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6-Shogaol, an active constituent of ginger, attenuates neuroinflammation and cognitive deficits in animal models of dementia

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ABSTRACT

Recently, increased attention has been directed towards medicinal extracts as potential new drug candidates for dementia. Ginger has long been used as an important ingredient in cooking and traditional herbal medicine. In particular, ginger has been known to have disease-modifying effects in Alzheimer's disease (AD). However, there is no evidence of which constituents of ginger exhibit therapeutic effects against AD. A growing number of experimental studies suggest that 6-shogaol, a bioactive component of ginger, may play an important role as a memory-enhancing and anti-oxidant agent against neurological diseases. 6-Shogaol has also recently been shown to have anti-neuroinflammatory effects in lipopolysaccharide (LPS)-treated astrocytes and animal models of Parkinson's disease, LPS-induced inflammation and transient global ischemia. However, it is still unknown whether 6-shogaol has anti-inflammatory effects against oligomeric forms of the Aβ (AβO) in animal brains. Furthermore, the effects of 6-shogaol against memory impairment in dementia models are also yet to be investigated. In this study, we found that administration of 6-shogaol significantly reduced microgliosis and astrogliosis in intrahippocampal AβO-injected mice, ameliorated AβO and scopolamine-induced memory impairment, and elevated NGF levels and pre- and post-synaptic marker in the hippocampus. All these results suggest that 6-shogaol may play a role in inhibiting glial cell activation and reducing memory impairment in animal models of dementia.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by cognitive dysfunction, neuronal loss and neuroinflammation [1]. The major pathological features in AD are thought to be primarily mediated by amyloid beta (Aβ) plaques [1]. In addition, it has been also shown that oligomeric forms of the Aβ (AβO) are mainly responsible for AD-related pathology [2]. Some recent studies focus on natural products in an effort to find new AD drug candidates [3]. It has been suggested that natural compounds and their derivatives have clinical efficacy, or at least, promising clinical evidence [4]. Therefore, searching for a potential dementia drug among natural compounds is an attractive strategy.

Ginger (*Zingiber officinale*) is worldwide consumed as a flavoring agent and a spice. Several studies have shown that ginger has disease-modifying effects in AD [5–9] and improves memory function in animal models of cognitive disorder [10–12]. Ginger is known to have a number of different components, including diarylheptanoids, paradols, gingerols and shogaols [13]. Therefore, further study is necessary to identify the potential active constituent in ginger for AD treatment and memory-enhancing effects. It has been known that 6-shogaol is one of the major active components in ginger [14,15]. In particular, 6-shogaol was shown to protect endothelial cells from Aβ-induced damage [16]. A huge number of studies have demonstrated that 6-shogaol has a strong ability to inhibit inflammation [17–24]. The 6-shogaol has been also reported to be the most potent anti-inflammatory and anti-oxidant

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component in ginger [25]. Furthermore, it has been demonstrated that 6-shogaol exerts anti-neuroinflammatory effects by inhibiting glial cell activation and proinflammatory molecule production in both lipopolysaccharide (LPS)-treated astrocytes and animal models of different pathologies such as Parkinson's disease, LPS-induced inflammation, and transient global ischemia [26–28]. However, there is no evidence that 6-shogaol also has an anti-inflammatory or memory protective effects in an animal model of AD. In this present study, we investigated the effects of 6-shogaol on glial activation induced by AβO and memory loss mediated by AβO and scopolamine.

2. Materials

2.1. Materials and reagents

6-Shogaol was purchased from Wako Pure Chemical (Osaka, Japan). Rabbit monoclonal anti-gilal fibrillary acid protein (GFAP) was purchased from Millipore Bioscience Research (Bedford, MA, USA). Rat monoclonal anti-CD11b (Mac-1) was purchased from Chemicon International (Temecula, CA, USA). Biotinylated goat anti-rabbit antibody, biotinylated goat anti-rat antibody, normal goat serum, avidin–biotin complex were purchased from Vector Laboratories (Burlingame, CA, USA). Paraformaldehyde, 3,3-diaminobenzidine (DAB), sodium chloride, sucrose, ethanol, phosphate buffer (PB), and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Donepezil hydrochloride was supplied by Eisai Korea Co. Ltd. (Aricept®; Seoul, Korea). $A\beta_{1-42}$ peptide was purchased from American Peptide (Sunnyvale, CA, USA).

2.2. Preparation of $A\beta O_{1-42}$ solution

Soluble oligomers were produced using the previously described method with minor modifications [29]. In short, at room temperature for 3 days, $A\beta_{1-42}$ was diluted in 1,1,1,3,3,3-hexafluoro-2-propanol to a final concentration of 1 mg/mL. An aliquot of the peptide was dried under vacuum for 1 h. It was then dissolved into DMSO to a final concentration of 1 mM. To determine the protein concentration, Bradford assay was performed. The $A\beta_{1-42}$ in DMSO was directly diluted into PBS to 10 μM. Then, it was stored at 4 °C for 24 h resulting in $A\beta O_{1-42}$.

2.3. Animals and surgery procedure

The injection of Aβ peptide into rodents' brain has been well known to be a efficient and useful tool to examine whether some agents act at earlier stages of AD pathogenesis [30]. Male ICR mice (27–30 g, 8 weeks) were bought from the Daehan Biolink Co. Ltd. All animal procedures were conducted in accordance with National Institutes of Health guidelines and the Animal Care and Use Guidelines of Kyung Hee University, Seoul, Korea. Mice were anesthetized by a mixture of Rompun® and Zoletil 50® solution (1:3 ratio, 1 mL/kg, i.p.,) and then mounted on a stereotaxic apparatus (myNeuroLab, St. Louis, MO, USA). The $A\beta O_{1-42}$ (10 μM, 3 μL) was injected unilaterally (at a rate of 0.5 μL/min) into the hilus of dentate gyrus (DG) in hippocampus of each mouse (bregma coordinates in mm: AP-2.0, ML 1.5, DV 2.0), according to the stereotaxic atlas of mouse brain [31]. For control mice, the same amount of saline was injected using the same method.

2.4. Drug administration

The total of 24 mice were randomly divided into 4 different groups (6 mice per group); (1) Sham group (sham-operation with oral administration of saline), (2) $A\beta O_{1-42}$ group ($A\beta O_{1-42}$-lesion with oral administration of saline), (3) $A\beta O_{1-42}$ + donepezil 2 mg/kg/day group ($A\beta O_{1-42}$-lesion with oral administration of donepezil 2 mg/kg/day), (4) $A\beta O_{1-42}$ + 6-shogaol 10 mg/kg/day group ($A\beta O_{1-42}$-lesion with oral administration of 6-shogaol 10 mg/kg/day). The dose of 6-shogaol administered in this experiment, 10 mg/kg/day, was determined according to a previous study demonstrating the anti-inflammatory effect of 6-shogaol in mice [26]. 6-Shogaol diluted in 10% DMSO was administered once daily for 5 consecutive days after the stereotaxic injection of $A\beta O_{1-42}$. Since donepezil is known to significantly inhibit AβO-induced inflammation and memory loss [32], we used donepezil as a positive control agent.

2.5. Step-through passive avoidance test

Learning and memory function was assessed using a two-compartment step-through passive avoidance apparatus. The bright compartment was shined by a 50 W electric lamp. Either donepezil or vehicle was administered to mice one hour before an acquisition trial. Then, they were placed in the bright compartment. 10 s later, the guillotine door was opened. As soon as the hind legs of a mouse entered the dark room, the door was closed, and electrical foot shock (0.6 mA) was simultaneously delivered throughout the grid floor for 3 s. 24 h after this acquisition trial, the mouse was again placed in the bright room for the retention trial. The latency time was defined as the time it takes for the mouse to enter the dark chamber after the door opening. The latency time was recorded for up to 300 s.

2.6. Novel object recognition test

The tests were performed in a black open field test box (45 cm × 45 cm × 50 cm). All animals had habituation period for 5 min without objects. After a habituation period, animals were placed into the test box with two identical objects and allowed to search for 3 min. The time spent by the mice exploring each object was recorded (defined as the training session). After a 24-h delay, animals were allowed to explore the objects for 3 min, in which original object used in the training session and a novel object. The time spent by the mice exploring the novel and the familiar objects were measured (defined as the test session). The mice were regarded to be exploring when they were facing, sniffing or biting the object.

2.7. Immunohistochemistry and quantification

The free floating sections were rinsed in PBS and treated with 1% hydrogen peroxide for 15 min to remove endogenous peroxidase activity. Then, they were incubated with a rabbit anti-GFAP antibody (1:3000 dilution) and a rat anti-Mac-1 (1:1000 dilution) overnight at 4 °C in the presence of normal goat serum and 0.3% triton X-100. Next, they were subjected to another incubation with biotinylated anti-rabbit or anti-rat (all 1:200 dilution) for 90 min, followed by incubation in avidin–biotin complex (1:100 dilution) for 1 h at room temperature. Peroxidase activity was visualized by DAB in 0.05 M tris-buffered saline (pH 7.6). The images were captured at 200× magnification in an optical light microscope to quantify the immunoreactivity of GFAP and Mac-1 in the dentate gyrus (DG). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the average area fractions of GFAP and Mac-1-stained regions. Selection of GFAP and Mac-1-positive area was conducted by manual threshold adjustment. The fraction of immunoreactive region was calculated as the selected area divided by captured total area. The data were expressed as percentages of the value compared to the vehicle-treated sham group.

2.8. NGF ELISA

The mouse brain hippocampal tissue was homogenized in ice-cold homogenization buffer and then homogenates were centrifuged. The samples were incubated with sheep anti-mouse NGF polyclonal antibody pre-coated for overnight. The plate was washed at four times and incubated with an anti-mouse NGF monoclonal antibody (1:100 dilution) for 2 h at room temperature. After the plate was washed, the plate was incubated with a donkey anti-mouse IgG polyclonal antibody (1:1000 dilution) for 2 h at room temperature, and then it was incubated with TMB/E substrate for 10 min and added stop solution to each well. The resulting plate was read at 450 nm by using spectrophotometer (Versamax microplate reader, Molecular Device; Sunnyvale, CA, USA) and concentrations of NGF were determined in the sample using a NGF standard calibration curve.

2.9. Western blotting

Hippocampal tissues of sacrificed mice were lysed with a triple-detergent lysis buffer to detect synaptophysin and PSD95. The lysates were separated by 12% SDS–polyacrylamide gel electrophoresis and gels were processed for antigens and blotted onto Immobilon®-P Transfer Membranes (Millipore, Bedford, MA, USA) for 1 h 30 min. Membranes were incubated with 5% skim milk in TBST for 1 h and then with the primary antibodies (1:500 dilution of SYN; 1:2000 dilution of PSD95 and β-actin) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 1 h. Blots were detected using an ECL detection kit and a LAS-4000 mini system (Fujifilm Corp., Tokyo, Japan) was used for visualization.

2.10. Statistical analysis

Graphpad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to calculate all statistical parameters. Values were described as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) following with Turkey's *post hoc* test were used to analyze the data. Only the differences with p values less than 0.05 were considered to have statistical significance.

3. Results

To examine the effect of 6-shogaol on glial cell activation *in vivo*, we administered 6-shogaol to mice with intra-hippocampal

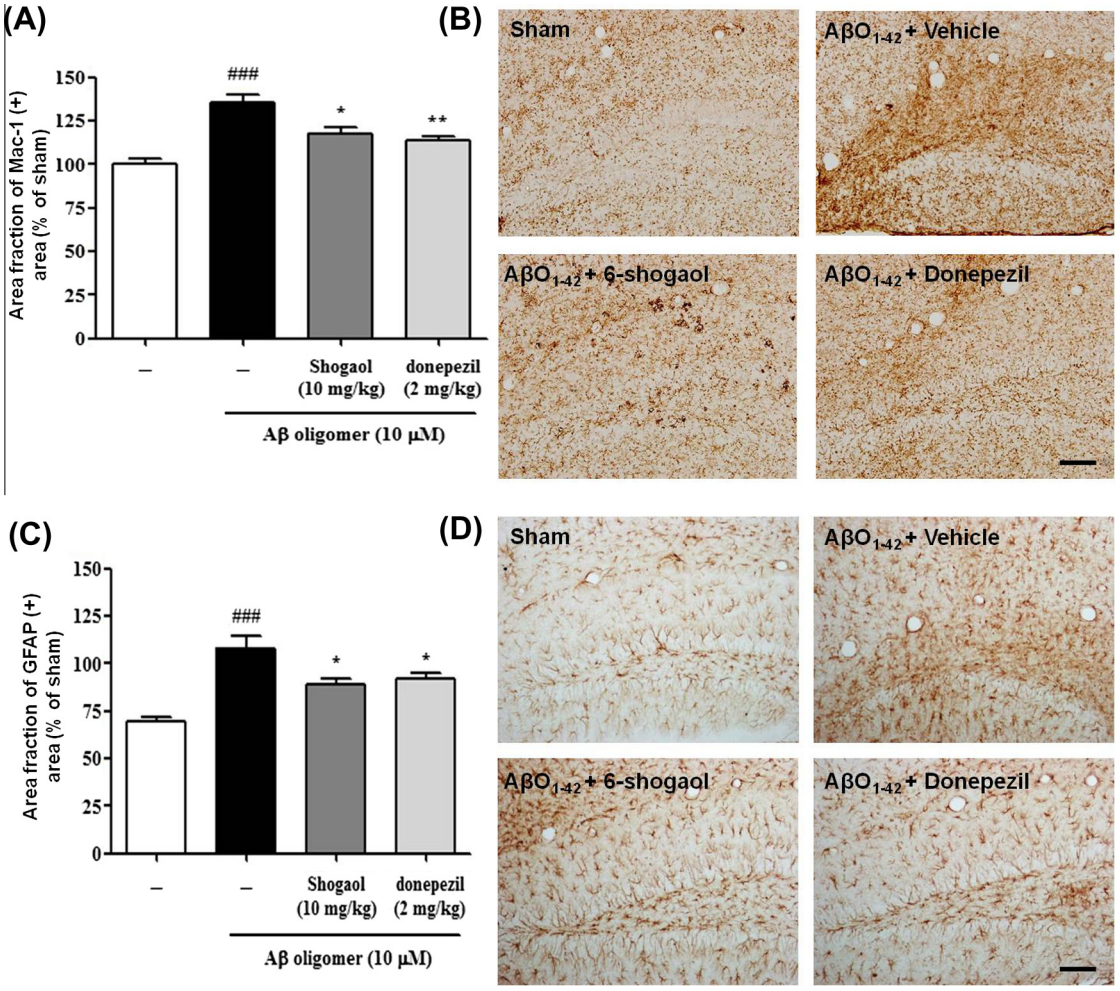

Fig. 1. Inhibitory effect of 6-shogaol on glial activation induced by $A\beta O_{1-42}$ in the mouse brain. After stereotaxic injection of $A\beta O_{1-42}$ (10 μM, 3 μL), mice were orally administered with vehicle, donepezil (2 mg/kg/day), or 6-shogaol (10 mg/kg/day) for 5 consecutive days. Activation of microglia and astrocyte was visualized by Mac-1 (B) and GFAP (D) staining. Quantification analysis of the Mac-1 (A) and GFAP (C) -stained cells was conducted by measuring the fractions of Mac-1 and GFAP-immunoreactive areas in the DG. Scale bar = 50 μM. Values are expressed as mean ± SEM. $^{###}p < 0.001$ indicates that mean value was significantly different from the sham group. $^{**}p < 0.01$ and $^{*}p < 0.05$ indicate that mean value was significantly different from the $A\beta O_{1-42}$-only treated group.

injection of $A\beta O_{1-42}$, and performed the immunohistochemical analysis. The percentage of the Mac-1-positive area, the degree of microgliosis, in DG exhibited a significant increase in the $A\beta O_{1-42}$-injected group vs. the sham group. However, with the treatment of 6-shogaol, the Mac-1-positive area was significantly decreased in the $A\beta O_{1-42}$-injected group vs. the sham group (Fig. 1A and B). These results indicate that 6-shogaol has the microglial deactivating effect *in vivo*. Since the 6-shogaol is known to suppress the inflammatory responses in LPS-treated astrocytes *in vitro* [28], we examined the effect of 6-shogaol on astrogliosis triggered by AβO in the brain using immunohistochemistry for GFAP. The quantification data demonstrated the GFAP-positive area was significantly increased in the $A\beta O_{1-42}$-injected group vs. the control group, while 6-shogaol treatment decreased the area fraction of GFAP-positive areas in the DG of hippocampus (Fig. 1C and D). Thus, our data indicate that 6-shogoal represses the activation of microglial cells and astrocytes induced by $A\beta O_{1-42}$ *in vivo*.

To investigate the effects of 6-shogaol on cognitive function in mice with intra-hippocampal injection of $A\beta O_{1-42}$, a passive avoidance test was performed. The $A\beta O_{1-42}$-injected group showed significantly reduced retention time, while 6-shogaol treatment group exhibited a significant recovery (Fig. 2). Passive avoidance latency during the acquisition trial showed no difference among all the groups. Therefore, 6-shogaol improved memory function in mice with $A\beta O_{1-42}$-lesion, suggesting that 6-shogaol-mediated inhibition of glial activation in the AβO-injected brain seems to be, in part, associated with recovery of cognitive dysfunction.

To confirm the protective effect of 6-shogaol on memory loss, we used a scopolamine model of dementia. The scopolamine-injected group showed significantly reduced retention time, while 6-shogaol treatment groups exhibited a significant recovery (Fig. 3A). It has not been examined whether 6-shogaol has a memory-enhancing effect under normal condition. Compared with control mice, 6-shogaol-treated mice spent more time exploring the novel object during the test session (Fig. 3B). For the first time, we found that administration of 6-shogaol can improve the cognition in normal experimental animals in novel object recognition test.

It is demonstrated that 6-shogaol increases production of NGF, which plays critical roles in accelerating neurite outgrowth and enhancing cognitive function [33,34]. To elucidate the mechanism by which 6-shogaol enhance cognitive functions under normal condition, we examined whether the 6-shogaol administration can affect the expression of NGF and synaptic molecules in the hippocampus, which are related with memory process in the brain. We performed NGF ELISA analyses and western blot analysis for PSD-95 and synaptophysin and using the brain hippocampal tissues. Data from ELISA and western blotting showed that 6-shogaol treatment significantly elevated the expression level of NGF, PSD-95 and synaptophysin in the hippocampal tissues, compared with vehicle-treated group (Fig. 4).

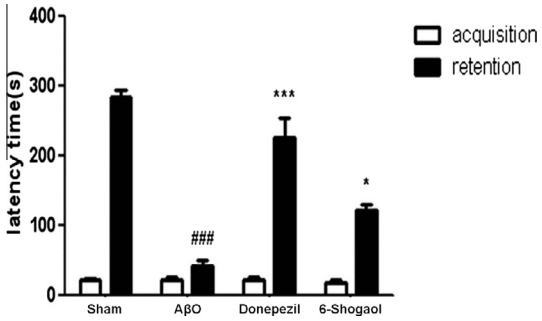

Fig. 2. Effect of 6-shogaol on memory impairment in an AD mouse model with intrahippocampal injection of $A\beta O_{1-42}$. After stereotaxic injection of $A\beta O_{1-42}$ (10 μM, 3 μL), mice were orally administered with vehicle, donepezil (2 mg/kg/day), or 6-shogaol (10 mg/kg/day) for 5 consecutive days. Passive avoidance test was conducted at 5 (acquisition) and 6 (retention) days after $A\beta O_{1-42}$ injection. Values are expressed as mean ± SEM. $^{###}p < 0.001$ indicates that mean value was significantly different from the sham group. $^{***}p < 0.001$ and $^{*}p < 0.05$ indicates that mean value was significantly different from the $A\beta O_{1-42}$-only treated group.

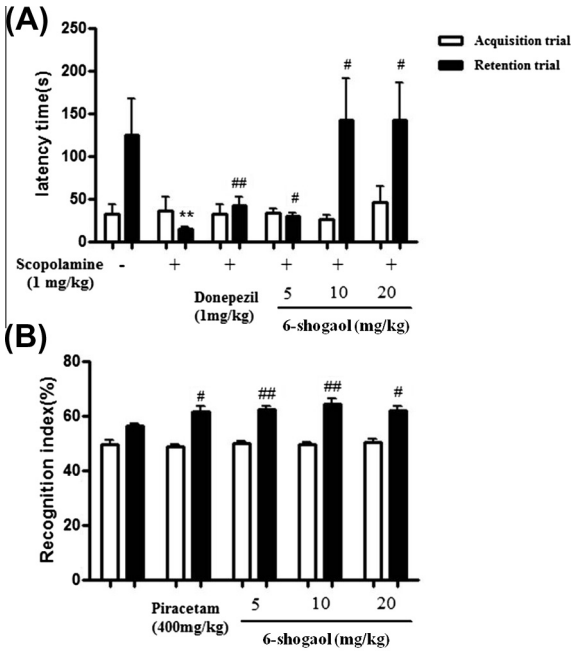

Fig. 3. (A) Effects of 6-shogaol on the scopolamine-induced memory impairment. Scopolamine-induced memory impairment was determined by passive avoidance test. Mice were treated with 6-shogaol and donepezil at the concentration of 5, 10, and 20 mg/kg/day and 1 mg/kg, respectively. Values are expressed as the mean ± S.E.M. $^{**}p < 0.01$ as compared to the vehicle-treated control group. $^{#}p < 0.05$ and $^{##}p < 0.01$ as compared to the scopolamine-treated group during retention session. (B) Direct effects of 6-shogaol on cognitive function. Mice were treated with 6-shogaol at 5, 10, and 20 mg/kg/day and piracetam (positive control) at 400 mg/kg/day. White bar (□) and black bar (■) indicate the percentage of novel object recognition time during training and test session, respectively. Values are expressed as the mean ± S.E.M. $^{#}p < 0.05$, $^{##}p < 0.01$ and $^{###}p < 0.001$ as compared to the vehicle-treated control group in test session.

4. Discussion

Present study demonstrated that 6-shogaol significantly inhibits its $A\beta O_{1-42}$-induced activation of microglial cells and astrocytes. The 6-shogaol could ameliorate memory deficit mediated by intrahippocampal injection of $A\beta O_{1-42}$ and scopolamine administration. In addition, the 6-shogaol treatment significantly enhanced the expression of NGF and synaptic molecules in the brain. These findings suggest that 6-shogaol, a major bioactive constituent of ginger, might reduce cognitive dysfunction by inhibiting inflammatory responses, up-regulating NGF level, and enhancing synaptogenesis in the brain with AD.

We found that oral administration of 10 mg/kg 6-shogaol significantly ameliorates the inflammation triggered by $A\beta O_{1-42}$ in the hippocampus of mice (Figs. 2 and 3). Numerous studies have revealed that 6-shogaol may be a potent inhibitor of inflammation [23]. In macrophages, 6-shogaol could result in suppression of gene expression of inflammatory iNOS and COX-2 [21,24]. The 6-shogaol was also reported to exert a strong anti-inflammatory effect in an experimental model for gouty arthritis [22]. Moreover, it was shown that 6-shogaol could attenuate LPS-elicited increase of prostaglandin E2 (PGE2) in murine macrophages (RAW 264.7)

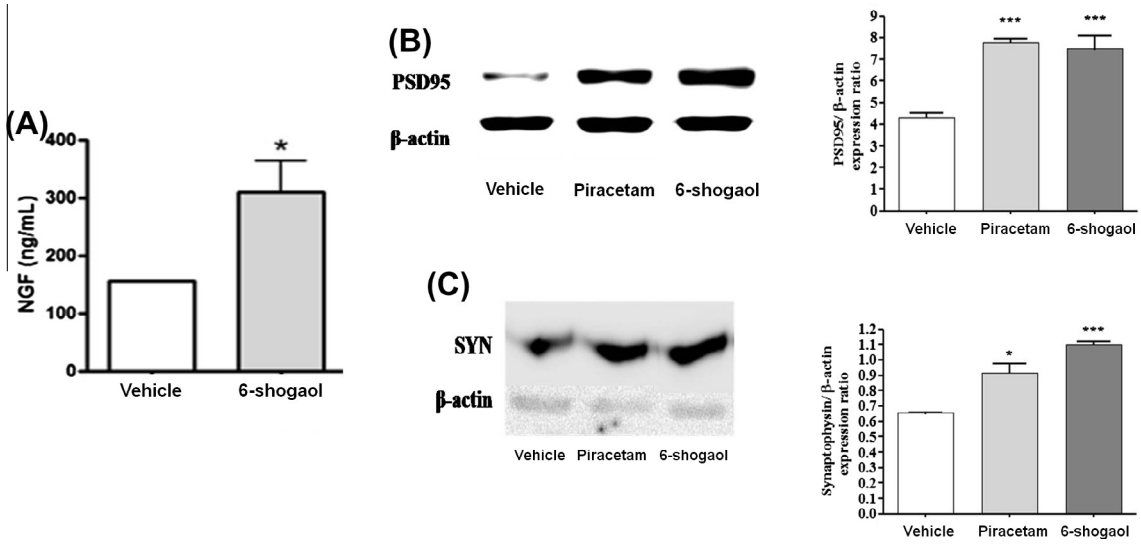

Fig. 4. Effects of 6-shogaol on NGF production and pre- and post-synaptic density in the mouse hippocampus. ICR mice were administrated with 6-shogaol at the concentration of 10 mg/kg per 5 days. (A) Expression of NGF was assessed using the NGF sandwich enzyme-linked immunosorbent assay. Expression of synaptophysin (SYN) and PSD-95 was assessed by western blot analysis using PSD-95 (B) and SYN (C) and antibodies. Values are expressed as the mean ± S.E.M. $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared to the vehicle-treated group.

[18]. 6-Shogaol also reduced mast cell-mediated allergic responses by inhibiting the production of proinflammatory mediators with the involvement of modulation of NF-κB and phosphorylation of JNK [19]. Additionally, 6-shogaol was shown to have much stronger inhibitory effects on nitric oxide synthesis and arachidonic acid release than 6-gingerol in LPS-stimulated RAW 264.7 cells [20]. Furthermore, 6-shogaol was found to exhibit more potent anti-inflammatory properties than other ingredients in ginger, such as 6-gingerol, 8-gingerol, and 10-gingerol [25]. In particular, 6-shogaol has the ability to suppress the neuroinflammation: attenuating a variety of neuroinflammatory responses in LPS-treated astrocytes; inhibiting the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced microglial activation and increases in the levels of TNF-α, NO, iNOS, and COX-2; reducing the microglial activation mediated by LPS and ischemic injury [26–28]. However, to date, it is unknown whether 6-shogaol administration can ameliorate inflammation mediated by $A\beta O_{1-42}$ in the brain. Our data for the first time suggest that 6-shogaol might reduce the neuroinflammation in an animal model of AD.

The pro-inflammatory substances have been known to act as suppressor of induction of long-term potentiation (LTP) [35]. Moreover, it is suggested that systemic inflammation in patients with AD results in increased production of proinflammatory molecules that speed up memory deficits [36]. In addition, previous studies have shown that soluble AβO may cause memory deficits by inducing glial activation [30,37]. Based on these findings, we hypothesized that anti-inflammatory compound 6-shogaol would inhibit cognitive decline associated with reactive gliosis triggered by AβO. Our findings in the present study show that inhibition of the inflammatory response by 6-shogaol administration is accompanied by memory improvement, suggesting that ameliorating the activation of glial cells may contribute to memory improvement in AD.

Ginger has been very well known to be a memory-enhancing agent [12]. However, there has been no evidence of which ingredients of ginger exhibit memory-enhancing effects. We found that 6-shogaol administration significantly enhanced learning and memory in both memory-impaired and normal mice (Figs. 2 and 3). Therefore, this is the first study to show the effect of 6-shogaol on cognitive function. Our data suggests that 6-shogaol seems to directly contribute to ginger-mediated enhancement of cognitive function. Our data suggests that 6-shogaol seems to, in part, contribute to ginger-mediated enhancement of cognitive function.

In summary, our study first demonstrates the efficacy of 6-shogaol on inhibiting AβO-mediated inflammation and cognitive decline in mouse models of dementia. All these findings suggest that 6-shogaol can be a good therapeutic candidate in AD due to its anti-inflammatory and memory-enhancing properties.

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