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Theracurmin Ameliorates Cognitive Dysfunctions in 5XFAD Mice by Improving Synaptic Function and Mitigating Oxidative Stress

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Abstract

As the elderly population is increasing, Alzheimer's disease (AD) has become a global issue and many clinical trials have been conducted to evaluate treatments for AD. As these clinical trials have been conducted and have failed, the development of new theraphies for AD with fewer adverse effects remains a challenge. In this study, we examined the effects of Theracurmin on cognitive decline using 5XFAD mice, an AD mouse model. Theracurmin is more bioavailable form of curcumin, generated with submicron colloidal dispersion. Mice were treated with Theracurmin (100, 300 and 1,000 mg/kg) for 12 weeks and were subjected to the novel object recognition test and the Barnes maze test. Theracurmin-treated mice showed significant amelioration in recognition and spatial memories compared those of the vehicle-treated controls. In addition, the antioxidant activities of Theracurmin were investigated by measuring the superoxide dismutase (SOD) activity, malondialdehyde (MDA) and glutathione (GSH) levels. The increased MDA level and decreased SOD and GSH levels in the vehicle-treated 5XFAD mice were significantly reversed by the administration of Theracurmin. Moreover, we observed that Theracurmin administration elevated the expression levels of synaptic components, including synaptophysin and post synaptic density protein 95, and decreased the expression levels of ionized calcium-binding adapter molecule 1 (Iba-1), a marker of activated microglia. These results suggest that Theracurmin ameliorates cognitive function by increasing the expression of synaptic components and by preventing neuronal cell damage from oxidative stress or from the activation of microglia. Thus, Theracurmin would be useful for treating the cognitive dysfunctions observed in AD.

Key Words: Theracurmin, Alzheimer's disease, Recognition memory, Spatial memory, Antioxidative activity, Synaptic component

INTRODUCTION

Epidemiological studies have indicated that the life expectancy of human being is increasing, and therefore, age-related diseases have become of great interest (Brown, 2015; Kingston *et al*., 2017). Dementia is a degenerative age-related disease, and 7.7 million people are newly diagnosed with dementia every year (Gulland, 2012). Alzheimer's disease (AD) is one of the most prevalent types of dementia and is characterized by the deficit of cognitive function caused by the accumulation of amyloid β (Aβ) protein plaques. Many clinical trials have been conducted to attempt to clear Aβ proteins from brain tissues, but unfortunately, positive outcomes have not been obtained (Karran and De Strooper, 2016). In the clinic, therefore, acetylcholinesterase (AChE) inhibitors, including donepezil or noncompetitive NMDA receptor antagonists, such as memantine, are still prescribed to treat AD. Despite significant improvements in the pharmacotherapy for AD, patients experience several drug-associated adverse effects, such as diarrhea, nausea or insomnia (Shintani and Uchida, 1997; Hashimoto *et al*., 2000). Therefore, the efforts to find novel therapies with few adverse effects and high efficacy to treat AD have emerged as a major interest.

Preventive interventions are options for AD therapy and, therefore, recent studies have focused on the antioxidative or anti-inflammatory agents (Shadfar *et al*., 2015; Giubilei, 2016; Bakhtiari *et al*., 2017). As a result, many possible antioxidants have been explored and suggested as potential options for AD prevention or treatment. *Curcuma longa* L. (Zingiberaceae) is one of the most popular dietary supplements because of its

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antioxidative function (Toda *et al*., 1985; Tharakan *et al*., 2010; Canales-Aguirre *et al*., 2012). In East Asian countries and India, it has been used as a medication for improving digestion, improving circulation and reducing inflammation. Recent studies have suggested that curcumin, the major active polyphenolic compound from *C. longa*, improves memory and cognitive function (Mishra and Palanivelu, 2008; Yu *et al*., 2013). However, curcumin has low bioavailability, and its mechanism of action has not been clearly elucidated (Anand *et al*., 2007). If the bioavailability of curcumin could be improved, it would be a potential candidate for AD prevention and therapy. Theracurmin is a newly generated form of curcumin, which is generated using the submicron colloidal dispersion technique, and its bioavailability is improved to at least 27 times compared that of curcumin (Sasaki *et al*., 2011). Therefore, Theracurmin could be effective for the treatment or prevention of AD.

Here, we investigated whether Theracurmin ameliorates cognitive impairments in a mouse model. We employed 5X familial AD (5XFAD) transgenic mice as an AD mouse model. The 5XFAD mouse has a mutation in human amyloid precursor protein (695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) and PSEN1 (M146L and L286V) mutatations, and 5XFAD mice show a rapid accumulation of $A\beta_{1-42}$ protein from 6 weeks of age and show cognitive impairment at approximately 16 weeks (Oakley *et al*., 2006; Jawhar *et al*., 2012). In addition, we studied the mechanism of action of Theraurmin using Western blot and antioxidant activity assays.

MATERIALS AND METHODS

Animals

B6SJL-transgenic (APPSwFlLon, PSEN1*M146L*L286V) male mice (6-7 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Every mouse was housed in each cage with environmental enrichments under a 12 h light and 12 h dark cycle (lights on at 7:00 AM and lights off at 7:00 PM). C57BL/6 mice (6-7 weeks) were purchased from Orient Bio (Seongnam, Korea) and used as a naïve control group. Temperature (23 ± 1°C) and humidity (60 ± 10%) were kept constant. Mice were allowed to freely access to food and water. All experimental protocols for animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Kyung Hee University (No. KHUASP(SE)-18-046).

Materials

Theracurmin was donated by Handok Co (Seoul, Korea). Theracurmin contains curcumin (30%) as an active compound within a vegitable gum (70%) as submicron form. Donepezil and protease inhibitor were purchased from Sigma Aldrich (St. Louise, MO, USA). Enhanced chemiluminescence reagent was purchased from GE Healthcare Life Sciences (Chicago, IL, USA). Mouse monoclonal anti-post synaptic protein (PSD) 95 and anti-β actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-synaptophysin and goat polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba-1) antibodies were purchased from Abcam (Cambridge, UK). Vectorstain avidin biotinyled enzyme complex (ABC) kit were purchased from Vector Lab (Burlingame, CA, USA). A pierce bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher (Waltham, MA, USA). Superoxide dismutase (SOD) assay kit and malondialdehyde (MDA) assay kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). A glutathione (GSH) reaction kit was purchased from BioVision (Mountainview, CA, USA). All of the chemicals were of highest grade commercially available.

Drug administration

Theracurmin was administrated to 5XFAD mice once a day (100, 300 or 1,000 mg/kg, p.o.) for 12 weeks from the age of 12 or 13 weeks. Age-matched wild-type mice were treated with the same volume of a vehicle solution. Donepezil (5 mg/kg) was used as a positive control.

Novel object recognition test

Novel object recognition test consisted of a 2-day habituation session, a training session and a probe session, as described elsewhere (Kim *et al*., 2014). The mice underwent the habituation sessions for 10 min during the first 2 days in the black polyvinyl plastic testing box (25×25×25 cm) without any objects. On day 3, the mice were placed in the same box without any objects for 2 min and then identical objects were placed at the diagonal edges of the same boxes and the mice were allowed to explore the objects for 5 min. The boxes and the objects were cleaned with 70% ethanol to remove odors and were completely dried before placing new mice in the box. The probe session was performed 24 h after the training session, and the mice were placed in the same boxes without objects for 2 min. Then, the objects were placed into the box after the 2 min of habituation. Only one object was changed, with a different shape but a similar texture. The exploration of the mice was recorded for 5 min by a video camera-based on EthoVision system (Noldus, Wageningen, Netherland), and the exploration time of the novel object (T_{novel}) and exploration time of the familiar object ($T_{familiar}$) was measured. The discrimination index was calculated with the following equation: $(T_{novel}–T_{familiar})/(T_{novel}+T_{familiar})×100$.

Barnes maze test

The Barnes maze test is widely used to examine a spatial learning and memory ability with less stress (Barnes, 1979; Morellini, 2013). The Barnes maze is a circular platform (92 cm in diameter and elevated 105 cm from the floor) consisting of 20 holes at regular distances (5 cm in diameter, 38 cm from the center and 3 cm from the edge of the platform). Four different shapes of visual cues were placed on the wall of the test room constantly during the entire experiments. An escape box (6×12×6 cm) was installed under one of the holes at a fixed position. The day before the training session, in the presence of light, mice were placed on the center of the platform and allowed to freely move around the platform for 10 s. After 10 s, the mice were gently guided to enter the escape box, and then the entrance hole was covered by a black polyvinyl plastic plate for 60 s so that the mice could adapt to the apparatus. During the 4 days of training sessions, the mice were placed into a polyvinyl transparent cylinder (11.3 cm in diameter, 15 cm in height) that was located at the center of the platform with absence of light. Light was given when removing cylinder. Mice were allowed to explore the platform and find the escape box for 180 s, and the time that it took for the mouse to enter the escape box was recorded by a video camera-based EthoVision system (Noldus) and was recorded as the escape latnecy. If the mice did not enter the escape box within 180 s,

the mice were guided to enter the escape box, and latency time was considered to be 180 s. When the mice entered the escape box, the light was immediately turned off, and the mice were kept inside the escape box for an additional 60 s. Three training tirals were performed during each training session, seperated 20 min intervals when the mice were moved to their home cage and allowed to rest. Twenty-four hours after the last training session, the escape box was removed, and a probe session was conducted for 90 s, which was recorded by a video camera-based EthoVision system (Noldus). The time spent in each of the quadrants, which were named left, right and opposite based on its position around the target quadrant, was measured and then analyzed.

Western blotting analysis

The mice were sacrificed 12 weeks after the Theracurmin administration (100, 300 or 1,000 mg/kg, p.o.) and the brain was isolated. Whole cortical and hippocampal tissues were isolated from both hemispheres and immediately frozen in liquid nitrogen for Western blot analysis. The tissues were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM orthovanadate, 1 mM sodium fluoride, 1 mM EDTA, 0.32 M sucrose and complete protease inhibitor (1 tablet per 50 mL of buffer). The tissue homogenates were centrifuged (4°C, 14,000 rpm, 20 min), and the supernatants were collected and then quantified with the BCA protein assay kit (Walker, 1994). The protein samples (15 μg of protein) were subjected to SDS-PAGE (8% or 12%) under reduced conditions. Proteins were transferred to PVDF membranes (300 mA, 2 h) in transfer buffer (25 mM Tris-HCl buffer containing 192 mM glycine and 20% v/v methanol, pH 7.4). The membranes were incubated in blocking agent (5% skim milk) for 2 h at room temperature and then with primary antibodies against PSD95 (1:1,000 dilution), synaptophysin (1:200 dilution), Iba-1 (1:1,000 dilution) or β-actin (1:3,000 dilution) at 4°C overnight. The membranes were washed with fresh Tris-buffered saline with Tween 20 (TBST) 3 times and incubated with the appropriate secondary antibody (1:5,000 dilution) for 2 h at room temperature. The membranes were washed again with TBST with the same protocol described above. The immunoblots were developed with an enhanced chemiluminescence reagent and analyzed using a Molecular Imager Gel Doc XR+ system (Bio-Rad Laboratory, Berkeley, CA, USA).

Immunohistochemistry

The mice were sacrificed 12 weeks after the Theracurmin administration (100, 300 or 1,000 mg/kg, p.o.) and the mice were subjected to perfusion with phosphate-buffered saline (PBS, 50 mM, pH 7.4) and fixation (4% paraformaldehyde) process. The brains were isolated and cut into 30 μm slice in a cryostat at –20°C (Leica, Nussloch, Germany). Free floating sections were incubated for 24 h in PBS containing goat anti-Iba-1 antibody (1:1,000 dilution) with 0.3% Triton X-100, 1% BSA and 1.5% normal rabbit serum. The sections were then incubated with biotinylated secondary antibody (1:2,000 dilution) for 90 min and with ABC solution (1:100 dilution) for 1 h at room temperature. The tissues were reacted with 0.02% DAB and 0.01% hydrogen peroxide for approximately 3 min. After three more washing step with the PBS, the tissues were mounted on gelatin-coated slides and dehydrated in ascending alcohol series (30% ethanol, 70% ethanol and 100% ethanol), then cleared in xylene.

Antioxidant activity assay

After the Theracurmin administration (100, 300 or 1,000 mg/kg) for 12 weeks, the mouse was immediately perfused with PBS (50 mM, pH 7.4) under isoflurane anesthesia, and the brain was isolated. The removed brain was homogenized in PBS (50 mM, pH 7.4) and centrifuged at 7,000 rpm for 10 min. Then, the supernatant was collected. The supernatants were used as enzyme source to measure the SOD activity and MDA levels. To measure the GSH level, the brain was homogenized in glutathione reaction buffer and centrifuged at 10,000 rpm for 10 min. The supernatants of the centrifugates were collected, and the extra proteins were precipitated by adding sulfosalicylic acid. The supernatants were used as enzyme sources to measure GSH level. The amount of protein was quantified by using BCA protein assay kit, and the antioxidant activities were measured using SOD, MDA and GSH assay kits. The absorbances were measured shortly after the reactions completed (OPTIZEN 2120UV, Mecasys Co. Ltd., Daejeon, Korea).

Statistical analysis

The results of the novel object recognition test, Barnes maze test, Western blot analysis and antioxidant activity analysis were analyzed by one-way analysis of variance (ANOVA) with the Newman-Keuls's multiple comparison. Two-way ANOVA followed by Bonferroni's *post hoc* test was used to analyze the object preference ratio during the novel object recognition test and the latency during the Barnes maze training session. Data were expressed as mean ± standard error of the mean (SEM). All statistical analyses were analyzed with the Prism 5.0 software (GraphPad, La Jolla, CA, USA).

RESULTS

Theracurmin ameliorates recognition memory in the novel object recognition test

To examine the effect of Theracurmin on recognition memory, the novel object recognition test was conducted using 5XFAD mice. Two-way ANOVA revealed that significant group effects were observed in the object preference ratio [preference to each object, $F_{1,102}$=26.84, p<0.0001; treatment, $F_{5,102}$=8.76, p<0.0001; interaction $_{\text{preference×treatment}}$ $F_{5,102}$=0, p=1] (Fig. 1A). In addition, one-way ANOVA revealed that significant group effects were observed in the discrimination indexes [$F_{5, 51}$=3.964, p=0.0041] (Fig. 1B). The impaired preference for novel object over familiar object was observed in vehicle-treated 5XFAD mice and was significantly reversed through Theracurmin administration (p<0.001), as observed in the donepezil-treated group (p<0.05). The decreased interest on novel object observed in 5XFAD mice was significantly reversed by Theracurmin administration (p<0.01), as observed in donepezil-treated group (p<0.05). There was no significant difference in the total exploration time among all groups [$F_{5, 51}$=1.114, p=0.3645] (Fig. 1C).

Theracurmin ameliorates spatial memory in the Barnes maze test

To determine whether impaired spatial memory could be reversed by the administration of Theracurmin, 5XFAD mice were subjected to the Barnes maze test. Two-way ANOVA re-

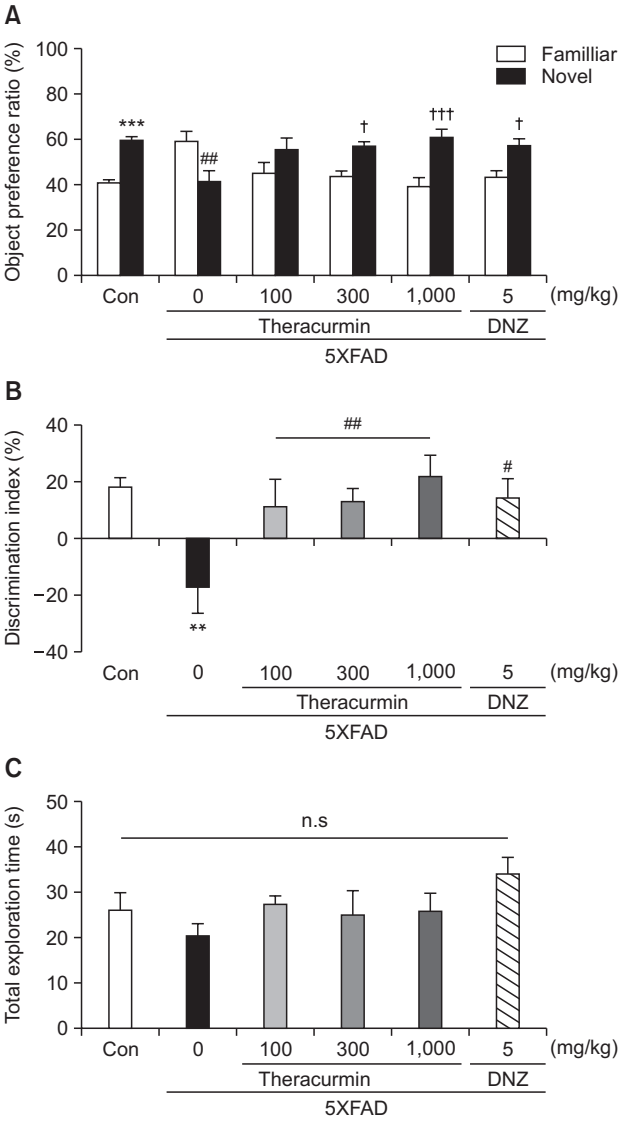

Fig. 1. The effects of Theracurmin against impaired recognition memory in 5XFAD mice during the novel object recognition test. 5XFAD mice were administrated of Theracurmin (100, 300 or 1,000 mg/kg) or the same volume of a vehicle solution for 12 weeks. Donepezil was used as a positive control. The mice were subjected to a training session 1 h after drug administration. A probe session was performed 24 h after the training session without drug administration. The effects of Theracurmin on the object preference ratio (A) and discrimination index (B) are presented, along with total exploration time (C). The data represent mean ± SEM (n=8-10/group). (A) ***p<0.001, $^{\#\#}p$<0.01, $^{\dagger}p$<0.05, $^{\dagger\dagger\dagger}p$<0.001, familiar vs. novel; (B) **p<0.01 when compared to control; $^{\#}p$<0.05, $^{\#\#}p$<0.01 when compared to the vehicle-treated group. Con, control; DNZ, donepezil.

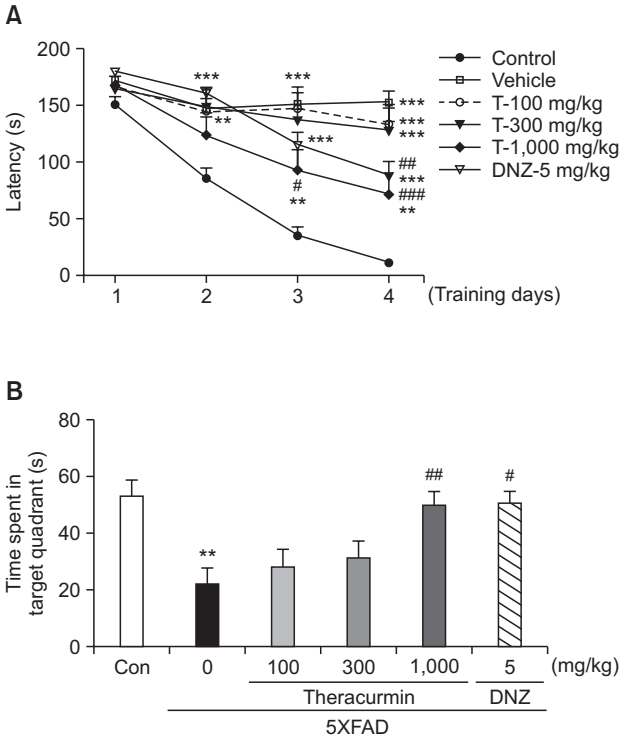

Fig. 2. The effects of Theracurmin against impaired spatial memory in 5XFAD mice during the Barnes maze test. 5XFAD mice were administrated of Theracurmin (100, 300 or 1,000 mg/kg) or the same volume of vehicle solution for 12 weeks, and donepezil was treated during the training sessions. The last drug administration was performed 1 h after each training session. The effects of Theracurmin on latency time to enter the escape box during the training session (A) and time spent in the target quadrant during the probe trial session (B) are presented. The data represent mean ± SEM (n=8-10/group). **p<0.01, ***p<0.001 when compared to the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 when compared to the vehicle-treated group. Con, control; DNZ, donepezil.

vealed that the latency for entering the escape box during the training sessions was significantly different among each group [latency, $F_{1,126}$=174.57, p<0.0001; treatment, $F_{5,126}$=18.66, p<0.0001; interaction $_{\text{latency×treatment}}$ $F_{5,126}$=0, p=1] (Fig. 2A). The latency of the wild-type control group was significantly decreased from day 2 of the training session compared to that of vehicle-treated 5XFAD group (p<0.01). Theracurmin-treated group (1,000 mg/kg) showed significant decrease of its latency on day 3 (p<0.05) and day 4 (p<0.001), compared to the vehicle-treated 5XFAD group. Donepezil-treated group showed a significant decrease in latency time on day 4 when compared to that of the vehicle-treated 5XFAD group (p<0.01). During the probe-trial session, there were significant differences in group effects in the time spent in the target quadrant [one-way ANOVA, $F_{5,48}$=5.663, p<0.001] (Fig. 2B). A significant decreased time spent in the target quadrant was observed in the vehicle-treated 5XFAD group compared to that of the wild-type control group (p<0.01), and a significant amelioration was observed in the high dose Theracurmin-treated group (1,000 mg/kg, p<0.01) and the donepezil-treated group (p<0.05) compared to that of wild-type control group.

The oxidative stress levels are normalized by Theracurmin administration in 5XFAD mice

Oxidative stress markers, including GSH, SOD and MDA, were measured in the brain tissues. One-way ANOVA revealed that there were significant group effects in the SOD [$F_{4,16}$=11.51, p<0.0001], MDA [$F_{4,16}$=3.665, p<0.05] and GSH levels [$F_{4,16}$=6.832, p<0.01]. The decreased SOD lev-

els in 5XFAD mice were ameliorated by the administration of Theracurmin (1,000 mg/kg, p<0.01, Fig. 3A). The marked increase in MDA levels in the 5XFAD mouse brain was reversed with the administration of Theracurmin (1,000 mg/kg, p<0.05, Fig. 3B). In addition, the GSH level, which was decreased in 5XFAD mice, was increased by the Theracurmin treatment (1,000 mg/kg, p<0.01, Fig. 3C).

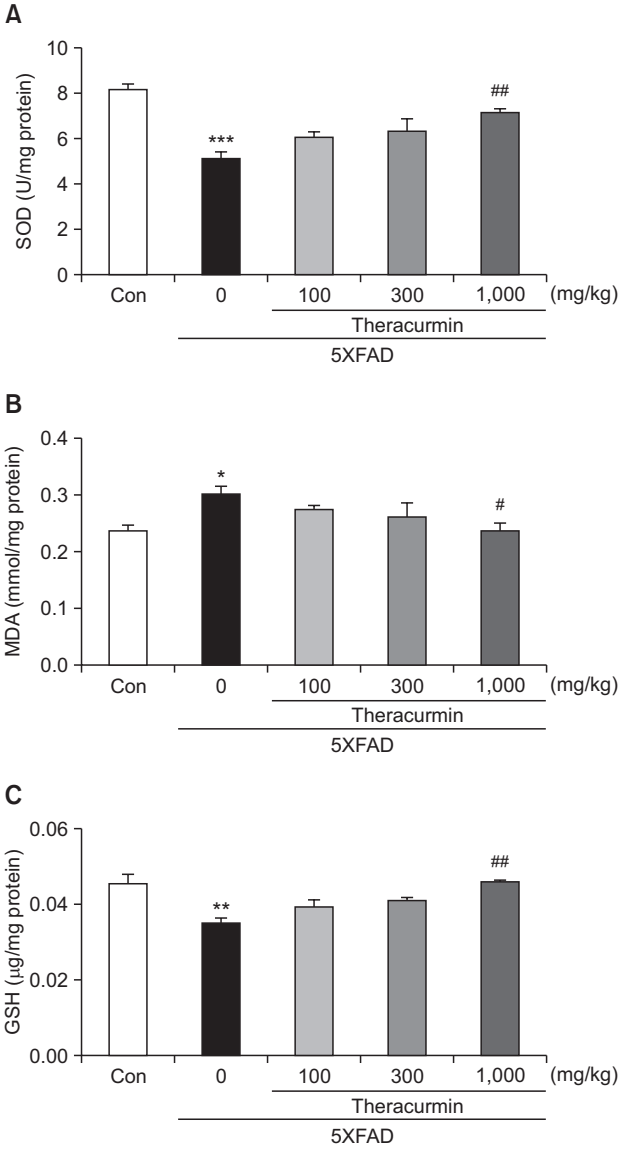

Fig. 3. Antioxidant activity assays in the whole brains of 5XFAD mice. 5XFAD mice were administrated of Theracurmin (100, 300 or 1,000 mg/kg) or the same volume of vehicle solution for 12 weeks. The last administration of Theracurmin (100, 300 or 1,000 mg/kg) or vehicle solution was performed 1 h before sacrifice. Superoxide dismutase (SOD) levels (A), malondialdehyde (MDA) levels (B) and glutathione (GSH) levels (C) were measured as markers of the antioxidative activity of Theracurmin. The data represent mean ± SEM (n=4-5/group). *p<0.05, **p<0.01, ***p<0.001 when compared to the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 when compared to the vehicle-treated group.

Synaptophysin and PSD95 in 5XFAD mice after Theracurmin administration

To examine the effects of Theracurmin on synaptic components, synaptophysin and PSD95 were measured by Western blotting. Regarding synaptophysin, significant differences between groups were observed in the cortex [$F_{5,28}$=3.378, p<0.05] (Fig. 4A) and in the hippocampus [$F_{5,28}$=4.289, p<0.01] (Fig. 4B). Significantly decreased levels of synaptophysin were observed both in the cortex (p<0.05, Fig. 4A) and the hippocampus (p<0.01) of 5XFAD mice compared to those of the wild-type controls. Theracurmin treatment significantly increased synaptophysin levels both in the cortex (p<0.05) and the hip-

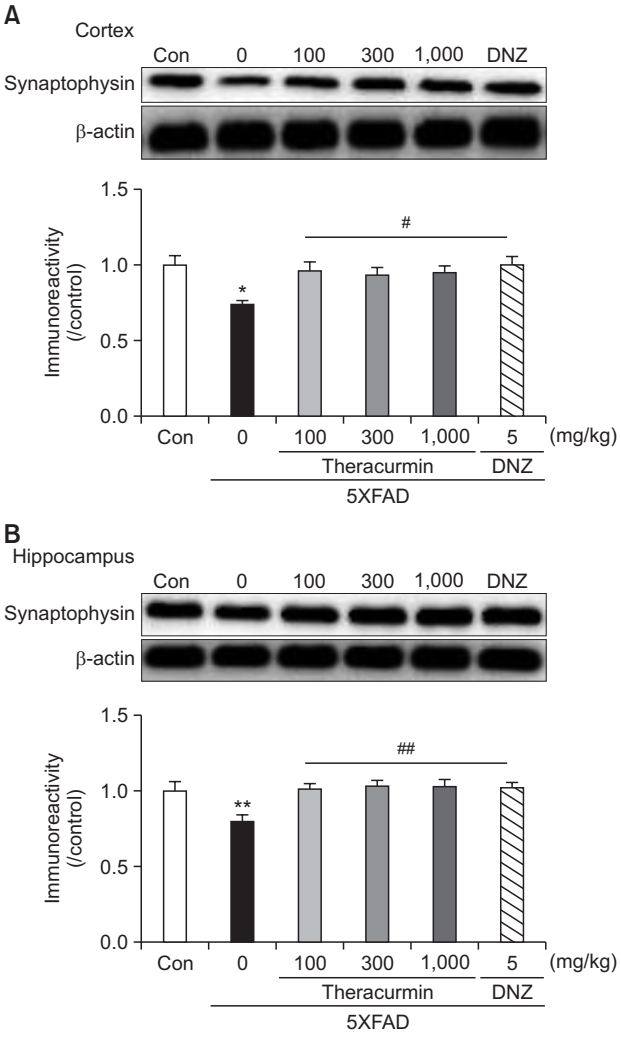

Fig. 4. The expression levels of synaptophysin in 5XFAD mice. Western blot analysis was conducted 12 weeks after the Theracurmin administration. The expression levels of synaptophysin were measured in the cortex (A) and the hippocampus (B). The last administration of Theracurmin (100, 300 or 1,000 mg/kg) or vehicle solution was performed 1 h before sacrifice. The decreased expression levels of synaptophysin, a pre-synaptic vesicle protein, in 5XFAD mice were reversed by Theracurmin administration. Immunoreactivity was normalized to controls is presented as mean ± SEM (n=5-6/group). *p<0.05, **p<0.01 when compared to the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 when compared to the vehicle-treated group.

pocampus (p<0.01) compared to that of the vehicle-treated 5XFAD mice, as observed in the donepezil-treated group. Regarding PSD95, significant differences between groups were observed in the cortex [$F_{5, 28}$=3.677, p<0.05] (Fig. 5A). The expression level of PSD95 was significantly decreased in the vehicle-treated 5XFAD mice compared to those of the wild-type control mice (p<0.05), and the PSD95 level was significantly increased by the administration of Theracurmin (100, 300 and 1,000 mg/kg, p<0.05) but not by donepezil compared to that of the vehicle-treated 5XFAD mice. However, no significant differences in PSD95 levels between groups were observed in the hippocampus [$F_{5, 28}$=1.582, p>0.05] (Fig. 5B).

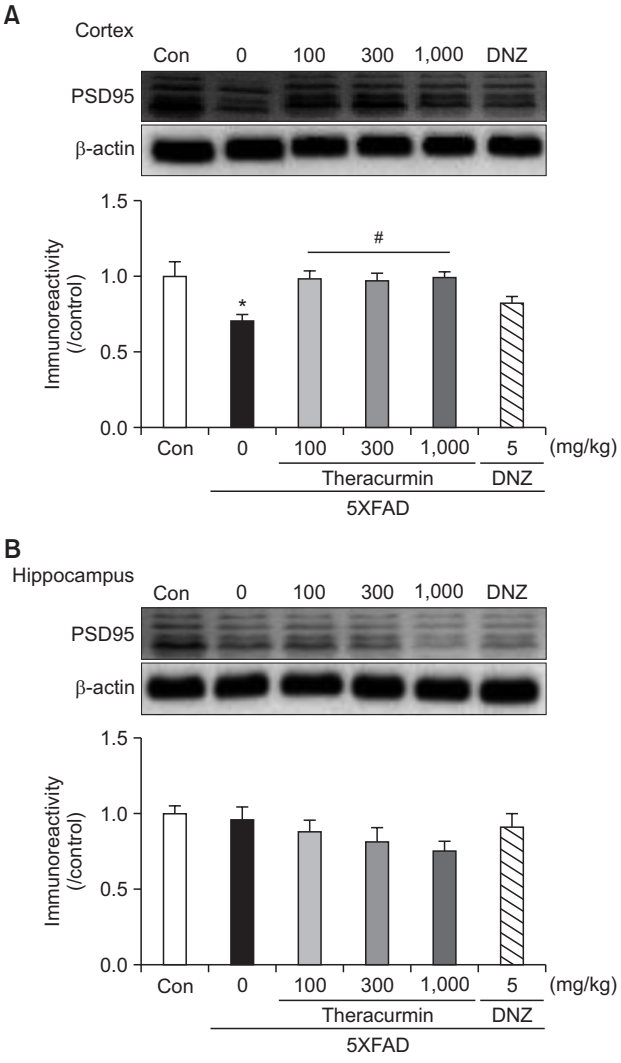

Fig. 5. The expression levels of post-synaptic density protein 95 (PSD95) in 5XFAD mice. Western blot analysis was conducted 12 weeks after the Theracurmin administration. The expression levels of PSD95 were measured in the cortex (A) and the hippocampus (B). The last administration of Theracurmin (100, 300 or 1,000 mg/kg) or vehicle solution was performed 1 h before sacrifice. The decreased expression levels of PSD95 were reversed by Theracurmin administration. Immunoreactivity was normalized to the controls is presented as mean ± SEM (n=5-6/group). *p<0.05 when compared to the control group; $^{\#}p$<0.05 when compared to the vehicle-treated group.

Iba-1 immunoreactivity in 5XFAD mice after Theracurmin administration

It is well acknowledged that microglia are activated in 5XFAD mouse brain tissue (Gyoneva *et al*., 2016). In the present study, we measured Iba-1, a marker of activated microglia, immunoreactivity using the western blot and immunohistochemistry in the cortical and hippocampal tissues. Significant group effects in the level of Iba-1 immunoreactivity were observed in the cortex [$F_{5, 34}$=4.608, p<0.01] (Fig. 6A). The increased level of Iba-1 immunoreactivity in the vehicle-treated 5XFAD mice compared to that of the wild-type control mice was significantly reversed by Theracurmin but not by donepezil treatment (p<0.05). Similar results were also observed in the immunohistochemical testing. However, no significant differences in Iba-1 levels between groups were observed in the hippocampus [$F_{5, 28}$=1.142, p>0.05] (Fig. 6B).

DISCUSSION

In the present study, we observed that Theracurmin ameliorated cognitive dysfunction in an AD mouse model, as measured by the Barnes maze and the novel object recognition tests. Moreover, Theracurmin enhanced the expression levels of synaptophysin and PSD95, which are presynaptic and postsynaptic markers, respectively. Furthermore, Theracurmin exhibited antioxidative activities, as measured by the SOD, MDA or GSH levels and decreased Iba-1 immunoreactivity in the cortex.

It is well known that curcumin contained in *C. longa* exerts antioxidative and anti-inflammatory activities (Cousins *et al*., 2007). Based on its pharmacological activities, curcumin has been used as a dietary supplement or as a drug. In addition, it has been reported that curcumin ameliorates cognitive impairments in AD (Yu *et al*., 2013; McClure *et al*., 2015). Although many studies have been conducted to determie the remedial effects of curcumin in several degenerative diseases, such as rheumatoid arthritis, asthma and AD, its usage has been limited because of its low bioavailability. The submicron colloidal dispersion technique is applied to prepare the Theracurmin which contains 30% curcumin as an active compound, and the bioavailability of Theracurmin has been reported to be 27 times higher in plasma compared to that of curcumin (Sasaki *et al*., 2011). Recently, Barrio's group suggested that daily intake of Theracurmin for 18 months improves memory and attention in adults who do not have dementia (Small *et al*., 2018). Therefore, in this study, we attempted to investigate the effects of Theracurmin on AD-associated cognitive impairment and its mechanism of action. We used the novel object recognition test to measure recognition memory, which appears to be delayed and impaired in patients with neurodegenerative diseases such as AD (Grady *et al*., 2001; Rosenbaum *et al*., 2010). We also employed the Barnes maze test to examine spatial memory performance because AD patients are reported to have poor spatial memory and navigation (Yiu *et al*., 2011). These behavioral tests are less stressful than the passive avoidance test or the Morris water maze task (Dere *et al*., 2007). We observed that the mice treated with Theracurmin tended to explore novel object over familiar object as observed in the donepezil-treated group, suggesting that the mice treated with Theracurmin had improved recognition memory. Furthermore, during the Barnes maze test, we ob-

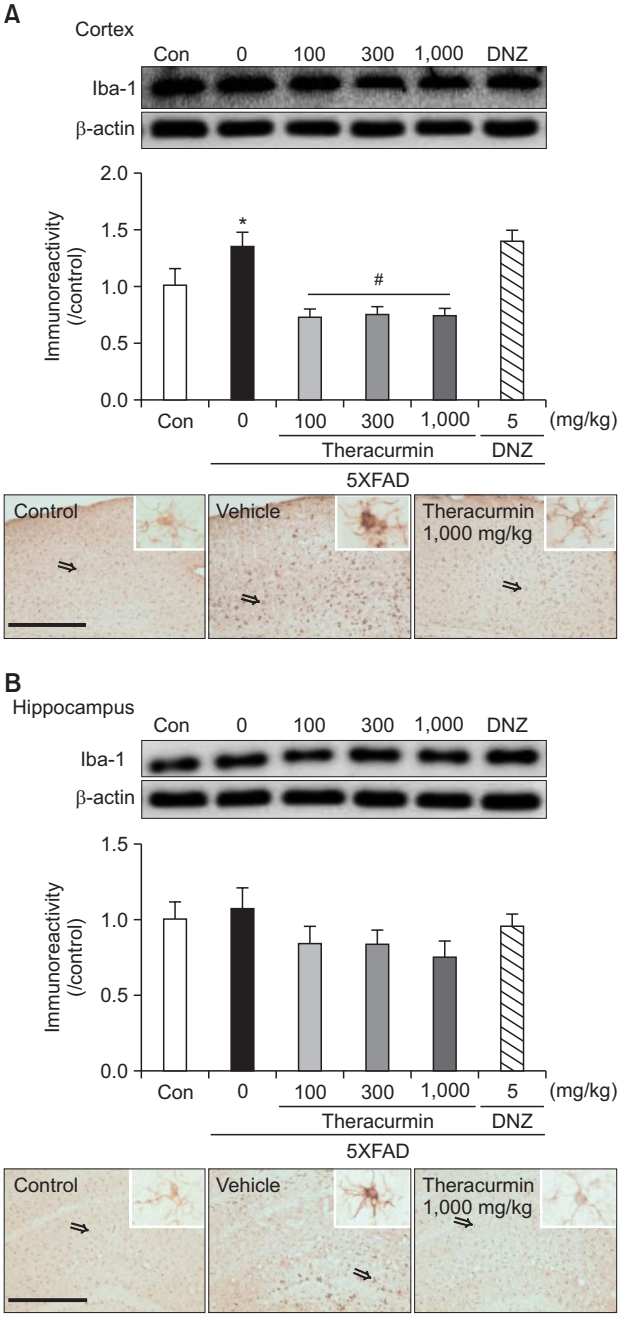

Fig. 6. The expression level of Iba-1 in 5XFAD mice. Western blot analysis and immunostaining were conducted 12 weeks after the Theracurmin administration. The expression levels of Iba-1 and the representative photomicrographs of Iba-1 immunostaining in the cortex (A) and the hippocampus (B) were presented. The last administration of Theracurmin (100, 300 or 1,000 mg/kg) or vehicle solution was performed 1 h before sacrifice. The increased expression levels of Iba-1, a marker of active microglia, in 5XFAD mice were reversed by Theracurmin administration. The photomicrographs of the magnified Iba-1 positive microglia (⇒) were shown in the upper panel. Immunoreactivity in the western blotting was normalized to controls is presented as mean ± SEM (n=5-6/group). *p<0.05 when compared to the control group; $^{\#}p$<0.05 when compared to the vehicle-treated group. Magnification: 100× and 400× (right upper side of each 100×), respectively. Bar=100 μm.

served that the mice treated with Theracurmin had a tendency to find the escape box faster during the training sessions and to explore in the target quadrant for a longer time during the probe session, indicating that Theracurmin ameliorates spatial memory. Thus, it could be concluded that Theracurmin ameliorates recognition memory and spatial memory in AD.

The loss of synapses is another hallmark of AD, along with the accumulation of Aβ protein plaques (Tampellini *et al*., 2010). Synaptophysin, a glycoprotein of pre-synaptic vesicles, shows high selectivity and specificity with synapses so that it can be used for the quantitation of synapses (Calhoun *et al*., 1996). Moreover, it has been reported that mice showed impaired recognition memory and spatial memory when synaptophysin was knocked out genetically (Schmitt *et al*., 2009). In addition to synaptophysin, PSD95 is the most abundant structural protein of the post-synaptic density, which plays important roles in memory formation, especially in synaptic plasticity (Meyer *et al*., 2014). Recently, Zheng *et al* (2017) reported that curcumin increases the expression levels of synaptophysin in the brains of 5XFAD mice. In this study, we also found that 5XFAD mice had significantly lower levels of synaptophysin and PSD95 compared to those of wild-type comtrol mice, similar to previous observations (Grinan-Ferre *et al*., 2018). Such decreased levels of synaptophysin expression were significantly increased by the administration of Theracurmin. Moreover, we also observed that the expression levels of PSD95 increased with the administration of Theracurmin compared to the vehicle-treated 5XFAD mice. These results suggest that Theracurmin attenuates presynaptic and postsynaptic structure degradation, as suggested by Zheng *et al* (2017). It is well known that the mitogen-activated protein kinase pathway is involved in learning and memory behaviors. In addition, Zhang *et al* (2015) reported that the extracellular signal regulated kinase (ERK)-cAMP response element binding protein (CREB) signaling pathway is attenuated by curcumin treatment in the $A\beta_{1\text{-}42}$ protein injected mouse model. In the present study, we observed that the immunoreactivity of phosphorylated ERK and CREB in the cortex or hippocampus of 5XFAD mice was decreased compared to that of wild-type control mice. However, the lowered levels of phosphorylated ERK or CREB were not ameliorated by the administration of Theracurmin (data not shown). Therefore, Theracurmin could ameliorate the impaired cognitive function in AD by normalizing synaptic function.

Recently, several studies have suggested that an up-regulation in the immune activity in the brain results in a chronic inflammatory response that exacerbates cognitive impairment (Kitazawa *et al*., 2011; Park *et al*., 2015). For example, the activation of microglia, which produces neuroinflammatory factors, could lead to cognition decline by disrupting synaptic structure (Hauss-Wegrzyniak *et al*., 2002; Ziehn *et al*., 2010). In addition, the elimination of microglia prevented neuronal loss and improved dendritic spines in 5XFAD mice (Vilalta and Brown, 2014; Spangenberg *et al*., 2016). Thus, it is likely that anti-inflammatory or antioxidative agents may attenuate microglial activation and ameliorate cognitive decline. We found that Iba-1 immunoreactivity was markedly increased in the 5XFAD mouse brain compared to that of wild-type control mice, and Iba-1 immunoreactivity decreased with the administration of Theracurmin. We also investigated whether Theracurmin attenuates astrocyte activation by measuring glial fibrillary acidic protein (GFAP). The immunoreactivity of GFAP was

markedly increased in cortical and hippocampal tissues of the 5XFAD mouse. However, Theracurmin slightly decreased the level of GFAP expression compared to that of vehicle-treated 5XFAD mice, but the decrease was not significant (data not shown). In addition, as expected, oxidative markers, including the SOD, MDA and GSH levels, were normalized by Theracurmin administration, suggesting that Theracurmin could protect neuronal cells from oxidative damage or from activated microglia. Yang *et al* (2005) reported that curcumin administration for 5 months reduced the plaque burden and the levels of the detergent-insoluble $A\beta_{1-42}$ protein. However, we observed that Theracurmin did not change the levels of $A\beta_{1-42}$ protein in the 5XFAD mouse brain compared to that of the vehicle-treated 5XFAD mice (data not shown). The reasons why our observations and the previous report by Yang *et al* (2005) are different must be investigated further. Here, we speculate that the duration of Theracurmin treatment (3 months vs. 5 months) might have caused these differences. Similarly, in a pilot study, the cognitive ameliorative activities of Theracurmin were observed with long-term administration but not with short-term treatment.

In summary, Theracurmin ameliorates cognitive function by increasing synaptic components, inhibiting microglia activation, and enhancing antioxidative activities. Although we did not examine the pharmacokinetic properties of Theracurmin in the present study, Theracurmin is recommended as a long-term intake for cognitive function, as suggested by Small *et al* (2018). Collectively, our present findings suggest that Theracurmin would be potential for the prevention or treatment of cognitive dysfunction, observed in AD.

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