Water-Soluble Fraction of *Rheum Undulatum* Attenuates Amyloid-β-induced Neuronal Death and Microglial Activation *in Vitro*

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Oxidative stressand glia-associated chronic inflammation have been linked to the pathophysiology of Alzheimer's disease. Rhei rhizoma has been commonly used as a purgative and a haemostatic agent in traditional oriental medicine. Recently, the methanol extract from a dried root of *Rheum undulatumhas* been shown to have anti-allergic and anti-inflammatory effects. In this study, we tested the potential of the extract of *Rheum undulatum* for neuroprotective agent. The aqueous extract of *Rheum undulatum* reduced cell death and p53 phosphorylation in neuronal cells and attenuated levels of cyclooxygenase-2 and inducible nitric oxide synthase mRNAs in BV2 microglial cells treated with amyloid-β

Key words : Alzheimer's disease, amyloid-β, microglia, neuron, *Rheum undulatum*

Introduction

Alzheimer's disease (AD) is a neurodegenerative illness characterized by progressive decline of cognitive function accompanied by neuronal loss. Extracellular deposition of the amyloid-b (Aβ) peptide in the brain has been suggested to be a primary cause of Alzheimer's disease (AD). Ab, a 40-42 amino acid peptide derived from proteolysis of the amyloid precursor protein, can be directly toxic to neurons in vitro and in vivo[1,2]. Yet, several studies suggest that chronic microglial activation can contribute to AD pathogenesis by the release of neurotoxic substances such as glutamate and proinflammatory mediators[3].

Extensive effort has been made to develop drugs that limit neurodegeneration in AD. The primary treatment for the disease is acetylcholinesterase inhibitors that temporarily improve cognitive function. Strategies are also underway to minimize Aβ-mediated neurotoxicity, which include antioxidants, blockers of glutamate-mediated excitotoxicity, and antiinflammatory drugs[4].

Rhei rhizoma has been commonly used in traditional oriental medicine for cathartic, febrifugal, and antidotal purposes. Recently, the methanol extract from rhizome of *Rheum undulatum* has been shown to have anti-allergic[5] and anti-inflammatory[6] effects. The aim of this study was to determine whether the aqueous extract of *R. undulatumcan* provide neuroprotective functions against Aβ.

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Materials and Methods

1. Preparation of *R. undulatum* extract

The dried roots of *R. undulatumwas* purchased from Kyung Hee University Medical Center (Seoul, South Korea). Two hundred grams of the dried roots were boiled in 2 L of distilled water for 2 h. The extract was concentrated with a rotary evaporator, and lyophilized. The powder was dissolved in distilled water to obtain a working stock solution of 1 mg/ml.

2. Neuronal culture and treatment

Mouse cortical neurons were prepared as previously described[7]. All animal use procedures conformed to the animal care guidelines of the Korean Academy of Medical Sciences. Cerebral cortices were removed from brains of the 14-day-old fetal mice, gently triturated and dissociated into individual cells by passing through a Pasteur pipette, and seeded on 24- or 96-well plates (five hemispheres per plate). The plates were precoated with 100 mg/ml poly-D-lysine and 4 mg/ml laminin (Sigma, St. Louis, MO). Plating medium consisted of Eagle's minimal essential media (MEM; Gibco, Gaithersberg, MD) supplemented with 5 % horse serum, 5 % fetal bovine serum (FBS; Gibco), 2 mM glutamine, and 20 mM glucose (Sigma). Cultures were maintained at 37 ℃ in a humidified 5 % CO_2

atmosphere. Cytosine arabinofuranoside (10 mM ; Sigma) was added to the cultures at day 7. Two days later, cultures were shifted into growth medium that was identical to the plating medium but lacking FBS. Experiments were performed at day 11-14. PC12 cells, rat pheochromocytoma cells were maintained in RPMI 1640 medium (Gibco) supplemented with 5 % FBS and 10 % horse serum (Gibco). Cells were plated on poly-D-lysine-coated plates, and grown for 24 h. For the experiments, cells were then treated for 24 h either with 2 mM Aβ (1-42) (U.S. Peptide, Rancho Cucamonga, CA) in medium containing 0.5 % FBS. The extract was added to the cultures just before the treatment with Ab. After the incubation, the cells were subjected to cell viability or Western blot analysis.

3. Cell viability assay

To assess cell viability, the cultures were incubated with MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 1 mg/ml; Sigma) at 1:2 volume of culture medium for 1 h at 37℃. At the end of the incubation, the MTT solution was removed, and the cells were dissolved in 150 ml of 0.04 M HCl in isopropyl alcohol. The optical density of samples was then measured at 570 nm using a microplate reader.

4. Western blot analysis

Cells were washed with PBS and incubated on ice in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM EDTA, 1 % protease inhibitor cocktail; Sigma). The lysates containing an equal amount of protein were fractionated by SDS10 %polyacrylamide gel electrophoresis, and then electroblotted to a nitrocellulose membrane. The membranes were blocked, incubated with monoclonal anti-phospho-p53 (Ser15) (Cell Signaling, Beverly, MA) or monoclonal anti-a-tubulin antibody (Sigma), and then reacted with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The blots were developed using enhanced chemiluminescence detection reagents (Amersham Biosciences). Prestained molecular weight standards were purchased from Invitrogen (Carlsbad, CA).

5. Microglia culture and treatment

The BV2 murine microglial cell line, initially generated by Blasi et al.[8], was obtained from Dr. E. J. Choi at Korea University, Seoul, Korea. BV2 cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 5% FBS. Cells were then treated for 24 h with 100 nM Ab(1-42) alone or in the presence of 10 ng/ml IFN-gin medium containing 0.5% FBS. After incubation, the cells were subjected to RT-PCR.

6. Reverse transcription (RT)-PCR

Total cellular RNA was isolated from microglial cells by using Trizol reagent (Gibco). RNA (1 mg) was reverse transcribed in a 20 ml reaction mixture using Moloney murine leukemic virus reverse transcriptase (Gibco). The cDNA (0.2 ml) was amplified using gene specific primers. The amounts of amplified products were determined using an image documentation system (ImageMaster VDS; Pharmacia, Uppsala, Sweden) with image analysis software (ImageMaster TotalLab; Pharmacia). DNA size markers (MBI, Amherst, NY) were run in parallel to validate the predicted sizes of the amplified bands. The primer sequences specific for the genes examined and predicted product sizes are shown in Table 1.

Table. 1 Primers and expected sizes of PCR products with each primer pair

Gene		Primer	Size (bp)
GAPDH	sense	5′ atcccatcaccatcttccag 3′	579
	antisense	5′ cctgcttcaccaccttcttg 3′	
COX2	sense	5′ ccagatgctatctttggggagac 3′	249
	antisense	5′ gcttgcattgatggtggctg 3′	
iNOS	sense	5′ cccttccgaagtttctggcagcagc 3′	496
	antisense	5′ ggctgtcagagcctcgtggctttgg 3′	

Results

1. Reduction of Aβ-induced cell death by *R. undulatum* extract in neuronal cultures

We tested whether the aqueous extract of *R. undulatum* can protect rat PC12 neuronal cells and mouse cortical cultures against Aβ. Exposure of the cells to Aβ (1-42) decreased cell viability to about 50 % of control cells. Treatment with the extract significantly prevented the cytotoxicity of Aβ (1-42) in a dose-dependent manner (Fig. 1).

2. Reduction of Aβ-induced phosphorylation of p53 by *R. undulatum* extract in PC 12 cells

The p53 tumor suppressor protein is a transcription factor that plays a key role in cellular response to DNA damage. It is activated through phosphorylation at specific amino acid residues, which then leads to growth arrest or apoptosis[9]. Serine 15 (Ser15) is one of major phosphorylation sites involved in regulation of p53 transcriptional activities in response to genetic damage[10]. Thus we examined p53 activation by probing with antibody that detects p53 phosphorylated at Ser15. As shown in Fig. 2, phosphorylation at Ser15 was prominently increased in PC12 cells treated with Aβ, which was reduced by *R. undulatum* extract.

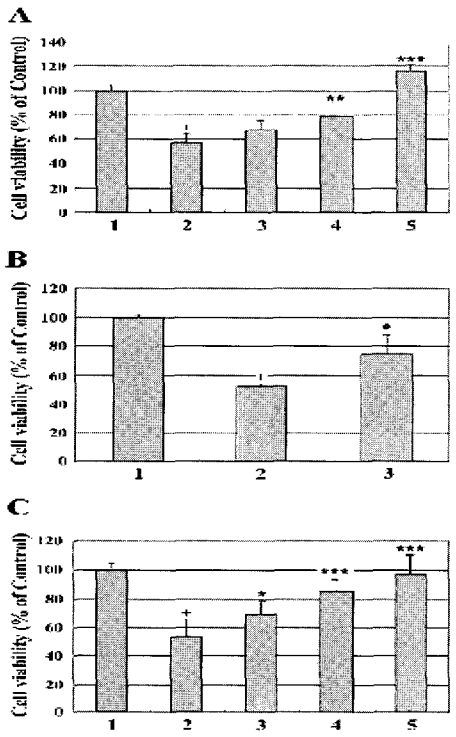

Fig. 1. Effect of the aqueous extract of *R. undulatum* on neuronal cell viability. PC12 cells (A, B) and primary mouse cortical neurons (C) exposed to Aβ. Cultures were treated for 24 h with 2 mM of Ab(1-42) in the presence of the aqueous extract of *R. undulatum*. Cell viability was then assessed by measuring MTT reduction. Results are expressed as percent control S.D. of three plates per group in three repeated experiments. (A) and (C) Column 1, untreated control cells; column 2, treated with Aβ(1-42) alone; column 3-5, treated with Aβ(1-42) in the presence of 5, 10, or 25 mg/ml of the aqueous extract of *R. undulatum*. (B) Column 1, untreated control cells; column 2, treated with Aβ(1-42) alone; column 3, treated with Aβ(1-42) in the presence of 500 mM ascorbic acid. Statistical analysis was evaluated by Student's t-test. +p<0.001, compared to untreated control; *p0.05, **p0.01, ***p0.001, compared to treated with Aβ(1-42) alone.

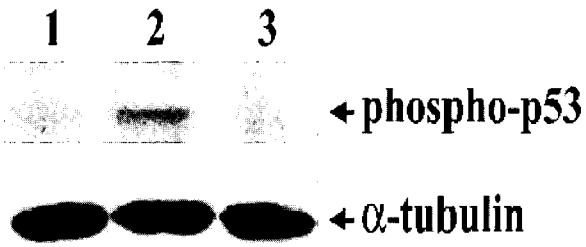

Fig. 2. Effect of the aqueous extract of *R. undulatum* on Aβ-induced activation p53 in PC12 cells. Cultures were treated for 24 h with 2 mM of Aβ(1-42) in the presence of the aqueous extract of *R. undulatum* (25 mg/ml). Phosphorylation of p53 was then assessed by Western blotting. Lane 1, untreated control cells; lane 2, treated with Aβ(1-42) alone; lane 3, treated with Aβ(1-42) in the presence of the aqueous extract of *R. undulatum*. Results are representative of three independent experiments.

3. Reduction of Aβ-induced expression of proinflammatory genes by *R. undulatum* extract in microglial cultures

Cyclooxygenase (COX)-2 is thought to be an inducible enzyme whose expression is up-regulated in pathological states, most notably those associated with inflammation[11]. COX-2 expression is up-regulated in several neurological disease, including stroke, AD, and seizures[12,13]. Glial cells in the brain respond to various toxins with an increased expression of inducible nitric oxide synthase (iNOS). This change have been reported to cause immune-mediated cytotoxicity as observed in neuron/glia cultures[14,15] and this alterations may form the basis of various disease in the central nervous system[16,17]. In this line, we tested whether the aqueous extract of *R. undulatum* can attenuate microglial activation against Aβ. As shown in Fig. 3, the levels of COX-2 and iNOS mRNAs were enhanced in BV2cells treated with Aβ, which was reduced by *R. undulatum* extract.

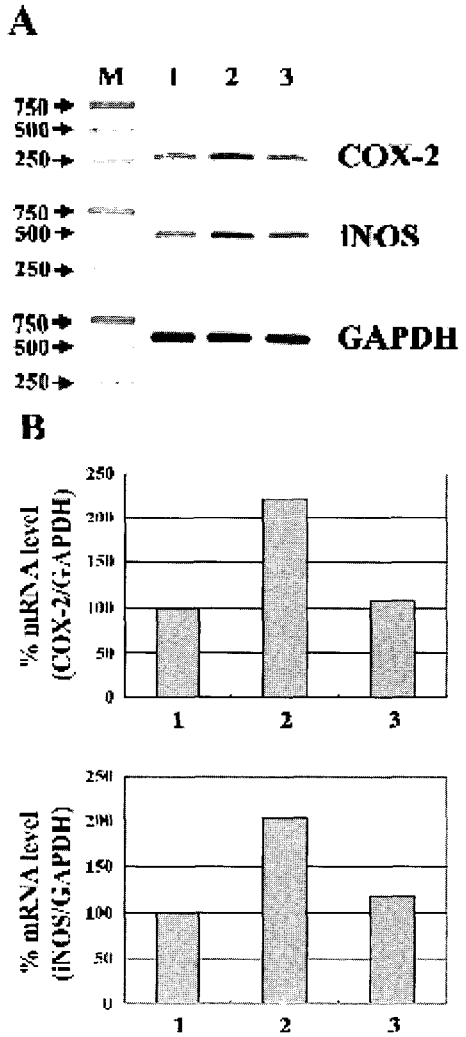

Fig. 3. Effects of the aqueous extract of *R. undulatum* on the levels of COX-2 and iNOS mRNAs in BV2 cells. (A) Cultures were treated for 24 h with 100 nM of Aβ in the presence of the aqueous extract of *R. undulatum* (10 mg/ml). Lane 1, untreated control cells; lane 2, treated with Aβ(1-42) alone; lane 3, treated with Aβ(1-42) in the presence of the aqueous extract of *R. undulatum*. For iNOS, cells were stimulated with Aβ(1-42) plus 10 ng/ml INF-g. Total RNA was extracted and analyzed for COX-2, iNOS and GAPDH mRNA expression. M, molecular size standards (in base pairs). (B) The intensities of amplified bands were determined from the gel shown in (A). Then, the amounts of target cDNA were normalized against GAPDH cDNA in the corresponding sample and shown in the graph. Values for control were set to 100%. The data presented are from one of three independent experiments that produced similar results.

Discussion

Neurodegeneration in AD appears to occur via neuronal apoptosis[18]. Several in vitro studies reported that Abinduced apoptosis in different models of cultured neuronal cells[19]. AD is accompanied by an inflammatory reaction which is considered a response to Aβ-deposition in the brain and represents an important pathogenic factor for the disease[20]. In this line the agents that target neuronal cell death and glial activation have been expected as neuroprotectors.

Extensive studies to search for new active extracts or compounds derived from various natural plants that can be used in the treatment of brain diseases have been carried out recently, in attempts to obtain advanced therapeutic drugs that possess both high efficacy and safety. Although the action mechanisms of plant extracts that have been used medicinally and traditionally should be investigated further, it is thought that they might have various active components responsible for the prevention of diverse brain diseases.

A dried root of *R.undulatum*, has been used as the remedy for the blood stagnation syndrome as well as a purgative agent. Recently, anti-platelet aggregation[21], anti-allergic[5], and anti-inflammatory[6] activities of stilbenes from the rhizome of *R. undulatum* have beenreported. Anti-oxidant effects of the methanol extract from *R. undulatum* and its stilbene constituents on lipid peroxidation have also been reported by using erythrocyte membrane ghost system[22]. In the study searching a new neuroprotective constituent from natural source, the aqueous extract of *R. undulatum* suppressed the cell death of PC12 cells and primary mouse cortical neurons in response to Aβ. and reduced p53 phosphorylation caused by Aβ. Moreover, the aqueous extract of *R. undulatum* reduced the levels of COX-2 and iNOS mRNAs in Aβ-stimulated BV2 cells. These findings suggest the possibility that this extract may be developed as protective means against neuronal death and microglia activation in Alzheimer's disease. Active components of the extract should be further identified and tested for application of repairing neuronal cell death.

Acknowledgments

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