Full Paper

***Aralia cordata* Protects Against Amyloid β Protein (25–35)–Induced Neurotoxicity in Cultured Neurons and Has Antidementia Activities in Mice**Soon Ock Cho^{1,†}, Ju Yeon Ban^{2,†}, Joo Youn Kim¹, Ha Yeon Jeong¹, Ik Soo Lee³, Kyung-Sik Song⁴, KiHwan Bae^{3,*a}, and Yeon Hee Seong^{1,*b}¹College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea²School of Dentistry, Dankook University, Cheonan, Chungnam, 330-714, Korea³College of Pharmacy, Chungnam National University, Taejeon, 305-764, Korea⁴College of Agriculture and Life-Sciences, Kyungpook National University, Daegu, 702-701, Korea

Received October 4, 2008; Accepted June 26, 2009

Abstract. The present study investigated an ethanol extract of the aerial part of *Aralia cordata* Thunb. (Araliaceae) for possible neuroprotective effects on neurotoxicity induced by amyloid β ($A\beta$) protein (25–35) in cultured rat cortical neurons and antidementia activity in mice. Exposure of cultured cortical neurons to 10 μ M $A\beta$ (25–35) for 36 h induced neuronal apoptotic death. At 1–10 μ g/ml, *A. cordata* inhibited neuronal death, elevation of intracellular calcium ($[Ca^{2+}]_i$), glutamate release into the medium, and generation of reactive oxygen species (ROS) induced by $A\beta$ (25–35) in primary cultures of rat cortical neurons. Memory loss induced by intracerebroventricular injection of ICR mice with 15 nmol $A\beta$ (25–35) was inhibited by chronic treatment with *A. cordata* (50 and 100 mg/kg, p.o. for 7 days) as measured by a passive avoidance test, and corresponding reductions were observed in brain cholinesterase activity and neuronal death measured histologically in the hippocampal region. Oleanolic acid isolated from *A. cordata* also inhibited neuronal death, elevation of $[Ca^{2+}]_i$, glutamate release, and generation of ROS induced by $A\beta$ (25–35) in cultured rat cortical neurons, suggesting that the neuroprotective effect of *A. cordata* may be, at least in part, attributable to this compound. From these results, we suggest that the antidementia effect of *A. cordata* is due to its neuroprotective effect against $A\beta$ (25–35)-induced neurotoxicity and that *A. cordata* may have a therapeutic role in preventing the progression of Alzheimer's disease.

Keywords: *Aralia cordata*, oleanolic acid, amyloid β protein, cultured neuronal death, memory impairment

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by cognitive impairment and pathologically by the appearance of senile plaques and neurofibrillary tangles (1). Amyloid β protein ($A\beta$), a 39- to 43-amino-acid peptide fragment derived from amyloid precursor protein, is thought to

be closely related to the pathogenesis of AD as the major component of the senile plaques that characterize this disease (2). Although the precise mechanism of $A\beta$ -induced cell death is not well understood, $A\beta$ neurotoxicity has been speculated to be due to various factors, including oxidative stress, excessive increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), and glutamate accumulation, and induction of neurotoxic cascades (3–5).

An experimental model that mimics the progression of AD was developed using an intracerebroventricular (i.c.v.) injection of $A\beta$ in mice (6). A continuous infusion of full-length $A\beta$, $A\beta$ (1–40), into the cerebral ventricle in rats resulted in learning and memory deficits

[†]These authors equally contributed to this work.

Corresponding authors.

*baekh@cnu.ac.kr, *bvepharm@chungbuk.ac.kr

Published online in J-STAGE

doi: 10.1254/jphs.08271FP

that were accompanied by a reduction of choline acetyltransferase activity, suggesting that accumulation of $A\beta$ disrupted cholinergic activity and caused the cognitive impairments of AD (7). Oxidative stresses are also involved in the mechanism of $A\beta$ -induced neurotoxicity and AD pathogenesis (8, 9). $A\beta$ has been reported to produce hydrogen peroxide and lipid peroxide in neurons and superoxide and proinflammatory cytokines in astrocytes and microglial cells (10, 11). Antioxidants such as α -tocopherol protect against cytotoxicity induced by $A\beta$ in vitro as well as against learning and memory deficits in vivo (12). Also, α -tocopherol and anti-inflammatory agents such as indomethacin reportedly slow the progression of AD (13, 14). $A\beta(25-35)$, the core toxic fragment of $A\beta(1-40)$, produces similar neurodegenerative properties as $A\beta(1-40)$, including oxidative damage, inflammatory responses, and memory impairment (15, 16).

Aralia species belong to the family Araliaceae and have long been recognized in China, Japan, and Korea as therapeutic herbs with antinociceptive, antidiabetic, antioxidant, and anti-inflammatory activities. The root of *Aralia cordata* Thunb. has been used as a traditional Chinese medicine for rheumatism, lumbago, and lameness (17). It exhibits pharmacological activities such as analgesia, hypothermia, and prolongation of pentobarbital-induced anesthesia (18). Diterpenes have been elucidated as analgesic and anti-inflammatory agents of this plant root (18, 19). In addition to diterpenes, polyacetylenes, a lipid glycerol, and sterols having cyclooxygenase (COX) inhibitory activity have been isolated from the root (20); and diterpenes, triterpenes, and a saponin have been isolated as COX-1 and COX-2 inhibitors from the aerial part of this plant (21). Since reactive oxygen species (ROS) and inflammation are part of the complex series of pathophysiological events that contribute to neurodegenerative diseases such as AD and stroke (22, 23), free radical scavengers and anti-inflammatory agents have attracted considerable attention as potential neuroprotective agents. We hypothesized that the aerial part of *A. cordata* might protect neurons against neurodegenerative diseases such as AD and ischemia on the basis of its antioxidant and anti-inflammatory activities. The aim of our study was to determine whether *A. cordata* has a protective effect against $A\beta(25-35)$ -induced memory deficits in mice and $A\beta(25-35)$ -induced neuronal damage in cultured rat cortical neurons. In addition, we identified oleanolic acid as an active component contributing to the neuroprotective effect of *A. Cordata* against $A\beta(25-35)$ -induced neurotoxicity.

Materials and Methods

Plant material, preparation, and identification

The aerial parts of *A. cordata* were collected in Daejeon, Korea and identified by one of the authors (Dr. KiHwan Bae). A voucher specimen (CNU 1499) has been deposited in the herbarium at the College of Pharmacy, Chungnam National University, Daejeon, Korea. The dried aerial parts of *A. cordata* (4 kg) were extracted 3 times with ethanol at room temperature for 3 days, filtered, and concentrated to yield an ethanol extract (300 g, yield: 7.5%), which was then stored at -20°C until required. *A. cordata* was standardized based on its oleanolic acid contents. Oleanolic acid was determined using reverse-phase high-performance liquid chromatography (HPLC; Shimadzu, Kyoto) equipped with a UV detector. Separation was carried out using a YMC-pack pro C_{18} column ($5\text{ }\mu\text{m}$, $10 \times 250\text{ mm}$; Shisheido, Tokyo) at 30°C with a linear gradient of acetonitrile:water (20:80, v/v) and acetonitrile:water (30:70, v/v) as the mobile phase at a flow rate of 1.5 ml/min. The detector wavelength was set at 210 nm. Oleanolic acid was found in the aerial parts of *A. cordata* at a mean level of $2.20 \pm 0.08\%$ ($n = 3$, Fig. 1).

The ethanol extract of *A. cordata* was suspended in H_2O (2 L) and then partitioned successively with hexane ($2\text{ L} \times 3$), ethyl acetate ($2\text{ L} \times 3$), and butanol ($2\text{ L} \times 3$) for activity-guided purification. Since the hexane-soluble fraction exhibited considerable activity, this fraction was investigated extensively. The hexane-soluble fraction (85 g) was chromatographed on a silica gel column ($30 \times 10.0\text{ cm}$) and eluted with a gradient of hexane-acetone (100:1 $\rightarrow$ 1:2) to produce 4 fractions. Repeated silica gel column chromatography ($30 \times 5.0\text{ cm}$) of fraction 4 using hexane-acetone (4:1) gave compound **1** (25 mg).

Fig. 1. Representative HPLC chromatogram of standard oleanolic acid (top trace) and the ethanol extract of *A. cordata* (bottom trace). Oleanolic acid was detected at around 22 min in this system.

Properties of Compound 1 (oleanolic acid):

white powder

mp: 196°C – 198°C.

IR (KBr) $\nu_{\max}$ cm⁻¹: 3430 (OH), 1705 (COOH).

¹H-NMR (300 MHz, CDCl₃) δ (ppm): 0.74, 0.79, 0.90, 0.92, 0.93, 0.99, and 1.12 (each 3H, s), 3.22 (1H, dd, J = 4.0, 9.5 Hz, H-3), 5.28 (1H, m, H-12).

¹³C-NMR (75 MHz, CDCl₃) (ppm): 38.9 (C-1), 28.4 (C-2), 79.8 (C-3), 39.6 (C-4), 56.0 (C-5), 19.0 (C-6), 33.3 (C-7), 40.0 (C-8), 48.4 (C-9), 37.5 (C-10), 23.8 (C-11), 122.8 (C-12), 144.2 (C-13), 42.0 (C-14), 28.4 (C-15), 23.7 (C-16), 46.5 (C-17), 42.0 (C-18), 46.5 (C-19), 31.1 (C-20), 34.5 (C-21), 33.2 (C-22), 28.7 (C-23), 16.5 (C-24), 16.0 (C-25), 18.0 (C-26), 26.0 (C-27), 184.2 (C-28), 33.2 (C-29), 23.8 (C-30).

Experimental animals

Pregnant Sprague-Dawley rats for primary neuronal culture and male ICR mice for the passive avoidance test were supplied by Daehan BioLink Co., Ltd. (Chungbuk, Korea) and housed in an environmentally controlled room at 22 ± 2°C, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water ad libitum. The procedures involving experimental animals complied with the animal care guidelines of the National Institutes of Health and the animal ethics committee of Chungbuk National University.

Induction of neurotoxicity in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 – 16 Sprague-Dawley rat fetuses, as described previously (24, 25). Neurotoxicity experiments were performed on neurons after 3 – 4 days in culture. Cultured neurons were treated with 10 μ M A β (25–35) (Bachem, Bubendorf, Switzerland) in serum-free DMEM (Sigma, St. Louis, MO, USA) at 37°C for 36 h (unless otherwise indicated) to produce neurotoxicity. An A β (25–35) stock solution of 2 mM was prepared in sterile distilled water, stored at –20°C, and incubated for more than 2 days at 37°C to aggregate before use. *A. cordata* and oleanolic acid were dissolved in DMSO at concentrations of 10 mg/ml and 10 mM, respectively, and further diluted in experimental buffers. The final concentration of DMSO was $\leq$ 0.1%, which did not affect cell viability. For each experiment, *A. cordata*, oleanolic acid, *N*^G-nitro-L-arginine methyl ester (L-NAME; Sigma), (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801; RBI, Natick, MA, USA), and verapamil (Sigma) were applied 15 min prior to treatment with 10 μ M A β (25–35). They were also present in the medium during A β (25–

35) incubation.

Measurements of A β (25–35)-induced neuronal death and intracellular biochemical changes

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death 36-h after exposure of cultured neurons to 10 μ M A β (25–35), as previously described (24, 25). Changes in [Ca²⁺]_i were measured with Fluo-4 AM (Molecular Probes), a calcium-sensitive fluorescent dye, using a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg GmbH, Germany) with a 488-nm excitation argon laser and 515-nm long-pass emission filters (24, 25). To measure glutamate secreted into the medium, cells were treated with 10 μ M A β (25–35) in a HEPES buffer containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, and 10 mM glucose at pH 7.4; and glutamate secreted over 24 h was quantified by HPLC with an electrochemical detector (MF series; BAS, West Lafayette, IN, USA) (24). The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Molecular Probes), and a laser scanning confocal microscope (TCS SP2 AOBS, Leica) with 488-nm excitation and 510-nm emission filters were used to monitor the generation of ROS in neurons treated with 10 μ M A β (25–35) for 36 h (24, 25).

Examination of learning and memory

Passive avoidance task: On day 1, *A. cordata*, donepezil (generously provided by Eisai Co., Ltd., Tokyo), and verapamil were administered to 5-week-old ICR mice and 30 min later, the aggregated form of A β (25–35) was administered i.c.v. using a microsyringe with a 27-gauge stainless-steel needle (Hamilton, Reno, NV, USA), the tip of which had been adjusted to a depth of 3 mm. The needle was inserted unilaterally 2 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears, and perpendicular to the plane of the skull (26). The injection volume of A β (25–35) was 15 μ l (15 nmol). *A. cordata* (50 and 100 mg/kg, p.o.) suspended in distilled water, donepezil (2 mg/kg, p.o.), and verapamil (20 mg/kg, p.o.) were administered daily for 7 days. At 30 min after administration of *A. cordata*, donepezil and verapamil on day 7, mice were trained on a one-trial step-through passive avoidance task. The passive avoidance box was divided into two chambers separated by a guillotine door and equipped with a grid floor and a shock generator (Gemini Avoidance System, San Diego, CA, USA). During the acquisition trial, each mouse was placed

into the start chamber and kept in the dark until it was illuminated. After 50 s, the start chamber was illuminated and the door was opened for the mouse to move into the dark chamber freely. Immediately after the mouse entered the dark chamber, the door was closed and the mouse received an inescapable electric shock (0.3 mA, 2 s). In the retention trial given 24 h after the acquisition trial, the mouse was again placed in the chamber and illuminated 50 s later, and the time it took until it re-entered the dark chamber was measured (the step-through latency, maximum testing limit was 300 s). To test for any possible effect of $A\beta(25-35)$ on motor function, the locomotor activity of the animals was measured using a photobeam monitoring system (AM1051; Benwick Electronics, Benwick, UK), and motor coordination was measured using a rota-rod apparatus (Daejong Inc., Seoul, Korea). Each mouse was placed in the center of the activity cage and the total number of beam interruptions was registered for 5 min. Then the mouse was put on the rota-rod for 2 min and the number of falls from the rod was noted.

Brain cholinesterase activity measurement: After the retention trial of the passive avoidance test, the mice were anesthetized deeply with diethyl ether and their brains were removed. Whole brain homogenates of cholinesterase preparation were prepared in a 50-fold volume of 10 mM phosphate buffer on ice. The concentration of acetylcholine (ACh) added as substrate of and hydrolyzed by cholinesterase was determined by the method of Hestrin (27) with a slight modification to measure brain cholinesterase activity. Briefly, aliquots (0.5 ml) of brain homogenates were mixed with 1 mL of 8 mM ACh, 0.4 ml of 0.1 mM phosphate buffer, and 0.1 ml distilled water. After adequate blending, the mixtures were incubated at 37°C for 30 min. The reaction was stopped by the addition of 2 ml alkalinized hydroxylamine, and 0.5 ml of 4 N HCl and 0.5 ml of 0.37 M $FeCl_3$ were added to the reaction mixture. Absorbance was read at 540 nm and calibrated with a blank and an ACh standard.

Histological examination: After the retention trial of the passive avoidance test, five mice in each group were anesthetized deeply with diethyl ether, and then the brains were perfused transcardially with cold heparinized phosphate-buffered saline (PBS, pH 7.2), removed, and fixed in 10% (v/v) formalin solution in PBS for 72 h. The brains were then embedded in paraffin and representative coronal sections (5- μ m-thick) that included the dorsal hippocampus were obtained using a rotary microtome (HM 315; Microm GmbH, Walldorf, Germany). The tissue sections were stained with hematoxylin and eosin (H&E). The sections were dehydrated, mounted in Canada balsam, and then analyzed using a

brightfield microscope (CX21; Olympus, Tokyo). The number of pyramidal cells in the CA1 region of the hippocampus was counted.

Statistical analyses

Data are expressed as the mean $\pm$ S.E.M. and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. $P < 0.05$ was considered significant.

Results

A. cordata and oleanolic acid inhibit $A\beta(25-35)$ -induced neuronal cell death

The concentration of 10 μ M was used for determining $A\beta(25-35)$ -induced neuronal cell damage in the present experiments based on our previous results (28). When cortical neurons were exposed to 10 μ M $A\beta(25-35)$ for 36 h, absorbance in the MTT assay was $67.8 \pm 1.3\%$ of the control level (untreated cells) (Fig. 2), indicating that $A\beta(25-35)$ caused neuronal cell death. In cultures treated with *A. cordata* (1, 5, or 10 μ g/ml), the $A\beta(25-35)$ -induced neuronal death was significantly reduced (absorbance: $84.4 \pm 1.7\%$ of the control with 10 μ g/ml *A. cordata*). To determine which components of *A. cordata* exhibit neuroprotective activity, we examined several compounds including diterpenes, triterpenes such as oleanolic acid, and saponins isolated from *A. cordata* (data not shown). Of these, only oleanolic acid protected against $A\beta(25-35)$ -induced neurotoxicity

Fig. 2. Inhibitory effects of *A. cordata* (AC) and oleanolic acid (OA) on $A\beta(25-35)$ -induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100%. Results are expressed as the mean $\pm$ S.E.M. of data obtained from 3 independent experiments performed in triplicate. ## $P < 0.01$ vs. control; * $P < 0.05$, ** $P < 0.01$ vs. 10 μ M $A\beta(25-35)$.

Fig. 3. Inhibitory effects of *A. cordata* (AC) and oleanolic acid (OA) on $A\beta(25-35)$ -induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. Results are apoptotic cells as a percentage of the total number of cells expressed as the mean $\pm$ S.E.M. of data obtained from 4 independent experiments performed in 2 to 3 wells. ## $P < 0.01$ vs. control, ** $P < 0.01$ vs. $10 \mu\text{M } A\beta(25-35)$.

in cultured cortical neurons. Pretreatment of cortical neurons with 1 and $5 \mu\text{M}$ oleanolic acid reduced the neuronal death induced by $10 \mu\text{M } A\beta(25-35)$ (absorbance: $76.2 \pm 1.2\%$ and $81.1 \pm 1.4\%$ of control, respectively; Fig. 2). MK-801 ($10 \mu\text{M}$), verapamil ($5 \mu\text{M}$), and L-NAME (1 mM) also inhibited the $10 \mu\text{M } A\beta(25-35)$ -induced neuronal death (absorbance: $83.2 \pm 3.9\%$, $88.3 \pm 4.0\%$, and $88.7 \pm 3.0\%$ of the control, respectively; Fig. 2).

Hoechst 33342 staining was used to detect condensed or fragmented DNA, which is indicative of $A\beta(25-35)$ -induced neuronal apoptotic death. Treatment of neurons with $10 \mu\text{M } A\beta(25-35)$ -induced apoptosis in $32.4 \pm 0.8\%$ of cultured cortical neurons, compared with $11.1 \pm 0.7\%$ in control cultures. The addition of *A. cordata* ($10 \mu\text{g/ml}$) or oleanolic acid ($5 \mu\text{M}$) significantly decreased $A\beta(25-35)$ -induced apoptotic cell death to $20.1 \pm 2.0\%$ and $18.7 \pm 2.0\%$ of all neurons, respectively (Fig. 3). Apoptotic cell death induced by $10 \mu\text{M } A\beta(25-35)$ was also blocked by pretreatment with MK-801 ($10 \mu\text{M}$), verapamil ($5 \mu\text{M}$), and L-NAME (1 mM) to $10.3 \pm 1.4\%$, $14.5 \pm 1.4\%$, and $16.0 \pm 2.0\%$, respectively (Fig. 3).

A. cordata and oleanolic acid inhibit $A\beta(25-35)$ -induced $[\text{Ca}^{2+}]_i$ elevation

Increases in $[\text{Ca}^{2+}]_i$ have been associated with $A\beta$ -induced cell death. In our cell cultures, $[\text{Ca}^{2+}]_i$ rapidly increased in response to treatment with $10 \mu\text{M } A\beta(25-35)$ and then showed a slow and gradual decrease over 10 min (Fig. 4). In contrast, pretreatment with

Fig. 4. Inhibitory effects of *A. cordata* (AC) and oleanolic acid (OA) on $A\beta(25-35)$ -induced $[\text{Ca}^{2+}]_i$ elevation in cultured cortical neurons. $[\text{Ca}^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[\text{Ca}^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least 3 independent experiments.

A. cordata ($10 \mu\text{g/ml}$), oleanolic acid ($5 \mu\text{M}$), MK-801 ($10 \mu\text{M}$), verapamil ($5 \mu\text{M}$), and L-NAME (1 mM) significantly inhibited the increase of $[\text{Ca}^{2+}]_i$ induced by $10 \mu\text{M } A\beta(25-35)$ throughout the measurement period. *A. cordata* and the other compounds did not affect basal $[\text{Ca}^{2+}]_i$.

A. cordata and oleanolic acid inhibit $A\beta(25-35)$ -induced elevation of glutamate release

We next quantified the glutamate released into the extracellular medium after treatment with $10 \mu\text{M } A\beta(25-35)$ for 24 h. As shown in Fig. 5, $10 \mu\text{M } A\beta(25-35)$ elevated the basal glutamate level from $3.39 \pm 0.29 \mu\text{M}$ in control neurons to $6.77 \pm 0.41 \mu\text{M}$. *A. cordata* (5 and $10 \mu\text{g/ml}$) significantly blocked the $A\beta(25-35)$ -induced elevation of glutamate release, resulting in maximal values of 4.17 ± 0.39 and $3.78 \pm 0.24 \mu\text{M}$, respectively. Oleanolic acid (1 and $5 \mu\text{M}$) also inhibited the $A\beta(25-35)$ -induced elevation of glutamate release, resulting in maximal values of 4.16 ± 0.97 and $3.70 \pm 0.55 \mu\text{M}$, respectively.

A. cordata and oleanolic acid inhibit $A\beta(25-35)$ -induced ROS generation

To clarify the involvement of oxidative stress in $A\beta$ neurotoxicity, we measured the accumulation of ROS after the exposure of the cells to $A\beta(25-35)$ for 36 h. In $\text{H}_2\text{DCF-DA}$ -loaded cerebral cortical neurons, $10 \mu\text{M } A\beta(25-35)$ increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with

Fig. 5. Inhibitory effects of *A. cordata* (AC) and oleanolic acid (OA) on $A\beta(25-35)$ -induced glutamate release in cultured cortical neurons. The amount of glutamate released over 24 h was measured by HPLC with an electrochemical detector. Results are expressed as the mean $\pm$ S.E.M. of data obtained from 3 independent experiments performed in 2 to 3 wells. ## P <0.01 vs. control; * P <0.05, ** P <0.01 vs. 10 μ M $A\beta(25-35)$.

Fig. 6. Inhibitory effect of *A. cordata* (AC) and oleanolic acid (OA) on $A\beta(25-35)$ -induced ROS generation in cultured cortical neurons. Results are expressed as the mean $\pm$ S.E.M. of RFI obtained from 3 independent experiments performed in 2 to 3 wells. ## P <0.01 vs. control; ** P <0.01 vs. 10 μ M $A\beta(25-35)$.

10 μ M $A\beta(25-35)$, the relative fluorescence increased approximately 2.5-fold to 108.8 ± 7.0 compared with the value in control neurons (43.3 ± 2.7 ; Fig. 6). The $A\beta(25-35)$ -induced increase in ROS generation was significantly inhibited by *A. cordata* (5 and 10 μ g/ml) and oleanolic acid (0.5, 1, and 5 μ M). MK-801 (10 μ M), verapamil (5 μ M), and L-NAME (1 mM) also significantly blocked the $A\beta(25-35)$ -induced increase of ROS generation (Fig. 6).

Fig. 7. Protective effect of *A. cordata* (AC) against $A\beta(25-35)$ -induced memory impairment in mice. The learning and memory performance was assessed by the passive avoidance test. Values are expressed as the mean $\pm$ S.E.M. of step-through latency ($n = 10$). ## P <0.01 vs. control; * P <0.05, ** P <0.01 vs. 10 μ M $A\beta(25-35)$.

A. cordata inhibits $A\beta(25-35)$ -induced memory impairment and neuronal death in mice

In the initial acquisition trial of the passive avoidance task, the step-through latency did not differ among the 6 groups [control, 15 nmol $A\beta(25-35)$, 15 nmol $A\beta(25-35)$ + 50 mg/kg *A. cordata*, 15 nmol $A\beta(25-35)$ + 100 mg/kg *A. cordata*, 15 nmol $A\beta(25-35)$ + 2 mg/kg donepezil, and 15 nmol $A\beta(25-35)$ + 20 mg/kg verapamil; data not shown]. The step-through latency of the $A\beta(25-35)$ -treated group in the retention trial decreased significantly to 47.6 ± 3.8 s, compared with 239.5 ± 24.5 s in the control group, indicating that $A\beta(25-35)$ impaired memory in mice. Chronically administered *A. cordata* markedly protected against the memory impairment produced by $A\beta(25-35)$. The step-through latency in groups administered *A. Cordata* was 182.1 ± 26.3 s and 199.6 ± 24.8 s at doses of 50 and 100 mg/kg, respectively (Fig. 7). Chronic administration of donepezil (2 mg/kg), a cholinesterase inhibitor, and verapamil (20 mg/kg) also protected against $A\beta(25-35)$ -induced memory impairment (step-through latency: 143.3 ± 31.2 and 199.6 ± 31.3 , respectively; Fig. 7). MK-801 (0.1 mg/kg, i.p.) and L-NAME (50 mg/kg, s.c.), however, failed to inhibit $A\beta(25-35)$ -induced memory impairment (data not shown). To elucidate whether *A. cordata* affects general motor functions, we measured spontaneous locomotor activity and motor coordination in the mice. Neither *A. Cordata* nor $A\beta(25-35)$ significantly affected locomotor and rota-rod activity (data not shown), indicating that the observed improvement of memory by *A. cordata* was not due to immobility.

Exposure to 15 nmol $A\beta(25-35)$ produced a signifi-

Table 1. Effect of *A. cordata* (AC) on brain cholinesterase activity in mice

Group	Dose	Cholinesterase activity ^a [$\mu\text{mol/h}$ per brain (1 g)]
Control	—	446.80 $\pm$ 2.66
$A\beta(25-35)$	15 nmol/animal	463.06 $\pm$ 1.78 [#]
+AC	50 mg/kg	456.87 $\pm$ 4.73
+AC	100 mg/kg	443.17 $\pm$ 2.60**

^aResults are expressed as the mean $\pm$ S.E.M. of acetylcholine concentration hydrolyzed by the cholinesterase preparation of brain homogenate for 1 h (n = 5 mice/group). [#] $P < 0.05$ vs. control, ** $P < 0.01$ vs. $A\beta(25-35)$ alone.

cant increase in cholinesterase activity in the brain. In the group consuming 100 mg/kg *A. cordata*, the cholinesterase activity was significantly decreased compared with the $A\beta(25-35)$ group (Table 1).

We examined the subsequent histological changes in the hippocampus of $A\beta(25-35)$ -injected mice. As compared with vehicle-treated mice (Fig. 8A: a and f, cell number 247.5 ± 10.7), the number of CA1 pyramidal cells decreased in $A\beta(25-35)$ -treated mice (Fig. 8A: b and g, cell number 146.5 ± 5.7). Administration of 50 and 100 mg/kg *A. cordata*, 2 mg/kg donepezil,

and 20 mg/kg verapamil for 7 days protected the hippocampus against the $A\beta(25-35)$ -induced neuronal loss (cell number: 167.5 ± 3.7 , 207.8 ± 3.6 , 180.0 ± 7.1 , and 227.5 ± 10.5 , respectively; Fig. 8B).

Discussion

As an active partial fragment of $A\beta$, $A\beta(25-35)$ forms a β -sheet structure and induces neuronal cell death, neuritic atrophy, synaptic loss, and memory impairment, although it is not found in the AD brain (15, 26, 29, 30). The present study demonstrated that $A\beta(25-35)$ causes $[\text{Ca}^{2+}]_i$ increase, glutamate release, ROS generation, and neuronal cell death in cultured cortical neurons, all of which were blocked by treatment with MK-801, an NMDA antagonist; verapamil, an L-type Ca^{2+} channel blocker; and L-NAME, a NOS inhibitor. These results supporting the involvement of NMDA glutamate-receptor activation, increased Ca^{2+} influx, and generation of ROS in $A\beta(25-35)$ -induced neurotoxicity in cultured neurons are consistent with the results of other studies (3, 31–33). Regardless of the relative contribution of these events to $A\beta(25-35)$ -induced neurotoxicity, the primary event following $A\beta(25-35)$ treatment of cultured neurons has been suggested to be Ca^{2+} influx,

Fig. 8. Representative photomicrographs of the hippocampus and CA1 region (A) and histogram of CA1 pyramidal cell numbers (B) representing the inhibitory effect of *A. cordata* (AC) on $A\beta(25-35)$ -induced hippocampal damage in mice. Brain sections were stained with H&E. Pathological features of brain sections 7 days after i.c.v. injection of 15 nmol $A\beta(25-35)$ were observed using a brightfield microscope. Panels a and f represent the control group; panels b and g, $A\beta$ -injected group; panels c and h, $A\beta$ + 100 mg/kg AC group; panels d and i, $A\beta$ + 2 mg/kg donepezil group; panels e and j, $A\beta$ + 20 mg/kg verapamil group. Panels a–e represent the hippocampus ($\times 40$); panels f–j, CA1 region ($\times 400$). Values are expressed as the mean $\pm$ S.E.M. of cell numbers from five mice. ## $P < 0.01$ vs. control; * $P < 0.05$, ** $P < 0.01$ vs. $A\beta(25-35)$.

apparently via L-type voltage-dependent Ca^{2+} channels (L-VDCC), because blockade of this channel or Ca^{2+} chelation prevents other consequences (5, 32). Vitamin E, an antioxidant, blocks the $\text{A}\beta$ -induced generation of ROS but not Ca^{2+} influx, whereas reduction of extracellular Ca^{2+} inhibits both the $\text{A}\beta$ -induced increase in intracellular Ca^{2+} and the generation of ROS, indicating that ROS generation is a consequence of Ca^{2+} accumulation (3). Many experiments (5, 31, 34), however, have demonstrated that free radicals are responsible for the increase of $[\text{Ca}^{2+}]_i$. The ROS-induced membrane damage causes further Ca^{2+} influx and the resulting increase in Ca^{2+} influx in turn induces the generation of further ROS. In the present study, both verapamil and L-NAME inhibited the $\text{A}\beta(25-35)$ -induced $[\text{Ca}^{2+}]_i$ increase and ROS generation. We therefore hypothesized that $[\text{Ca}^{2+}]_i$ increase and ROS generation were almost simultaneous early events induced by $\text{A}\beta(25-35)$ and were responsible for $\text{A}\beta(25-35)$ -induced neuronal death in this culture system. *A. cordata* inhibited the $\text{A}\beta(25-35)$ -induced $[\text{Ca}^{2+}]_i$ increase, glutamate release, ROS generation, and neuronal cell death in cultured cortical neurons in the current study. This result indicates that *A. cordata* might prevent Ca^{2+} entry through VDCC- and/or NMDA-receptor-coupled channels to inhibit $\text{A}\beta(25-35)$ -induced neuronal death, although the mechanism by which *A. cordata* blocks the channels is not clear. *A. cordata* has been reported to possess antioxidant principles such as those of diterpenes and triterpenes (35, 36), suggesting that inhibition of $\text{A}\beta(25-35)$ -induced neuronal death by *A. cordata* might be due to their ROS-scavenging activity. Furthermore, diterpenes and triterpenes have been demonstrated to inhibit Ca^{2+} entry through Ca^{2+} channels (37, 38), indicating that these compounds, including oleanolic acid, may contribute to the inhibitory activity of *A. cordata* on Ca^{2+} entry through VDCC- and/or NMDA-receptor-coupled channels to inhibit $\text{A}\beta(25-35)$ -induced neuronal death. Further study to elucidate the precise mechanism should be performed.

Many researchers have demonstrated that $\text{A}\beta$ triggers apoptotic degeneration in in vitro neuronal experiments (3, 39). In the present work, cultured cortical neurons exposed to $\text{A}\beta(25-35)$ for 36 h showed increased chromatin condensation, a typical feature of apoptotic cell death, which was reduced by *A. cordata*. $\text{A}\beta$ -induced apoptosis was also associated with COX-2 upregulation, and COX has been suggested to be an important source of ROS in the pathologic brain (40, 41). Therefore, the protective effect of *A. cordata* against the $\text{A}\beta$ -induced neurotoxicity might result from the inhibition of COX-1 and COX-2 (20, 21). *A. cordata*

inhibits chondrocyte apoptosis through the downregulation of JNK and p38 MAP kinase signaling and the inhibition of caspase-3 activity (42). *A. cordata* could therefore prevent any step of the apoptotic cascade. The molecular mechanism for the prevention of neuronal apoptosis by *A. cordata* should be further clarified.

I.c.v. injection of $\text{A}\beta(25-35)$ into experimental rodents induces memory impairment in different behavioral paradigms, including spontaneous alternation, the water maze, and passive avoidance (26, 43). $\text{A}\beta(25-35)$ preferentially impairs spatial and non-spatial short-term memory, and these effects remain evident up to 6 months after even a single i.c.v. injection of the peptide (44). Memory impairment in the passive avoidance test was also confirmed in mice 7 days after the i.c.v. injection of $\text{A}\beta(25-35)$ in the present work. Chronic treatment with *A. cordata* (50 and 100 mg/kg, p.o.) effectively protected the mice against $\text{A}\beta(25-35)$ -induced memory deficit. Moreover, *A. cordata* treatment significantly prevented $\text{A}\beta(25-35)$ -induced neuronal damage in the hippocampus, as observed by histological examination. Especially, $\text{A}\beta(25-35)$ induced a significant cell loss in the CA1 pyramidal cell layer, as evidenced by others (45, 46), and *A. cordata* protected against this. These results were consistent with its protective effect against $\text{A}\beta(25-35)$ -induced neurotoxicity in vitro. $\text{A}\beta$ accumulation associated with cognitive impairment in AD is accompanied by an increase in cholinesterase activity (47); consistent with this, the increase of cholinesterase activity by $\text{A}\beta(25-35)$ in the brain was inhibited by *A. cordata* in the present study. The present study also demonstrated the protection by donepezil, a cholinesterase inhibitor, against $\text{A}\beta(25-35)$ -induced memory impairment. In addition, *A. cordata* administration significantly prevented scopolamine-induced learning and memory impairment, reducing escape latency during training in the Morris water maze test, a spatial task that is sensitive to impaired cholinergic function in the hippocampus (48) (data not shown). Taken together, these results suggest that *A. cordata* increases cholinergic activity.

On the other hand, other studies have indicated that oxidative stress is responsible for the onset of the cognitive dysfunction as well as the progression of AD (31, 49). Elevated levels of $\text{A}\beta$ induce oxidative stress, increasing the appearance of ROS such as superoxide (O_2^-) and NO and subsequently producing ONOO $^-$ by a rapid interaction, could mediate the damage seen in AD (49, 50). A scavenger of ONOO $^-$ protects against $\text{A}\beta(25-35)$ -induced memory impairment (51), and antioxidants such as α -tocopherol protect against cytotoxicity in vitro as well as against learning and memory deficits induced by $\text{A}\beta$ (12). In the present study, 10 μM

$A\beta(25-35)$ significantly increased the ROS level in cultured neurons, and this was inhibited by *A. cordata*. In addition, *Aralia* species contain antioxidant components (35, 36). Therefore, it is possible that the favorable effect of *A. cordata* on $A\beta(25-35)$ -induced cognitive deficits can be attributed to the inhibition of ROS generation. The attenuation of memory impairment by *A. cordata* is likely to be a corollary of its inhibition of $A\beta(25-35)$ -induced $[Ca^{2+}]_i$, because memantine was demonstrated to have therapeutic benefits on AD due to its affinity for the NMDA-receptor Ca^{2+} channel (52). Moreover, the possible contribution of the blocking effect of donepezil, an acetylcholinesterase inhibitor, on the voltage-gated Ca^{2+} channels to the neuroprotective effect in AD was reported (53). In support of this hypothesis, verapamil, an L-type Ca^{2+} channel blocker, inhibited $A\beta(25-35)$ -induced memory impairment in the present study. Quartermain and Garcia deSoria also reported enhancing effects of Ca^{2+} -channel antagonists on passive avoidance and maze learning (54, 55), although there are controversial reports about the mnemonic effect of Ca^{2+} -channel antagonists (56, 57). However, we failed to demonstrate the inhibitory effects of MK-801 and L-NAME on $A\beta(25-35)$ -induced memory deficits. These results are consistent with the reports showing the impairment of learning and memory by NMDA-receptor antagonists and the role of NO on learning and memory in animals (58–61).

Oleanolic acid isolated from the aerial part of *A. cordata* also protected against $A\beta(25-35)$ -induced neuronal damage. Oleanolic acid was able to reduce the $A\beta$ -induced $[Ca^{2+}]_i$ increase, glutamate release, and ROS generation and, as a result, to attenuate neuronal apoptotic death in primary cultures of rat cortical neurons. Therefore, the inhibition by *A. cordata* of the $A\beta(25-35)$ -induced memory deficit as well as neuronal damage in cultures may be attributable, at least in part, to the beneficial effects of oleanolic acid. Oleanolic acid is found naturally in various medicinal herbs traditionally used for anti-inflammatory, analgesic, hepatoprotective, and cardioprotective effects (62) and in rats has been reported to protect against myocardial ischemia–reperfusion injury by enhancing antioxidant activity of the mitochondria mediated by glutathione and α -tocopherol (63). Oleanolic acid has also been reported to have inhibitory activity against COX-1 and COX-2 (21). Although there has been no evidence up until now to show that oleanolic acid antagonizes $A\beta(25-35)$ -induced neurotoxicity, the present study demonstrated a novel pharmacological activity of oleanolic acid in neurons.

In conclusion, the protection against $A\beta(25-35)$ -induced neuronal cell damage in culture and $A\beta(25-35)$ -

induced memory deficit in vivo may explain the inhibitory action of *A. cordata* on the progression of AD. Further studies should determine the specific components in *A. cordata* that are responsible for preventing the cognitive deficits.

Acknowledgment

This work was supported by a grant (Code 20080401034050) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

- 1 Price DL, Tanzi RE, Borchelt DR, Sisodia SS. Alzheimer's disease: Genetic studies and transgenic models. *Annu Rev Genet.* 1998;32:461–493.
- 2 Ivins KJ, Ivins JK, Sharp JP, Cotman CW. Multiple pathways of apoptosis in PC12 cells. Crma inhibits apoptosis induced by beta-amyloid. *J Biol Chem.* 1999;274:2107–2112.
- 3 Ekinci FJ, Linsley MD, Shea TB. Beta-amyloid-induced calcium influx induces apoptosis in culture by oxidative stress rather than tau phosphorylation. *Brain Res Mol Brain Res.* 2000;76:389–395.
- 4 Gray CW, Patel AJ. Neurodegeneration mediated by glutamate and beta-amyloid peptide: A comparison and possible interaction. *Brain Res.* 1995;691:169–179.
- 5 Ueda K, Shinohara S, Yagami T, Asakura K, Kawasaki K. Amyloid beta protein potentiates Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels: A possible involvement of free radicals. *J Neurochem.* 1997;68:265–271.
- 6 Van Dam D, De Deyn PP. Drug discovery in dementia: The role of rodent models. *Nat Rev Drug Discov.* 2006;5:956–970.
- 7 Nitta A, Fukuta T, Hasegawa T, Nabeshima T. Continuous infusion of beta-amyloid protein into the rat cerebral ventricle induces learning impairment and neuronal and morphological degeneration. *Jpn J Pharmacol.* 1997;73:51–57.
- 8 Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: Potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med.* 2002;32:1050–1060.
- 9 Butterfield DA, Reed T, Newman SF, Sultana R. Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. *Free Radic Biol Med.* 2007;43:658–677.
- 10 Gitter BD, Cox LM, Rydel RE, May PC. Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells. *Proc Natl Acad Sci U S A.* 1995;92:10738–10741.
- 11 McDonald DR, Brunden KR, Landreth GE. Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. *J Neurosci.* 1997;17:2284–2294.
- 12 Yamada K, Tanaka T, Han D, Senzaki K, Kameyama T, Nabeshima T. Protective effects of idebenone and α -tocopherol on beta-amyloid-(1–42)-induced learning and memory deficits in rats: Implication of oxidative stress in beta-amyloid-induced neurotoxicity in vivo. *Eur J Neurosci.* 1999;11:83–90.

- 13 Rogers J, Kirby LC, Hempelman SR, Berry DL, McGeer PL, Kaszniak AW, et al. Clinical trial of indomethacin in Alzheimer's disease. *Neurology*. 1993;43:1609–1611.
- 14 Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, et al. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's disease cooperative study. *N Engl J Med*. 1997;336:1216–1222.
- 15 Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW. Structure-activity analyses of beta-amyloid peptides: Contributions of the beta 25-35 region to aggregation and neurotoxicity. *J Neurochem*. 1995;64:253–265.
- 16 Richardson JS, Zhou Y, Kumar U. Free radicals in the neurotoxic actions of beta-amyloid. *Ann NY Acad Sci*. 1996;777:362–367.
- 17 Perry LM. Medicinal plants of east and southeast Asia: Attributed properties and uses. Cambridge, MA: The MIT Press; 1980.
- 18 Okuyama E, Nishimura S, Yamazaki M. Analgesic principles from *Aralia cordata* thunb. *Chem Pharm Bull*. 1991;39:405–407.
- 19 Han BH, Han YN, Han LA, Park MH, Lee EO. Studies on the anti-inflammatory activity of *Aralia contineltalis*. *Arch Pharm Res*. 1983;6:17–23.
- 20 Dang NH, Zhang X, Zheng M, Son KH, Chang HW, Kim HP, et al. Inhibitory constituents against cyclooxygenases from *Aralia cordata* thunb. *Arch Pharm Res*. 2005;28:28–33.
- 21 Lee IS, Jin W, Zhang X, Hung TM, Song KS, Seong YH, et al. Cytotoxic and COX-2 inhibitory constituents from the aerial parts of *Aralia cordata*. *Arch Pharm Res*. 2006;29:548–555.
- 22 Hoehn BD, Palmer TD, Steinberg GK. Neurogenesis in rats after focal cerebral ischemia is enhanced by indomethacin. *Stroke*. 2005;36:2718–2724.
- 23 Pitchumoni SS, Doraiswamy PM. Current status of antioxidant therapy for Alzheimer's disease. *J Am Geriatr Soc*. 1998;46:1566–1572.
- 24 Ban JY, Jeon SY, Bae K, Song KS, Seong YH. Catechin and epicatechin from *Smilacis chinae* rhizome protect cultured rat cortical neurons against amyloid beta protein (25-35)-induced neurotoxicity through inhibition of cytosolic calcium elevation. *Life Sci*. 2006;79:2251–2259.
- 25 Ban JY, Cho SO, Choi SH, Ju HS, Kim JY, Bae K, et al. Neuroprotective effect of *Smilacis chinae* rhizome on NMDA-induced neurotoxicity in vitro and focal cerebral ischemia in vivo. *J Pharmacol Sci*. 2008;106:68–77.
- 26 Maurice T, Lockhart BP, Privat A. Amnesia induced in mice by centrally administered beta-amyloid peptides involves cholinergic dysfunction. *Brain Res*. 1996;706:181–193.
- 27 Zhu MC, Xin YB, Sun MJ, Fang YZ. Purification and properties of acetylcholinesterase from human brain. *Sci China B*. 1993;36:1207–1215.
- 28 Lee BY, Ban JY, Seong YH. Chronic stimulation of GABA_A receptor with muscimol reduces amyloid beta protein (25-35)-induced neurotoxicity in cultured rat cortical cells. *Neurosci Res*. 2005;52:347–356.
- 29 Grace EA, Rabiner CA, Busciglio J. Characterization of neuronal dystrophy induced by fibrillar amyloid beta: Implications for Alzheimer's disease. *Neuroscience*. 2002;114:265–273.
- 30 Tohda C, Matsumoto N, Zou K, Meselhy MR, Komatsu K. A beta (25-35)-induced memory impairment, axonal atrophy, and synaptic loss are ameliorated by M1, a metabolite of proto-panaxadiol-type saponins. *Neuropsychopharmacology*. 2004;29:860–868.
- 31 Butterfield DA, Drake J, Pocernich C, Castegna A. Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid beta-peptide. *Trends Mol Med*. 2001;7:548–554.
- 32 Mattson MP, Chan SL. Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium*. 2003;34:385–397.
- 33 Harkany T, Hortobagyi T, Sasvari M, Konya C, Penke B, Luiten PG, et al. Neuroprotective approaches in experimental models of beta-amyloid neurotoxicity: Relevance to Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry*. 1999;23:963–1008.
- 34 Huang HM, Ou HC, Hsieh SJ. Antioxidants prevent amyloid peptide-induced apoptosis and alteration of calcium homeostasis in cultured cortical neurons. *Life Sci*. 2000;66:1879–1892.
- 35 Suh SJ, Jin UH, Kim KW, Son JK, Lee SH, Son KH, et al. Triterpenoid saponin, oleanolic acid 3-O-beta-d-glucopyranosyl(1→3)-alpha-l-rhamnopyranosyl(1→2)-alpha-l-arabinopyranoside (oa) from *Aralia elata* inhibits LPS-induced nitric oxide production by down-regulated NF-kappaB in raw 264.7 cells. *Arch Biochem Biophys*. 2007;467:227–233.
- 36 Xi M, Hai C, Tang H, Chen M, Fang K, Liang X. Antioxidant and antiglycation properties of total saponins extracted from traditional chinese medicine used to treat diabetes mellitus. *Phytother Res*. 2008;22:228–237.
- 37 Tirapelli CR, Ambrosio SR, da Costa FB, de Oliveira AM. Evidence for the mechanisms underlying the effects of pimaradienoic acid isolated from the roots of *Viguiera arenaria* on rat aorta. *Pharmacology*. 2004;70:31–38.
- 38 Cherng JM, Lin HJ, Hung MS, Lin YR, Chan MH, Lin JC. Inhibition of nuclear factor kappaB is associated with neuroprotective effects of glycyrrhizic acid on glutamate-induced excitotoxicity in primary neurons. *Eur J Pharmacol*. 2006;547:10–21.
- 39 Yan XZ, Qiao JT, Dou Y, Qiao ZD. Beta-amyloid peptide fragment 31-35 induces apoptosis in cultured cortical neurons. *Neuroscience*. 1999;92:177–184.
- 40 Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. *J Cereb Blood Flow Metab*. 2001;21:2–14.
- 41 Jang JH, Surh YJ. Beta-amyloid-induced apoptosis is associated with cyclooxygenase-2 up-regulation via the mitogen-activated protein kinase-NF-kappaB signaling pathway. *Free Radic Biol Med*. 2005;38:1604–1613.
- 42 Baek YH, Huh JE, Lee JD, Choi DY, Park DS. Effect of *Aralia cordata* extracts on cartilage protection and apoptosis inhibition. *Biol Pharm Bull*. 2006;29:1423–1430.
- 43 Um MY, Choi WH, Aan JY, Kim SR, Ha TY. Protective effect of *Polygonum multiflorum* thunb on amyloid beta-peptide 25-35 induced cognitive deficits in mice. *J Ethnopharmacol*. 2006;104:144–148.
- 44 Stepanichev MY, Moiseeva YV, Lazareva NA, Onufriev MV, Gulyaeva NV. Single intracerebroventricular administration of amyloid-beta (25-35) peptide induces impairment in short-term rather than long-term memory in rats. *Brain Res Bull*. 2003;61:197–205.
- 45 Holscher C, Gengler S, Gault VA, Harriott P, Mallot HA. Soluble beta-amyloid[25-35] reversibly impairs hippocampal synaptic plasticity and spatial learning. *Eur J Pharmacol*. 2007;561:85–90.
- 46 Huang HJ, Liang KC, Chen CP, Chen CM, Hsieh-Li HM. Intrahippocampal administration of a beta(1-40) impairs spatial

- learning and memory in hyperglycemic mice. *Neurobiol Learn Mem.* 2007;87:483–494.
- 47 Atack JR, Perry EK, Bonham JR, Perry RH, Tomlinson BE, Blessed G, et al. Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: Selective loss of the intermediate (10s) form. *Neurosci Lett.* 1983;40:199–204.
 - 48 Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods.* 1984;11:47–60.
 - 49 Kontush A. Amyloid-beta: An antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. *Free Radic Biol Med.* 2001;31:1120–1131.
 - 50 Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci.* 1997;17:2653–2657.
 - 51 Alkam T, Nitta A, Mizoguchi H, Itoh A, Nabeshima T. A natural scavenger of peroxynitrites, rosmarinic acid, protects against impairment of memory induced by Abeta(25-35). *Behav Brain Res.* 2007;180:139–145.
 - 52 Wenk GL. Neuropathologic changes in Alzheimer's disease: Potential targets for treatment. *J Clin Psychiatry.* 2006;67 Suppl 3:3–7; quiz 23.
 - 53 Solntseva EI, Bukanova JV, Marchenko E, Skrebitsky VG. Donepezil is a strong antagonist of voltage-gated calcium and potassium channels in molluscan neurons. *Comp Biochem Physiol C Toxicol Pharmacol.* 2007;144:319–326.
 - 54 Quartermain D, deSoria VG, Kwan A. Calcium channel antagonists enhance retention of passive avoidance and maze learning in mice. *Neurobiol Learn Mem.* 2001;75:77–90.
 - 55 Quartermain D, Garcia deSoria V. The effects of calcium channel antagonists on short- and long-term retention in mice using spontaneous alternation behavior. *Neurobiol Learn Mem.* 2001;76:117–124.
 - 56 Borroni AM, Fichtenholtz H, Woodside BL, Teyler TJ. Role of voltage-dependent calcium channel long-term potentiation (LTP) and NMDA LTP in spatial memory. *J Neurosci.* 2000;20:9272–9276.
 - 57 Lashgari R, Motamedi F, Zahedi Asl S, Shahidi S, Komaki A. Behavioral and electrophysiological studies of chronic oral administration of L-type calcium channel blocker verapamil on learning and memory in rats. *Behav Brain Res.* 2006;171:324–328.
 - 58 Harooni HE, Naghdi N, Sepehri H, Rohani AH. The role of hippocampal nitric oxide (NO) on learning and immediate, short- and long-term memory retrieval in inhibitory avoidance task in male adult rats. *Behav Brain Res.* 2009;201:166–172.
 - 59 Jafari-Sabet M. NMDA receptor blockers prevents the facilitatory effects of post-training intra-dorsal hippocampal NMDA and physostigmine on memory retention of passive avoidance learning in rats. *Behav Brain Res.* 2006;169:120–127.
 - 60 Majlessi N, Choopani S, Bozorgmehr T, Azizi Z. Involvement of hippocampal nitric oxide in spatial learning in the rat. *Neurobiol Learn Mem.* 2008;90:413–419.
 - 61 Milton AL, Lee JL, Butler VJ, Gardner R, Everitt BJ. Intra-amygdala and systemic antagonism of NMDA receptors prevents the reconsolidation of drug-associated memory and impairs subsequently both novel and previously acquired drug-seeking behaviors. *J Neurosci.* 2008;28:8230–8237.
 - 62 Liu J. Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol.* 1995;49:57–68.
 - 63 Du Y, Ko KM. Oleanolic acid protects against myocardial ischemia-reperfusion injury by enhancing mitochondrial antioxidant mechanism mediated by glutathione and alpha-tocopherol in rats. *Planta Med.* 2006;72:222–227.