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Amelioration of Cognitive Dysfunction in APP/PS1 Double Transgenic Mice by Long-Term Treatment of 4-*O*-Methylhonokiol

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Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disease without known ways to cure. A key neuropathologic manifestation of the disease is extracellular deposition of beta-amyloid peptide (Aβ). Specific mechanisms underlying the development of the disease have not yet been fully understood. In this study, we investigated effects of 4-*O*-methylhonokiol on memory dysfunction in APP/PS1 double transgenic mice. 4-*O*-methylhonokiol (1 mg/kg for 3 month) significantly reduced deficit in learning and memory of the transgenic mice, as determined by the Morris water maze test and step-through passive avoidance test. Our biochemical analysis suggested that 4-*O*-methylhonokiol ameliorated Aβ accumulation in the cortex and hippocampus *via* reduction in beta-site APP-cleaving enzyme 1 expression. In addition, 4-*O*-methylhonokiol attenuated lipid peroxidation and elevated glutathione peroxidase activity in the double transgenic mice brains. Thus, suppressive effects of 4-*O*-methylhonokiol on Aβ generation and oxidative stress in the brains of transgenic mice may be responsible for the enhancement in cognitive function. These results suggest that the natural compound has potential to intervene memory deficit and progressive neurodegeneration in AD patients.

Key Words: Alzheimer's disease, 4-*O*-methylhonokiol, APP/PS1 double transgenic, Antioxidant, Beta-site APP-cleaving enzyme

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease and a significant cause of mortality and morbidity among elderly people. A prime neuropathologic manifestation of the neurodegenerative disease is extracellular deposition of amyloid-beta (Aβ) (Hardy and Allsop, 1991). Aβ is produced from amyloid precursor protein (APP) by β- and γ-secretases through their sequential proteolytic actions (Hardy and Allsop, 1991). Beta-site APP-cleaving enzyme (BACE) 1 cleaves APP to form Aβ N-terminus, APPβ and a C-terminal fragment, C99 and γ-secretase subsequently generates Aβs with two variants, $A\beta_{1-40}$ and $A\beta_{1-42}$. Presenilins (PSs) have been identified to be associated with γ-secretase *in vivo* and *in vitro* (Kimberly *et al*., 2000). PS1 knockout mice showed markedly reduced γ-secretase cleavage of APP (De Strooper *et al*., 1998). In addition, knockout of both PS1 and PS2 completely abolished γ-secretase activity (Herreman *et al*., 2000).

Several mutations in the APP and presenilin 1 and 2 (PS1, PS2) genes were found to be linked to familial form of AD (Price and Sisodia, 1998). These mutations are associated with alterations of APP processing with enhancement of $A\beta_{1-42}$ production (Octave *et al*., 2000). Trangsgenic mice that overexpress mutant APP showed Aβ amyloid deposition in the brain (Hsiao *et al*., 1996), and occurrence of Aβ amyloid plaques became earlier when they were crossed with mutant PS1 transgenics (Borchelt *et al*., 1997, McGowan *et al*., 1999).

Compounds from Magnolia species have exhibited various pharmacological activity such as antimicrobial (Ho *et al*., 2001), anxiolytic (Seo *et al*., 2007), neurotrophic (Lee *et al*., 2009) and cholinergic (Matsui *et al*., 2009) effects. To define these effects, researchers isolated many bioactive components such as honokiol, obovatol, magnolol and 4-*O*-methylhonokiol. In previous studies, we showed that 4-*O*-methylhonokiol amelio-

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rated memory impairment in several different animal models including a systemic LPS-induced dementia model (Lee *et al*., 2012a), presenilin 2 mutant transgenic mice (Lee *et al*., 2011) and Tg2576 mice (Lee *et al*., 2012b). Another compound from Magnolia species, obovatol potently blocked Aβ aggregation (Choi *et al*., 2012a) and enhanced learning and memory in Tg2576 mice by blocking neuroinflammatory responses and Aβ formation (Choi *et al*., 2012a).

In the present study, we investigated whether administration of 4-*O*-methylhonokiol could attenuate cognitive dysfunction in mice expressing the mutant APP (K670N, M671L) and the mutant PS1 (M146L). Here, we shows 4-*O*-methylhonokiol significantly ameliorates memory deficit and pathologic Aβ deposition in the animals.

MATERIALS AND METHODS

Preparation of 4-*O*-methylhonokiol

4-*O*-methylhonokiol was isolated from the bark of *Magnolia officinalis* according to previous description (Lee *et al*., 2009). Briefly, the air-dried bark of *Magnolia officinalis* (3 kg) was cut into small pieces and extracted with 95% (v/v) ethanol for 3 days at room temperature. After filtration through the 400-mesh filter cloth, the filtrate was re-filtered through filter paper (Whatman, No. 5) and concentrated under reduced pressure. The extract (450 g) was then suspended in distilled water, and the aqueous suspension was extracted with n-hexane, ethyl acetate, and n-butanol, respectively. The n-hexane layer was evaporated to dry, and the residue (70 g) was chromatographed on silica gel with n-hexane:ethyl acetate (9:1) solution to extract a crude fraction that included 4-*O*-methylhonokiol. This fraction was repeatedly purified by silica gel chromatography using n-hexane:ethyl acetate as the eluent to obtain pure 4-*O*-methylhonokiol. The purity was identified to be more than 99.5%.

Animals and treatment

Male double transgenic APP/PS1 mice expressing human amyloid precursor protein (HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed with female C57Bl/6 mice to generate double transgenic and non-transgenic littermates. The animals were maintained in accordance with the Korea Food and Drug Administration guideline for the humane care and use of laboratory animals. All of the experimental procedures in this study were approved by IACUC of Chungbuk National University (approval number: CBNUA-144-1001-01). Animals were housed in a room that was automatically maintained at 21-25°C and relative humidity (45-65%) with a controlled light-dark cycle. The mice were under free access to food and water. 5 month old male APP/PS1 mice were treated with either 4-*O*-methylhonokiol (1 mg/kg, n=10) or vehicle (0.5% ethanol, n=10) for 3 months.

The Morris water maze test

The Morris water maze test was performed as described in the previous studies with minimal modification (Choi *et al*., 2012a; Choi *et al*., 2012b). Briefly, mice were placed in the pool and allowed to swim freely. Swimming traces of animals were recorded until they reached to the hidden platform and the time length was defined as latency. Each trial lasted for 60 seconds or ended as soon as the mouse reached the submerged platform and was allowed to remain on the platform for 10 seconds. Escape latency, escape distance, swimming speed, and swimming pattern of each mouse were monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain). A quiet environment, consistent lighting, constant water temperature, and fixed spatial frame were maintained throughout the period of the experiment. Test trial was performed for 8 days after the last training trial. A probe trial to assess memory consolidation was performed 24 hrs after the 8-day acquisition tests. In this trial, the platform was removed from the tank, and the mice were allowed to swim freely. For these tests, percentage of time in the target quadrant and target site crossings within 60 seconds was recorded. The time spent in the target quadrant was taken to indicate the degree of memory consolidation that had taken place after learning.

Passive avoidance test

Mice were subject to passive avoidance test 24 hrs after the Morris water maze test as described previously (Choi *et al*., 2012a; Choi *et al*., 2012b). Briefly, for the training trial, individual animal was placed in the light compartment. When the animal entered the dark chamber, the door was closed and an electrical foot shock (0.4 mA) with 2 second-long duration was delivered through the stainless steel rods. The test trial was conducted 24 hrs after training trial. Latency was defined as time mice spent before enter the dark compartment. Maximum latency was set to 600 seconds.

Immunohistochemistry

After behavioral tests, 5 out of 10 animals were intracardially perfused with 4% paraformaldehyde in PBS under the deep anesthesia (Na pentobarbital, 100 mg/kg). After perfusion, the brains were removed from the skull and post-fixed for 24 hr in the same fixative at 4°C, and were then cryoprotected in 30% sucrose prepared in phosphate buffer. Serial coronal sections of brain (30 μm) were cut with a freezing slide microtome (Thermo Fisher Scientific, Waltham, MA, USA). The sections were incubated overnight at 4°C with antibody to $A\beta_{1-42}$ (1:2000, Covance, Berkeley, CA, USA) or 4-hydroxynonenal (4-HNE) (1:2000, Abcam, Cambridge, MA, USA). After multiple washings in PBS, the sections were incubated in biotinylated IgG (1:1000, Vector Laboratories Inc., Burlingame, California, USA) for 1 hr at room temperature. The sections were subsequently washed and incubated with avidin-conjugated peroxidase complex (ABC kit, Vector Laboratories Inc.) for 60 min. The immunocomplex was visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Korea, Seoul, Korea) as the chromogen. Finally, the brain sections were rinsed and mounted on poly-glycine-coated slides and analyzed under the light microscopy.

Western blot

Animals were sacrificed after behavioral tests and five half brains were subject to the Western blot analysis. The analysis was performed as described previously with slight modification (Choi *et al*., 2012a). Briefly, equal amount of proteins (30 μg) were electrophoresed on a 10 or 15% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 hr at room temperature in 5% (w/v)

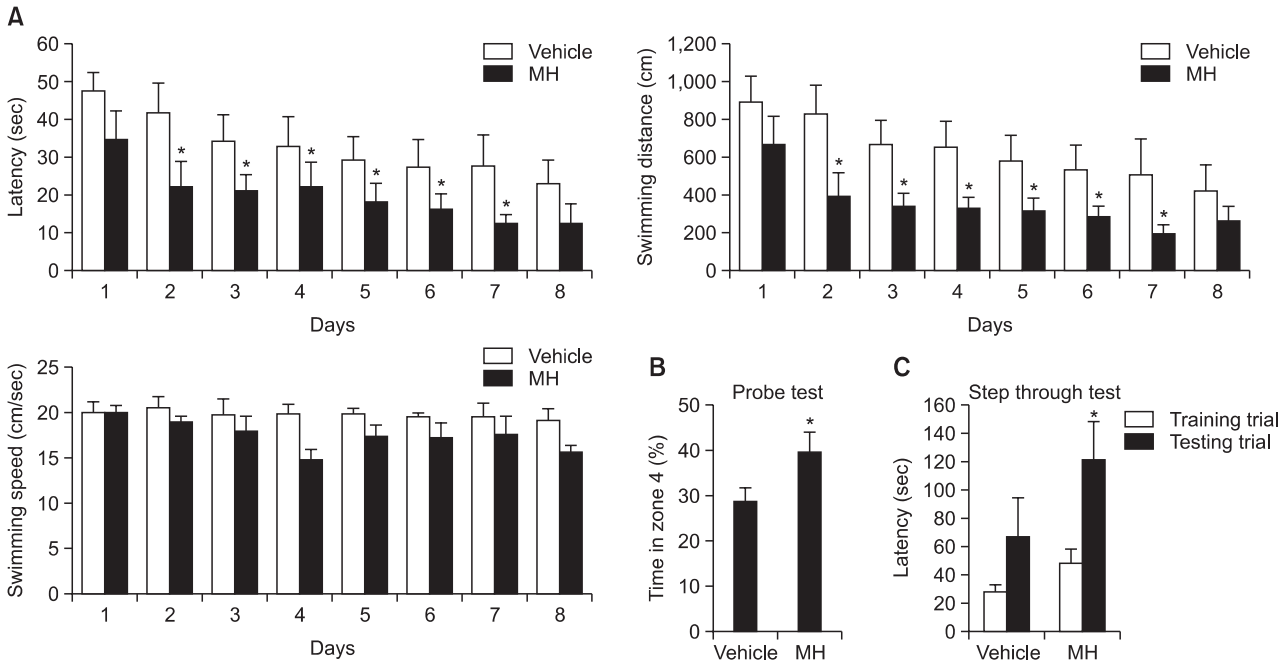

Fig. 1. 4-*O*-methylhonokiol ameliorates memory impairments in APP/PS1 double transgenic mice. Animals were treated with the natural compound for 3 months and the Morris water maze and passive avoidance tests were performed. With the Morris water maze test, 4-*O*-methylhonokiol appears to enhance cognitive function of the double transgenic mice (A). There is no significant difference between vehicle-treated animals and the compound-treated animals. The memory consolidation is shown to be better in 4-*O*-methylhonokiol-treated animals as determined by probe test (B). The step-through passive avoidance memory test reveals that the compound improves contextual memory in the transgenic mice (C). Values are presented as mean ± SD from 10 mice. **p*<0.05 vs. vehicle treatment. MH=4-*O*-methylhonokiol.

non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] containing 0.05% tween-20. The membrane was then incubated for 1 hr at room temperature with specific primary antibody against APP (1:500, ABR-affinity Bioreagents, Golden, CO, USA), BACE1 (1:500, Sigma-Aldrich), $A\beta_{1-42}$ (B-4) (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 4-HNE (1:000, Abcam) or β-actin (1:5000, Sigma-Aldrich Korea). The blots were then incubated in the corresponding horseradish peroxidase-conjugated immunoglobulin G (Santa Cruz Biotechnology Inc.). Immunoreactive protein was detected with the ECL western blot detection system. We used Super Signal West Femto Chemiluminescent Substrate (Thermo Fisher scientific) to detect Aβ. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA).

Assay for glutathione peroxidase activity

Five half brains were used to assay glutathione peroxidase activity. Brain tissue was sonicated in PBS for 15 seconds on ice and the homogenate was subject to centrifugation at 1,000 ×*g* at 4°C for 5 min to obtain supernatant. The supernatant was used in glutathione peroxidase assay. Briefly, the GSSG produced during the glutathione peroxidase enzyme reaction is immediately reduced by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Therefore, the rate of NADPH consumption (monitored as a decrease in absorbance at 340 nm) is proportional to formation of GSSG during the glutathione peroxidase reaction. The reaction buffer contained 20 mM potassium phosphate, (pH 7.0), 0.6 mM ethylenediaminetetraacetic acid, 0.15 mM NADPH, 4 units of glutathione reductase, 2 mM GSH, 1 mM sodium azide, and 0.1 mM H_2O_2. This assay was performed at 25°C. 1 unit of glutathione peroxidase activity is defined 1 μmol NADPH consumed per minute.

Statistical analysis

The data were analyzed using GraphPad Prism 4 software (Version 4.03, GraphPad Software Inc., San Diego, CA, USA). Difference between groups was assessed by one-way analysis of variance (ANOVA) followed by *post hoc* Dunnet's test. When a value of p is less than 0.05, it was considered to be statistically significant.

RESULTS

4-*O*-methylhonokiol-mediated cognitive improvement in transgenic mice

We determined learning ability by carrying out the Morris water maze test. The animals were trained for 3 days (twice/day) prior to test trials, and their learning for location of hidden platform was examined daily for 8 days. Cognitive function was rated by distance and time that animals took till they located to the platform. Treatment of 4-*O*-methylhonokiol or vehicle did not show any significant effects on the swimming speed during the tests (Fig. 1A). In contrast, the escape latency was gradually decreased in training sessions with significant difference between vehicle-treated and 4-*O*-methylhonokiol-treated groups (Fig. 1A). The compound administration

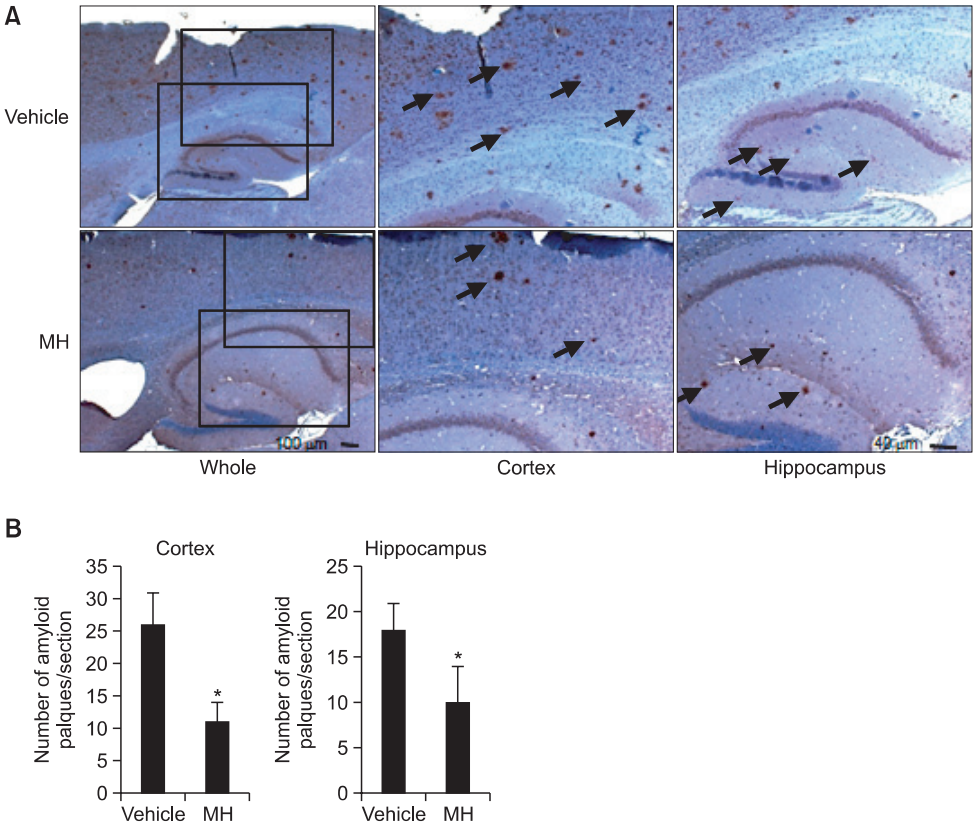

Fig. 2. 4-*O*-methylhonokiol attenuates Aβ accumulation in the cortex and hippocampus of the double transgenic mice. Immunohistochemical analysis shows that Aβ is markedly deposited in the brains of the transgenic mice and long-term treatment of 4-*O*-methylhonokiol alleviates accumulation of the pathogenic peptide (A). Quantification of Aβ accumulation shows that 4-*O*-methylhonokiol significantly attenuates Aβ deposition both in the cortex and hippocampus (B). **p*<0.05 vs. vehicle MH=4-*O*-methylhonokiol.

significantly decreased the escape latency and distance compared with vehicle treatment at test trials. At day 8, there was no significant difference in latency between vehicle-treated and the compound-treated mice. Probe test confirmed that memory retention was significantly consolidated only in 4-*O*-methylhonokiol-administered animals (Fig. 1B).

To determine 4-*O*-methylhonokiol-induced improvement of the contextual memory in the transgenic mice, we performed step-through passive avoidance tests. Vehicle-treated animals did not show significant memory retention as performance on test trial was not statistically different from that on training sessions, suggesting that the transgenic mice had a cognitive dysfunction (Fig. 1C). In contrast, there was significant memory retention in 4-*O*-methylhonokiol-administered animals. In addition, the result showed that the treatment significantly attenuated the memory deficit when it was compared with vehicle-treated control (Fig. 1C).

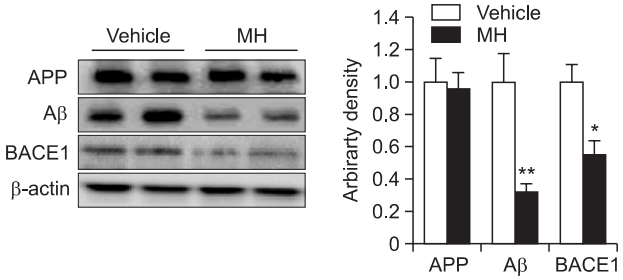

Fig. 3. 4-*O*-methylhonokiol suppresses BACE1 expression and Aβ generation. Western blot analysis shows that 4-*O*-methylhonokiol significantly decreases BACE1 expression in the brain. Aβ level is significantly lower in the 4-*O*-methylhonokiol-treated brains. Values are presented as mean ± SD from 5 independent blots. **p*<0.05 vs. vehicle, ***p*<0.01 vs. vehicle. MH=4-*O*-methylhonokiol.

4-*O*-methylhonokiol-mediated amelioration in Aβ generation in transgenic mice

We assessed effects of 4-*O*-methylhonokiol on Aβ formation in the transgenic animals using western blot and immunohistochemical analysis. Immunostaining for Aβ clearly showed that Aβ was accumulated in the cortex and hippocampus of the animals (Fig. 2). Aβ-positive plaques were observed throughout the cortex and hippocampus areas both in vehicle-treated and 4-*O*-methylhonokiol-treated mice. However, long-term treatment of 4-*O*-methylhonokiol significantly attenuated Aβ deposition in the brain areas as shown by immunohistochemistry (Fig. 2). To clarify how Aβ deposition was attenuated by the natural compound, we analyzed levels of APP, Aβ and BACE1 in the brain. Our data showed that 4-*O*-methylhonokiol treatment significantly reduced expression of BACE1, while APP production was not affected by the treatment (Fig. 3).

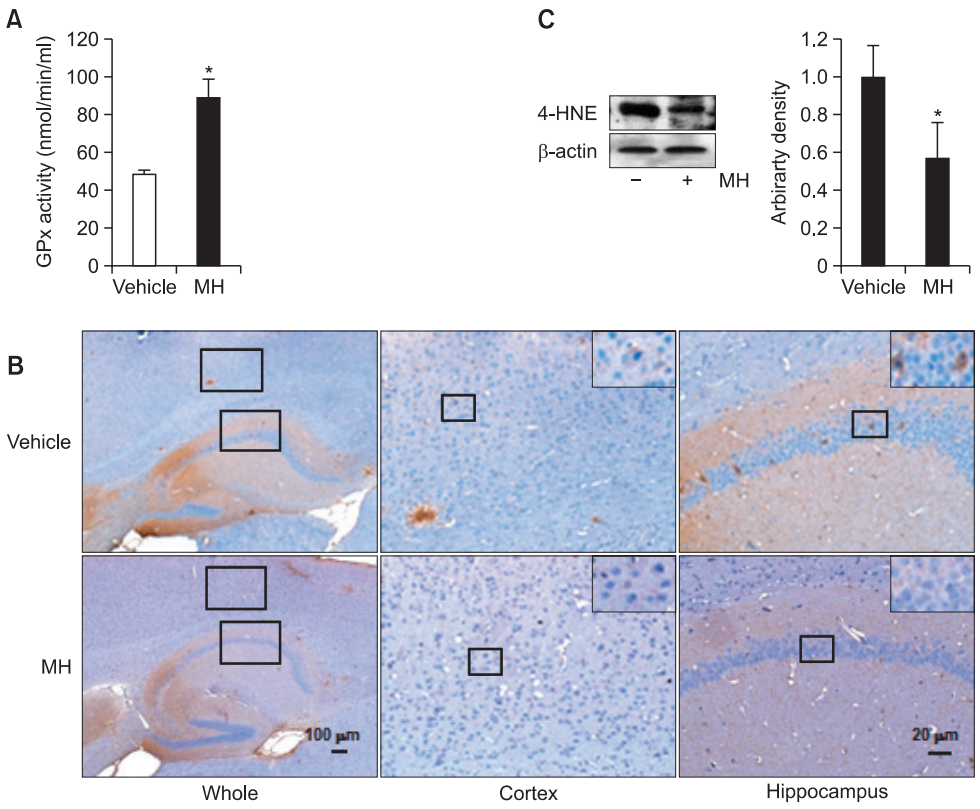

Fig. 4. 4-*O*-methylhonokiol increases antioxidant capacity and decreases lipid peroxidation. Long-term treatment of 4-*O*-methylhonokiol raises the activity of glutathione peroxidase in the brains (A). 4-HNE level is significantly lower in the 4-*O*-methylhonokiol as assessed by immunohistochemistry (B) and western blots (C). Values are presented as mean ± SD from 5 independent experiments. **p*<0.05 vs. vehicle, MH=4-*O*-methylhonokiol, GPx=glutathione peroxidase.

Furthermore, administration of 4-*O*-methylhonokiol alleviated level of Aβ in the brain implying that reduced expression of BACE1 by the compound might be relevant to attenuated Aβ accumulation in the cortex and hippocampus (Fig. 3).

Attenuation in oxidative stress by 4-*O*-methylhonokiol

Finally, we assessed effects of 4-*O*-methylhonokiol on lipid peroxidation level in the brain, since previous study had shown that the compound improved the cognitive function through antioxidant properties (Choi *et al*., 2011). Our assessment revealed that the compound significantly elevated glutathione peroxidase activity (Fig. 4A). In addition, 4-*O*-methylhonokiol significantly suppressed lipid peroxidation in the transgenic brain as shown by immunostanings and western blot for 4-HNE (Fig. 4B). Thus, these results imply that the compound alleviated oxidative stress in the brains and this effect might be associated with protection from cognitive malfunction.

DISCUSSION

AD is the most prevalent neurodegenerative disorder pathologically characterized by extracellular deposition of Aβ and intracellular deposition of hyper-phosphorylated tau protein. Despite of significant advances in understanding molecular mechanisms by which neurons are degenerated in Alzheimer's brains, the cause of the disease remains to be elucidated. Furthermore, there is no way to cure or slow down the neurodegeneration up to now. Because current approved medications have only limited or marginal efficacy on AD, development of a novel therapy for the disease is necessary. In this investigation, we clearly demonstrated that 4-*O*-methylhonokiol attenuated cognitive impairments in APP/PS1 double transgenic mice. Our results suggest that the improvement in cognitive function might come from decrease in Aβ generation *via* suppressing BACE1 expression. In addition, the beneficial effects of 4-*O*-methylhonokiol might be partially attributable to decrease in oxidative stress.

We observed that treatment of 4-*O*-methylhonokiol significantly attenuated deterioration in learning and memory, and this effect was coincided with reduction in Aβ deposition in the hippocampus and cortex. Aβ is derived from APP by sequential proteolytic actions of BACE1 and γ-secretase. BACE1 is necessary for initiation of Aβ generation and the enzyme expression is up-regulated in AD brains (Holsinger *et al*., 2002; Cole and Vassar, 2008). Activation of NF-κB upregulates expression of BACE1 and enhances Aβ formation (Sambamurti *et al*., 2004; Chen *et al*., 2012; Ai *et al*., 2013). Further support for the assumption comes from a report that the mutation on the NF-κB binding site of BACE1 promoter decreases the promoter activity resulting in reduced expression of BACE1 (Bourne *et al*., 2007). In previous studies, we demonstrated that 4-*O*-methylhonokiol suppressed DNA-binding activity of NF-κB and BACE1 expression in the brains of AD animal

models, suggesting that the compound may reduce BACE1 level by way of blunting NF-κB signaling pathway (Lee *et al*., 2012a).

Accumulated evidence suggests that neuroinflammatory events are related to regulation of BACE1 expression (Sastre *et al*., 2008). Although we did not demonstrated anti-neuroinflammatory properties of 4-*O*-methylhonokiol in this investigation, previous studies revealed that this compound had a potent anti-neuroinflammatory activity which was associated with cognitive enhancement in the AD animal models (Lee *et al*., 2012a; Lee *et al*., 2013). We suggested that the anti-inflammatory activity was mediated by diminishing NF-κB signaling pathway. Recently, Schuehly *et al* showed that 4-*O*-methylhonokiol acts like a cannabinoid type 2 (CB_2) receptor ligand (Schuehly *et al*., 2011). They suggest that effects of the compound on CB_2 receptors are associated with anti-neuroinflammatory effects, since the receptors are primarily associated with a broad range of inflammatory processes (Gertsch and Anavi-Goffer, 2012). In general, CB_2 receptors do not exist in the central nervous system under normal conditions, but are expressed in microglial cells and astrocytes upon neuroinflammatory stimulation (Ashton and Glass, 2007). Overall, it seems that multiple action mechanisms of 4-*O*-methylhonokiol are involved in its anti-neuroinflammatory activity which might be responsible for downregulation of BACE1 expression.

Oxidative damage has been shown to induce β-secretase activity (Tamagno *et al*., 2002). Oxidative damage in brain tissue of sporadic AD patients was detected which was significantly correlated with β-secretase activity (Guglielmotto *et al*., 2010). In Tg2576 mice, deletion of 12/15-lipoxygenase decreased oxidative stress and amyloid formation *via* β-secretase downregulation (Yang *et al*., 2010). Furthermore, deficiency in cytochrome oxidase c in neurons significantly reduces both oxidative stress and amyloid genesis in an animal model for AD, which is related to lowered β-secretase activity (Fukui *et al*., 2007). Our previous study showed that 4-*O*-methylhonokiol suppressed oxidative stress in neurons with concurrent reduction in β-secretase activity (Choi *et al*., 2011). Consistent with previous studies, our results demonstrated that 4-*O*-methylhonokiol reduced oxidative stress as 4-HNE level was decreased in the brain. Additionally, 4-*O*-methylhonokiol mediated rise in glutathione peroxidase activity. These results suggest that reduction in BACE1 expression might be related to antioxidant effects of the natural compound.

In conclusion, this study showed that 4-*O*-methylhonokiol stabilized cognitive function in APP/PS1 double transgenic mice. Population of AD patients will be growing rapidly, because of increase in life expectancy and aging of baby boomer generation. Thus, development of new therapeutics for AD treatment is an urgent issue. Novel compounds from plant sources such as 4-*O*-methylhonokiol could be valuable alternatives in the context of the treatment of AD due to its therapeutic effects and safety.

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