Suppression of β-Secretase (BACE1) Activity and β-Amyloid Protein-Induced Neurotoxicity by Solvent Fractions from Petasites japonicus Leaves

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Abstract
Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by neuronal loss and extracellular senile plaques containing β-amyloid peptide (Aβ). The deposition of the Aβ peptide following proteolytic processing of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase is a critical feature in the progression of AD. Among the plant extracts tested, the ethanol extract of Petasites japonicus leaves showed novel protective effect on B103 neuroblastoma cells against neurotoxicity induced by Aβ, as well as a strong suppressive effect on BACE1 activity. Ethanol extracts of P. japonicus leaves were sequentially extracted with methylene chloride, ethyl acetate and butanol and evaluated for potential to inhibit BACE1, as well as to suppress Aβ-induced neurotoxicity. Exposure to Aβ significantly reduced cell viability and increased apoptotic cell death. However, pretreatment with ethyl acetate fraction of P. japonicus leaves prior to Aβ (50 μM) significantly increased cell viability (p<0.01). In parallel, cell apoptosis triggered by Aβ was also dramatically inhibited by ethyl acetate fraction of P. japonicus leaves. Moreover, the ethyl acetate fraction suppressed caspase-3 activity to the basal level at 30 ppm. Taken together, these results demonstrated that P. japonicus leaves appear to be a useful source for the inhibition and/or prevention of AD by suppression of BACE1 activity and attenuation of Aβ induced neurotoxicity.

Key words: Petasites japonicus, Alzheimer’s disease, β-amyloid protein, β-secretase, neuroprotection

INTRODUCTION
Alzheimer’s disease (AD) is a neurodegenerative disorder primarily marked by the progressive depositions of insoluble amyloid plaques and neurofibrillary tangles (1), followed by oxidative damage to neurons that ultimately results in dementia (2). The major component of the amyloid plaques is the β-amyloid peptide (Aβ), which is a 40~42 residue internal peptide segment of amyloid precursor protein (APP).

The APP is initially cleaved by the aspartic protease β-secretase to yield the N-terminus of Aβ at the Asp+1 residue of the Aβ sequence (1). Following β-secretase cleavage, C99 is the substrate of a second protease, γ-secretase, which cleaves the APP to generate the C-terminus of Aβ, and the mature peptide is proteolytically secreted from the cell (3).

Both in vitro and in vivo studies have indicated that aggregated Aβ initiates a pathogenic cascade that ultimately leads to neuronal loss and dementia. The mechanism underlying β-amyloid-stimulated toxicity are complex, but may involve N-methyl-D-aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, increment of intracellular Ca2+ concentration and oxidative stresses (3,4). In particular, abundant evidence suggests that enhanced oxidative stress is involved with the pathogenesis and/or progression of the apoptotic mechanism of AD (4,5).

Petasites japonicus is a culinary vegetable in eastern Asia. The roots of P. japonicus have long been used as a traditional medicine in European countries for the treatment of migraines, asthma and gastric ulcers in (6). Previous studies have revealed several antioxidants such as petasinophenol, phenylprophenoyl sulfonic acid and fukinolic acid from P. japonicus leaves (7,8). Recent research demonstrated a neuroprotective effect of petasignolide A from the butanol fraction of the aerial part of P. japonicus on the oxidative damage in the brain of mice challenged with kainic acid (9). Studies regarding bioactivity of P. japonicus are limited.

In the course of screening natural products with anti-dementia property, the ethanol extract of P. japonicus leaves not only suppressed BACE1 activity but also exerted a strong neuroprotective effect against Aβ(25-35)-induced toxicity in neuroblastoma B103 cells. In the present study, therefore, we examined the fractions from P. japonicus and investigate their protective effects against Aβ-induced neurotoxicity and BACE1 activity.
MATERIALS AND METHODS

Chemicals and cell lines

All solvents used for extraction, such as ethanol, methylene chloride, ethyl acetate, n-butanol, and water, were of analytical grade and purchased from Duksan Chemical (Ansan, Gyeong-gi-do, Korea). Aβ(25-35) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyldrazotium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum and N2 supplement were purchased from Gibco Co. (Grand Island, NY, USA). Hoechst 33342 dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA), was obtained from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were purchased from Sigma Chemical Co.

Aβ(25-35), the most toxic peptide fragment from APP, was reconstituted in distilled water at a concentration of 1 mM and stored at -20°C. The stock solution was diluted with phosphate buffer saline (PBS) to appropriate concentration and incubated for 48 hr at 37°C for aggregation prior to each experiment (10). The aged and aggregated Aβ(25-35) showed higher cytotoxicity toward B103 cells than the freshly diluted one when examined by the MTT reduction assay (data not shown).

Plant material, extraction and fractionation

The aerial parts of *P. japonicus* (30 kg) were purchased from an agricultural farm in Ul-eum-gol, Milyang, Korea in April, 2007. The voucher specimen (No. PJ 0401) has been deposited at Biomaterials Lab., Dong-A University, Busan, Korea. In brief, the plants were washed, leaves separated and dried at RT. The dried leaves of PJ (2 kg) were chopped and extracted with 95% ethanol (10 L) for 1 week in a dark place (×3). The EtOH extract was concentrated to dryness under reduced pressure by a rotary evaporator. The concentrate (180 g) was suspended in 500 mL water and consecutively fractionated with methylene chloride, ethyl acetate, and n-butanol.

Determination of BACE1 activity

A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera (Madison, WI, USA). The assay was carried out according to the manufacturer’s instructions with minor modifications (11). Briefly, a mixture of 10 µL of assay buffer (50 mM sodium acetate, pH 4.5), 10 µL of BACE1 (1.0 U/mL), 10 µL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 µL of sample dissolved in MeOH or DMSO were incubated for 60 min at 18°C. The mixture was excited at 545 nm and the light emitted at 585 nm was collected. The inhibition ratio was obtained using the following equation:

\[
\text{Inhibition} \% = \left(1 - \frac{S - S_0}{C - C_0}\right) \times 100
\]

where C was the fluorescence of control (enzyme, assay buffer, and substrate) after 60 min of incubation, C0 was the fluorescence of control at time zero, S was the fluorescence of tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S0 was the fluorescence of the tested samples at time zero.

Cell culture

Mouse neuroblastoma B103 cells were cultured routinely in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, JRH biosciences, Lenexa, KS, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in humidified atmosphere of 5% CO2/95% air. The medium was changed every other day and cells were plated at an appropriate density according to each experimental scale.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldrazotium cell viability assay

Cell viability was determined by the conventional 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyldrazotium bromide (MTT) reduction assay (12). The assay depends primarily on mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state (13). Therefore, the more formazan produced from the reduction of MTT, the greater the cell viability. Briefly, B103 cells were plated in 96-well plates at a density of 5 × 10³ cells, allowed to adhere at 37°C for 2 hr and then the culture medium was switched to serum-free N2 medium. Cells were then incubated for 1 hr in the N2 medium and further incubated in the presence of 50 µM Aβ (25-35) with or without PJ fractions for 24 hr. The fractions were dissolved in DMSO and diluted with buffer to desired concentration and applied 30 min prior to the Aβ(25-35) treatment for each experiment. The final concentration of DMSO was always adjusted to less than 0.01%, a concentration found to have no effect on the cell viability (data not shown).

After 24 hr incubation with Aβ(25-35), cells were treated with MTT solution (final concentration 1 mg/mL) and further incubated for 2 hr. After incubation, the medium was removed, and the dark blue formazan crystals formed in intact viable cells were solubilized with DMSO and absorbance at 570 nm was measured with a microplate reader (ELX808, Bio-Tek, Winooski, VT, USA). Data were shown as the percentage of MTT reduction to the absorbance of control cells.

Identification and quantification of apoptotic neuronal cell death

Hoechst 33342, bis-benzimidazole dye, penetrates the plasma membrane and homogeneously stains nuclei of
normal cells, whereas highly condensed and/or fragmented stained chromatin are key morphological indicatives of apoptotic cells (14). As described in MTT reduction assay, cells on glass coverslips were treated with Aβ(25-35) in serum-free N2 medium for 24 hr, washed with cold PBS twice and then fixed with 4% paraformaldehyde at 4°C for 30 min. Fixed cells were washed with cold PBS (Ca\(^{2+}\) and Mg\(^{2+}\) free) and treated with Hoechst 33342 at a concentration of 1 μg/mL in incubation buffer for 15 min. The morphological changes were examined under UV illumination by a fluorescence microscope (Olympus, Tokyo, Japan) with excitation centered at 340 nm and emission filtered with 510 nm (10,15). For quantification of apoptotic cells, one hundred cells were assessed in each field at 200× magnification and ten randomized fields for each experimental condition were chosen. Results were expressed as percentage of apoptotic cells to total cells.

**Determination of caspase-3 activity**

The caspase-3 activation in B103 cells treated with 50 μM Aβ(25-35) was examined using commercially available caspase-3 colorimetric assay kit (R&D Systems, Inc., Minneapolis, MN, USA). The assay procedure followed in accordance with the protocol supplied by the manufacturer with minor modification. Briefly, B103 cells that have been induced to undergo apoptosis by the treatment with Aβ(25-35) were collected by centrifugation (250×g, 10 min). The cell pellets were resuspended in lysis buffer and incubated on ice for 10 min. The cells were centrifuged (10,000×g, 1 min) and then the supernatant was transferred. The cytosolic protein (150 μg) was incubated with reaction buffer for 1 hr at 37°C. The activity of caspase-3 was measured with caspase-3 colorimetric substrate peptide (DEVD-pNa) using a microplate reader at 405 nm wavelength light (ELX808, Bio-Tek). Results were shown as the relative percentage of caspase-3 activity over only Aβ(25-35)-treated cells.

**Statistical analysis**

All experiments were performed in triplicate. For each experiment, data are expressed as the mean±SE (n=3) and statistical significance was assessed by one-way analysis of variance (ANOVA) with unpaired Student’s t-tests. p values of <0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

**BACE1 inhibitory activities by solvent fractions from P. japonicus leaves**

The solvent fractions from ethanol extract of P. japonicus leaves were assayed for their BACE1 inhibitory activity. The BACE1 inhibitory activity of each solvent fraction was compared with resveratrol as a positive control. The EtOAc fraction displayed the most potent BACE1 inhibitory activity with 95.3±3.7% inhibition at the concentration of 30 ppm (Fig. 1). Other solvent fractions such as methylene chloride and butanol fraction also exhibited strong BACE1 inhibitory activities at the concentration of 30 ppm (78.2±2.3 and 72.1±1.5%, respectively), although they were not as strong as ethyl acetate fraction.

**Cellular Protection against Aβ(25-35)-induced cell death by solvent fractions from P. japonicus leaves**

The accumulation of Aβ aggregates is a primary event in the pathological cascade of AD and the neurotoxic effect of Aβ is attributable to the presence of amino acid located in positions 25~35 of the full-length β-amyloid (13). Aβ(25-35) has a neurotoxic property itself and generates free radical spin adducts in aqueous solutions (16). In addition, Aβ increases neurotoxicity induced by glutamate or free radicals and neuronal susceptibility to injury by glucose deprivation (17,18).

To assess the protective effects of the solvent fractions from P. japonicus leaves against Aβ(25-35)-induced neuronal toxicity, the viability of B103 cells was evaluated by the MTT reduction assay. The conventional MTT assay is widely used as a sensitive and early index of Aβ-induced impairment of the cellular redox activity (19). The concentration of 50 μM was used for determining Aβ(25-35)-induced neuronal cell damage in the present experiments based on our preliminary results (not...
shown). With 24 hr incubation with Aβ(25-35) at a concentration of 50 μM, cell viability significantly decreased to 56.7±2.3%, compared with that of unstimulated cells (p<0.01, the absorbance of control was regarded as 100%). However, pretreatment of B103 cells with each solvent fraction from *P. japonicus* leaves significantly recovered cellular viability (Fig. 2). The ethyl acetate fraction of *P. japonicus* leaves significantly prevented the Aβ(25-35)-stimulated decrease of the MTT reduction rate, showing 95.3±3.8% and 72.1±2.5% with the concentration of 30 and 3 ppm, respectively (p<0.01). The methylene chloride and butanol fractions also suppressed such cellular damage significantly both at 3 and 30 ppm concentration, but revealed somewhat lower protection than that of the ethyl acetate fraction.

**Inhibitory activity of Aβ(25-35)-induced apoptotic cell death and caspase-3 activation**

Many *in vitro* neuronal experiments have demonstrated that β-amyloid induces apoptotic degeneration (20,21). An additional experiment was done with Hoechst 33342 staining to evaluate the neurotoxicity of Aβ. The ratio of apoptotic cells was shown in Fig. 3. The 24 hr of treatment of 50 μM Aβ(25-35) raised the number of apoptotic cells significantly to 38.8±1.1% of the total population of B103 cells compared to 8.2±1.1% of apoptotic cells in the control without treatment of Aβ (25-35) (p<0.01). However, such increase in damaged cells was significantly restored by the pretreatment of the each solvent fraction of *P. japonicus* leaves at the concentration of 3 or 30 ppm. The ethyl acetate fraction of *P. japonicus* leaves incredibly reduced cellular apoptotic damage to 20±1.3% and 12±1.5% at the concent-

tration of 3 and 30 ppm, respectively (p<0.01). The addition of methylene chloride fraction of *P. japonicus* leaves at 3 ppm reduced Aβ-induced apoptotic damage but did not significantly alter the damage rate.

Apoptosis is associated with activation of the caspase family, aspartic acid-specific cystein proteases (22,23). The caspase family consists of at least 14 homologues and caspase-3 has been suggested to play a predominant role in the final execution phase of apoptosis and is associated with the converging point of different apoptotic signaling pathways (23).

In this study, the activity of caspase-3 was significantly up-regulated to 22.3±1.0% after 24 hr incubation of Aβ (25-35) at 50 μM in B103 cells (p<0.01, Fig. 4). When B103 cells were treated with the solvent fractions of *P. japonicus* leaves prior to Aβ(25-35) exposure, the activity of caspase-3 was significantly suppressed (Fig. 4). Notably, the ethyl acetate fraction at both 3 and 30 ppm suppressed caspase-3 activity to 70±1.7% and 75±2.3% which is less than that of the control.

AD is a complex neurodegenerative disorder caused by various factors including β-amyloid aggregation, cholinergic synapse degeneration and active oxygen species (3-5). The immune cells in the brain respond to the plaques and tangles and try to eliminate the debris. In the process of digesting the material within plaques and tangles, microglia release pro-inflammatoryary proteins and free radicals, which cause secondary damage (24). In addition, the antioxidant defense system in the elderly loses its ability to neutralize oxidative species and subsequent
oxidative stress can act as a risk factor for the initiation and progression of AD (25).

The results of this study suggest that the protective action of the solvent fractions of P. japonicus leaves against Aβ(25-35)-induced apoptotic death in B103 cells may, at least in part, be attributed in inhibition of the caspase cascade. Additional studies relating intracellular mechanism in response to Aβ-induced neurotoxicity and their inhibition by the solvent fractions of P. japonicus and its isolated compounds may be needed for further insight into the anti-dementia related effects of natural products. In conclusion, this study provides evidence that Aβ(25-35)-induced cellular injury was prevented by the solvent fractions of P. japonicus, with the ethyl acetate fraction of P. japonicus leaves exerting the strongest neuroprotective properties against BACE1 and Aβ-induced damage in B103 cells. The cellular protection against Aβ-stimulated neurotoxicity by the ethyl acetate fraction of P. japonicus might provide the pharmacological basis of its usage in prevention and/or treatment of neurodegeneration in AD.

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