INTRODUCTION

Dementia is a neurodegenerative disease characterized by the loss of intellectual ability, and the dementia patient suffers severe interference with occupational and social performance. With increasing age, the prevalence of dementia increases, and the overall number is increasing worldwide. According to the causes and symptoms, dementia is classified into Alzheimer’s disease (AD), vascular dementia or dementia with Lewy’s bodies (Burns and Iliffe, 2009). In AD, the most prevalent dementia, one of the main pathological hallmarks is severe cholinergic dysfunction, such as decreased choline acetyltransferase (ChAT) and increased acetylcholinesterase (AChE) activities, in the basal forebrain cholinergic neurons (Schliebs and Arendt, 2011). Hence, numerous researchers have investigated ways to increase the cholinergic neurotransmitter system. As a result, AChE inhibitors (AChEIs) were developed for AD therapy. Donepezil, an approved drug targeting AChE inhibition, prevents the decomposition of acetylcholine in the synapse and is prescribed clinically (Tune and Sunderland, 1998; Schneider, 2000). However, these AChEIs have some adverse effects such as diarrhea, insomnia or vomiting. Therefore, it would be necessary to explore new therapeutic agents that increase cognitive function with reduced side effects.

Biflorin Ameliorates Memory Impairments Induced by Cholinergic Blockade in Mice

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Abstract

To examine the effect of biflorin, a component of Syzygium aromaticum, on memory deficit, we introduced a scopolamine-induced cognitive deficit mouse model. A single administration of biflorin increased latency time in the passive avoidance task, ameliorated alternation behavior in the Y-maze, and increased exploration time in the Morris water maze task, indicating the improvement of cognitive behaviors against cholinergic dysfunction. The biflorin-induced reverse of latency in the scopolamine-treated group was attenuated by MK-801, an NMDA receptor antagonist. Biflorin also enhanced cognitive function in a naive mouse model. To understand the mechanism of biflorin for memory amelioration, we performed Western blot. Biflorin increased the activation of protein kinase C-ζ and its downstream signaling molecules in the hippocampus. These results suggest that biflorin ameliorates drug-induced memory impairment by modulation of protein kinase C-ζ signaling in mice, implying that biflorin could function as a possible therapeutic agent for the treatment of cognitive problems.

Key Words: Biflorin, N-methyl D-aspartate receptor, Cognition, Protein kinase C-ζ
does not exert neurotoxicity, it would be a promising candidate for AD therapy. Recently, we observed that biflorin, a quinine compound, exhibits binding affinity to NMDA receptor.

Biflorin is an α-naphthoquione, contained in various herbal materials including Syzygium aromaticum. According to the American Herbal Products Association (AHPA), Syzygium aromaticum is classified as one of Class I herb, which means safe and able to eat (McSuffin et al., 1997). Several reports revealed that biflorin has antioxidant and protective effects against cytotoxicity, genotoxicity, mutagenicity, and intracellular lipid peroxidation (Cai and Wu, 1996; Vasconcellos et al., 2005; Wisintosh et al., 2014). However, the effects of biflorin on cognitive functions remain unknown. Here, we investigated whether biflorin has ameliorating effects on scopolamine-induced memory impairment using the passive avoidance, the Y-maze or the Morris water maze tasks. In addition, we employed an antagonism study and Western blot analysis to investigate the changes in memory-related signaling molecules.

MATERIALS AND METHODS

Animals

ICR male mice (6 weeks old, 25-30 g) were purchased from the Orient Co (branch of the Charles River Laboratories, Gyeonggi, 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 h) at a constant temperature (23 ± 1°C) and relative humidity (60 ± 10%). Animal treatment and maintenance were conducted in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University (Seoul, Korea). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (approval number: KHP-2013-01-04).

Materials

Biflorin was provided by one of the authors (D.S. Jang) and suspended in 10% Tween 80 solution. Donepezil hydrochloride monohydrate, scopolamine hydrobromide, and dizocilpine (MK-801) were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C-ζ (PKC-ζ), phosphorylated PKC-ζ at Thr 410, cAMP response element-binding protein (CREB) at Ser 133, extracellular signal-regulated kinase (ERK), and phosphorylated ERK at Thr202/Tyr204 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against phosphorylated CaMKII at Thr 286 (pCaMKII) was purchased from Abcam (Cambridge, UK). All other materials were obtained from normal commercial sources and were of the highest grade available. All drugs were freshly made on the day of testing. Donepezil, a positive control, and scopolamine were dissolved in a 0.9% saline solution.

Passive avoidance task

For the assessment of the passive avoidance task, male ICR mice were trained (acquisition trial) 24 h prior to the retention trial. The acquisition trial was performed in a box consisting of two identical chambers (20x20x20 cm), one illuminated with a 50 W bulb and another non-illuminated chamber, separated by a guillotine door (5x5 cm), as described elsewhere (Lee et al., 2013). Mice were administered either biflorin (0, 0.1, 0.3, 1, or 3 mg/kg, p.o.), or donepezil (5 mg/kg, p.o.) 1 h before the acquisition trial. The control group received the vehicle solution (10% Tween 80 solution).

Mice were initially placed in the illuminated compartment during the acquisition trial. The door between the two compartments was opened 10 s later. After the mice entered the non-illuminated compartment, the door automatically closed, and a 3-s electrical foot shock (0.5 mA) was delivered through the stainless steel rods. Mice that did not enter the non-illuminated compartment within 60 s after the opening of the door were excluded from the retention trial. The retention trial was conducted 24 h after the acquisition trial by returning individual mice to the illuminated compartment. Scopolamine (1 mg/kg, i.p.) or MK-801 (0.1 mg/kg, i.p.) was administered 30 min after the treatment of biflorin. The time for the mouse to enter the dark compartment after the door opening was defined as latency in both trials. Latencies of up to 300 s were recorded.

In the memory enhancing study, mice were administered only biflorin (0, 0.3, 1, or 3 mg/kg, p.o.) or the same volume of 10% Tween 80 solution 1 h before the acquisition trial. When the mice entered the non-illuminated compartment, a 3-s electrical foot shock (0.25 mA) was delivered through the stainless steel rods to avoid ceiling effects. Latencies of up to 600 s were recorded. Other procedures were the same as described above.

For an antagonism study, biflorin (1 mg/kg) was administered 1 h before the acquisition trial, and a sub-effective dose of MK-801 (0.1 mg/kg, i.p.) (Kim et al., 2009), an NMDA receptor antagonist, was administered 30 min after the administration of biflorin. Scopolamine (1 mg/kg) was administered 5 min after the treatment with MK-801. The acquisition trial was conducted 25 min after the administration of scopolamine. The dose of MK-801 in this study did not impair passive avoidance task performance when administered alone. Other procedures were the same as described above.

Y-maze

The Y-maze is a horizontal maze with three arms (40 cm-long×3 cm-wide×12 cm-high) symmetrically disposed at 120° angles from each other. The maze was constructed of dark opaque polyvinyl plastic, as described elsewhere (Jung et al., 2014). Mice were initially placed within one arm, and the sequence (e.g., ABC, CAB) and number of arm entries were recorded manually for each mouse over an 8 min period. An actual alternation was defined as entry into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). One hour before the test, mice were orally administered either biflorin (0, 0.1, 0.3, 1, or 3 mg/kg) or donepezil (5 mg/kg). The control group received 10% Tween 80 solution rather than biflorin or donepezil. Scopolamine (1 mg/kg) was introduced to induce memory impairment 30 min before the test. Maze arms were thoroughly cleaned with water spray between each test to remove residual odors and residues. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries) minus two multiplied by 100, as shown by the following equation: % Alternation=[(Number of alternations)/(Total arm entries−2)]×100.

Morris water maze

The Morris water maze consists of a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner sur-
face. The pool was filled to a depth of 30 cm with water containing 500 mL of black pigment (24 ± 1°C). The tank was placed in a dimly lit, soundproof test room with visual cues. A black platform (6 cm in diameter and 29 cm high) was then placed in one of the pool quadrants. 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 two trials per session per day with the platform in place. The time interval between each trial per session was 30 min. When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 60 s, it was forced into the platform and placed on it for an additional 10 s. During each trial session, the time taken and distance moved to find the hidden platform (latency time) were recorded using a video camera-based EthoVision System (Noldus, Wageningen, Netherlands). One 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, allowing the mice to swim for 60 s to search for it. A record of the time taken to locate the platform was recorded for 25 min. The horizontal locomotor activity was estimated in the presence of the total ambulatory distance.

Western blot analysis
Mice were sacrificed 1 h after biflorin administration for Western blotting. The vehicle group received 10% Tween 80 solution only. The isolated hippocampal tissues were 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, 1 mM sodium orthovanadate, and one protease inhibitor tablet per 50 ml buffer. The homogenates (15 μg total protein) were then subjected to SDS-PAGE (10% gel) under reducing conditions. The proteins were transferred to PVDF membranes in modified 10 mM Tris-HCl, pH 7.4 using standard techniques. A 10 μg aliquot of membrane was incubated with 4 nM [3H]TCP for 45 min at 25°C. Non-specific binding was estimated in the presence of 1.0 μM MK-801. Membranes were filtered and washed 3 times, and the filters were counted to determine specifically bound [3H]TCP. Biflorin was screened at 100 μM.

Acetylcholinesterase inhibition assay
In our ex-vivo study, mice were administered biflorin (1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) and sacrificed 1 h after each administration (Kim et al., 2007b). Donepezil (5 mg/kg) was used as a positive control. An analysis of AChE activity was conducted using acetylthiocholine iodide substrate in a colorimetric method (Ellman et al., 1961). Whole mouse brain except cerebellum was homogenized in a glass Teflon homogenizer (Eylaa, Tokyo, Japan) including 3.5 ml (10 times of each tissue weight) volumes of homogenizer buffer (0.1 M phosphate buffer, pH 8.0), and then centrifuged at 3000 g for 10 min at 4°C. The supernatant was used as enzyme source for the assay. The supernatant solution was mixed with 144 μl of Buffer A (0.1 M phosphate buffer, pH 8.0), 22 μl of buffered Ellman’s reagent (10 mM 5, 5′-dithiobis [2-nitrobenzoic acid] and 15 mM sodium bicarbonate) and 1.1 μl of acetylthiocholine iodide solution, and then mixed with 4.4 μl of neostigmine solution in 96 well after then incubated at room temperature for 10 min. Absorbance was measured at 412 nm using a UV spectrophotometer (OPTIZEN 2120UV, Mecasys Co., Ltd., Daejeon, Korea).

Radioligand binding assay
In the receptor-binding assay, we found that biflorin only showed binding affinity to NMDA receptors among 27 receptors (AB23234) that are known to be involved in learning and memory processes. NMDA receptor binding studies using [3H]TCP, an antagonist radioligand for NMDA receptor (Cat no. 233000), were performed (Custom Screen by Eurofins Panlab, formerly Ricerca Biosciences, LCC, Seattle, WA, USA). Briefly, CHO cells stably transfected with a plasmid encoding the Wistar rat NMDA receptor were used to prepare membranes in modified 10 mM Tris-HCl, pH 7.4 using standard techniques. A 10 μg aliquot of membrane was incubated with 4 nM [3H]TCP for 45 min at 25°C. Non-specific binding was estimated in the presence of 1.0 μM MK-801. Membranes were filtered and washed 3 times, and the filters were counted to determine specifically bound [3H]TCP. Biflorin was screened at 100 μM.

Statistics
Data are expressed as the mean ± standard error of mean (SEM). Data from behavioral tests were analyzed by one-way analysis of variance (ANOVA) with Tukey’s post hoc comparison or by two-way ANOVA with Bonferroni’s post hoc test. Western blot data were analyzed by one-way ANOVA followed by Tukey’s post hoc analysis for multiple comparisons. Statistical significance was set at p<0.05. All statistical analyses were performed using the Prism 5.0 software (GraphPad, La Jolla, CA, USA).

RESULTS
The effects of biflorin on the cognitive dysfunction induced by scopolamine or cognitive enhancement in the passive avoidance task
The passive avoidance task was performed to evaluate the ameliorating effects of biflorin on the cognitive dysfunction. Significant step-through latency effects were observed between the groups in the retention trial [F (6, 68)=12.53, p<0.05] (Fig. 1A). The reduction in the step-through latency
The effect of biflorin on spontaneous alternation behavior. A significant group effect was observed in spontaneous alternation behavior upon the administration of biflorin [F (6, 69)=8.821, p<0.05]. The percentage of spontaneous alternations in the scopolamine-treated group was significantly lower than in the vehicle-treated control group (p<0.05, Fig. 2A), and the reduction in spontaneous alternation was significantly ameliorated by biflorin (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) (p<0.05, Fig. 2A). However, the mean numbers of arm entries were similar across all experimental groups (Fig. 2B), suggesting that general locomotor activity was not affected by biflorin.

The ameliorating effect of biflorin on scopolamine-induced cognitive impairment in the Morris water maze task

The Morris water maze task was performed to evaluate the effect of biflorin (1 mg/kg, p.o.) on spatial learning and memory. As shown in Fig. 3A, the scopolamine-treated group exhibited longer escape latencies than the vehicle-treated control group throughout the training days. However, the escape latencies of both the biflorin (1 mg/kg, p.o.) and donepezil-treated (5 mg/kg, p.o.) groups were significantly shortened compared with the scopolamine-treated group during training trial sessions 3 and 4 [trial session 3, F (3, 39)=8.348, p<0.05; trial session 4, F (3, 39)=11.65, p<0.05]. On the day after the final day of the training sessions (5th day), the effects on swimming time within...
the target quadrant and the number of crossings of the target zone in the scopolamine-treated group were significantly reversed by biflorin (1 mg/kg, p.o.) or donepezil administration [F (3, 39)=5.834, p<0.05] (Fig. 3B, 3C). However, there were no significant differences in swimming speed across all groups (Fig. 3D).

The effect of biflorin on general locomotor activity in the open field test

Because the stimulatory effect of biflorin on exploratory behavior also affects cognitive behavior, the open field test was performed, and spontaneous locomotor activity was observed. There were no significant changes in total ambulatory distances across all groups (p>0.05, Fig. 4).

The ameliorating effects of biflorin against MK-801-induced cognitive impairment in the passive avoidance task

We conducted a receptor-binding assay to investigate the receptor signaling(s) involved in the cognitive function effects

Fig. 3. Effects of biflorin on scopolamine-induced memory dysfunction in the Morris water maze task. The escape latency time throughout training trial sessions for 4 days (A), the swimming time in the target quadrant (B), the number of crossings (C) in the area where the hidden platform was located, and the swimming speed (D) during the probe trial section on day 5 in the Morris water maze task were measured. The training sessions were conducted for 4 days, and biflorin (1 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween solution) was administered to the mice 60 min before the first training trial of each session. Memory impairment was induced by the administration of scopolamine (1 mg/kg, i.p.) 30 min before the first training trial. The training trial and probe trial sessions were performed over 60 s, as described in Section 2.5. Data represent means ± SEM (n=10/group) (*p<0.05, versus the vehicle-treated controls; #p<0.05, versus the scopolamine-treated group). Con, control.

Fig. 4. Effects of biflorin on locomotor activity in the open field test. The exploratory behaviors of mice in the open field test were observed for 25 min. The mice were administered biflorin (0.3, 1 or 3 mg/kg, p.o.), donepezil (5 mg/kg) or the same volume of vehicle (10% Tween 80 solution) 1 h before the test. Data are expressed as means ± SEM (n=10/group).
of biflorin. Among the 27 targeted receptors associated with learning and memory, biflorin significantly inhibited the binding activity of \(^{3}H\)TCP, a radioligand for NMDA receptor, by approximately 60% at 100 \(\mu\)M. Based on the result that biflorin acts as an NMDA receptor ligand, we had a clue that the effect of biflorin on learning and memory was related to NMDA receptor.

To confirm whether the memory-ameliorating effect of biflorin is mediated via NMDA receptor signaling, a sub-effective dosage of MK-801 (0.1 mg/kg, i.p.) was co-administered with scopolamine in biflorin-treated mice (1 mg/kg), and the passive avoidance task was conducted. The reduced step-through latency induced by scopolamine was significantly reversed by biflorin administration (1 mg/kg) \([F (5, 55)=178.2, p<0.05]\) (Fig. 5). The ameliorating effect of biflorin on scopolamine-induced memory impairment was inhibited by the administration of MK-801, and the step-through latency was similar to the results in the group treated with scopolamine alone. In the acquisition trial, there were no significant differences in step-through latency between the groups.

Fig. 5. The role of NMDA receptor signaling in biflorin-induced cognitive function. To test the antagonistic effect of a sub-effective dose of MK-801 on the effect of biflorin on scopolamine-induced memory impairment, biflorin (1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered 60 min before the acquisition trial, and a sub-effective dose of MK-801 (0.1 mg/kg, i.p.) was given to the mice 30 min later. Scopolamine (1 mg/kg, i.p.) was administered 5 min after MK-801 injections. The retention trial was conducted 24 h after the acquisition trial. Data represent means ± SEM (n=8-10/group) (*\(p<0.05\), versus the vehicle-treated controls; \(^{+}\)\(p<0.05\), versus scopolamine-treated groups; \(^{\#}\)\(p<0.05\), versus biflorin plus scopolamine-treated group). Con, control.

Fig. 6. Effects of biflorin on memory-related proteins in the hippocampus. The mice were administered biflorin (0.3 or 1 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) and sacrificed 1 h after drug administration. The immunoreactivity and quantitative analysis of PKC-\(\zeta\), phosphorylated PKC (pPKC-\(\zeta\)) (A), CaMKII, phosphorylated CaMKII (pCaMKII) (B), ERK, phosphorylated ERK (pERK) (C), CREB, and phosphorylated CREB (pCREB) (D) were measured in the hippocampal tissue. Data represent the means ± SEM (n=3-4/group) (*\(p<0.05\), versus the vehicle-treated controls).
Inhibitory effects of biflorin on acetylcholinesterase (AChE) activity in an ex vivo assay. In mice, biflorin (1 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered 1 h before, and then, the mice were sacrificed. Whole brains were removed and homogenized using Buffer A. AChE activities were determined as described in Materials and Methods. Data represent means ± SEM (n=4/group) (*p<0.05, versus the vehicle-administered control group).

**Fig. 7.** Inhibitory effects of biflorin on acetylcholinesterase (AChE) activity in an ex vivo assay.
biflorin did not exhibit cytotoxic activity in the primary hippocampal culture system, and the IC50 value was over 300 µM.

This evidence suggests that PKC-ζ and CaMKII activation play an important role in learning and memory. The administration of biflorin increased the phosphorylation level of PKC-ζ as well as of CaMKII in the hippocampus. Additionally, biflorin slightly facilitated LTP without affecting basal synaptic transmission (data not shown). In this study, we could not fully examine the exact mechanism by which biflorin activates PKC-ζ and CaMKII signaling; however, we assumed that enhancing the NMDA receptor agonist property of biflorin would induce those signaling cascades.

Several studies have suggested that the activity of ERK1/2, a mitogen-activated protein kinase, is required for the establishment of synaptic activity and the development of several forms of memory. When a neuron is exposed to synaptic activation, the intracellular calcium level is elevated, and several kinases, including PKC, phosphoinositide-3 kinase (PI3K) and ERK1/2 are activated (Sutton and Chandler, 2002; Cohen-Matsliah et al., 2007; Zheng et al., 2009). In addition, CREB, which is located downstream of the signal transduction pathways of cAMP and calcium (Silva et al., 1998), is well established to act as a positive regulator during the cognitive process. The activation of CREB is mediated by several kinases such as CaMKII, ERK or cAMP-dependent PKA (Deisseroth and Tsien, 2002) and depends on its phosphorylation at serine 133 (Gonzalez and Montminy, 1989; Gonzalez et al., 1989). Activated CREB targets the transcription of memory-related genes, such as c-fos, activity-regulated cytoskeleton-associated protein or brain-derived neurotrophin factor, and is considered to enhance memory consolidation or LTP by regulating long-term memory-relevant excitability changes. J. Neurosci. 27, 12584-12589.

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