Carbohydrate Polymers 117 (2015) 879–886

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A polysaccharide from *Polygonatum sibiricum* attenuates amyloid-β-induced neurotoxicity in PC12 cells

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ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form 8 October 2014
Accepted 9 October 2014
Available online 23 October 2014

Keywords:
Polygonatum sibiricum
Polysaccharide
Alzheimer's disease
Neuroprotection
Akt
PC12 cells

ABSTRACT

One of the pathological hallmarks of Alzheimer's disease (AD) is the progressive accumulation of beta-amyloid (Aβ) in the form of senile plaques, and Aβ induced neurotoxicity has been identified as a major cause of the onset of AD. In this study, we investigated the protective effects of a polysaccharide (PS-WNP) from *Polygonatum sibiricum* against the $A\beta_{25-35}$-induced neurotoxicity in PC12 cells and explored the underlying mechanism. The results showed that pretreatment with PS-WNP significantly attenuated cell death and the elevated Bax/Bcl-2 ratio evoked by $A\beta_{25-35}$, and subsequently inhibited mitochondrial dysfunction and cytochrome c release into the cytosol. Moreover, PS-WNP significantly inhibited $A\beta_{25-35}$ induced caspase-3 activation and enhanced the protein levels of phosphorylated Akt (p-Akt) in PC12 cells. Additionally, pretreatment with the PI3K inhibitor (LY294002) completely abolished the protective effects of PS-WNP against $A\beta_{25-35}$-induced neuronal cell apoptosis. These observations unambiguously suggested that the protective effect of PS-WNP against $A\beta_{25-35}$-induced apoptosis in PC12 cells was associated with the enhancement of PI3K/Akt signaling pathway.

1. Introduction

Alzheimer's disease (AD), one most frequent form of age-related dementia, is a serious and progressive neurodegenerative disorder characterized by degeneration and loss of neurons in the brain that usually begins with memory loss (Cuenco et al., 2008). Much accumulated evidence shows that accumulation of beta-amyloid (Aβ) fibrillar deposition in senile plaques and neurofibrillary tangles lesions in specific areas of brain correlates with the progression of cognitive dysfunction in AD patients (Jang & Surh, 2003; Kayed et al., 2003). Aβ peptide and its aggregates are the main components of senile plaques and evidences from *in vivo* and *in vitro* experiments have shown that Aβ-induced neurotoxicity contributes to the pathogenesis and progression of AD (Billings, Oddo, Green, McGaugh, & LaFerla, 2005; Hamnér, Skoglösa, & Lindholm, 1999; Walsh & Selkoe, 2004). The underlying mechanism of Aβ-induced neurotoxicity is not yet fully understood but appears to involve several pathways associated with apoptosis, which in turn may lead to the neuronal degeneration in AD (Loo et al., 1993; Yao, Nguyen, & Pike, 2005). Therefore, inhibition of Aβ-induced neuronal apoptosis may provide a plausible approach for AD prevention and treatment.

At present, there is no definitive treatment or cure for AD. Increasing evidence indicates that traditional Chinese herbal medicine may be a promising source of effective drugs for treating AD, which have been demonstrated to effectively enhance cognitive function and brain function, and ameliorate other symptoms in patients with AD (Howes, Perry, & Houghton, 2003; May et al., 2009; Zhou, Yang, Xu, Li, & Hu, 2009). The rhizoma of *Polygonatum sibiricum*, known as "Huang-Jing" in Chinese, is a common Chinese medicinal herb and has been used traditionally for more than hundreds of years (Sun, Li, & Wang, 2005). Its therapeutic functions in Chinese medicine are to reinforce qi and nourish yin, invigorate the function of the spleen, moisten the lung, and benefit the kidney (China Pharmacopoeia Committee, 2010). Pharmacological studies indicate that *P. sibiricum* may enhance immune system, lower blood glucose and lipid levels, and prevent aging (Liu, Dong, Dong, Fang, & Ding, 2007). Moreover, *P. sibiricum* have been widely used as an ingredient or supplement in food industry, because of its sweet fragrance and taste, as well as health-improving activity (Baek et al., 2012). In recent years, many chemical constituents have been isolated from *P. sibiricum*, including alkaloids (Sun et al., 2005),

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http://dx.doi.org/10.1016/j.carbpol.2014.10.034
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flavones (Chopin, Dellamonica, & Besson, 1977), steroid saponins (Son & Du, 1990), lignins (Sun & Li, 2001), and polysaccharides (Liu et al., 2007). Among them, polysaccharides isolated from *P. sibiricum* have been reported to significantly reverse cognitive deficits in a scopolamine-induced mouse model of dementia (Zhang, Zhang, Wang, & Mao, 2008), implying that *P. sibiricum* polysaccharide may possess the potential therapeutic effect against AD. Accumulating evidences proved that Aβ-induced neuronal cell damage in PC12 cells has been widely and extensively used as a well established cellular model of AD *in vitro* to test the neuroprotective effect of drugs (Ji & Liu, 2001; Zhou et al., 2009). Therefore, the present study aims to purify the water-soluble neutral polysaccharide (PS-WSNP) from this plant and observe its effect on PC-12 cell damage induced by Aβ peptide. Furthermore, the molecular signaling pathway involved in the neuroprotective effect of PS-WSNP on Aβ-induced neurotoxicity in PC12 cells was also explored.

2. Materials and methods

2.1. Materials and chemicals

The rhizome of *Polygonatum sibiricum* was purchased from Beijing Tong Ren Tang Pharmacy Store (Beijing, China). All other chemicals and solvents were of analytical grade from China.

2.2. Isolation and purification of a polysaccharide from P. sibiricum

The dried rhizome of *P. sibiricum* (900 g) was cut into small pieces and further ground into fine powder (20 meshes) using a high speed disintegrator, and then was refluxed in a Soxhlet apparatus with 95% ethanol twice for 8 h at 75 °C to remove some small molecule materials. The residue was then boiled with 10 vol. of distilled water at 90 °C for 3 h thrice and filtered with linen cloth. After centrifugation (1700 × *g* for 10 min), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (1:4 (v/v)) at 4 °C for overnight. The precipitate collected by centrifugation was dissolved in distilled water and treated with Sevag reagent (1:4 *n*-butanol:chloroform, v/v, 400 ml) to remove proteins (Staub, 1965). The final deproteinized solution was dialyzed against distilled water for 24 h, and then freeze-dried to give the deproteined polysaccharide fraction PS-CP (22.3 g).

The PS-CP was dissolved in distilled water, centrifuged, and then subjected to a DEAE-cellulose column (2.0 cm × 40 cm, Cl^-) (Amersham Pharmacia Biotech. Sweden) pre-equilibrated with distilled water. The column was eluted first with 3 column volumes of distilled water at a flow rate of 2 mL/min to obtain the neutral fraction (PS-CWNP), followed by 0.2, 0.5, 0.8 and 2 M NaCl solutions to obtain the anion-charged fractions (PS-CWAPA, PS-CWAPB, PS-CWAPC, PS-CWAPD). The eluate was monitored by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The PS-CWNP was concentrated, dialyzed, lyophilized, and further fractionated on a Sepharose CL-6B column (2.0 cm × 90 cm) (Amersham Pharmacia Biotech. Sweden) eluted with 0.1 M NaCl at 0.5 ml/min, yielding one completely separated water-soluble neutral polysaccharide fraction (PS-WNP, 1.2 g, 5.4% of the crude polysaccharide PS-CP), which was used for further study.

2.3. Physicochemical and structural analysis of polysaccharide

2.3.1. Measurement of protein, carbohydrate and uronic acid contents

Protein content was measured by the method of Bradford (1976). Total carbohydrate content was determined by the phenol–sulfuric acid method (Dubois et al., 1956). Uronic acid content was determined by the carbazole–sulfuric acid method (Bitter & Muir, 1962).

2.3.2. Homogeneity and molecular weight

The molecular weight and purity of polysaccharide PS-WNP were determined by high performance gel permeation chromatography (HPGPC) on a Waters HPLC system fitted with one TSK-G3000 PWXL column (Ø 7.8 mm × 300 mm ID) and a 2410 differential refractive index detector (RAD).

2.3.3. Monosaccharide composition analysis

The identification and quantification of the monosaccharides of polysaccharide was achieved by gas chromatography (GC) analysis as described by Liu et al. (2011). The resulting alditol–acetates were analyzed by GC on a Vavian 3400 instrument (Hewlett-Packard, Component, USA) with DM-2330 capillary column (30 m × 0.32 mm × 0.2 μm) and detected with a flame-ionization detector (FID) at 260 °C.

2.3.4. Methylation analysis

PS-WNP (2 mg) was dissolved in DMSO, followed by treatment with iodomethane (1 ml) and powdered NaOH (20 mg) by the modified method (Kalyan & Paul, 1992). The reaction mixture was extracted with $CHCl_3$ and the solvent was then removed by evaporation. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were then hydrolyzed with formic acid and 2 M TFA at 100 °C for 6 h, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed products were reduced with $NaBH_4$ for 24 h and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions as above.

2.4. $A\beta_{25-35}$ preparation

$A\beta_{25-35}$ (Sigma, St. Louis, MO. USA), which is the most toxic peptide fragment derived from amyloid precursor protein, was dissolved in deionized distilled water and incubated with constant oscillation at 37 °C for 3 days to induce its aggregation (Hao, Zhang, Yu, Cheng, & Ji, 2011). After aggregation, the solution was stored at −20 °C until use. The stock solution was diluted to desired concentrations immediately before use and added to cell culture medium.

2.5. Cell culture and treatment

The PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). They were routinely maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO, USA) medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was changed every other day. In all experiments, PC12 cells were seeded into 96-well multiplates (2×10^5 cells/well). After 24 h, cells were pretreated in the absence or in the presence of PS-WNP (10, 20, 50, 100 and 200 μg/ml) for a period of 24 h, followed by incubating with $A\beta_{25-35}$ (20 μM) for an additional 24 h.

2.6. MTT assay

Cell viability was estimated by the MTT reduction assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Following incubation

for 24 h at 37 °C and 5% CO_2, the supernatant was removed and 100 μl dimethyl sulfoxide added to each well to dissolve the formazan. Absorbance was measured using a microplate reader at a wavelength of 570 nm. Cell viability was normalized as relative percentages in comparison with untreated controls.

2.7. Flow cytometric analysis

Apoptosis was detected using the Annexin V-FITC/PI double staining Kit (Becton Dickinson, San Jose, CA) according to manufacturer's instruction. The apoptotic cells were measured with a FACScan flow cytometer (Becton Dickinson, USA). In some experiments, P13K inhibitor LY294002 (Calbio-chem-Behring, Za Jolla, CA) was added to the cells at a final concentration of 20 μM 30 min before $A\beta_{25-35}$ and PS-WNP treatments.

2.8. Determination of mitochondrial membrane potential (MMP)

A unique fluorescence probe rhodamine 123 (Rh123, Sigma) was used to measure the loss of MMP in PC12 cells, which selectively enters the mitochondrial matrix with an intact membrane potential (Lemasters et al., 1987). After treatment, the PC12 cells were incubated with 5 mg/l rhodamine 123 for 30 min at 37 °C in the dark. After incubation, cells were washed three times with PBS and the fluorescence was measured on a microplate reader at 510 nm with excitation at 488 nm. MMP was expressed as percentage of the non-treated control.

2.9. Mitochondrial cytochrome c release assay

Cytochrome c (Cyt c) release from mitochondria into the cytosol was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) within 96-well plates according to procedure given by the manufacturer. The results were expressed as ng/ml by reference to the standard curve.

2.10. Caspase enzyme activity assay

Upon completion of the treatment, PC12 cells were collected, washed, and subjected to caspase-3 enzyme activity measurement using caspase-3 assay kit (Sigma–Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. The absorbance values are detected by the absorbance at 405 nm wavelength. The activities were normalized using the total protein concentrations as determined according to the method of Bradford (Bradford, 1976).

2.11. Western blot

For western blot analysis, treated or untreated cells were homogenized in a cell lysis buffer (Beyotime, Jiangsu, China) to prepare protein extracts as previously described (Napolitano et al., 2011) and the protein concentrations were determined using the Bradford protein assay (Bradford, 1976). Each equal amount of total cell proteins (40 μg) were loaded and separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes (Millipore, MA, USA) by electroblotting. After the membrane were blocked in 5% non-fat milk for 1 h at room temperature, the membranes were probed with primary antibodies (anti-Bcl-2, 1:200; anti-Bax, 1:200; anti-β-actin, 1:1000; anti-p-Akt, 1:1000; anti-Akt, 1:1000; anti-cleaved caspase 3, 1:1000; anti-PARP [poly(ADP-ribose) polymerase] 1:1000, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. β-Actin was used for a control for protein loading in each lane. The blots were washed, incubated with horseradish-peroxidase-conjugated secondary antibody (1:2000) for 2 h at room temperature and then

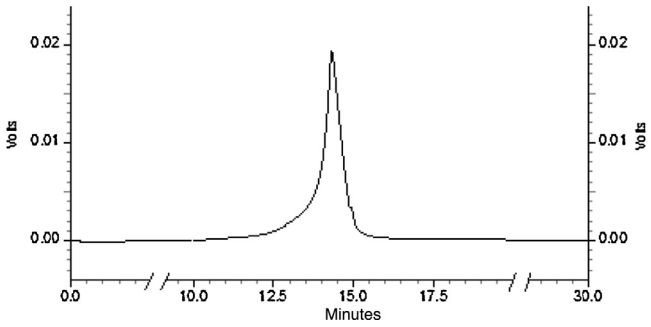

Fig. 1. High-performance gel-permeation chromatography (HPGPC) of the polysaccharide PS-WNP from the rhizome of *P. sibiricum*.

subjected to autoradiography using an ECL Western blotting Detection kit from Amersham Pharmacia (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The optical density (ODOD) was analyzed by the Gel Image Analysis System (Tanon 2500R, Shanghai, PR China).

2.12. Statistical analysis

Results are expressed as the means ± standard error of the mean (SEM), and were analyzed using SPSS 13.0 software. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey test when appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of PS-WNP from the rhizome of P. sibiricum and its physicochemical characteristics

The water-soluble crude polysaccharide (PS-CP) was prepared from the rhizome of *P. sibiricum*, with a yield of 2.5% of the dried material. The PS-CP was then purified by DEAE-cellulose anion-exchange chromatography with a successive elution manner. One water-soluble neutral fraction (PS-CWNP) and four acidic polysaccharide fractions (PS-CWAPA, PS-CWAPB, PS-CWAPC and PS-CWAPD) were obtained from 0.2, 0.5, 0.8 and 2 M NaCl eluent, respectively. The PS-CWNP fraction was further purified through Sepharose CL-6B gel-permeation chromatography, leading to the isolation of a major purified polysaccharide PS-WNP, with its yield being 1.35 ‰ of the dried material.

The PS-WNP had a negative response to the Bradford's method and had no absorbance peak at 280 nm in the UV spectrophotometer, indicating the absence of protein. Results from phenol–sulfuric acid assay showed that PS-WNP contained 95.5% total carbohydrate. As determined by m-hydroxydiphenyl colorimetric method and GC, no uronic acid was detected in PS-WNP. The HPGPC profile (Fig. 1) showed a single and symmetrically sharp peak, indicating that PS-WNP was a homogeneous polysaccharide. According to the calibration curve with standard dextran, the average molecular weight of PS-WNP was calculated as 76 kDa. The results of GC quantitative analysis of the acetylated of monosaccharides revealed that PS-WNP was composed of Gal and Man in a molar ratio of 12.1:5.4, indicating that PS-WNP is a mannogalactan. For the purpose of further determining the sugar linkage of PS-WNP, the GC–MS analysis revealed the presence of (1 → 6)-galactopyranosyl (Residue-A), (1 → 2,6)-D-galactopyranosyl (Residue-B) and terminal D-mannopyranosyl (Residue-C) moieties in a ratio of nearly 6.2:6.1:5.8, respectively (Table 1). This showed a good correlation between terminal and branched residues, and these molar ratios agreed with the overall monosaccharide composition described above. Therefore, it is likely to conclude that Residue-A and

Table 1
GC–MS data for methylation analysis of the polysaccharide PS-WNP from the rhizome of *P. sibiricum*.

Peak no.	Methylated sugars	Molar ratios	Linkage type
Residue A	2,3,4,-Me_3-Gal*p*	6.2	6→)-Galp-(1→
Residue B	3,4,-Me_2-Gal*p*	6.1	2,6→)-Galp-(1→
Residue C	2,3,4,6-Me_4-Man*p*	5.8	Manp-(1→

Residue-B were major components of the backbone structure, which are terminated with Residue-C in O-2 position of Residue-B.

3.2. Effect of PS-WNP on cell viability and apoptosis in $A\beta_{25-35}$-treated PC12 cells

$A\beta_{25-35}$, the highly toxic peptide of Aβ protein (Yankner & Lu, 2009), was used as a neurotoxicant. As shown in Fig. 2A, a 24-h incubation with $A\beta_{25-35}$ induced cell death to 56.68% when compared with that of control group ($P < 0.01$). However, pretreatment with different concentrations of PS-WNP for 24 h prior to $A\beta_{25-35}$ exposure significantly reversed $A\beta_{25-35}$-treated PC12 cell death in a dose-dependent manner, which was significant beyond the concentration of 50 μg/ml ($P < 0.05$). Therefore, we selected 50–200 μg/ml concentration of PS-WNP for subsequent experiments. At the same time, we also detected the effect of PS-WNP on the cell viability in PC12 cells without $A\beta_{25-35}$ incubation for 24 h. Even at high concentration, PS-WNP showed no toxicity to PC12 cells (Fig. 2B, $P > 0.05$).

To examine whether or not the decrease in cell viability of $A\beta_{25-35}$-treated PC12 cells in our studies occurred through an apoptotic-like mechanism, the percentage of apoptotic cells was quantified on a FACScan flow cytometer by staining cells with annexin-VFITC/PI. This method is well-established for measuring apoptosis for many model systems (Bacsó, Everson, & Eliason, 2000). As shown in Fig. 2C, treatment of PC12 cells with 20 μM $A\beta_{25-35}$ for 24 h significantly increased both early and late apoptotic death in PC12 cells as indicated by the percentage of Annexin V-positive cells. Similar to the preventive effects of PS-WNP on the cell death, pretreatment of PC12 cells with PS-WNP (50, 100 and 200 μg/ml) for 24 h, prior to $A\beta_{25-35}$ exposure, dose-dependently and significantly decreased apoptosis compared to $A\beta_{25-35}$ alone ($P < 0.05$). The apoptotic rate in $A\beta_{25-35}$-treated PC12 cells was 50.2%, but was reduced by PS-WNP pretreatment to 36.2% at 50 μg/ml, 25.2% at 100 μg/ml, and 18.5% at 200 μg/ml. These results suggested that PS-WNP protected PC12 cells from $A\beta_{25-35}$ induced cytotoxicity in PC-12 cells through inhibiting apoptosis.

3.3. Effect of PS-WNP on the ratio of Bcl-2/Bax in $A\beta_{25-35}$-treated PC12 cells

As the proteins of Bcl-2 family are important modulators of PC12 cells apoptosis induced by Aβ (Lee et al., 2005), alterations in the protein levels of Bax and Bcl-2 were analyzed by Western blotting (Fig. 3A) using antibodies against Bax and Bcl-2. Exposure of the PC12 cells to 20 μM $A\beta_{25-35}$ for 24 h caused a significant upregulation of the expression of proapoptotic Bax protein and downregulation of anti-apoptotic Bcl-2 protein in PC12 cells, leading to the rise of Bax/Bcl-2 ratio (Fig. 3B). However, pretreatment with various concentrations of PS-WNP prior to $A\beta_{25-35}$ exposure reversed these responses. Thus, the ratio of Bax/Bcl-2 protein decreased compared to cells treated with $A\beta_{25-35}$ alone ($P < 0.05$, $P < 0.01$).

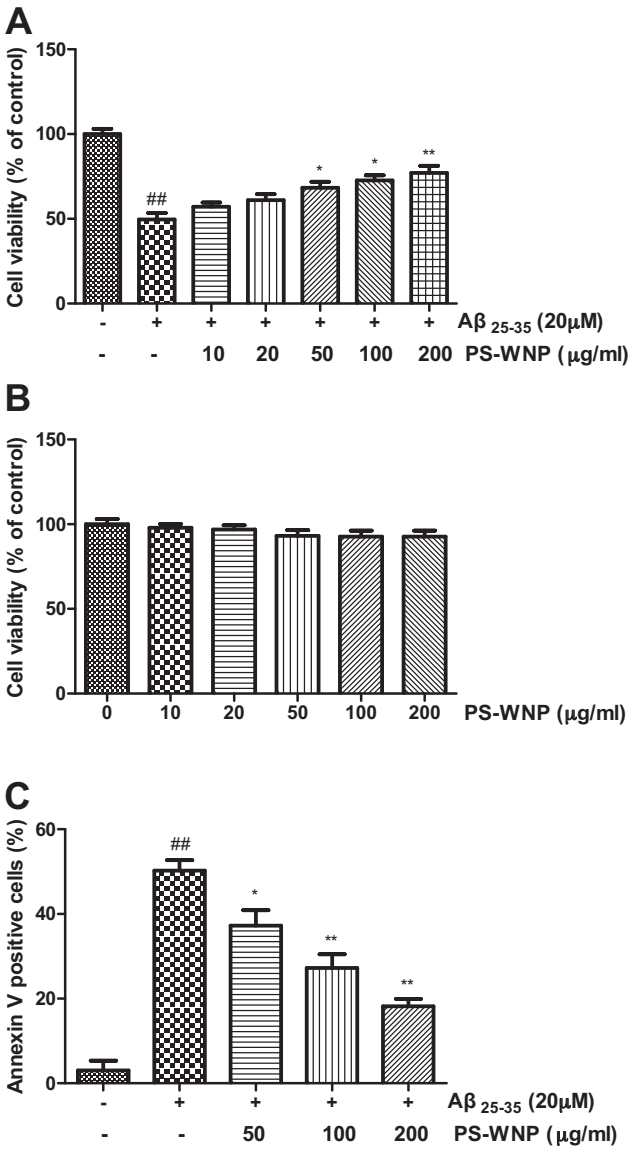

Fig. 2. Effects of PS-WNP on the viability of PC12 cells in (A) $A\beta_{25-35}$-induced or (B) basal conditions, as analyzed by the MTT assay. (C) Effects of PS-WNP on apoptosis in $A\beta_{25-35}$-induced PC12 cells, as evaluated by flow cytometric analysis. For all conditions, PC12 cells were treated with the indicated concentrations (0–200 μg/ml) of PS-WNP for a period of 24 h. This treatment was followed by exposure to $A\beta_{25-35}$ (20 μM) for an additional 24 h, in (A) and (C) conditions. Data shown are representative of three independent experiments. $^{\#\#}P < 0.01$ compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to $A\beta_{25-35}$ group.

3.4. Effect of PS-WNP on mitochondrial dysfunction and cytochrome c release in $A\beta_{25-35}$-treated PC12 cells

Increasing evidence suggests that a decrease in MMP and subsequently cytochrome c release constitutes a central event of the apoptotic process (Green & Reed, 1998; Favaloro, Allocati, Graziano, Di Ilio, & De Laurenzi, 2012). We first used the mitochondrial probe Rh 123 to measure MMP change. As shown in Fig. 4A, exposure of PC12 cells to 20 μM $A\beta_{25-35}$ for 24 h significantly decreased MMP to 62% of the control value. When the cells were pretreated with PS-WNP at the concentrations of 50, 100 and 200 μg/ml for 24 h, the $A\beta_{25-35}$-induced decrease of MMP was significantly increased to 78, 82 and 89% of the control, respectively, raising the possibility that PS-WNP may act at least in part by protecting mitochondrial function. To assess for this, we next measure cytochrome c concentration in cytosolic fractions using a commercial ELISA kits

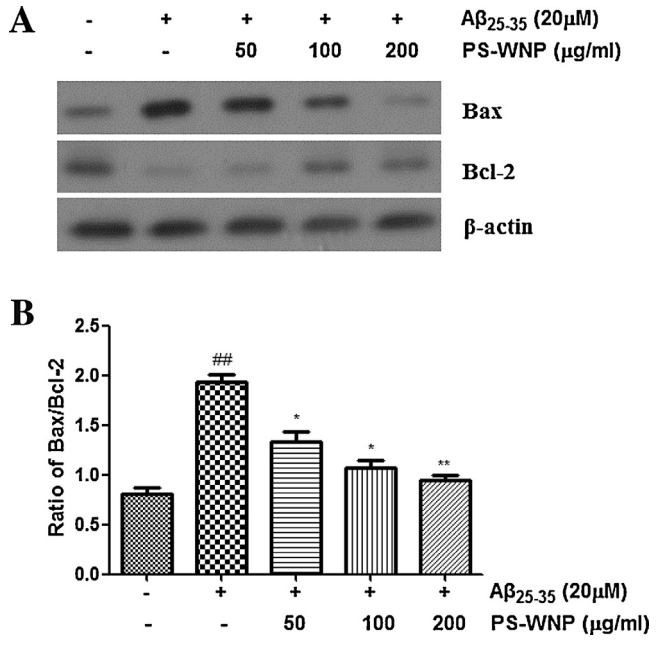

Fig. 3. (A) Effects of PS-WNP on the expression of Bcl-2 and Bax in $A\beta_{25-35}$-treated PC12 cells analyzed by Western blot analysis. PC12 cells were treated with the indicated concentrations (0, 50, 100 and 200 μg/ml) of PS-WNP for 24 h, followed by incubating with $A\beta_{25-35}$ (20 μM) for an additional 24 h. (B) Ratio of Bax/Bcl-2 proteins. Data shown are representative of three independent experiments. $^{\#\#}P < 0.01$ compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to $A\beta_{25-35}$ group.

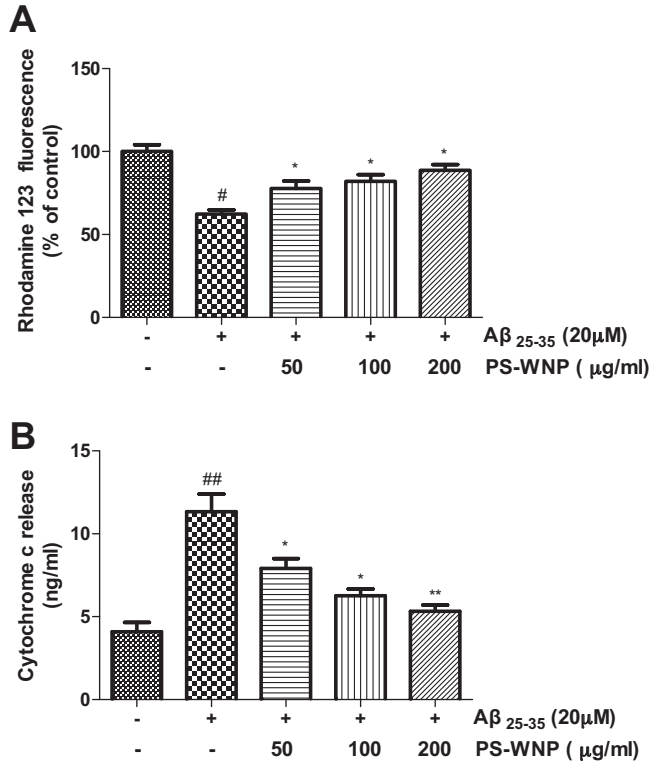

Fig. 4. Effect of PS-WNP on (A) mitochondrial membrane potential (MMP) in $A\beta_{25-35}$-treated PC12 cells, as analyzed by Rh123 method or (B) cytosolic cytochrome c levels in $A\beta_{25-35}$-treated PC12 cells, as analyzed by ELISA. PC12 cells were exposure to the indicated concentrations (0, 50, 100 and 200 μg/ml) of PS-WNP for a period of 24 h, followed by treatment with $A\beta_{25-35}$ (20 μM) for additional 24 h. Data shown are representative of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to $A\beta_{25-35}$ group.

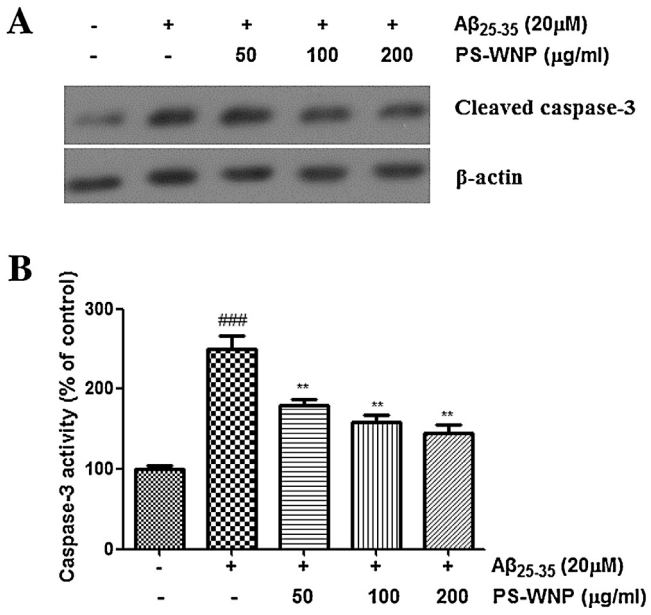

Fig. 5. Effects of PS-WNP on (A) active caspase-3 expression in $A\beta_{25-35}$-treated PC12 cells, as analyzed by Western blot analysis or (B) caspase-3 activity in $A\beta_{25-35}$-treated PC12 cells, as measured by a caspase-3 assay kit. PC12 cells were exposure to the indicated concentrations (0, 50, 100 and 200 μg/ml) of PS-WNP for a period of 24 h, followed by treatment with $A\beta_{25-35}$ (20 μM) for additional 24 h. Data shown are representative of three independent experiments. $^{\#\#\#}P < 0.001$ compared to control group; $^{**}P < 0.01$ compared to $A\beta_{25-35}$ group.

(Fig. 4B). Treatment of PC12 cells with $A\beta_{25-35}$ for 24 h showed an increase in the cytosolic cytochrome c levels. Pretreatment with PS-WNP significantly attenuated cytochrome c release induced by $A\beta_{25-35}$ in a dose-dependent manner ($P < 0.01$), as compared with $A\beta_{25-35}$-treated control group. These observations suggested that PS-WNP prevented $A\beta_{25-35}$-induced mitochondrial dysfunction in PC12 cells.

3.5. Effect of PS-WNP on caspase-3 activation in $A\beta_{25-35}$-treated PC12 cells

Caspase-3, a key downstream effector of the cysteine protease family, is the final executor of apoptosis and its activation has been recognized as an important factor for apoptosis (Mazumder, Plesca, & Almasan, 2008). Consequently, we determined the expression of active form of caspase-3 by western blot analysis (Fig. 5A). Consistent with the inhibitory effect of PS-WNP on cytochrome c release from the mitochondria to the cytosol, $A\beta_{25-35}$-induced increase of cleaved caspase-3 protein expression was significantly depressed by PS-WNP pretreatment.

To examine whether the observed increase in the cleavage of caspase-3 correlated with an increase in its activity, we measured the caspase-3-like protease activity using the substrate DEVD-pNA, a colorimetric peptide substrate specific for caspase-3. As shown in Fig. 5B, the enzyme activity of caspase-3 was significantly elevated in $A\beta_{25-35}$-treated PC12 cells. However, pretreatment with PS-WNP at concentrations of 50, 100 and 200 μg/ml significantly blocked $A\beta_{25-35}$-induced caspase-3 activation in PC12 cells in a dose-dependent manner ($P < 0.01$).

3.6. Effect of PS-WNP on P13K/Akt pathway in $A\beta_{25-35}$-treated PC12 cells

We further explored whether intracellular signaling mechanisms responsible for the protective effects of PS-WNP against $A\beta_{25-35}$-induced PC12 cell death is through PI3K/Akt activation.

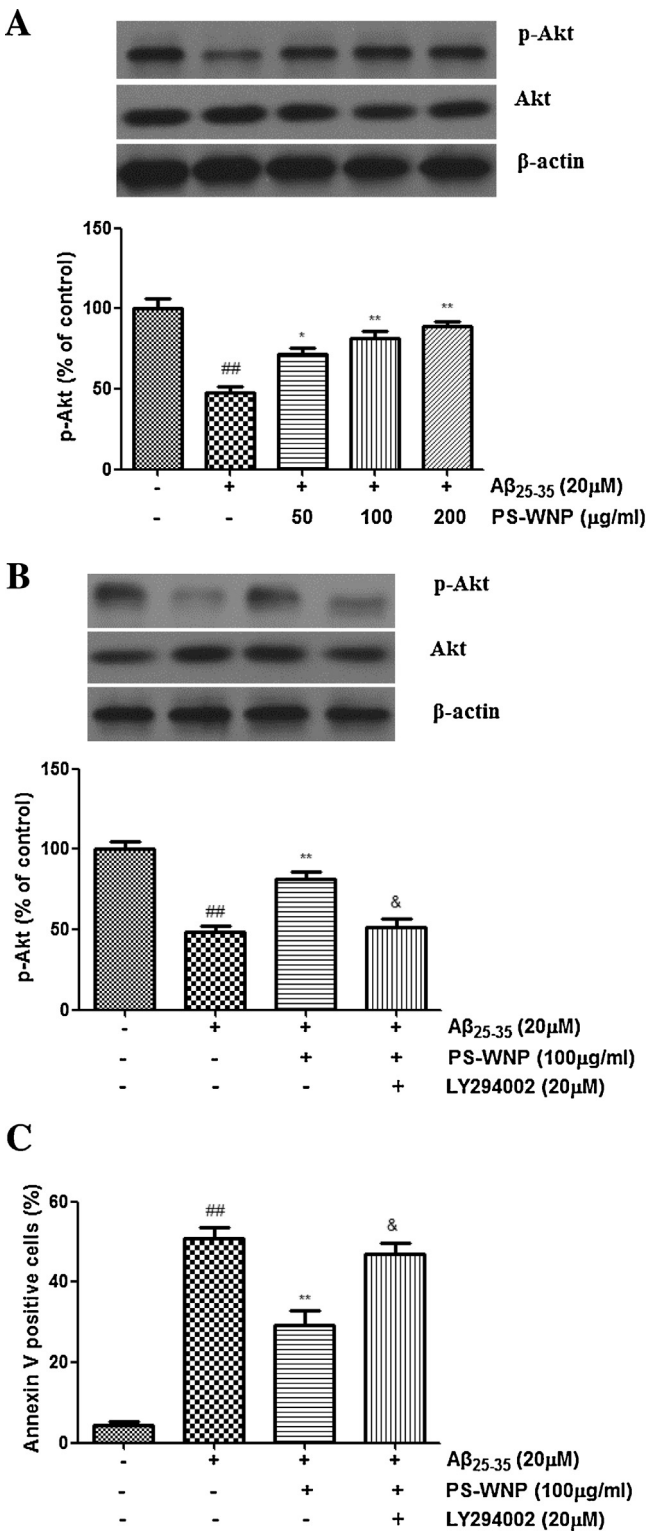

Fig. 6. (A) Effects of PS-WNP on p-Akt and Akt protein expression in $A\beta_{25-35}$-treated PC12 cells analyzed by Western blot analysis and quantitated results of p-Akt. PC12 cells were treated with the indicated concentrations (0, 50, 100 and 200 μg/ml) of PS-WNP for 24 h, followed by incubating with $A\beta_{25-35}$ (20 μM) for an additional 24 h. (B) Effects of PI3 K/Akt pathway inhibitor LY294002 on PS-WNP-mediated p-Akt and Akt protein expression in $A\beta_{25-35}$-treated PC12 cells analyzed by Western blot analysis and quantitated results of p-Akt. PC12 cells were treated with or withoutP13 K inhibitor LY294002 (20 μM) for 30 min, then with PS-WNP for 24 h, followed by incubating with $A\beta_{25-35}$ (20 μM) for an additional 24 h. (C) Effects of PI3 K/Akt pathway inhibitor LY294002 on PS-WNP-mediated apoptosis in $A\beta_{25-35}$-treated PC12 cells analyzed by flow cytometric analysis. PC12 cells were treated with or withoutP13K inhibitor LY294002 (20 μM) for 30 min, then with PS-WNP for 24 h, followed by incubating with $A\beta_{25-35}$ (20 μM) for an additional 24 h. Data shown are representative of three independent experiments. $^{\#\#}P < 0.01$ compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to $A\beta_{25-35}$ group; $^{\&}P < 0.01$ compared to PS-WNP plus $A\beta_{25-35}$-treated group.

As shown in Fig. 6 A, 20 μM $A\beta_{25-35}$ significantly reduced Akt phosphorylation (p-Akt), whereas PS-WNP pretreatment upregulated Akt phosphorylation, with higher doses producing a larger effect.

To further assess the contribution of Akt, the PI3K inhibitor LY294002 (20 μM) was used. Activation of Akt by the PS-WNP was blocked when the PC12 cells were pre-incubated with LY294002 for 30 min before $A\beta_{25-35}$ and PS-WNP treatments, while total Akt levels did not change. What is more, the addition of LY294002 completely neutralized the neuroproetcive effects of PS-WNP treatment ($P < 0.01$; Fig. 6B), with an increased percentage of apoptotic cells.

4. Discussion and conclusions

Despite advances in drug developments and clinical trials for the treatment of AD, only a small amount of drugs were permitted by the U.S. Food and Drug Administration (FDA), such as acetylcholinesterase inhibitor and *N*-methyl-D-aspartic acid receptor (NMDA) antagonist. What is more, due to the complexity of pathology, AD is not very sensitive to current approved drugs (Francis, Palmer, Snape, & Wilcock, 1999). Additionally, these drugs usually showed marginal therapeutic benefits for AD (Tayeb, Yang, Price, & Tarazi, 2012). Thus, there is a great need to search for a novel way of AD treatment.

Apoptosis in neurons may contribute to the neuronal degeneration in AD. It is well known that neuronal apoptosis is a leading pathway for Aβ-induced neurotoxicity and prevention of Aβ-triggered apoptosis is viewed as a reasonable therapeutic strategy for AD (Bachurin, 2003; Hardy & Selkoe, 2002). In recent decades, much attention has been paid on natural compounds with advantages of anti-apoptotic activity and low toxicity to explore neuroprotective agents (Li, Tian, Feng, & Yang, 2006; Xing, Chen, & Ma, 2008). The purpose of this study was to investigate neuroprotective effects of PS-WNP, a polysaccharide from the rhizome of *P. sibiricum*, against $A\beta_{25-35}$ neurotoxicity and to explore the signaling pathways involved. First we demonstrated that cell viability was significantly decreased in response to $A\beta_{25-35}$, but was almost completely restored by the addition with PS-WNP in a concentration-dependent manner. The effects of PS-WNP in attenuating $A\beta_{25-35}$-induced cell death were also confirmed by results of double staining PC12 cells with Annexin V-FITC/PI on a FACScan flow cytometer. Consistent with cytoprotective effect of PS-WNP, Annexin-V positive cells, which are well-established indicative of apoptotic cell death, were also decreased following pretreatment with PS-WNP in $A\beta_{25-35}$-treated PC12 cells. These findings suggest that PS-WNP affords protection against $A\beta_{25-35}$-induced neuronal cell death.

It is well known that Bcl-2 family plays a central role in regulating distinct intracellular apoptotic signaling pathways and is associated with various neurodegenerative disorders including AD (Desagher & Martinou, 2000). Bcl-2 family proteins consist of several homologous proteins including anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins including Bax (Kosten, Galloway, Duman, Russell, & D'Sa, 2008). Moreover, the ratio of Bax/Bcl-2 has also been suggested to determine the apoptotic state of neuronal cells following Aβ insult (Gu et al., 2009). In the present study, we found that $A\beta_{25-35}$ treatment caused the upregulation of Bax and downregulation of Bcl-2 protein expression in PC12 cells, thus

leading a high ratio of Bax/Bcl-2. However, pretreatment with PS-WNP significantly reserved these changes induced by $A\beta_{25-35}$ in PC12 cells, supporting a neuroprotective role for PS-WNP acting *via* modulating Bcl-2 family protein levels.

The regulation of mitochondrial permeability and the release of cytochrome c from the mitochondria to the cytosol are pivotal in the apoptotic repertoire, and the events are tightly controlled by the Bcl-2 protein family (Mullauer, Kessler, & Medema, 2009). It has been demonstrated that Aβ peptide may activate the mitochondrial apoptosis pathway. The Aβ peptide is known to induce mitochondrial dysfunction (Dragicevic et al., 2010), and the impairment of the mitochondrial membrane integrity would lead to the release of cytochrome c from the mitochondria to the cytosol. Once released, cytosolic cytochrome c will form an apoptosome that activates the initiating protease caspase-9, which in turn can then activate the executioner caspases-3, causing the cell to undergo apoptosis (Twiddy et al., 2004; Wu, Hsu, & Chan, 2007). Increased neuronal caspase-3 expression is observed in brain tissue derived from AD patients (Abrahamson et al., 2006). Our finding indicated that treating PC12 cells with $A\beta_{25-35}$ for 24 h markedly reduced the MMP, which was detected by Rh123 staining, and reduced MMP by $A\beta_{25-35}$ were dose-dependently reversed by pretreatment with PS-WNP for 24 h. Similarly, cytosolic cytochrome c levels in $A\beta_{25-35}$-treated PC12 cells were significantly higher than that in the control cells. Interestingly, the cytochrome c levels in cytosolic significantly decreased after PS-WNP treatment. We further determined the expression of activated caspase-3 and show that $A\beta_{25-35}$ increased the level of caspase-3, while PS-WNP efficiently attenuated $A\beta_{25-35}$-induced caspase-3 activation, reducing the elevation of caspase-3 activity from 250% to 180% for 50 μg/ml, to 158% for 100 μg/ml, to 145% for 200 μg/ml, respectively. These findings imply that the involvement of mitochondrial apoptosis pathway is associated with the protective effect of PS-WNP against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells.

The exact mechanism of PS-WNP against $A\beta_{25-35}$-induced neurotoxicity is not yet clear, but inhibition of PI3K/Akt signaling pathway should be considered. Many studies have suggested that the activation of PI3K/Akt signaling pathway would prevent Aβ-induced neuronal death (Allen, Eldadah, Huang, Knoblach, & Faden, 2001; Franke, Hornik, Segev, Shostak, & Sugimoto, 2003; Kihara et al., 2001; Liu, Jin, Sun, Xu, & Hu, 2010; Yan, Fraser, Qiu, & Tsang, 2006; Zhao et al., 2011). Therefore, we examined whether P13K/Akt pathway are involved in the protective effect of PS-WNP against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells. The finding showed that $A\beta_{25-35}$ treatment significantly decreased the protein expression of p-Akt in PC12 cells, whereas pretreatment with PS-WNP could reverse this response. Also, the addition of PI3K inhibitor LY294002 (20 μM) into PC12 cells 30 min before treatment completely abolished the effect of PS-WNP on the activation of Akt, thus resulting in an increase of apoptosis, which accounted for the protective mechanism of PS-WNP against $A\beta_{25-35}$-induced neurotoxicity. These findings strongly suggested that PS-WNP-mediated protection against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells involves the activation of the PI3K/Akt signaling pathway.

From our results and those found in the literature, it can be explained that cytoprotective effects of PS-WNP may be attributed, at least in part, to their anti-apoptosis properties *via* enhancement of PI3K/Akt pro-survival pathway (Lou, Fan, Perez, & Lou, 2011; Xian et al., 2013; Xing et al., 2011). Further studies will be required to elucidate the full spectrum of cellular mechanisms underlying the neuroprotective effects of PS-WNP, especially in AD animal models, which may ultimately give us more ideas about the application of PS-WNP as a novel therapeutic for the treatment of AD in the clinic.

Acknowledgements

This research is funded by National Natural Science Foundation of China (grant No. 81171122 and No. 81371324), the Key Program of National Natural Science Foundation of the Heilongjiang Province (grant No. ZD201208), and Specialized Research Fund for the Doctoral Program of High Education (Grant No. 20132307110008).

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