Administration of Phytoceramide Enhances Memory and Up-regulates the Expression of pCREB and BDNF in Hippocampus of Mice

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Abstract
This study was aimed at investigating the possible effects of phytoceramide (Pcer) on learning and memory and their underlying mechanisms. Phytoceramide was orally administered to ICR mice for 7 days. Memory performances were assessed using the passive avoidance test and Y-maze task. The expressions of phosphorylated cAMP response element binding protein (pCREB), brain-derived neurotrophic factor (BDNF) were measured with immunoblot. The incorporation of 5-bromo-2-deoxyuridine (BrdU) in hippocampal regions was investigated by using immunohistochemical methods. Treatment of Pcer enhanced cognitive performances in the passive avoidance test and Y-maze task. Immunoblotting studies revealed that the phosphorylated CREB and BDNF were significantly increased on hippocampus in the Pcer-treated mice. Immunohistochemical studies showed that the number of immunopositive cells to BrdU was significantly increased in the hippocampal dentate gyrus regions after Pcer-treatment for 7 days. These results suggest that Pcer contribute to enhancing memory and BDNF expression and it could be secondary to the elevation of neurogenesis.

Key Words: Memory, Neurogenesis, Phytoceramide, Y-maze

INTRODUCTION
It has been emphasized the importance of sphingolipids as bioactive molecules that regulate various cellular processes such as proliferation, growth, differentiation, migration, and apoptosis (Posse de Chaves, 2006). In sphingolipid pathway, the phosphorylation of sphingosine by sphingosine kinase generated sphingosin-1-phosphate (S1P). The fatty acyl-CoA is connected to dihydrosphingosine or phytosphingosine (Pso) by amide bond to generate dihydroceramide or phytoceramide (Pcer) (Garcia et al., 2008). Phytosphingosine is the primary sphingoid base which is produced by hydroxylated dihydroceramide at C4 (Garcia et al., 2008). Phytoceramide generated from acylated Pso by many fatty acids and Pcer has the structural backbone with trans double bond at C4 it can be hydrolyzed to Pso by ceramidase (Mao et al., 2001). The possible physiological role of these complicated molecules for neuronal function has been extensively studied. The S1P concentration increasing mostly promotes cell proliferation and survival (Hait et al., 2006; Hannun and Obeid, 2008). An anti-apoptotic and pro-autophagic effects of S1P could modulate the progress of senescence (Patschan et al., 2008), but correctly how S1P functioned in this system was not clear. A direct connection to the sphingolipid metabolism with the neuronal function has not been established, and it is not understood how this lipid metabolites lead to neuronal function. Phytoceramide showed neuroprotective activity in the glutamate-induced neurotoxicity in cultured cells and recovered the scopolamine-induced reduction of memory in the previous research (Jung et al., 2011).

In the present study, we wanted to investigate whether the Pcer demonstrate memory enhancement and modulate the memory-involving signals as well as neurogenesis. To achieve the aim of this study, the memory performance was examined in memory impaired mice by scopolamine treatment using passive avoidance test and Y-maze task. Passive avoidance test is greatly dependent on long-term memory (Ambrogi Lorenzini et al., 1997). And it is well known that learning and memory are closely related to the cholinergic and glutamatergic neu-
rotransmitter systems in brain; (Bartus et al., 1982; Durand et al., 1996). The scopolamine, a muscarinic receptor antagonist, blocked the cholinergic neurotransmitter and damaged learning and memory in mice (Bartus et al., 1982; Renner et al., 2005). The scopolamine-induced amnesic animal models have been used to evaluate for potential drug treatments for cognitive function (Ennaceur and Meliani, 1992). Y-maze task is used for screening working memory, a form of short-term memory (Sarter et al., 1988). In addition, neurogenesis in the hippocampus is also accompanied in learning and memory (Gould et al., 1999; Shors et al., 2001).

Immature neurons in hippocampus are accepted to play a role in the composition of new synaptic connections which are required for memory consolidation (van Praag et al., 2002). And adult hippocampal neurogenesis is involved in memory and affected by many factors which are connected with either memory enhancement or memory formation (Gould et al., 1999; Dupret et al., 2008). In addition, many previous researches demonstrated that neurotrophic factors, including BDNF, are critically demanded for neuronal survival during development and neurogenesis (Goldberg and Barres, 2000). Collectively, it indicates that BDNF signaling and neurogenesis indicate a mechanism of memory enhancement. In this study, we investigated whether Pcer affects hippocampal neurogenesis and hippocampus-dependent learning and memory performance in mice.

MATERIALS AND METHODS

Animals
Male ICR mice (28-30 g) were purchased from Orient Laboratory Animal (Seoul, Korea). Mice allowed access to water and food ad libitum were grouped 5-6 per cage, and maintained at an ambient temperature of 23°C and a 12 h diurnal light cycle (light on 07:00-19:00). All behavioral experiments were carried out in a room adjacent to that in which the mice were housed under the same conditions of temperature and light cycle. All the experiments were carried out according to the guidelines of the Animal Care and Use Guidelines of School of Medicine, Ewha Womans University, Korea.

Passive avoidance test
Passive avoidance test was carried out in identical light and dark boxes (Gemini Avoidance System, San Diego, CA, USA). The light compartment (20×20×20 cm) contained a 100 W bulb, and the floor of dark compartment (20×20×20 cm) was composed of 2 mm grid stainless steel bars spaced 1 cm apart. These compartments were separated by a small guillotine door (5×5 cm). One hour before the acquisition trial, mice were administered Pcer (5-50 mg/kg, p.o.). Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.) 30 min after the administration of Pcer. For the acquisition trial, mice were initially placed in the light compartment and the door between the two compartments was opened 10 s later. When mice entered the dark compartment, the door closed and the mouse stepped down onto the grid floor, it received an electrical foot shock (0.25 mA, 2.5 sec). After twenty four hours, the mice were again placed in the light compartment for the retention trials. The time taken for a mouse to enter the dark compartment was measured after door opening as latency times in both acquisition and retention trials. If a mouse did not enter the dark compartment within 300 sec, it was assumed that the mouse had remembered the single training trial.

Y-Maze task
The Y-maze is a three-arm triangle-shaped maze (40 cm-long and 3 cm-wide with 12 cm-high walls) in which the arms separated by 120° angles each other. The maze floor and walls were constructed from black polyvinyl plastic. Mice were initially placed on the central platform, and the sequence (i.e., ABCAB, etc.) and number of arm entries were recorded for each mouse with all four paws over 8-min period. An actual alternation was calculated as entries into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). Maze arms were thoroughly cleaned between tasks to remove residual odors. One hour after the last administration of Pcer or vehicle alone, memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). The percentage of alternations was defined according to the following equation: % alternation=[(number of alternations)/(total arm entries-2)] ×100. The number of arm entries served as an indicator of locomotor activity.

Bromodeoxyuridine (BrdU) administration and immuno-histochemistry
To evaluate the effect of cell proliferation, the thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) was injected intraperitoneally (50 mg/kg, dissolved in PBS) twice daily with a 2 h interval after the last drug administration. The animals were sacrificed 16 h after the last injection. All procedures were based on information from Cameron and McKay (2001). The animals were anesthetized and perfused first with ice-cold PBS followed by a ice cold 4% paraformaldehyde (PFA) solution (in 0.1 M phosphate buffer, pH 7.4). The brains were removed and postfixed in the same fixation solution for 24 h at 4°C. Following post fixation, brains were overnight in 30% sucrose solution (in 0.1 M PBS) at 4°C until they sank. For immunohistochemical staining of BrdU, frozen brains were coronally sectioned on a microtome at 30 μm-thick between 1.70 and 1.94 mm posterior to the bregma as defined in mouse brain atlas for each brain and then stored in storage solution (30% ethylene glycol and 30% glycerin in a 0.02 M phosphate buffer) at 4°C. Free-floating brain sections were incubated in 0.3% H2O2 at room temperature to eliminate endogenous peroxidase for 15 min. Then they were rinsed with PBS followed by 0.3% Triton-X for 15 min. To detect BrdU labeled cell, free-floating sections were rinsed with PBS, and to denature DNA, sections were incubated in 2N HCl at 37°C for 30 min. Sections were neutralized by incubating them for 15 min in PBS. Then the sections were incubated with rat anti-BrdU antibody (1:1,000, Abcam, USA) overnight at 4°C. After rinsed with PBS, the sections were incubated with biotinylated anti-rat IgG second antibody (1:200, Vector, CA, USA), and then with ABC complex (1:100, Vector, CA, USA) for 1 h at room temperature. BrdU-labeled cells were visualized with nickel ammonium sulfate in DAB solution for about 3 min. The number of BrdU-positive cells in the hippocampal granular layer of the DG was counted in four slides per mouse using a computerized image analysis system (Leica Microsystems AG, Wetzlar, Germany) and results were averaged.