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Inhibitory effect of ethanol extract of *Magnolia officinalis* and 4-O-methylhonokiol on memory impairment and neuronal toxicity induced by beta-amyloid

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ABSTRACT

The components of the herb *Magnolia officinalis* have exhibited antioxidant and neuroprotective activities. In this study, we investigated effects of ethanol extract of *M. officinalis* and its major component 4-*O*-methylhonokiol on memory dysfunction and neuronal cell damages caused by Aβ. Oral pretreatment of ethanol extract of *M. officinalis* (2.5, 5 and 10 mg/kg) and 4-*O*-methylhonokiol (1 mg/kg) into drinking water for 5 weeks suppressed the intraventricular treatment of $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.)-induced memory impairments. In addition, 4-*O*-methylhonokiol prevented the $A\beta_{1-42}$-induced apoptotic cell death as well as β-secretase expression. 4-*O*-methylhonokiol also inhibited H_2O_2 and $A\beta_{1-42}$-induced neurotoxicity in cultured neurons as well as PC12 cells by prevention of the reactive oxygen species generation. 4-*O*-methylhonokiol also directly inhibited β-secretase activity and Aβ fibrilization in vitro. Thus, ethanol extract of *M. officinalis* may be useful for prevention of the development or progression of AD, and 4-*O*-methylhonokiol may be a major active component.

1. Introduction

Beta-amyloid peptide (Aβ) is accumulated as insoluble extracellular deposits in senile plaques, the neuropathological hallmarks of Alzheimer's disease (AD). AD is an age-related neurodegenerative disease characterized by progressive degeneration and loss of neurons in the brain. Based on the strong association between Aβ and AD, it seems that therapeutic strategies to reduce the levels of Aβ in the brain are beneficial for the treatment of AD. Aβ, a hydrophobic polypeptide, is proteolytically produced from an amyloid precursor protein (APP) by proteases, called β- and γ-secretases (Ling et al., 2003). Compounds that alter the proteolytic cleavage of APP, including those that inhibit β- or γ-secretase activity, can reduce the production of Aβ peptides, and may have interesting therapeutic potential in the treatment of AD (Owens et al., 2003; Citron, 2004). Several lines of evidence have shown that Aβ is considered to have a causal role in the development and progress of AD through the induction of neuronal cell death (Hardy and Selkoe, 2002; Patel and Brewer, 2003; Pappolla et al., 1998; Lecanu et al., 2004). The mechanism underlying Aβ-induced neurotoxicity is complex, but increment of the vulnerability of neurons due to the increase of reactive oxygen species (ROS) generation may be implicated in the development and progress of AD (Pappolla et al., 1998; Anekonda and Reddy, 2005; Lee et al., 2006). Moreover, ROS can upregulate β- and γ-secretase to generate Aβ (Smith et al., 2004; Tamagno et al., 2006, 2008).

Magnolol, honokiol, and obovatol are well known bioactive constituents of the bark of *Magnolia officinalis* (Family Magnoliaceae), which has been used as traditional Chinese medicine for treatment of neurosis, anxiety, stroke, fever and headache (Song et al., 1989). Magnolol, obovatol, and honokiol have been known to have various pharmacological activities such as anti-inflammatory (Choi et al., 2007; Tse et al., 2007; Chen et al., 2006; Lee et al., 2005a,b; Park et al., 2004; Liou et al., 2003a) as well as anti-oxidative activity (Lin et al., 2006; Liou et al., 2003b; Haraguchi et al., 1997). Moreover, the effects of honokiol and magnolol on the central cholinergic system have recently been reported (Tsai et al., 1995; Fukuyama et al., 1992). Honokiol was known to promote potassium-induced release of acetylcholine in a rat hippocampal slice (Tsai et al., 1995) and to enhance neurite sprouting (Fukuyama et al., 1992). In addition, both compounds exhibited acetylcholine esterase (AChE) inhibitory property in rat spleen microsomes and human polymorphonuclear leukocytes (Yamazaki et al., 1994). We also recently found that obovatol has strong anxiolytic activity (Seo et al., 2007). Among various constituents of ethanol extract of *M. officinalis*, we have isolated a novel major compound and have

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identified that it is 4-*O*-methylhonokiol. In this study, we investigated whether ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol could inhibit the memory impairment induced by Aβ through reduction of neurotoxicity and ROS generation.

2. Materials and methods

2.1. Materials

The bark of *M. officinalis* were purchased from KyungDong Market, Seoul, Korea, and taxonomically identified by Dr. Ban Yeon Hwang at the Research Institute of Drug Resource, Chungbuk National University (Cheongju, Korea). A voucher specimen (number) was deposited at the Herbarium of Chungbuk National University, Chungbuk, Korea. The ethanol extract of *M. officinalis* contained 16.6% methylhonokiol followed by 16.5% honokiol and 12.9% magnolol, and 42–45% others as similar to other studies (Shen et al., 2009; Wu et al., 2006). MH (Fig. 1) were isolated from the bark of *M. officinalis Rehd. et Wils*, by subsequently extraction with n-hexane, ethyl acetate and *n*-BuOH, and then identified by ^{1}H-NMR, and ^{13}C-NMR as described elsewhere (Lee et al., 2009; Oh et al., 2009). MH was supplied by Bioland Ltd. (Chungnam, Korea) as a white powder of 99.6% purity. Ethanol extract of *M. officinalis* (2.5, 5 and 10 mg/kg) and 4-*O*-methylhonokiol (1 mg/kg) was used in this study. The ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol dissolved in 0.05% ethanol were added to drinking water and mice were allowed access to it for 5 weeks *ad libitum* before induction of memory impairment.

2.2. Animals

Male ICR mice weighing 25–30 g (Samtako, Gyeonggi-do, Korea) were maintained in accordance with the guideline of National Institute of Toxicological Research of the Korea Food and Drug Administration for the care and use of laboratory animals. Animals were housed 3 per cage, allowed access to water and food *ad libitum*, and maintained on a 12-h light/dark cycle regulated at room temperature (23 °C). The experiments were performed at least 1 week after their arrival to the individual home cage. A total of 100 mice were used for memory tests. One group (50 mice) was used for water maze test, and the other group (50 mice) was used for step-through test. Each group was divided into five subgroups: control, three different doses of ethanol extract of *M. officinalis* treated subgroups and 4-*O*-methylhonokiol treated subgroups.

2.3. Preparation of beta-amyloid peptide 1–42 ($A\beta_{1-42}$) and fibrilization

The $A\beta_{1-42}$ (Sigma, St. Lous, MO, USA) was dissolved, and aliquots were stored at −20 °C until use. Aliquots of $A\beta_{1-42}$ at a concentration of 200 μg/ml prepared in distilled water (DW) were incubated for 5 days at 37 °C. At the end of the incubation period, 10 μl of $A\beta_{1-42}$ solution was mixed with 10 μl of DW for fibrilization analysis. To study the effect 4-*O*-methylhonokiol on fibril formation, 10 μl of 4-*O*-methylhonokiol dissolved in 0.05% ethanol was added to fibrillized $A\beta_{1-42}$ solution to reach final concentrations of 4-*O*-methylhonokiol 10 μg/ml. Same $A\beta_{1-42}$ fibrillized solution was used for in vivo study.

2.4. $A\beta_{1-42}$ infused mouse model

Several studies have demonstrated that injection of Aβ into brain caused impairment of learning and memory and thus used as an AD or anti-AD-agent screening model (Nitta et al., 1994; Jhoo et al., 2004). Chacon et al. (2004) injected $A\beta_{1-42}$ into hippocampus to induce the memory impairment in rat and investigated the anti-AD agent using the $A\beta_{1-42}$ infused animal. Therefore, administration of $A\beta_{1-42}$ into brain could be applicable for screening of anti-AD agents. Therefore, we used this method as an AD mice model. The same $A\beta_{1-42}$ solution described above, was diluted (final concentration of 0.5 μg/mouse) in saline. The $A\beta_{1-42}$ injection was performed according to the procedure established by Laursen and Belknap (1986). Briefly, The sterile saline (0.9% NaCl) containing $A\beta_{1-42}$ was injected directly into the third ventricle to be 0.25 mm posterior to the bregma of mice with 50 μl Hamilton syringe fitted with a 26 gauge needle, which was adjusted to be inserted to a depth of 2.4 mm (anteroposterior, −0.25 mm; mediolateral, 0 mm; dosalventral, −2.4 mm relative to bregma). The selection of the third ventricle was based on its vicinity to brain regions most affected in AD (Chauhan et al., 2001). The injection sites were verified by injection of same volume of methylene blue in the sites and were at the same position where it was injected using stereotaxic instrument. The injection rate was approximately 0.2 μl/s.

2.5. Passive avoidance performance test

The passive avoidance test is widely accepted as a simple and rapid method for memory test. The passive avoidance response was determined using a "step-through" apparatus (Med Associates Inc, Vermont, USA) that consists of an illuminated and a dark compartment (each 20.3×15.9×21.3 cm) adjoining each other through a small gate with a grid floor, 3.175 mm stainless steel rod

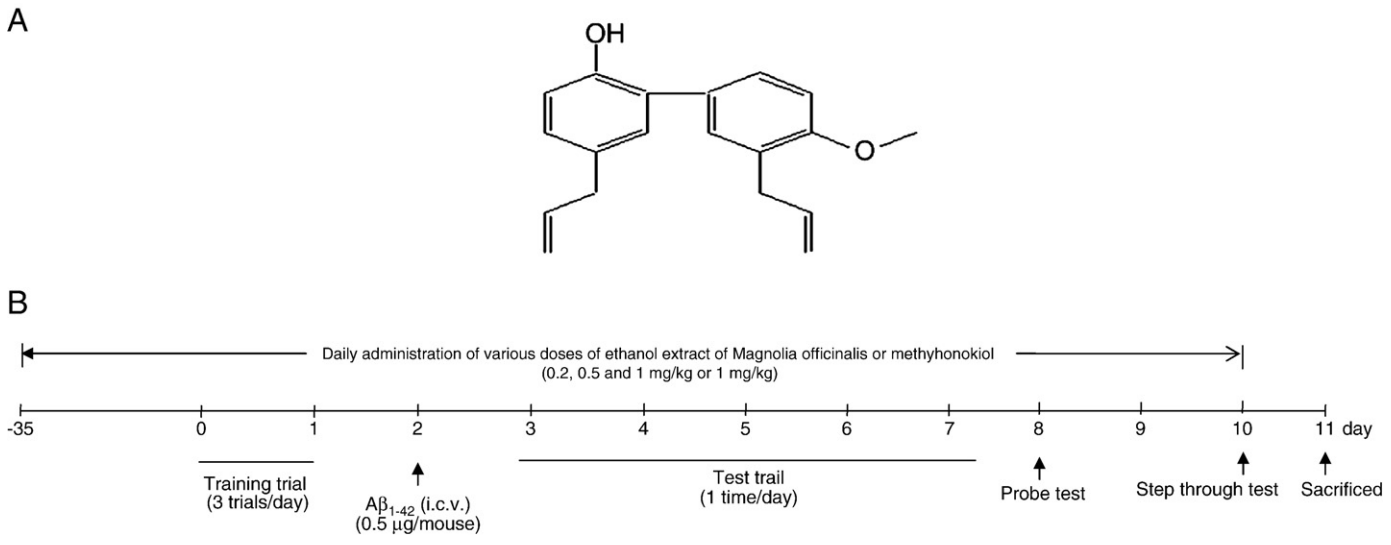

Fig. 1. Structure of 4-O-methylhonokiol (A) and experimental scheme (B).

set 8 mm apart. On a training trial, the ICR mice were placed in the illuminated compartment facing away from the dark compartment. When the mice moved completely into the dark compartment, they received an electric shock (1 mA, 3 s duration). Then, the mice were returned to their home cage. 24 h later, the mice were given $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.) or saline (i.c.v.). After 1-day treatment of $A\beta_{1-42}$, the mice were placed in the illuminated compartment and the latency period to enter the dark compartment defined as "retention" was measured. The time when the mice entered the dark compartment was recorded and described as step-through latency. The retention trials were set at a limit of 600 s of cut-off time.

2.6. Water maze test

The water maze test is also a widely accepted method for memory test, and we performed this test as the method described by Morris (1984). Maze testing was performed by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with milky water kept at 22~25 °C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 0.5~1 cm below the surface of the water in position. On training trials, the mice were placed in a pool of water and allowed to remain on the platform for 10 s and were then returned to the home cage during the second-trial interval. The mice that did not find the platform within 120 s were placed on the platform for 10 s at the end of trial. At 24 h after 6 trails (three times per day for 2 days), mice were given $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.) or saline (i.c.v.). 1 day after treatment of $A\beta_{1-42}$, each mouse was placed in the water pool in which the platform was taken out. They were allowed to swim until they sought escape platform. Escape latency, escape distance, swimming speed, and swimming pattern of each mouse was monitored for 5 days (one time/day) by a camera above the center of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain).

2.7. Probe trial test

A probe trial in order to assess memory consolidation was performed 24 h after the 5-day acquisition tests. In this trial, the platform was removed from the tank, and the rats were allowed to swim freely. For these tests, percentage time in the target quadrant and target site crossings within 60 s was recorded. The time spent in the target quadrant was measured to indicate the degree of memory consolidation that took place after learning. The time spent in the target quadrant was used as a measure of spatial memory. All time measurements were performed by an experimenter who used a stopwatch and was not told to which experimental group each animal belonged.

2.8. Brain collection and preservation

After the behavior test, animals were perfused with PBS under inhaled chloroform anesthetization. The brains were immediately collected in the same manner, and frozen stored at −20 °C, and separated into cortical and hippocampal regions. All the brain regions were immediately stored at −80 °C or used to measure apoptosis and western blotting.

2.9. Detection of apoptosis

One day after final step-through test, mice were sacrificed with diethyl ether and were perfused with 0.1 M PBS, and then the brains were collected from mice and immediately fixed in 4% paraformaldehyde for 24 hr. The brains were transferred successively to a 10%, 20% and 30% sucrose solution. Subsequently, brains were frozen in a cold stage and sectioned (40 μm-thick). DNA fragmentation was examined by terminal deoxynucleotydyl transferase-mediated FITC-dUDP nick-end labeling (TUNEL). TUNEL assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, MannheiM, Germany) according to manufacturer's instructions. Briefly, after fixation of 40 μm cryosections with 4% paraformaldehyde and treatment with 0.1% $NaBH_4$ and 0.1 Triton X, the slides were incubated at least for 1 h with a reaction mix containing deoxynucleotidyl transferase and FITC-dUDP (Roche, Reinach, Switzerland). For 4′-6′-diamidino-2-phenylindole digydrochloride (DAPI) staining slides were incubated for 15 min at room temperature in the dark with mounting medium for fluorescence with containing DAPI (Vector Laboratories, inc., Burlingame, CA). The tissues were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany). Staining the nuclei was visualized using DAPI. The number of DAPI-stained TUNEL-positive cells was counted in the given area, and the percentage of these cells was expressed as apoptotic cell death index.

2.10. Western blotting

Brain tissues were homogenized with protein extraction buffer, and lysed by 60 min incubation on ice. The lysate was centrifuged at 15,000 rpm for 15 min. Equal amount of proteins (40 μg) was separated on a SDS/10% or 20%-polyacrylamide gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Water & Process Technologies, Trevose, PA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-Buffered Saline Tween-20 [TBST: 10 mM Tris (pH 8.0) and 150 mM NaCl solution containing 0.05% tween-20]. After a short wash in TBST, the membrane was incubated for 12 h at room temperature with specific antibodies. Rabbit polyclonal antibodies against BACE (1:500, Sigma, St. Louis, MO, USA), C99 (1:500, ABR-affinity Bioreagents. Inc., Golden, CO, USA) and $A\beta_{1-42}$ (1:500 dilution, Covance, Berkeley, CA, USA) were used in study. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (1:4000 dilution, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) for 2 h. Immunoreactive proteins were detected with the BM Chemiluminescence blotting substrate (Roche Applied Science, Mannheim, Germany).

2.11. Thioflavin T fluorescence assay for Aβ fibril formation

The assay was done using a slight modification of a previously described method (LeVine, 1999). Briefly, $A\beta_{1-42}$ was dissolved and diluted with DW to 25 mM. Thioflavin T (Sigma, St. Louis, MO, USA) was prepared as a 5 mM stock solution in DMSO and stored at −20 °C. The solution was diluted in 50 mM glycine-NaOH buffer (pH 8.5) and used at a final concentration of 5 μM. To measure 4-*O*-methylhonokiol effect on $A\beta_{1-42}$ fibril formation, solution of fibrillized $A\beta_{1-42}$ was mixed with/without 4-O-methylhonokiol (0.05, 0.5, 5, 10 μg/ml in PBS). The mixtures were incubated for 30 min at room temperature. The resulting mixtures were transferred into the thioflavin T solution. Fluorescence measurements were recorded in a TECAN spectrofluorometer (TECAN, Grodig, Austria) at 25 °C using a 1 cm pathlength quartz cell. The excitation wavelength was set to 450 nm (slit width = 4 nm), and emission was monitored from 485 nm (slit width = 8 nm). The relative fluorescence at 482 nm was used as a measure of the amount of fibrillar aggregates formed in solution. The fluorescence intensities were calibrated to provide an estimate using the following equation: Aβ aggregation (% of control) = $(F_2 - F_0)/F_1 - F_0 \times 100$ (F_0, Dye alone fluorescence; F_1, Aβ fluorescence; F_2, Aβ + 4-O-methylhonokiol fluorescence).

2.12. β-secretase assay

The inhibitory activity 4-O-methylhonokiol on β-secretase in vitro was determined using commercially available β-secretase

fluorescence resonance energy transfer (BACE 1 FRET) assay kit (PANVERA, Madison, USA) according to the manufacture's protocols, respectively. To determine β-secretase, 10 μl of 4-*O*-methylhonokiol (1–10 μM) was mixed with 10 μl BACE1 substrate (Rh-EVNLDAEFK-Quencher), and then the reaction mixture was incubated for 1 h at room temperature in the 96 well flat bottom microtitre plate. The reaction was stopped by addition of 10 μl BACE1 stop buffer (2.5 M sodium acetate). The formation of fluorescence was read with Fluostar galaxy fluorometer (excitation at 545 nm and emission at 590 nm) with Felix software (BMG Labtechnologies, Offenburg, Germany). The enzyme activity was linearly related to the increase in fluorescence. The enzyme activity was expressed as nM produced substrate, which was determined by the formation of fluorescence per mg protein per min. All controls, blanks, and samples were run in triplicate.

2.13. Cell culture

PC12 cells were cultured routinely in DMEM and F-12 nutrient mixture medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air. All cells were cultured in poly-D-lysine-coated culture dishes. The medium was changed every 48 h, and cells were plated at an appropriate density (0.5×10^6 cells/cm^2). Cells were incubated with $A\beta_{1-42}$ in the absence or presence of 4-*O*-mertylhonokiol dissolved in 0.05% ethanol for 48 h for viability, expression of apoptotic or apoptotic signal proteins, and apoptotic assay, 4 h for reactive oxygen species. Cultures of dissociated cortical cells were prepared from day 18 embryos of ICR mice pups using methods similar to those previously described (Lee et al., 2006). Briefly, cerebral cortices were removed and incubated for 15 min in Ca^{2+}- and Mg^{2+}-free Hanks' balanced saline solution (Life Technologies, Inc.) containing 0.2% trypsin. Cells were dissociated by trituration and plated into polyethyleneimine-coated plastic or glass-bottom culture dishes containing minimum essential medium with Earle's salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 20 mM KCl, 10 mM sodium bicarbonate, and 1 mM Hepes (pH 7.2). Following cell attachment (3–6 h after plating), the culture medium was replaced with neurobasal medium containing B27 supplements (Life Technologies, Inc.). Experiments were performed with 6 to 8 day-old cultures; greater than 90% of the cells in these cultures were neurons, and the remainder were astrocytes, as judged by cell morphology and by immunostaining with antibodies against neurofilaments and glial fibrillary acidic protein. For calcium measurements, cortical neuronal cells were isolated from 1 day-old mice brain, and intracellular calcium levels were immediately determined.

2.14. Cell viability

The cells were plated in 96-well plates, and cell viability was determined by the conventional 4-(3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-terazolio)-1,3-benzene disulfonate (WST-1) reduction assay (Dojin Laboratory, Kumanoto, Japan) as described elsewhere (Park et al., 2005). The cells were exposed to $A\beta_{1-42}$ at various concentrations (1–50 μM) and fixed $A\beta_{1-42}$ at 5 μM and with/without various concentrations of 4-*O*-methylhonokiol (1–10 μM) for 24 h. The cells were treated with the WST-1 solution (final concentration, 1 mg/ml) for 2 h. The absorbance was measured with a microplate reader (Tecan, Sunrise, Salzburg, Austria) at 450 nm. Results were expressed as the percentage of WST-1 reduction.

2.15. Reactive oxygen species (ROS) generation

To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment with $A\beta_{1-42}$ (5 μM) for 24 h in the presence or absence of 4-*O*-methylhonokiol (1–10 μM), the cells were washed in modified Kreb's buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM $NaHCO_3$, 5.5 mM glucose, 10 mM Hepes, pH 7.4. The cell suspension was transferred into plastic tubes. Measurement was started by an injection of 5 μM DCF-DA in the dark. After 30 min of incubation at 37 C, and then ROS generation was determined by Fluorometer (Fmax, Molecular devices corp., San Diego, CA, USA) at Ex = 485 and Em = 538 nm.

2.16. Statistical analysis

Homogeneity of variances was assessed using a Bartlett test. If variances were homogeneous, differences between groups and treatments were assessed by one-way analysis of variance (ANOVA) followed by Dunnette's test using GraphPad Prism 4 software (Version 4.03, GraphPad software, Inc.). Data are presented as mean standard error of the mean (SEM). # Significantly different from control group ($p<0.05$). *Significantly different from Aβ-treated group ($p<0.05$).

3. Results

3.1. Effect of ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol on $A\beta_{1-42}$-induced memory impairment by Morris water maze test

We determined the effect of ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol on improvement of spatial memory function using Morris water maze. The mice were treated continuously with administration of ethanol extract of *M. officinalis* (2.5, 5 and10 mg/kg) and 4-*O*-methylhonokiol (1 mg/kg/) for 5 weeks and during the experiments into drinking water, and then $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.) was administrated into mice through i.c.v. injection 35 days after having started the administration of ethanol extract of *M. officinalis* or 4-*O*-methylhonokiol. The animals were then trained for three trials per day for 2 days, and were tested for 5 consecutive days to locate and escape onto the platform, and their spatial learning scores (latency in seconds and in length) were recorded. The control mice exhibited shorter and shorter escape latency by the training; however, the escape latency of $A\beta_{1-42}$-treated mice was not significantly reduced compared to control mice. In contrast, *M. officinalis* and 4-*O*-methylhonokiol-treated groups significantly inhibited the effects of $A\beta_{1-42}$ on escape latencies (cm and s) (Fig. 2). However, $A\beta_{1-42}$ treatment and the treatment of ethanol extract of *M. officinalis* and 4-O-methylhonokiol did not affect the swimming speed in all trials (data not shown). Mice treated only with ethanol extract of *M. officinalis* (10 mg/kg) showed no change in the pattern of memory function during the experiment period. Moreover, to further study the effect of ethanol extract of *M. officinalis* and 4-O-methylhonokiol on memory function, we also assessed memory consolidation performed 24 h after the acquisition tests. High-dose ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol-administered group performed better in probe trials suggesting an increase in memory consolidation. The treated mice remained significantly longer in the target quadrant compared with the $A\beta_{1-42}$-treated mice (Fig. 3A).

3.2. Effect of ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol on the $A\beta_{1-42}$-induced memory impairment by passive avoidance performance test

To further investigate whether ethanol extracts of *M. officinalis* and 4-O-methylhonokiol prevent Aβ-induced memory impairment, passive avoidance test was then performed. In passive avoidance test using step-through apparatus, neither $A\beta_{1-42}$ nor ethanol extract of *M. officinalis* and 4-O-methylhonokiol changed step through latency in training trial. $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.) significantly decreased the step-through latency of testing trial in comparison to that of control mice. However, ethanol extract of *M. officinalis* (2.5, 5 and 10 mg/kg) and 4-*O*-methylhonokiol (1 mg/kg/) administrated for 5 weeks into drinking water significantly suppressed $A\beta_{1-42}$

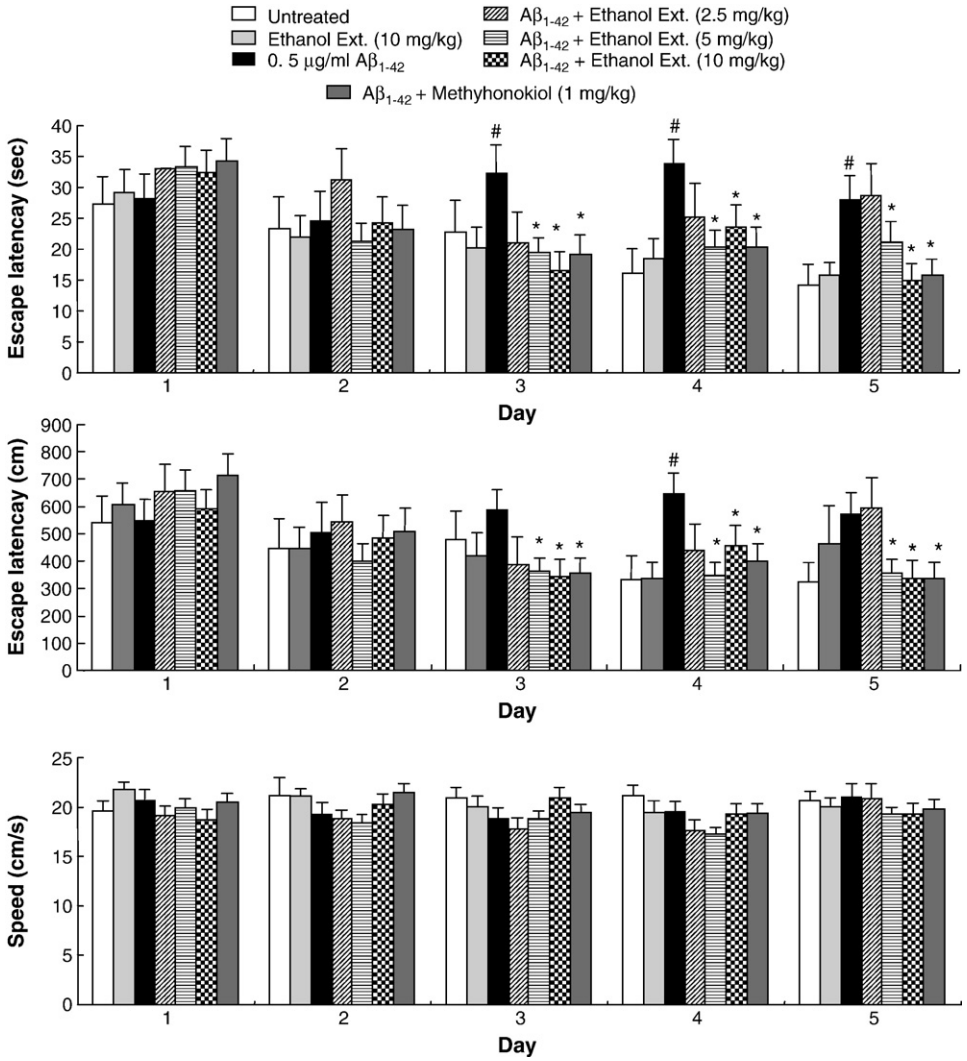

Fig. 2. Inhibitory effect of ethanol extract of *M. officinalis* and 4-O-methylhonokiol on memory impairment induced by $A\beta_{1-42}$ in the water maze test. The mice were treated continuously with administration of ethanol extract of *M. officinalis* (2.5, 5 and 10 mg/kg) and 4-O-methylhonokiol (1 mg/kg) into drinking water for 5 weeks. For the water maze test, the animals were trained for 2 days (3 times/day), treated with $A\beta_{1-42}$ (0.5 μg/mouse, i.c.v.) at one day after training trail. Then, the memory function was determined by the escape latencies (cm and s) at 1 day after administration of $A\beta_{1-42}$ for 5 days as described in the Materials and Methods. Each value is means ± S.D. from 10 mice. *Significantly different from $A\beta_{1-42}$ treated control ($p<0.05$). #Significantly different from control ($p<0.05$). *Significantly different $A\beta_{1-42}$ treated control ($p<0.05$).

(0.5 μg/mouse, i.c.v.)-induced decrease of step-through latency (Fig. 3B). Mice treated with ethanol extract of *M. officinalis* (10 mg/kg) alone showed no decrease in the step-through latency.

3.3. Effect of 4-O-methylhonokiol on Aβ-induced neuronal cell death

To investigate the effect of ethanol extract of *M. officinalis* and 4-O-methylhonokiol on neuronal cell death, we performed DAPI and TUNEL staining. Aβ-induced mice brain has a significantly increased TUNEL reactive cell number than control brain, but those were significantly decreased in 10 mg/kg ethanol extract of *M. officinalis* or 4-O-methylhonokiol administrated group (Fig. 4).

3.4. Effect of 4-O-methylhonokiol on Aβ fibrillization in vitro

To investigate whether the inhibitory effect of 4-O-methylhonokiol on Aβ fibrillization could be involved in the memory improving mechanism of ethanol extract of *M. officinali*, we determined *in vitro* fibrillization of Aβ in the presence and absence of 4-O-methylhonokiol by the thioflavin T method. 4-O-methylhonokiol prevented Aβ fibrillogenesis in a dose dependent manner (Fig. 5A). All controls (buffer, 4-O-methylhonokiol alone, 46 μM albumin as an irrelevant protein and thioflavin T) showed low constant fluorescence for the duration of each experiment (data not shown). In an attempt to determine IC_{50}, the concentration required for a 50% inhibition of Aβ fibrilization, assay was performed with various concentrations of 4-O-methylhonokiol. Assuming that the fluorescence intensity is proportional to the extent of Aβ fibrilization, the results indicate that 8.2 μM of 4-O-methylhonokiol inhibits the fibrilization about 52 ± 2%.

3.5. Effect of 4-O-methylhonokiol on the β-secretase activity in in vitro and on the expression in the brain

To further elucidate the memory improving mechanism of ethanol extract of *M. officinalis* and 4-O-methylhonokiol through reduced Aβ formation, we determined β-secretase (BACE) activity in *in vitro*. The activity of the β-secretase was significantly and dose dependently inhibited by 4-O-methylhonokiol with IC_{50} 10.3 μM (Fig. 5B). We then determined the expression of β-secretase (BACE) by western blotting because the inhibition of BACE has been widely known for reducing the Aβ production. The expression of the BACE level in the cerebral cortex and hippocampus of $A\beta_{1-42}$-treated mice was increased. The ethanol extract of *M. officinalis* and 4-O-methylhonokiol treatment, however, consistent with the lowering effect on $A\beta_{1-42}$ level,

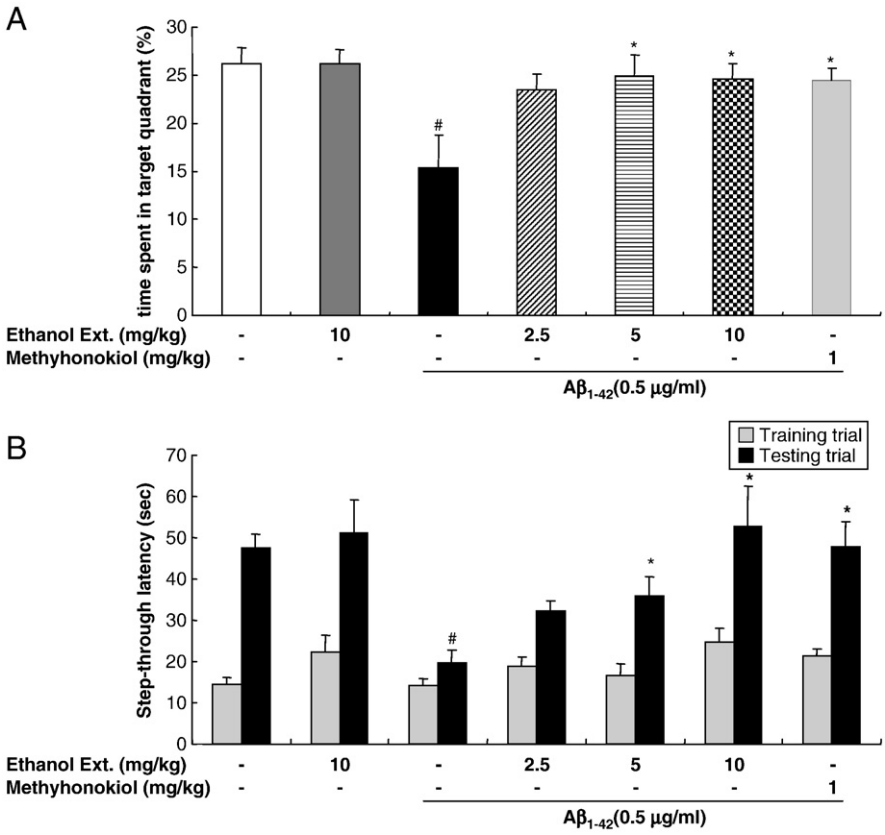

Fig. 3. Inhibitory effect of ethanol extract of *M. officinalis* and 4-O-methylhonokiol on memory impairment induced by $A\beta_{1-42}$ in the probe test (A) and the step-through type passive avoidance performance test (B). (A) For probe test, one day after final water maze test, mean percentage of the time spent in the target quadrant in which the platform had previously been located during acquisition was measured. The latency (spent time) was compared to control and treated groups. Each value is means ± S.D. from 10 mice. (B) For the passive avoidance performance test, mice were trained one time at 2 day after probe test, and then the mice were returned to their home cage. One day after training, passive avoidance performance was tested. The latency period was measured as described in the Materials and Methods. Each value is means ± S.D. from 10 mice. #Significantly different from control ($p<0.05$). *Significantly different from $A\beta_{1-42}$ treated control ($p<0.05$).

inhibited expression of BACE in a dose dependent pattern in both cortex and hippocampus (Fig. 6).

3.6. Effect of 4-O-methylhonokiol on Aβ-induced cell viability in in vitro

To study the neuroprotective effect of 4-O-methylhonokiol in neuronal cells, we treated 4-O-methylhonokiol in human PC12 cells and cultured cortical neurons. In the cells treated with $A\beta_{1-42}$, the cell viability was about 20–60%. However, we found dose dependent recovery of cell viability in both cultured PC12 cells and cultured cortical neurons treated with 4-O-methylhonokiol in dose dependent manner (Fig. 7A). To determine whether recovery effect of 4-O-methylhinokiol from $A\beta_{1-42}$-induced neurotoxicity is involved with anti-oxidative damage, we also treated the neuroprotective effect of 4-O-methylhinokiol on H_2O_2 treated cells. Similar dose dependent recovery effect of 4-O-methylhonokiol was found in the H_2O_2 (150 μM)-damaged PC12 cells and cultured cortical neurons (Fig. 7A).

3.7. Effect of 4-O-methylhonokiol on $A\beta_{1-42}$-induced reactive oxygen species (ROS) generation

Since cell death of neuronal cells may be related with over release of ROS, which can act as an implicated contributor in the neuronal cell death, we tried to determine whether 4-O-methylhonokiol inhibits ROS generation in $A\beta_{1-42}$-treatment in PC12 cells and cultured cortical neurons. The level of ROS was measured in the culture medium. After cotreatment with $A\beta_{1-42}$ (5 μM) and 4-O-methylhonokiol (1–10 μM) for 24 h, $A\beta_{1-42}$-induced elevation of ROS generation was decreased in a concentration-dependent manner in both PC12 cells and cultured cortical neurons (Fig. 7B).

4. Discussion

Our study showed that ethanol extract of *M. officinalis* and its major component 4-*O*-methylhonokiol suppressed the $A\beta_{1-42}$-induced memory impairment. Moreover, 4-O-methylhonokiol reduced $A\beta_{1-42}$-induced apoptotic cell death in *in vivo*. 4-O-methylhonokiol also showed direct inhibition of Aβ aggregation/fibrillization as well as β-secretase activity induced by $A\beta_{1-42}$ in vitro. Further in vitro study demonstrated that 4-O-methylhonokiol inhibited $A\beta_{1-42}$-induced ROS generation, and $A\beta_{1-42}$ and H_2O_2 induced neurocytotoxicity in cultured PC12 cells and cortical neurons. Therefore, these results suggest that ethanol extract of *M. officinalis* may be useful for ameliorating the memory impairment in AD, and that 4-O-methylhonokiol is a major active component.

Aβ peptide in the early stages of AD is represented by the fibrillary peptide form (Drouet et al., 2000). It is well known that the aggregated Aβ is toxic in vitro to cultured neurons, finally resulting in neuronal dysfunction and death (Yankner, 1996). Interplay between oxidative stress and Aβ peptides has been explicitly shown as a matter of Aβ-induced neurotoxicity in vitro and in vivo (Behl et al., 1994; Yatin et al., 1998; Harkany et al., 2000) Fibrillization of Aβ is more toxic to the neurons than Aβ itself, and this effect could be related with the generation of ROS (Drake et al., 2003; Boyd-Kimball et al., 2004). Generation of ROS in the development of AD is also well documented in many studies (Pappolla et al., 1998; Jhoo et al., 2004; Lee et al., 2006). Thus, the inhibitory ability of 4-O-methylhonokiol on Aβ fibrillization might be related with the

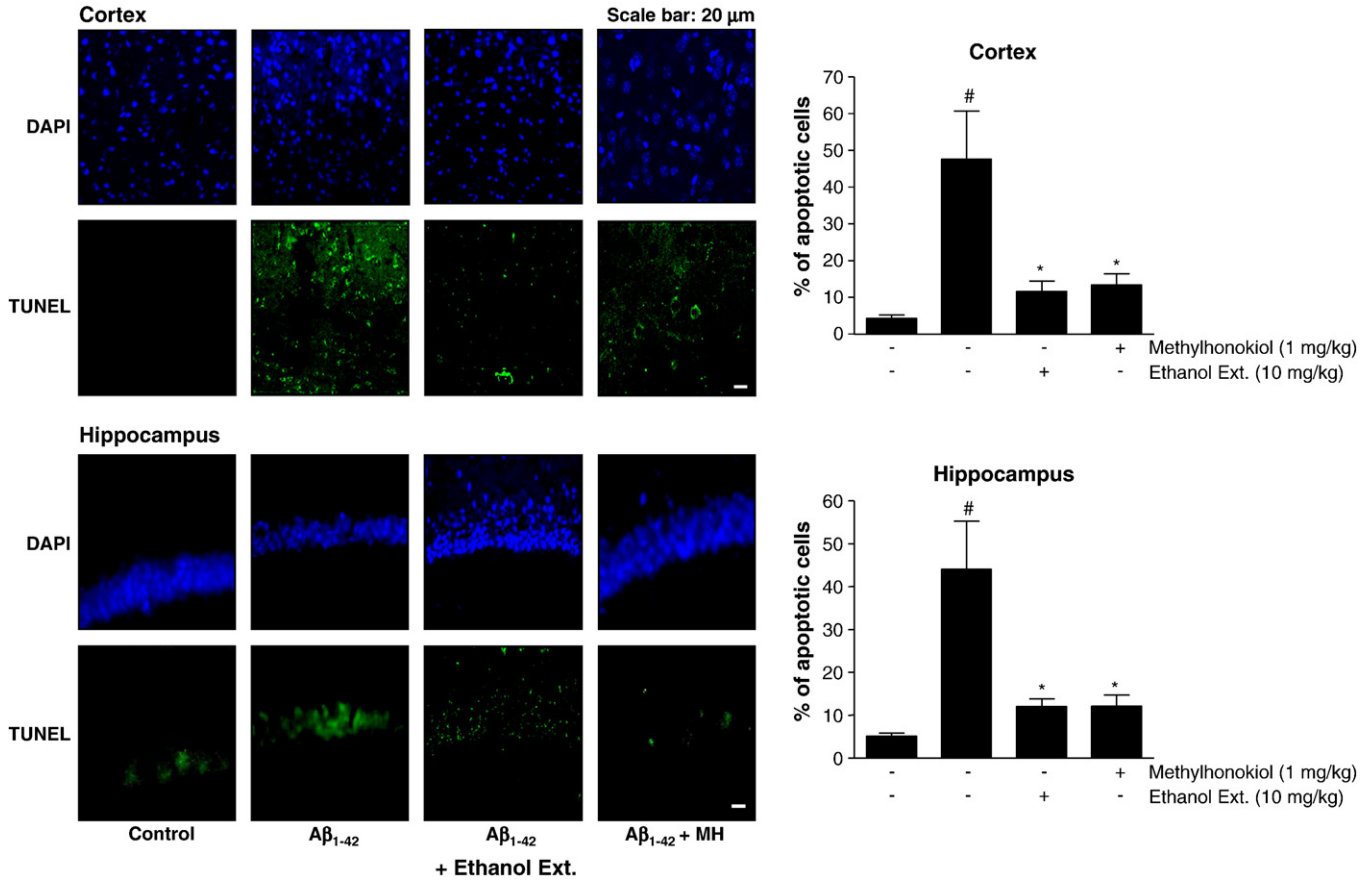

Fig. 4. Inhibitory Effect of 4-O-methylhonokiol on the $A\beta_{1-42}$ induced apoptosis. Inhibitory effect of 4-O-methylhonokiol on $A\beta_{1-42}$ induced apoptosis was determined by DAPI staining and TUNEL assay as described in Materials and methods. Apoptosis (%) was defined as number of TUNEL-positive stained cells divided by DAPI-stained corresponding total cell number (fluorescent microscopy, magnification 200×). Values are mean ± S.D. of four independent animal brain sections. $^{\#}p<0.05$ indicate significantly different from control group. $^{*}p<0.05$ indicate significantly different from $A\beta_{1-42}$ treated group.

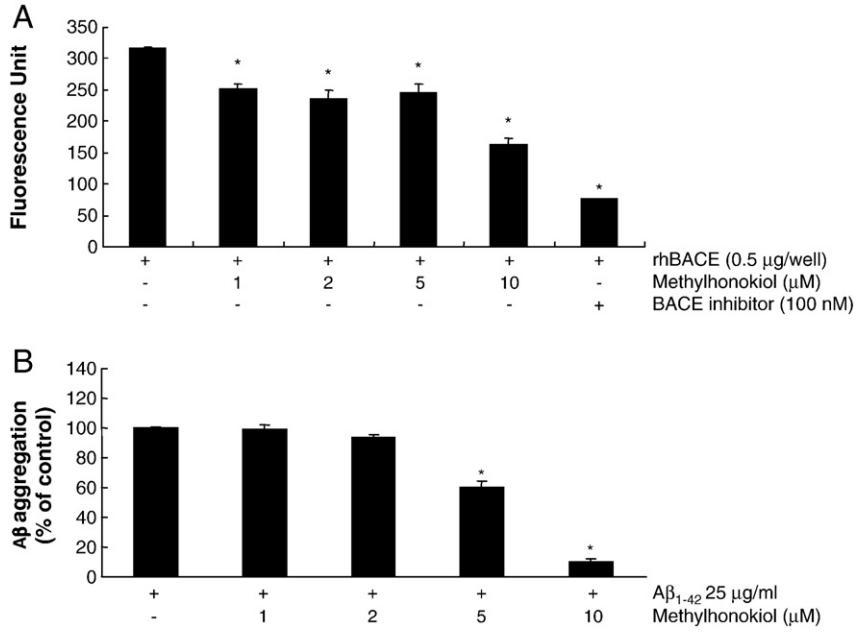

Fig. 5. Inhibitory effect of 4-O-methylhonokiol on the β-secretase activity (A) and Aβ fibrillogenesis (B) *in vitro*. (A) Inhibitory effect of 4-O-methylhonokiol on β-secretase activity. Effect of various concentrations of 4-O-methylhonokiol on β-secretase was determined in vitro, and the β-secretase activity was presented by the unit enzyme activity as described in Materials and methods. Value is mean ± S.D. of three individual experiments. *Significantly different from control (rhBACE) group ($p<0.05$). (B) To measure the 4-O-methylhonokiol effects on the Aβ fibrillogenesis, $A\beta_{1-42}$ was mixed with/without 4-O-methylhonokiol (1–100 μM) in the presence of fluorescence dye, thioflavin T. Fluorescence was measured within 5 s in a TECAN spectrofluorometer with the excitation and emission wavelengths of 450 and 485 nm, respectively. The effect of 4-O-methylhonokiol was tested in three independent experiments. *Significantly different from control group in which $A\beta_{1-42}$ is aggregated without 4-O-methylhonokiol ($p<0.05$).

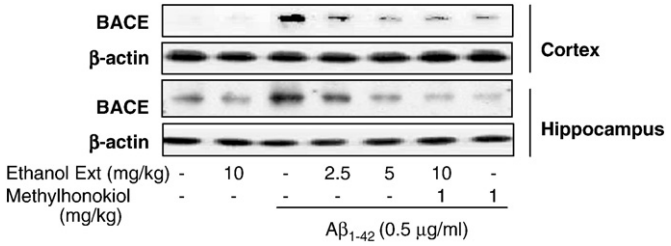

Fig. 6. Inhibitory effect of ethanol extract of *M. officinalis* and 4-O-methylhonokiol on the $A\beta_{1-42}$-induced BACE expression induced by $A\beta_{1-42}$ in the cortex and hippocampus. One day after step-through test, the mice were scarified, and then expression of BACE was detected in each brain region as described in the Materials and methods. The expression of BACE was detected by Western blotting using specific antibodies. β-actin protein was used here as an internal control. The BACE blot is the representative of three samples.

inhibitory effect on $A\beta_{1-42}$-induced ROS generation as well as neurotoxicity which could be implicated in the prevention of apoptotic cell death in vivo. Many studies have demonstrated that compounds having anti-oxidative properties such as spiro[imidazo-(1,2-azopyridie-3,2-indan)-2(3H)-one (Yamaguchi et al., 2006), melatonin (Shen et al., 2002), curcumin (Lim et al., 2001; Begum et al., 2008), and EGCG (Rasoolijazi et al., 2007) as well as natural product such as red mold rice (Lee et al., 2007), *Bacopa monniera* extract (Dhanasekaran et al., 2007; Holcomb et al., 2006), *Polygonum multiflorum* Thunb water extract (Um et al., 2006), *Glycyrrhiza uralensis* Fisch water extract (Ahn et al., 2006), and ethanolic extract of *Angelica gigas* Nakai (Umbelliferae) (Yan et al., 2004) have neuroprotective effects against oxidative stress by Aβ and enhance memory function. Together, the present data showed that neuroprotective effects through blocking the generation ROS and inhibiting Aβ formation and/or fibrillization could serve as important mechanisms for the memory enhancement capabilities of ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol.

Several cellular changes by oxidative stresses have been related with Aβ plaques formation and pathophysiological events of AD (Anekonda and Reddy, 2005). It has previously shown that fibrillar form of Aβ-induced oxidative stress can upregulate BACE-1 expression and activity in differentiated neuronal NT_2 cells (, and such an effect is also mimicked by other pro-oxidants (Elena et al., 2006). Thus, it is possible that anti-oxidative effect of 4-*O*-methylhonokiol could be related with the reducing effect on β-secretase activity in vitro. Ethanol extract of *M. officinalis* and 4-*O*-methylhonokiol also inhibited $A\beta_{1-42}$-induced BACE (beta-secretase, a beta-site APP cleaving enzyme) expression. The relationship between inhibition of β- and γ-secretase and reduction of Aβ level has been well demonstrated. The β- and γ-secretase inhibitors reduce brain concentrations of Aβ in human APP_{V717F} (PDAPP mice)

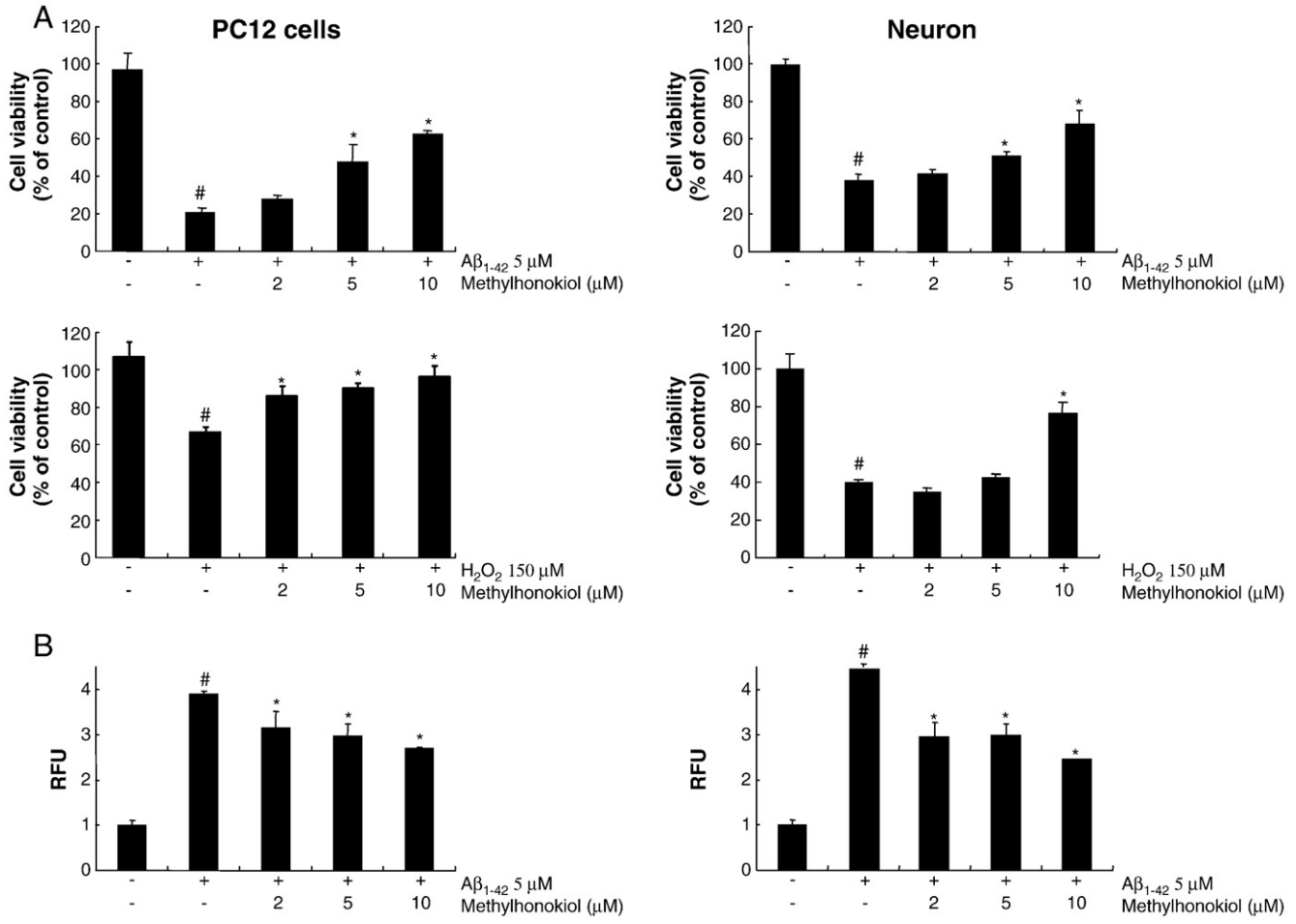

Fig. 7. Inhibitory effect of 4-O-methylhonokiol on $A\beta_{1-42}$ and H_2O_2-induced cytotoxicity (A) and reactive oxygen species (ROS) generation (B) in PC12 cells and cultured cortical neurons. (A) Cell viability was determined by MTT assay. Cells were treated with the indicated concentrations of H_2O_2 and $A\beta_{1-42}$ or co-treated with 4-O-methylhonokiol for 24 h. Values are the means ± SD of three separate experiments performed in triplicate. $^{\#}p<0.05$ indicate significantly different from control group. *Significantly different from treated group ($p<0.05$). (B) Inhibitory effect of 4-O-methylhonokiol on $A\beta_{1-42}$-induced ROS. Intracellular ROS levels were determined by measuring DCF fluorescence, as described in Materials and methods. Values are the means ± SD of three separate experiments performed in triplicate. $^{\#}$Significantly different from control group ($p<0.05$). *Significantly different from $A\beta_{1-42}$ treated group ($p<0.05$).

transgenic mice and "Swedish" mutation ($APP_{K670N/M671L}$) Tg2576 mice (Irizarry et al., 2002; Chang et al., 2004). In several transgenic mice such as APP mutation mice, and PS1 and 2 mutation mice, elevated Aβ level by increased β- and γ-secretase has been found (Borchelt et al., 1997; Hwang et al., 2002). It was also found that overexpression of β-secretase enhanced Aβ level (Lee et al., 2005a,b). 4-O-methylhonokiol, similar but to less extent (50% lower activity) to Z-VLL, a cell-permeable BACE-1 inhibitor, concentration-dependently inhibited BACE-1 activity. Because BACE-1 is the major enzyme in the amyloidogenic processing to release Aβ, the inhibitory effect of 4-O-methylhonokiol on BASE may be implicated as a mechanism lowering Aβ level as well as Aβ-induced neuronal cell death. In the alternative non-amyloidogenic pathway, APP is cleaved within the Aβ domain by α-secretase, releasing sAPPα into the extracellular medium and precluding the generation of Aβ (Fu et al., 2007). However, it may not likely be the major mechanism of the lowering of $Aβ_{1–42}$ level of the ethanol extract of *M. officinalis* and 4-O-methylhonokiol since we did not observe any elevating effects of the ethanol extract of *M. officinalis* and 4-O-methylhonokiol on α-secretase in the brain as well as in vitro assay system (data not shown). The close relationship between the cholinergic system and APP processing has been widely known. AChE has been found to induce the activation of the non-amyloidogenic pathway and thus to induce the Aβ formation (Kimura et al., 2005; Liskowsky and Schliebs, 2006). In fact, we previously found that ethanol extract of *M. officinalis* and 4-O-methylhonokiol inhibits AChE activity. Thus, indirect mechanism of the ethanol extract of *M. officinalis* and 4-O-methylhonokiol through cholinergic system may be, in part, involved in the inhibition of Aβ formation causing neuronal cell death.

Possible signal pathway in the ethanol extract of *M. officinalis* and 4-O-methylhonokiol inhibited secretase activity should be needed for further study. However, it is noteworthy that a secretory process of APP is dependent with the activation of the mitogen-activated protein (MAP) kinase pathway (Yogev-Falach et al., 2002). We also observed that Aβ-elicited β-secretase produces Aβ by activation of ERK pathway (unpublished data). In this regard, it is worthy to note that Honokiol, another major component of ethanol extract of *M. officinalis* induced neurite outgrowth promotion that depended on activation of extracellular signal-regulated kinases (ERK1/2) (Zhai et al., 2005). We previously found that obovatol inhibited c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) signal in LPS-induced NF-κB activation and inflammation of RAW macrophages (Choi et al., 2007). We recently also found that 4-O-methylhonokiol activated ERK pathway in neuronal cell differentiation as well as anti-inflammatory effect on LPS-induced Raw 264.7 cells via inhibition of ERK activation (unpublished data). Inhibition of these pathways has been linked to the reduction of ROS generation in Aβ treated neuronal cell death (Lee et al., 2006; Wasilewska-Sampaio et al., 2005; Marques et al., 2003; Ekinci et al., 1999). In fact, we also observed that 4-O-methylhonokiol prevented $Aβ_{1–42}$-induced activation of ERK and p38 pathway in neuronal cortex and hippocampus in the present study (data not shown). Therefore, it is possible that inhibition of MAP kinase pathway can block β-secretase activity via inhibition of ROS generation and thereby reduce the formation of Aβ. The reduction of Aβ burden could be useful for the treatment of AD.

In summary, our data shows that the ethanol extract of *M. officinalis* and 4-O-methylhonokiol has suppressed effect against $Aβ_{1–42}$-induced memory impairment function through inhibition of $Aβ_{1–42}$-induced ROS generation and neuronal cell death. This study therefore suggests that the ethanol extract of *M. officinalis* may be a useful agent for prevention of development or progress of AD, and 4-O-methylhonokiol may be one of the main active component of the ethanol extract of *M. officinalis*.

Acknowledgments

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