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The ameliorating effects of stigmasterol on scopolamine-induced memory impairments in mice

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ABSTRACT

Stigmasterol, a kind of phytosterol, is present in small amounts in various foods. In the present study, we investigated the effects of stigmasterol on scopolamine-induced memory impairments using the passive avoidance and the Morris water maze tasks in mice. In addition, changes in memory-related molecules, including extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB), were examined following the administration of stigmasterol. Scopolamine-induced memory impairments were significantly attenuated by the administration of stigmasterol (10 mg/kg) in the passive avoidance task. In the Morris water maze task, the escape latencies were significantly decreased in the stigmasterol-treated group compared to the scopolamine-treated group during the training phase. The swimming times within the target zone during the probe trial were significantly increased as compared to scopolamine-treated mice. Furthermore, the ameliorating effect of stigmasterol on scopolamine-induced memory dysfunction was blocked by a sub-effective dose of dizocilpine (MK-801), an NMDA receptor antagonist, and tamoxifen, an estrogen receptor antagonist, in the passive avoidance task. In addition, the expression levels of phosphorylated ERK and CREB in the hippocampus were significantly increased by stigmasterol, which was blocked by tamoxifen or MK-801 with scopolamine. These results suggest that stigmasterol-induced cognitive ameliorative effects are mediated by the enhancement of cholinergic neurotransmission system via the activation of estrogen or NMDA receptors.

1. Introduction

Dementia is characterized by the loss of cognitive abilities, such as memory, attention, language, and problem solving, and is mainly caused by Alzheimer's disease or vascular dementia. Alzheimer's disease is progressive neurodegenerative disorder that is caused by the accumulation of amyloid β-peptide, a sticky peptide prominent in brain plaques, and abnormalities in the tau protein, resulting in the formation of neurofibrillary tangles in the medial temporal lobe and cortical areas of the brain (Goedert et al., 1991; Hardy and Selkoe, 2002; Schmitz et al., 2004). In Alzheimer's disease brains, losses of choline acetyltransferase activity or basal forebrain cholinergic neurons are observed, and these losses are correlated with cognitive impairments (Bartus et al., 1982; Francis et al., 1999; Mufson et al., 2008). The current therapeutic approach to treat cognitive loss associated with Alzheimer's disease has been a cholinergic enhancement strategy using muscarinic or nicotinic receptor ligands and acetylcholinesterase (AChE) inhibitors, such as donepezil, rivastigmine, and galantamine (Terry and Buccafusco, 2003). Nevertheless, new drugs are necessary to treat Alzheimer's disease patients, because current drugs have unpleasant side effects, including diarrhea, nausea, vomiting, and bradycardia (Rountree et al., 2009).

Stigmasterol, the most common phytosterol, has a structure similar to that of cholesterol with some modifications (Awad and Fink, 2000) (Fig. 1). Stigmasterol is present in foods at low concentrations and detected in phytosterol-enriched spreads and oils such as peanut, sunflower, or corn oils (Awad et al., 2000). Previous studies on phytosterols focused on cholesterol metabolism due to the structural similarities between phytosterol and cholesterol (Yang et al., 2004). For example, phytosterols reduce the absorption of dietary cholesterol and thus offer protection against cardiovascular diseases (Ikeda et al., 1988; Nguyen, 1999). However, little is known about the role of stigmasterol in the brain and its therapeutic role in dementia. It has been reported that stigmasterol has an estrogenic effect (Newill et al., 2007), activates the glutamatergic neurotransmission system (Khabazian et al., 2002), or inhibits AChE activity (Sultana and Khalid, 2010), indicating that stigmasterol may have anti-amnesic property. Based on these reports, we investigated the effects of stigmasterol on scopolamine-induced cognitive impairments in mice using the step-through passive avoidance and the Morris water

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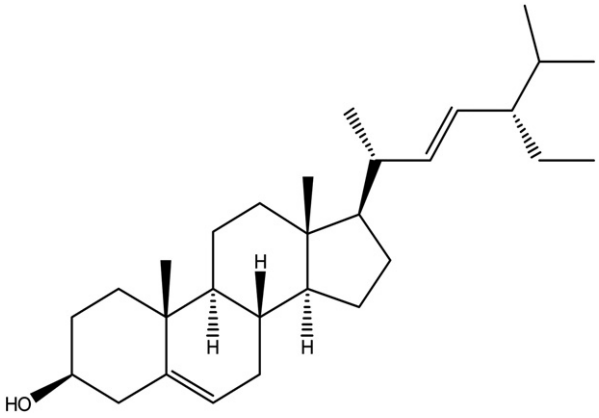

Fig. 1. The structure of stigmasterol.

maze tasks. Moreover, we confirmed the effects of stigmasterol on the levels of memory-related biochemical parameters, including phosphorylation of extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB) in the hippocampus.

2. Materials and methods

2.1. Animals

All experimental protocols were approved by the Institutional Animal Care and Usage Committee of Kyung Hee University. Male ICR mice (6 weeks old, 25–30 g) were purchased from the Orient Co. Ltd., a branch of Charles River Laboratories (Seoul, Korea). Mice were housed 5 per cage, provided with food and water ad libitum and, kept under a 12 h light/dark cycle (light on 07:30–19:30) at a constant temperature (23 ± 1 °C) and humidity (60 ± 10%). After delivery, animal maintenance and treatment were carried out in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University, Korea.

2.2. Materials

Stigmasterol, (−)-scopolamine hydrobromide, dizocilpine (MK-801), tamoxifen, and 9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride hydrate (tacrine, THA) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phosphorylated ERK (pERK) antibody was purchased from Cell Signaling Technology (Cell Signaling, MA). Anti-ERK, and anti-CREB antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An anti-phosphorylated CREB (pCREB) antibody was purchased from Chemicon (Temecula, CA). All other materials were of the highest grades available and were obtained from normal commercial sources. Tacrine, scopolamine, and MK-801 were dissolved in 0.9% saline solution. Stigmasterol was suspended in 10% Tween 80 solution. Tamoxifen was dissolved in 0.1% DMSO saline.

2.3. Passive avoidance task

Assessment of acquisition and retention of the passive avoidance task was carried out using identical illuminated and non-illuminated compartments (20 cm × 20 cm × 20 cm) containing a 50 W bulb, as previously described (Park et al., 2010b). In brief, the animals underwent two separate trials: an acquisition trial and a retention trial, conducted 24 h after the acquisition trial. For the acquisition trial, the mouse was initially placed in the light compartment, and after 10 s, the guillotine door (5 cm × 5 cm) between the two compartments was opened. When the mouse entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA, 3 s) was delivered through the grid floor. One hour before the acquisition trial, mice were administered stigmasterol (2.5, 5, 10, or 20 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.). The control group received 10% Tween 80 vehicle solution rather than stigmasterol. Thirty minutes after treatment with stigmasterol, tacrine, or saline, mice were treated with scopolamine (1 mg/kg, i.p.) or 0.9% saline solution. For the retention trial, the mice were placed in the light compartment again and the time taken to enter the dark compartment (latency) was recorded for each mouse. If the mouse did not enter the dark compartment within 300 s, we concluded that the mouse remembered the foot shock from the acquisition trial. In a separate antagonism study, stigmasterol (10 mg/kg, p.o.) or the same volume of vehicle was administered 60 min before the acquisition trial, and 30 min later, mice were injected with a sub-effective dose of MK-801 (0.0125 mg/kg, s.c.) or tamoxifen (1 mg/kg, i.p.). Scopolamine (1 mg/kg, i.p.) was administered 5 min after the MK-801 or tamoxifen treatment. The acquisition trial was carried out 25 min after the scopolamine administration. The dose of tamoxifen and MK-801 used in the antagonism study did not impair passive avoidance task performance when administered itself (Kim et al., 2010; Park et al., 2010b).

2.4. Morris water maze task

The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was filled to a depth of 30 cm with water containing black dye (24 ± 1 °C). The tank was placed in a dimly lit, soundproof test room with various visual cues. The pool was conceptually divided into quadrants. A white platform (6 cm in diameter and 29 cm high) was placed in one of the pool quadrants and submerged 1 cm below the water surface so that it was not visible. The test was conducted as previously described (Park et al., 2010b), with slight modifications. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the four subsequent days, the mice were given four training trials per session per day with the platform in place. When a mouse located the platform, it was allowed to remain on the platform for 10 s. If a mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animals were returned to their home cages and allowed to dry under an infrared lamp after each trial. The time between training-trials was 30 s. During each training session, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision system (Noldus, Wageningen, The Netherlands). For each training trial, the mice were placed in the water facing the pool wall in a randomly selected pool quadrant. The day after the last training trial session, the mice were subjected to a probe trial session in which the platform was removed from the pool, and the mice were allowed to search for it for 60 s. A record was kept of the swimming time in the pool quadrant where the platform had been located previously. Stigmasterol (10 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.) was administered daily 1 h before the first training trial of training session. Memory impairment was induced by scopolamine administration (1 mg/kg, i.p.) 30 min after stigmasterol treatment. The control group received 10% Tween 80 vehicle solution only.

2.5. Western blot analysis

Isolated hippocampal tissue was homogenized in ice-chilled Tris–HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate. Western blot analysis was conducted as described previously (Park et al., 2010a). Samples of homogenates (15 μg of protein) were subjected to SDS-PAGE (8% gel) under reducing conditions. Proteins were transferred onto PVDF membranes in transfer buffer [25 mM Tris–HCl (pH 7.4)

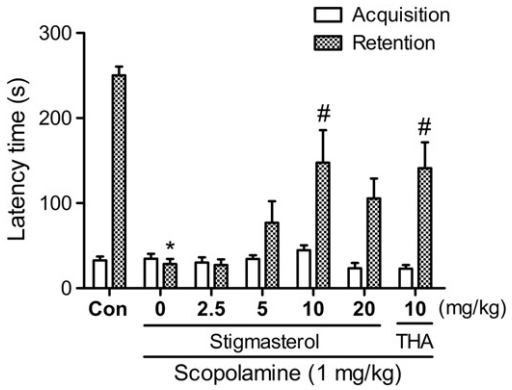

Fig. 2. The effects of stigmasterol on the scopolamine-induced memory impairments in the passive avoidance task. Stigmasterol (2.5, 5, 10, or 20 mg/kg, p.o.), tacrine (THA, 10 mg/kg, p.o.), or the same volume of vehicle (10% Tween 80 solution) was administered 60 min before the acquisition trial. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the acquisition trial. Twenty-four hours after the acquisition trial, a retention trial was conducted for 300 s. Data represent means ± S.E.M (n = 8–9 per group) $^{*}P<0.05$, versus the vehicle-treated controls, $^{\#}P<0.05$, versus the scopolamine-treated group. Con, control.

containing 192 mM glycine and 20% v/v methanol] and further separated at 100 V for 2 h at 4 °C to determine pERK, ERK, pCREB, and CREB levels. Western blots were incubated for 4 h in blocking solution (5% skim milk) at 4 °C, then incubated overnight in 1:3000 dilutions of anti-pERK, anti-ERK, anti-pCREB, and anti-CREB antibodies. Blots were washed twice with Tween 20/Tris-buffered saline (TTBS), incubated in 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed three times with TTBS, and developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Immunoreactivity was analyzed using the multi-gage, bio-imaging program on a LAS-4000 mini (Fujifilm Lifescience USA, Stamford, CT).

2.6. Statistics

The results of studies were expressed as means ± S.E.M. Data from the latencies observed in the passive avoidance task, the probe trial of the Morris water maze task, and the Western blot analysis were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis for multiple comparisons. Escape latency in the Morris water maze task was analyzed using a two-way repeated measures ANOVA followed by Bonferroni's test with day as the first variable and treatment as the second. The interactions between stigmasterol and tamoxifen or MK-801 in the passive avoidance task were analyzed by a two-way ANOVA, and Tukey's post-hoc test was used to perform pairwise comparisons to determine antagonist or agonist effects. Statistical significance was set at $P<0.05$.

3. Results

3.1. The effect of stigmasterol on the scopolamine-induced memory impairment in the step-through passive avoidance task

We assessed the effect of stigmasterol on the scopolamine-induced cognitive dysfunction using the step-through passive avoidance task. A significant group effect was observed for step-through latency during the retention trial [F (6, 51) = 13.943, $P<0.001$]. The step-through latency in the scopolamine-treated group was significantly shorter than in the vehicle-treated control group ($P<0.05$, Fig. 2). In addition, the reduced step-through latency caused by scopolamine was significantly ameliorated by stigmasterol (10 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.) in the retention trial ($P<0.05$, Fig. 2). However, during

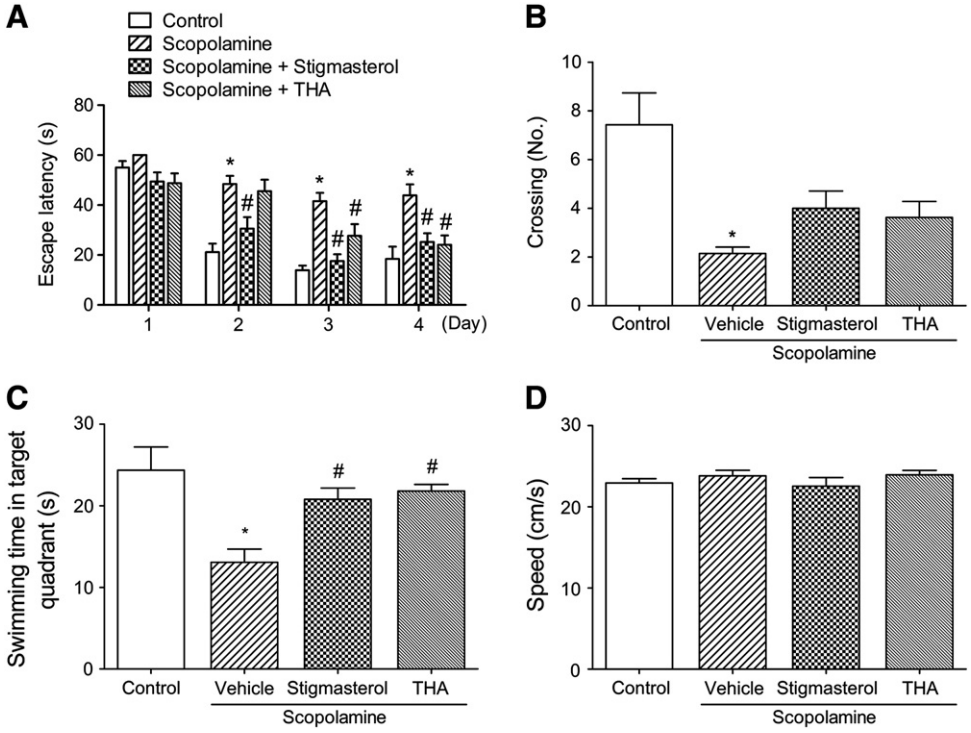

Fig. 3. The effects of stigmasterol on the scopolamine-induced memory dysfunction in the Morris water maze task. Escape latency during the training trial sessions (A), the number of platform crossings (B), the swimming time in the target quadrant (C), and the swimming speed (D) during the probe trial session. Sixty minutes before the first training trial of each session, stigmasterol (10 mg/kg, p.o.), tacrine (THA, 10 mg/kg, p.o.), or the same volume of vehicle (10% Tween 80 solution) was administered to the mice. Memory impairment was induced by administering scopolamine (1 mg/kg, i.p.) 30 min before the first training trial. Training trial and probe trial sessions were performed over 60 s as described in Materials and methods. Data represent means ± S.E.M (n = 8–9 per group) $^{*}P<0.05$, versus the vehicle-treated controls, $^{\#}P<0.05$, versus the scopolamine-treated group.

the acquisition trial, no significant inter-group differences in the step-through latency were observed [$F(6, 51) = 1.983$, $P > 0.05$].

3.2. The effect of stigmasterol on the scopolamine-induced cognitive impairment in the Morris water maze task

We investigated the effect of stigmasterol (10 mg/kg, p.o.) on scopolamine-induced spatial and working memory impairments using the Morris water maze task. A two-way ANOVA revealed that there were significant group effects for days [$F(3, 112) = 21.60$, $P<0.001$] and treatment groups [$F(3, 112) = 48.63$, $P<0.001$]. As shown in Fig. 3A, the escape latencies did not decrease in the scopolamine-treated group (1 mg/kg, i.p.) compared to the vehicle-treated controls. Additionally, the scopolamine-treated group showed similar escape latencies during the four training days, indicating memory impairment. However, stigmasterol plus scopolamine-treated group showed significantly shorter escape latencies compared to the scopolamine-treated group during the training sessions from day 2 through day 4 ($P<0.05$). In addition, the tacrine-treated group (10 mg/kg, p.o.) showed significantly decreased escape latencies compared to the scopolamine-treated group during training trial sessions 3 and 4 ($P<0.05$). Following the last day of the training sessions, the stigmasterol or tacrine plus scopolamine-treated groups significantly increased their swimming time in the target quadrant after the platform was removed compared to the scopolamine-treated group [$F(3, 35) = 6.630$, $P<0.05$] (Fig. 3C). However, no significant differences were observed between groups with respect to swim speeds, which indicates that stigmasterol did not change the locomotor activity of the mice (Fig. 3D).

3.3. The effects of MK-801 or tamoxifen on the memory ameliorating effects of stigmasterol against scopolamine-induced cognitive impairment in the passive avoidance task

To determine whether the ameliorating effect of stigmasterol on scopolamine-induced memory dysfunction was exerted via *N*-methyl-D-aspartate (NMDA) receptor signaling, stigmasterol (10 mg/kg, p.o)-treated mice were co-treated with scopolamine and a sub-effective dose of MK-801 (0.0125 mg/kg, s.c.) to block NMDA receptors. A significant group effect was observed in the step-through latency during the retention trial [$F(5, 54) = 21.116$, $P<0.001$] (Fig. 4A). The reduced latency induced by scopolamine (1 mg/kg, i.p.) was ameliorated by stigmasterol, and MK-801 administration eliminated this effect to the level of the scopolamine-treated group. Moreover, a two-way ANOVA revealed that the interaction between the stigmasterol and MK-801 treatments showed a significant group effect [$F(1, 35) = 6.376$, $P<0.05$]. However, in the acquisition trial, no significant differences in the step-through latency were observed among groups.

Because stigmasterol is reported to have agonistic activity at the estrogen receptor (Newill et al., 2007), we investigated whether tamoxifen, an estrogen receptor antagonist, inhibits the effects of stigmasterol on the step-through latency in the passive avoidance task. A significant group effect was observed for the step-through latency in the retention trial [$F(5, 56) = 20.784$, $P<0.001$] (Fig. 4B). The step-through latency was significantly increased in the stigmasterol (10 mg/kg, p.o.) plus scopolamine (1 mg/kg, i.p.)-treated group compared to the scopolamine-treated group ($P<0.05$, Fig. 4B), and this effect was eliminated to the level of the scopolamine-treated group by co-administration of tamoxifen. Furthermore, a two-way ANOVA revealed that stigmasterol and tamoxifen had significant group interactions [$F(1, 36) = 5.901$, $P<0.05$]. In the acquisition trial, there were no significant differences in the step-through latency among the groups.

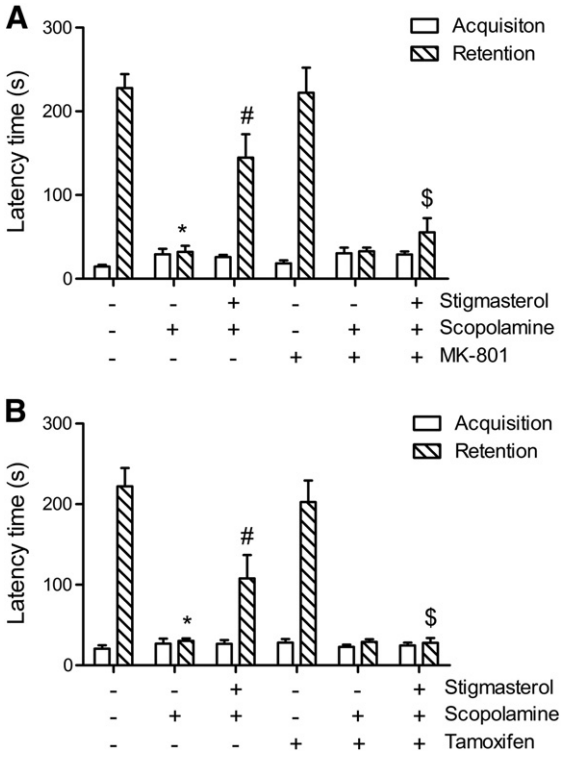

Fig. 4. The effects of stigmasterol on the memory impairment induced by scopolamine via NMDA receptor (A) or estrogen receptor (B). Stigmasterol (10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered 60 min before the acquisition trial. Thirty minutes later, the mice were injected with a sub-effective dose of MK-801 (0.0125 mg/kg, s.c.) or tamoxifen (1 mg/kg, i.p.). Scopolamine (1 mg/kg, i.p.) was administered 5 min after the MK-801 or tamoxifen treatment. The acquisition trial was conducted 25 min later. The retention trial was conducted 24 h after the acquisition trial. Increased passive avoidance latency by stigmasterol was reversed by a sub-effective dose of MK-801, an NMDA receptor antagonist, or tamoxifen, an estrogen receptor antagonist. Data represent means ± S.E.M (n = 9–10 per group) $^{*}P<0.05$, versus the vehicle-treated controls, $^{\#}P<0.05$, versus the scopolamine-treated groups, $^{\$}P<0.05$, versus the stigmasterol plus scopolamine-treated group.

3.4. The effects of stigmasterol on the expression of memory-related molecules including pERK and pCREB expression levels in the hippocampus

To determine the effect of stigmasterol on the levels of ERK and CREB phosphorylation in the hippocampus, Western blot analysis was conducted in normal, naive mice. Mice were sacrificed 1 h after the administration of stigmasterol (2.5, 5, 10, or 20 mg/kg, p.o.). Statistical analysis revealed that a significant group effect of stigmasterol was observed on the level of memory-related proteins in the hippocampus [pERK, $F(4, 15) = 5.053$, $P<0.05$; pCREB, $F(4, 15) = 2.902$, $P<0.05$]. The administration of stigmasterol (10 mg/kg, p.o.) significantly increased the phosphorylation levels of ERK and CREB compared to the levels observed in vehicle-treated controls in the hippocampus ($P<0.05$, Fig. 5A). Furthermore, we also found that the increased expression level of pERK or pCREB induced by stigmasterol (10 mg/kg) was blocked by a sub-effective dose of MK-801 (0.0125 mg/kg) or tamoxifen (1 mg/kg) with co-treatment with scopolamine [pERK, $F(6, 19) = 8.392$, $P<0.05$; pCREB, $F(6, 19) = 12.87$, $P<0.05$, Fig. 5B]. However, a sub-effective dose of MK-801 or tamoxifen did not affect the expression levels of pERK and pCREB in the hippocampus (Fig. 5B).

4. Discussion

In the present study, we observed the anti-amnesic effects of stigmasterol on scopolamine-induced memory impairments in the step-through passive avoidance and the Morris water maze tasks. We

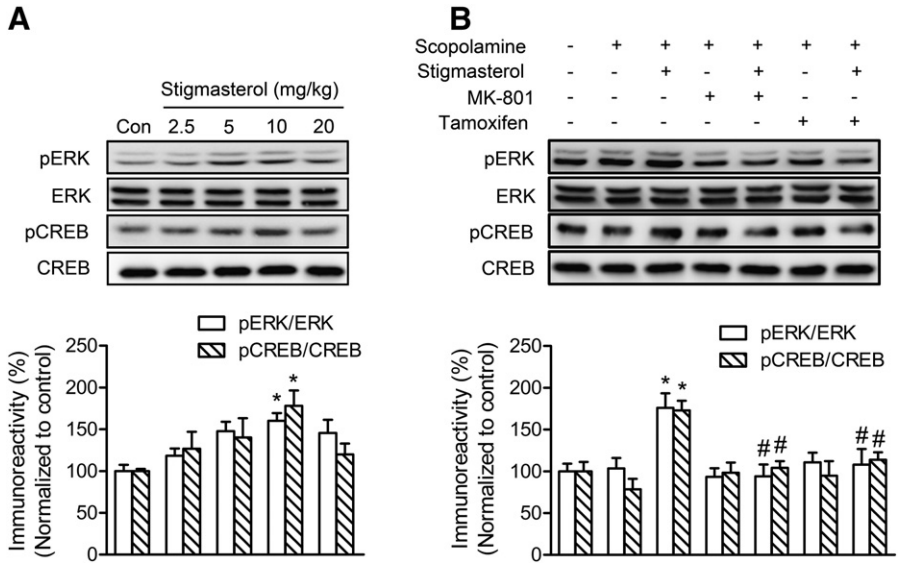

Fig. 5. Effect of stigmasterol alone (A), or with MK-801, tamoxifen and scopolamine (B) on the phosphorylation levels of ERK and CREB in the hippocampus. In the stigmasterol alone experiment (A), mice were administered stigmasterol (2.5, 5, 10, or 20 mg/kg, p.o.), and sacrificed 1 h after the administration. Immunoreactivity and quantitative analysis of pERK or pCREB expression were measured in the hippocampal tissue. In a separate experiment (B), stigmasterol (10 mg/kg) was administered 30 min before MK-801 (0.0125 mg/kg, s.c.) or tamoxifen (1 mg/kg, s.c.) treatment. Scopolamine (1 mg/kg, i.p.) was administered 25 min before sacrificed. Control group (Con) was treated with the same volume of vehicle solution. The administration of stigmasterol (10 mg/kg, p.o.) significantly increased the expression levels of pERK or pCREB in the hippocampus, and this effect was blocked by a sub-effective dose of MK-801 (0.0125 mg/kg, i.p.) or tamoxifen (1 mg/kg, i.p.) with scopolamine (1 mg/kg). Data represent means ± S.E.M. (n = 3–4 per group). $^{*}P<0.05$, versus the vehicle-treated control group; $^{\#}P<0.05$, versus the scopolamine plus stigmasterol-treated group.

also found that the memory ameliorating effect of stigmasterol was blocked by MK-801 and tamoxifen in the passive avoidance task. Furthermore, stigmasterol increased the phosphorylation levels of both ERK and CREB in the hippocampus.

Recently, it has been reported that stigmasterol inhibits AChE activity in vitro (Sultana and Khalid, 2010), suggesting that sitgmasterol might have a cholinomimetic property. These reports suggest that cognitive deficits caused by hypocholinergic neurotransmission could be attenuated by stigmasterol. In the present study, scopolamine-induced memory dysfunction was ameliorated by stigmasterol (10 mg/kg, p.o.) in the passive avoidance and the Morris water maze tasks. These results suggest that the anti-amnesic effects of stigmasterol might be due to the enhancement of cholinergic neurotransmission system. However, we cannot disregard the participation of other neurotransmission system as concentration that showed inhibitory effects on the AChE activity is high (Sultana and Khalid, 2010) and we did not observe any significant changes of AChE activity in ex-vivo experiments (data not shown).

It is well known that learning and memory are closely related to both cholinergic neurotransmission, which includes muscarinic and nicotinic receptors, and glutamatergic neurotransmission, which involves NMDA and AMPA receptors in the central nervous system (Bliss and Collingridge, 1993; Everitt and Robbins, 1997; Whitlock et al., 2006). In particular, the NMDA receptor plays a critical role in synaptic plasticity and long term potentiation (LTP), which have been implicated in learning and memory (Nakanishi, 1992). Activation of the NMDA receptor has been shown to induce various signaling processes, including memory-related pathways (Mattson, 2008). NMDA receptor antagonists, such as dizocilpine (MK-801), phencyclidine, or ketmamine, have been shown to produce learning and memory dysfunction in various experiments (Newcomer and Krystal, 2001; Riedel et al., 2003). Direct and/or indirect interactions between central cholinergic and glutamatergic neurotransmission systems have been shown to be involved in learning and memory processes (Abe et al., 2004; Monteiro Moreira et al., 2005). For example, endogenous acetylcholine has been shown to exert a positive modulatory action on NMDA responses via M1-like muscarinic receptors, which facilitate NMDA receptor-dependent LTP (Calabresi et al., 1998; Markram and Segal, 1990). Moreover, NMDA receptor agonists such as D-cycloserine, a partial agonist of the NMDA receptor-associated glycine site, are known to ameliorate learning and memory deficits induced by hippocampal muscarinic blockade, which is involved with LTP in the brain (Andersen et al., 2002; Burgdorf et al., 2011; Fishkin et al., 1993; Henneberger et al., 2010; Ohno and Watanabe, 1996; Pitkanen et al., 1995). In the present study, the ameliorating effects of stigmasterol on scopolamine-induced memory dysfunction were antagonized by a sub-effective dose of MK-801. Recently, it was also reported that NMDA receptor activation ameliorates cognitive dysfunction via enhancement of ERK-CREB signaling (Al Rahim et al., 2009). We observed that stigmasterol enhanced ERK-CREB signaling in the normal, naive mice, which would promote positive modulatory effects on learning and memory (Sweatt, 2004; Tully et al., 2003). We also found that the increased level of pERK or pCREB expression in the hippocampus was blocked by MK-801 with scopolamine. These results suggest that the ameliorating effects of stigmasterol on learning and memory may be due to the enhancement of glutamatergic and cholinergic signaling cascades.

It has been demonstrated that phytosterols, such as stigmasterol or β-sitosterol, bind to estrogen receptors (Zava et al., 1998), and stigmasterol has been shown to have an antitumor activity similar to 17β-estradiol via the activation of estrogen receptors (Jeon et al., 2005). The administration of estrogen receptor agonists, such as estrogen or 17β-estradiol, has been reported to improve cognitive performance and counteract the amnesic effects of scopolamine in rodents (Abraham et al., 2009; Dohanich et al., 1994; Luine et al., 1998). Recently, we also observed that daidzin or its aglycon daidzein, a type of isoflavone, acts on estrogen receptors to attenuate memory impairment induced by cholinergic dysfunction in male mice (Kim et al., 2010). These findings suggest that stigmasterol may be able to ameliorate memory impairments caused by cholinergic dysfunction through agonistic activity on estrogen receptors. To confirm this hypothesis, we administered the estrogen receptor antagonist tamoxifen to determine whether it blocked the ameliorating effects of stigmasterol on scopolamine-induced memory impairments in the passive avoidance task. Our results demonstrated that the effects of stigmasterol were blocked by tamoxifen. It has been suggested that the activation of estrogen receptors on the cell membranes triggered

the activation of intracellular signaling pathways through ERK, protein kinase A, and CREB signaling (Szego et al., 2006). These results suggest that stigmasterol attenuates cholinergic blockade-induced cognitive dysfunction through its estrogenic properties. In addition, we assumed that stigmasterol could also modulate expression of several genes including brain-derived neurotrophic factor which is involved in cognitive performance, after binding on nuclear estrogen receptors. However, it is unclear whether the increase of pERK or pCREB expression is derived from binding on the cell membranes or nucleus of estrogen receptors. Further studies are needed to clarify these issues.

Interestingly, memory performance in the passive avoidance task and the expression of pERK or pCREB in the hippocampus were not observed at high dose of stigmasterol (20 mg/kg). Several reports have suggested that high concentration of neurotransmitters such as acetylcholine or glutamate would suppress the synaptic transmission by an action at a presynaptic autoreceptor, which results in an inverted U-shaped dose–response curve (Baskys and Malenka, 1991; Calabrese, 2008; Luccini et al., 2007). Although we did not measure the neurotransmitter levels in the synaptic clefts, we suppose that the inverted U-shaped dose–response curve of stigmasterol is due to the activity of presynaptic autoreceptors activated by high concentration of neurotransmitters, such as acetylcholine or glutamate at synaptic cleft.

In summary, we found that stigmasterol ameliorated scopolamine-induced memory impairments in the passive avoidance and the Morris water maze tasks in mice. Moreover, these effects were blocked by MK-801 or tamoxifen in the passive avoidance task. In addition, stigmasterol enhanced ERK-CREB signaling in the hippocampus, and these effects also blocked by MK-801 or tamoxifen. These results suggest that the beneficial effects of stigmasterol are mediated by the enhancement of cholinergic neurotransmission via the activation of NMDA receptor or estrogen receptor signaling.

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