PS (25 μM) and PT (10, 25, and 50 μM) both showed observable anti-apoptosis activity against $A\beta_{25-35}$ in PC12 cells. Our study first provides evidence that PS repressed $A\beta_{25-35}$-induced PC12 cell apoptosis via the inhibition of ROS and promotion of Akt phosphorylation. Compared with PS, PT possesses more eminent anti-apoptosis and neuroprotective activity via a novel PI3K/Akt/Bad signaling pathway and downstream mitochondria-mediated and caspase-dependent signaling pathway. The results provide the basis for dietary intervention involved in the prevention and adjunctive therapy of AD.

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Conflict of interest

No conflicts of interest are declared for any of the authors.

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