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Attenuation of Scopolamine-induced Cognitive Dysfunction by Obovatol

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Alzheimer's disease (AD) is the most prevalent cause of dementia in the elderly people. The disease is pathologically characterized by extracellular deposition of beta-amyloid peptide (Aβ), cholinergic neurodegeneration and elevation of acetylcholine esterase (AChE) activity in the affected regions. In this study, we investigated the effects of obovatol on memory dysfunction, which was caused by scopolamine. Obovatol (0.2, 0.5 and 1 mg/kg for 7 day) attenuated scopolamine (1 mg/kg, i.p.)-induced amnesia in a dose-dependent manner, as revealed by the Morris water maze test and step-through passive avoidance test. Mechanism studies exhibited that obovatol dose-dependently alleviated scopolamine-induced increase in Aβ generation and β-secretase activity in the cortex and hippocampus. Obovatol also attenuated scopolamine-induced rise in AChE activity in the cortex and hippocampus. Obovatol might rescue scopolamine-mediated impaired learning and memory function by attenuating Aβ accumulation and stabilizing cholinergic neurotransmission, which suggests that the natural compound could be a useful agent for the prevention of the development or progression of AD neurodegeneration.

Key words: Alzheimer's disease, Memory, Obovatol, Scopolamine, Secretase, Acetylcholine esterase

Selected by Editors

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly people. The neurodegenerative disease is pathologically characterized by extracellular accumulation of amyloid-beta (Aβ). Aβ is generated from amyloid precursor protein (APP), by sequential proteolytic actions of β- and γ-secretases. Beta-site APP-cleaving enzyme (BACE) 1 cleaves APP to form Aβ N terminus, APPβ and a C-terminal fragment, C99. Subsequently, γ-secretase generates Aβs with two variants, $A\beta_{1-40}$ and $A\beta_{1-42}$. α-Secretase cleaves APP within the Aβ domain to produce APPα and C83, precluding the formation of Aβ by competing with BACE1 (Cole and Vassar, 2008). $A\beta_{1-42}$ is a highly neurotoxic and fibrillogenic form of Aβ, and is considered as a prime trigger of AD pathogenesis (Glabe, 2008).

Another consistent pathological hallmark of AD is a loss of cholinergic neurons in the basal forebrain (Auld et al., 2002). It has been suggested that the cognitive impairments in AD patients are attributable to demise of forebrain cholinergic neurons. In accordance with the suggestion, acetylcholine esterase (AChE) inhibitors attenuate symptoms that are related to the cognitive dysfunction in AD patients by increasing the concentration of ACh in the brain regions affected by AD (Messamore et al., 1993; Nordberg and Svensson, 1998). Thus, maintaining the ACh level in the brain might be beneficial to AD patients. AChE inhibitors, including donepezil, galantamine, and rivastigmine, are the current approved drugs for the treatment of AD patients. However, AChE inhibitors present some limitations, such as their short half-lives and excessive side effects caused by activation of peripheral cholinergic systems

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(Kaduszkiewicz et al., 2005). Furthermore, the medications are not able to halt or cure the neurodegeneration in AD. Due to these disadvantages, alternative and complementary therapies should be developed.

Treatment of scopolamine, a muscarinic receptor antagonist with animals and humans, causes impairments in learning and short-term memory (Petersen, 1977; Park et al., 2012). It seems that scopolamine induces amnesia by elevating AChE activity, as well as antagonizing the muscarinic receptor of ACh (Ahmed and Gilani, 2009; Lee et al., 2009b). Apart from causing cognitive deficit, scopolamine recapitulates some biochemical features of AD. For instance, studies showed that scopolamine administration increased the level of Aβ in the brain of Tg2576 mice (Liskowsky and Schliebs, 2006) and elicited oxidative stress in the mouse brain (Kwon et al., 2010). By virtue of the property, the drug has been used to create experimental animal model for dementia (Beatty et al., 1986; Preston et al., 1988).

Compounds from Magnolia species have exhibited various pharmacological activities, such as antimicrobial (Ho et al., 2001), anxiolytic (Seo et al., 2007), neurotrophic (Lee et al., 2009a) and cholinergic (Matsui et al., 2009) property. In search of novel compounds of Magnolia extract, with inhibitory effect on Aβ aggregation, we found that obovatol potently inhibits Aβ aggregation (Choi et al., 2012a). Significantly, we demonstrated that obovatol improved cognitive function in human mutant APP, overexpressing Tg2576 in mice, by blocking neuroinflammatory responses and Aβ formation (Choi et al., 2012a). In the present study, we investigated whether administration of obovatol could attenuate amnesia, induced by scopolamine.

MATERIALS AND METHODS

Isolation of obovatol

We isolated obovatol from leaves of *Magnolia obovata*, according to the method described by Lee et al. (Lee et al., 2008). We used obovatol with ~95.0% purity in the entire study.

Animals and treatment

Male ICR mice (20-25 g) were purchased from Samtako, and were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guideline for the humane care and use of laboratory animals. All of the experimental procedures in the present 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. Obovatol and scopolamine were treated with animals as depicted in Fig. 1.

Passive avoidance performance test

Passive avoidance test was conducted as described previously (Choi et al., 2012a, b). 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. Mice were given an injection of scopolamine and subject to test trial, 1, 3 and 7 days after the training session. Latency was defined as the time the mice spent before entering the dark compartment. Maximum latency was set to 600 seconds.

Water maze test

The Morris water maze test was performed, as described in the previous study with a slight modification (Choi et al., 2012a, b). Briefly, mice were placed in the pool, and were 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. The animals were given scopolamine (1 mg /kg, i.p.) or the same volume of saline and then test trial was performed 1, 3 and 7 days after the last training trial. Escape latency, escape distance, swimming speed, and swimming pattern of each mouse were monitored and analyzed by a video tracking system (SMART-LD program, Panlab).

Western blot

Western blot analysis was performed, as described previously with a slight modification (Choi et al., 2012a). Briefly, equal amounts of protein (40 μg) were electrophoresed on a 10 or 15% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Hybond ECL, Amersham Pharmacia Biotech Inc.). Blots were blocked for 2 h at room temperature, in a 5% (w/v) 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 h at room temperature, with specific primary antibody against APP (1:500, Santa Cruz Biotechnology Inc.), BACE1 (1:500, Santa Cruz Biotechnology Inc.), C99 (1:500, Sigma-Aldrich), Aβ (1:1000, Santa Cruz Biotechnology Inc.) or β-actin (1:5000, Sigma-Aldrich). 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 a Super Signal West Femto Chemiluminescent Substrate (Thermo

scientific) to detect Aβ and C99. The relative density of the protein bands was quantified by densitometry, using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Com).

β-Secretase activity assay

The total activity of β-secretase present in cortical and hippocampal regions were determined, using a commercially available β-secretase fluorescence resonance energy transfer (BACE1 FRET) assay kit (PANVERA), as described previously (Choi et al., 2012a). The activity was expressed as fluorescence units. All controls, blanks and samples were run in triplicate.

AChE activity assay

AChE activity was determined by Ellman's method with a slight modification (Ellman et al., 1961). Briefly, the animals were perfused with PBS, under anesthetization, induced by overdose of pentobarbital (100 mg/kg). The brains were immediately removed from the skull, and the cortex and hippocampus were dissected out on the ice. Brain tissues were homogenized in PBS, centrifuged at 15,000 g for 15 min, and the supernatant was then used for the assays. A 5 μL of sample was mixed with 200 μL of reaction buffer (0.5 mM PBS pH 7.4) containing 0.02% 5,5'-dithio-bis-2-nitrobenzoic acid, 0.02% acetylthiocholine, and 0.1 mM tetraisopropyl pyrophosphoramide (isoOMPA). Activity of the enzyme was determined 5 min after incubation, at 37°C, and stopped with 1 mM BW284c51, a potent selective inhibitor of AChE. The optical density was measured at 405 nm, and enzyme activity was expressed as a unit of the quantity (μM) of acetylcholine hydrolyzed to acetylthiocholine per min and mg protein (μM/min/mg protein).

Statistics

The data was analyzed using GraphPad Prism 4 software (Version 4.03, GraphPad Software, Inc.). The difference between the groups was assessed by one-way or two-way analysis of variance (ANOVA), followed by *post hoc* Dunnet's test. One-way ANOVA was used to analyze the data for AChE activity, while the data obtained from the Morris water maze (escape distance, escape latency, and average speed) was analyzed using a two-way ANOVA. Data from the step-through avoidance test was analyzed using the nonparametric test (Kruskal-Wallis test) followed by a Dunnet's test. When a value of p is less than 0.05, it was considered to be statistically significant.

RESULTS

Effect of obovatol on scopolamine-induced cognitive impairments

To determine whether the treatment of obovatol improve the contextual memory in scopolamine-injected mice, we performed step-through passive avoidance tests. Animals showed statistically identical performance, at the training sessions, regardless of the treatments (data not shown). However, there was a significant impairment in memory retention in scopolamine-injected animals, compared with vehicle-treated control, when the test trials were conducted (Fig. 1). Scopolamine-induced cognitive malfunction was gradually declined over the experiments. Importantly, the deficit in memory retention was significantly relieved by the obovatol treatment (Fig. 1).

The learning ability was determined by the Morris water maze test. The animals were trained for 3 days (twice/day) prior to the test trials, and their learning for location of a hidden platform was examined at 1, 3 and 7 days after the last obovatol treatment. Cognitive function was rated by the distance and time that the animals took to locate the platform. The escape latency was gradually decreased with training sessions without any difference among the groups (data not shown). However, scopolamine injection significantly increased the escape latency and distance, compared with the vehicle injection at the test trials (Fig. 2A and B). The scopolamine-mediated impairment in learning was gradually diminished over time. Obovatol treatment significant-

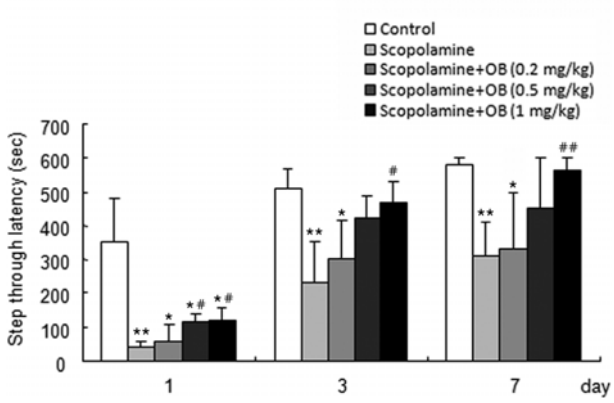

Fig. 1. Obovatol attenuates scopolamine-induced contextual memory impairments. Animals were treated with obovatol 1 week prior to scopolamine injection, and step-through passive avoidance memory tests were performed to determine contextual memory performance. Results show that scopolamine induces cognitive dysfunction over time and obovatol alleviates the deficits in a dose-dependent manner. Values are presented as mean ± S.E.M. from 7 mice. *$p < 0.05$ *vs* control, **$p < 0.01$ *vs* control, #$p < 0.05$ *vs* scopolamine, $^{\#\#}p < 0.01$ *vs* scopolamine. OB=obovatol.

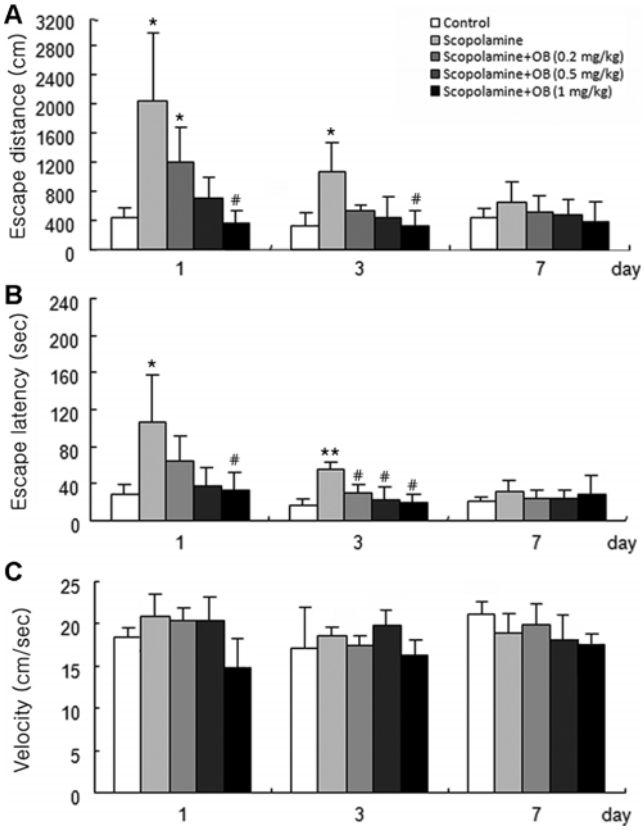

Fig. 2. Obovatol attenuates scopolamine-induced impairment in learning. Learning abilities of mice were determined by the Morris water maze test. Animals were treated with obovatol for 1 week prior to scopolamine injection, and the Morris water maze tests were performed. The learning ability was rated by escape distance (**A**) and escape latency (**B**). Swimming speed was also measured to confirm there was no locomotor dysfunction in animals (**C**). Results exhibit that scopolamine induces deficit in learning for hidden platform location over the tasks and obovatol improve the ability for learning. Values are presented as mean ± S.E.M. from 7 mice. *$p < 0.05$ *vs* control, **$p < 0.01$ *vs* control, #$p < 0.05$ *vs* scopolamine. OB=obovatol.

ly inhibited the scopolamine-mediated amnesia. Treatment of scopolamine or obovatol did not show any effects on the swimming speed during the tests (Fig. 2C).

Effect of obovatol on scopolamine-induced increase in Aβ generation

We determined the effect of obovatol on Aβ formation in scopolamine-treated animals, using a Western blot analysis, since there is a close relationship between Aβ level and AChE activity. Intriguingly, intraperitoneal injection of scopolamine increased the Aβ level in the cortex and hippocampus (Fig. 3A, B). The treatment of obovatol significantly attenuated Aβ generation in a dose dependent fashion. To clarify how $A\beta_{1\text{-}42}$ deposition had occurred, we analyzed the levels of APP, BACE1 and C99. There was a significant rise in the level of the proteins in the cortex and hippocampus, after a scopolamine injection. Obovatol exhibited its inhibitory effect on the increase, indicating that Aβ accumulation might result from the induction of APP and BACE1, and inhibitory effect of obovatol might prevent Aβ generation (Fig. 3A, B). To confirm the speculation, we next attempted to assay β-secretase activity. The activity of β-secretase significantly increased in both the cortex and hippocampus (Fig. 4). The alteration in β-secretase activity was attenuated by the obovatol treatment.

Effect of obovatol on scopolamine-induced elevation in AChE activity

AChE is an enzyme that is responsible for the degradation of ACh in the brain. Scopolamine-induced amnesia seems to be partially caused by an increase in AChE activity. Thus, we determined the enzyme activity in the cortex and hippocampus. AChE activity was significantly increased in the cortex and hippocampus by an injection of scopolamine, whereas, the increase in AChE activity was significantly suppressed by an oral administration of obovatol, in a dose dependent manner (Fig. 5).

DISCUSSION

AD is the most prevalent neurodegenerative disorder, pathologically, manifested by an extracellular deposition of Aβ and preferential demise of cholinergic neurons in the cortex and hippocampus. Much effort has been taken to discover the underlying mechanisms, by which the neurons are degenerated in the Alzheimer's brains, but the cause of the disease is still obscure. Furthermore, there is no way to cure or stop the AD neurodegeneration, to date. In the present study, we clearly demonstrated that obovatol attenuated scopolamine-induced impairments in cognitive function. The preventive effects might come from the inhibition of scopolamine-mediated rise in the AChE activity and Aβ generation. These results indicate that obovatol could be a potential therapeutic intervention for AD.

Neuroprotective and/or cognition-enhancing properties of natural compounds have been shown in different animal models, rendering potential agents for AD prevention or treatment. For instance, (-)-epigallocatechin-3-gallate (EGCG), a major polyphenol compound of green tea was revealed to promote cognitive function through antioxidative and iron chelating activity (Weinreb et al., 2009). Resveratrol is found in the skin of red grapes and in red wine, and the neuroprotective effect of the compound attributes to the activation of

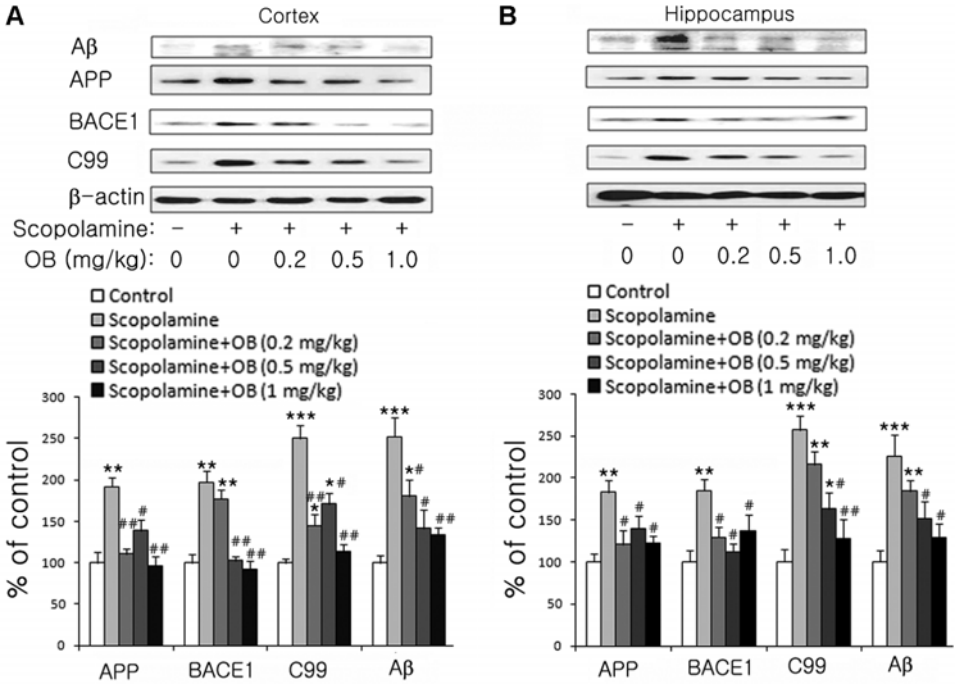

Fig. 3. Obovatol attenuates scopolamine-induced increase in levels of Aβ, APP, BACE1 and C99. Western blot analysis shows that scopolamine injection elevates levels of Aβ, APP, BACE1 and C99 in both cortex (A) and hippocampus (B). Obovatol treatment suppresses the increase in dose-dependent fashion. Values are presented as mean ± S.E.M. from 5 independent blots. $^{*}p < 0.05$ *vs* control, $^{**}p < 0.01$ *vs* control, $^{***}p < 0.001$ *vs* control, $^{\#}p < 0.05$ *vs* scopolamine, $^{\#\#}p < 0.01$ *vs* scopolamine. OB = obovatol.

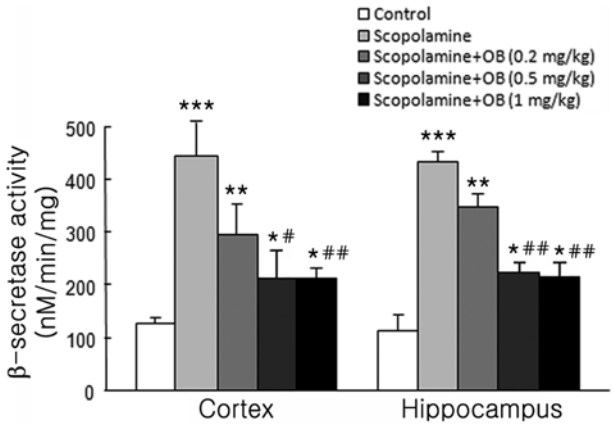

Fig. 4. Obovatol attenuates scopolamine-mediated increase in β-secretase activity. Results exhibit that scopolamine elevates the activity of β-secretase in both cortex and hippocampus, while obovatol treatment inhibits the elevation. Values are presented as mean ± S.E.M. from 5 independent experiments. $^{*}p < 0.05$ *vs* control, $^{**}p < 0.01$ *vs* control, $^{***}p < 0.001$ *vs* control, $^{\#}p < 0.05$ *vs* scopolamine, $^{\#\#}p < 0.01$ *vs* scopolamine. OB=obovatol.

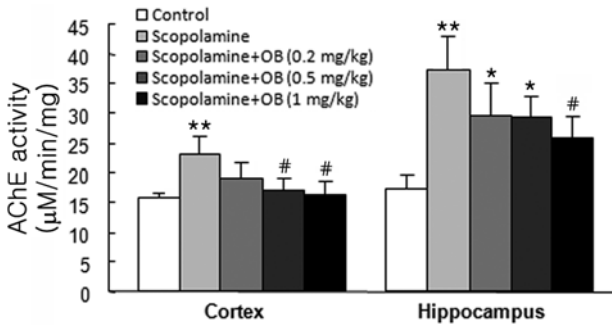

Fig. 5. Obovatol attenuates scopolamine-induced rise in AChE activity. Assay of AChE activity shows that scopolamine injection causes increase in AChE activity in both cortex and hippocampus. Values are presented as mean ± S.E.M. from 5 independent experiments. $^{*}p < 0.05$ *vs* control, $^{**}p < 0.05$ *vs* control, $^{\#}p < 0.05$ *vs* scopolamine. OB=obovatol.

silent mating type information regulation 2, homolog 1 (Huber and Superti-Furga, 2011). One study showed that obovatol exerted neuroprotection through the mitigation of neuroinflammation (Ock et al., 2010). Obovatol treatment, with embryonic neurons, induced the release of neurotrophic factors eliciting neurite outgrowth (Lee et al., 2010). In previous studies, we have shown that obovatol enhanced cognitive performance in different AD animal models by suppressing neuroinflammation and Aβ deposition (Choi et al., 2012a, b). Current investigation demonstrated that the treatment of obovatol alleviated the scopolamine-induced elevation of AChE activity and Aβ accumulation, which might be associated with a decline of cogni-

tive deficits. Taken together, obovatol exhibited neuroprotection and cognitive enhancement, via multi-pharmacological properties.

An intriguing finding here is that scopolamine administration induces Aβ production. It is an open question of how scopolamine stimulates Aβ generation. However, a body of evidence indicates that APP processing is regulated by cholinergic neurotransmission. There was a rise in sAPPα secretion when M1/M3, but not M2/M4 muscarinic ACh receptor, was selectively activated, which indicated an activation of M2/M4 muscarinic receptors is related to the reduction of Aβ production (Nitsch et al., 1992). In contrast, the muscarinic transmission decreased the total Aβ formation, both *in vitro* (Ensinger et al., 1993; Muller et al., 1997) and *in vivo* (Hock et al., 2000; Nitsch et al., 2000). Moreover, AChE inhibitor decreased Aβ and increased sAPPα, in the primary rat neurons (Bailey et al., 2011). Selective lesion of basal forebrain cholinergic neurons in the rat brain caused a cognitive deficit and an increase in APP level (Lin et al., 1998). These findings indicate that the reduction in muscarinic cholinergic activity tends to generate Aβ, which might be implicated in AD pathogenesis. In support, current study showed that scopolamine-induced inhibition of muscarinic receptors led to an elevation of Aβ level and accompanied cognitive dysfunction, which was blocked by obovatol treatment. Obovatol significantly inhibited the rise in AChE activity, induced by scopolamine injection. This inhibitory effect of the compound might contribute to the alleviation of amyloidogenesis, and the memory deficit in this investigation. At this point, specific mechanisms by which obovatol suppresses the scopolamine-mediated elevation in AChE activity are not clear. Several natural compounds have been revealed to directly inhibit the AChE activity improving learning and memory tasks of animals (Lee et al., 2009b, 2010; Kwon et al., 2010; Uriarte-Pueyo and Calvo, 2011). Significantly, we found that 4-O-methylhonokiol isolated from *Magnolia officinalis*, inhibited AChE activity *in vitro* with IC_{50} of 12 nM. We have not proved any antagonistic action of obovatol against AChE activity in the absence of scopolamine, but it would be possible that the agent may inhibit AChE activity. Alternatively, AChE expression could be decreased by obovatol, as the other study showed that scopolamine-mediated elevation of immunoreactivity for AChE is mitigated by Rehmannia glutinosa extract in the hippocampus (Lee et al., 2010). However, further study is required to elucidate the neuroprotective mechanism of obovatol in this setup.

In conclusion, this study showed that obovatol stabilized cognitive function against scopolamine-induced amnesia. The beneficial effects are attributed to the suppression of the rise in AChE activity and Aβ level. We assume that obovatol may directly inhibit AChE activity or down-regulate AChE expression by an unknown mechanism. The prevalence of AD would be rapidly increased in the near future, due to a fast growth of elderly population in the world. Thus, the development of new therapeutics for AD treatment is an urgent issue. Novel compounds from plant sources, such as obovatol, could be valuable alternatives in the context of the treatment of AD.

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