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Research Article

JOURNAL OF BIOTECHNOLOGY AND BIOMEDICINE

ISSN: 2642-9128

Bee Venom Phospholipase A2 Reduces Tau Phosphorylation through Inhibition of GSK3β Expression

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Abstract

Alzheimer's Disease (AD) is characterized by neuronal cell death and neuroinflammation. Neurofibrillary tangles (NFTs) are neuropathological hallmarks of AD. In this study, we investigated whether that phospholipase A2 (PLA2) reduces tau phosphorylation and neuroinflammation and thus ameliorates AD development. To validate pathological activities in vivo, we examined of the inhibitory effect of bee venom PLA2 (bvPLA2) on memory loss and tau phosphorylation as well as neuroinflammation by subcutaneous injection of bvPLA2 (0.5 mg/kg) in Tg2576 mice. For the in vitro study, we examined the effect of bvPLA2 on cell death, tau pathology and neuroinflammation by treating LPS-activated PC12 cells with bvPLA2. Our study showed that bvPLA2 mitigated memory impairment and spatial memory in Tg2576 mice, In association with memory improvement, tau levels and phosphorylation were decreased by bvPLA2 treatment. The expression levels of pro-inflammatory cytokines and inflammation-related proteins were also decreased in the brains of bvPLA2-treated Tg2576 mice. Considering the reduced tau levels and phosphorylation, GSK3β phosphorylation was also studied. Phosphorylation of GSK3β on Ser9 was significantly increased by treatment with bvPLA2, but phosphorylation of GSK3β on Tyr216 was significantly decreased in brains of Tg2576 mice. These data thus indicate that bvPLA2 prevents memory impairment through reduction of tau phosphorylation.

Keywords: Bee venom phospholipase A2; Alzheimer's disease; Tau phosphorylation; Neuroinflammation; GSK-3β

Introduction

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease [1, 2]. An estimated 6.2 million Americans aged 65 years and older are living with Alzheimer's dementia today. This number could grow to 13.8 million by 2060 barring the development of medical breakthroughs to prevent, slow, or cure AD [3, 4] . However, there are currently only six drugs approved by the United States Food and Drug Administration (FDA) for the treatment of AD: rivastigmine, galantamine, donepezil, memantine, memantine combined with donepezil, and tacrine, furthermore, it is recognized that none of these drugs can facilitate a full recovery from AD—they can only temporarily relieve symptoms [5]. Therefore, fundamental drugs for AD need to be developed. On a cellular level, AD is characterized by the accumulation of intracellular tau-containing neurofibrillary tangles (NFTs) and extracellular plaques of amyloid β protein (Aβ) peptides [6]. NFTs are primarily comprised of aggregation of the microtubule-associated protein tau [7]. Tau, a natively

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Citation: Seung Sik Yoo, Sang-Bae Han, Jaesuk Yun, In Jun Yeo, Hyeon Joo Ham, Yeonjoo Kim, Dong Ju Son, Eui Suk Park, Hae In Rhee, Dae-Youn Hwang, Pil-Hoon Park, Dong-Young Choi, Won-Kyu Lee, Jin Tae Hong. Bee Venom Phospholipase A2 Reduces Tau Phosphorylation through Inhibition of GSK3β Expression. Journal of Biotechnology and Biomedicine 6 (2023): 202-213.

Received: November 10, 2023
Accepted: November 16, 2023
Published: May 23, 2023

unfolded cytosolic protein, assists in microtubule stabilization and axonal transport [8]. Hyperphosphorylation of tau results in the failure of its microtubule-binding function, which leads to accumulation in the neurons and causing cell death [9]. Thus, a major hypothesis explaining the pathogenesis of AD is the tau hypothesis [10]. The presence of microglia is related to phosphorylated tau deposits in neurodegenerative diseases. Microglia, which are residential macrophages in the brain, serve several functions, such as inflammatory and immune surveillance; phagocytosis of evading microorganisms, cellular debris, and plaques; and assistance in the maintenance of synaptic plasticity [11]. Upon encountering aggregates and damaged neurons, microglia can increase phagocytosis of extracellular tau oligomers and release inflammatory cytokines [12]. Overactivation of microglia can also lead to memory loss and neuroinflammation in AD [11]. Patients with symptomatic AD pathology have also been found to show activation of microglia accompanied by increased tau accumulation [13]. Thus, neuroinflammation could be involved in the progression of AD through tau phosphorylation. Two proline-directed kinases, glycogen synthase kinase-3 (GSK3) and cyclin-dependent kinase-5, are thought to be key factors in abnormal tau phosphorylation [14]. Overexpression of GSK3β in adult Tet-OFF GSK3β transgenic mice induces tau phosphorylation, neurodegeneration, and learning deficits [15]. Lithium, a direct [16] and indirect [17, 18] inhibitor of GSK3, has been shown to suppress tau phosphorylation, enhance tau-microtubule binding, and promote microtubule assembly [17]. Previous studies [18] have reported that treating mice with a GSK3β-specific inhibitor significantly attenuates tau protein phosphorylation and prevents the tau-induced neurodegeneration. These data indicate that GSK3β could be a significant contributing kinase for tau phosphorylation and thus the progression of AD. In the present study, we investigated whether bee venom phospholipase A2 (bvPLA2) could reduce tau phosphorylation through activation of GSK3β and thus prevent the progression of AD.

Studies have shown that PLA2 has utility as a treatment for Parkinson's disease, atopic dermatitis, and asthma [19]. Abnormal membrane phospholipid metabolism has been documented in the AD brain, and reduced PLA2 activity is correlated with an increased number of senile plaques and neurofibrillary tangles as well as increased brain atrophy and clinical severity of dementia [10, 11]. Notably, in a Japanese individual, neuroaxonal dystrophy, which is an event causing AD, was associated with a novel compound heterozygous mutation in a splicing site of the PLA2G6 gene [20]. Inhibition of PLA2 has also been reported to increase tau phosphorylation at Ser214 in embryonic rat hippocampal neurons [21]. Increased iPLA2 activity and phosphorylated GSK3B levels in platelets are associated with donepezil treatment in patients with AD [22]. Bee venom is composed of various peptides and enzymes including PLA2 [23]. Our previous study found that bee venom offers neuroprotective effects in a lipopolysaccharide (LPS)-induced AD mouse model [21], and other researchers have also reported that bee venom has therapeutic effects against other neurological diseases such as Parkinson's disease [22]. Moreover our recent study showed that bee venom-derived PLA2 has anti-inflammatory and anti-amyloidogenic effects in a Tg2576 mouse model [24]. Therefore, we investigated the possible protective effect of bvPLA2 against tau pathology and memory dysfunction in a Tg2576 AD mouse model.

Materials and Methods

Materials

The bvPLA2 was supplied from INIST ST Co. (Gyeonggi-do, Republic of Korea) and was dissolved in phosphate-buffered saline (PBS; final concentration of 1 mg/mL) and stored at -20°C until use.

Animal and treatment

Twelve month old Tg2576 mice were maintained and handled in accordance with the humane animal care and use guidelines of the Ministry of Food and Drug Safety. Tg2576 mice harboring human APP695 with Swedish double mutation (hAPP; HuAPP695; K670N/M671L) were purchased from Taconic Farms (Germantown, NY, USA), and the strain was maintained in the animal laboratory at Chungbuk National University. The mice were randomly divided into five groups: (I) the wild type vehicle-treated group; (II) the wild type bvPLA2 (2 mg/kg) -treated group; (III) the Tg2576 control vehicle-treated group, (IV) the Tg2576 bvPLA2 (0.5 mg/kg)-treated group; and (V) the Tg2576 donepezil (0.5 mg/kg)-treated group. The bvPLA2 was administered subcutaneously once a week for 4 weeks. Control mice were alternatively given an equal volume of vehicle. The behavioral tests of learning and memory capacity were assessed using the water maze, probe, and object location memory tests. Mice were sacrificed after behavioral tests by CO2 asphyxiation.

PC12 cell culture

PC12 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI1640, penicillin, streptomycin, fetal bovine serum (FBS), horse serum (HS), and nerve growth factor (NGF) were purchased from Invitrogen (Carlsbad, CA, USA). PC12 cells were grown in RPMI1640 with 5% FBS, 10% HS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO2-humidified atmosphere.

Morris Water Maze

The water maze test is a commonly accepted method for memory test, and we performed this test as described by Morris et al [25]. Maze testing was carried out by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with water made opaque with skim milk kept at 22–

Yoo SS, et al., J Biotechnol Biomed 2023
DOI:10.26502/jbb.2642-91280084

25°C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 1-1.5 cm below the surface of the water in position. Testing trials were performed on a single platform and at two rotational starting positions. After testing trial, the mice were allowed to remain on the platform for 120 seconds and were then returned to their cage. Escape latency and escape distance of each mouse was monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain).

Probe test

To assess memory retention, a probe test was performed 24 hours after the water maze test. The platform was removed from the pool which was used in the water maze test, and the mice were allowed to swim freely. The swimming pattern of each mouse was monitored and recorded for 60 seconds using the SMART-LD program (Panlab, Barcelona, Spain). Retained spatial memory was estimated by the time spent in the target quadrant area.

Object Location Memory Task

The Object-Location Memory task is widely used in the study of cognition, specifically spatial memory and discrimination of novel object, in rodent models of CNS disorders. Testing occurs in an open field box [100 cm (L) × 100 cm (W) × 60 cm (H)], to which the mice are first habituated for 10 minutes. After 24 hours later, in the first trial the mice are allowed to explore the arena with the four objects for 10 minutes. In the second trial shortly thereafter, mice are again encounters the four objects, except that two of them have switched positions. The trials are recorded using a SMART-LD program (Panlab, Barcelona, Spain).

Collection and preservation of brain tissues

After behavioral tests, mice were perfused with PBS with heparin under inhaled CO2 anesthetization. The brains were immediately removed from the skulls, after that, only the hippocampus region was isolated and stored at -80°C until biochemical analysis.

Measurement of total tau levels

Lysates of brain tissue were obtained through a protein extraction buffer containing protease inhibitor. Total tau levels were assessed in each region utilizing a commercially available enzyme-linked immune-sorbent assay (ELISA) from (Camarillo, California, total tau: #KMB7011), (Sandiego, CA, USA, p-tau (S396): #MBS7269992). Protein was extracted from brain tissues using a protein extraction buffer (PRO-PREP; Intron Biotechnology, Kyungki-do, Korea), incubated on ice for 1 hour, and centrifuged at 13,000×g for 15 minutes at 4 °C. In brief, 50 µL of sample was added into a precoated plate and incubated for 2 hours at 37 °C. After removing any unbound substances, a biotin-conjugated antibody specific for total tau was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was then added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount tau bound in the initial step. Following the addition of stop solution the optical density was measured at 450 nm in a Molecular Devices VersaMax.

Immunohistochemistry

The brains were collected from mice following perfusion and immediately fixed in 4% paraformaldehyde for 24 hrs. The brains were transferred successively to 10%, 20% and 30% sucrose solutions. Subsequently, brains were frozen on a cold stage and sectioned in a cryostate (20 µm-thick). Sections were treated with endogenous peroxidase (3% H2O2 in PBS), followed by an additional two washes in PBS for 10 minutes each. The brain sections were blocked for 1 hour in 3% bovine serum albumin (BSA) solution and incubated overnight at 4°C with glial fibrillary acidic protein (GFAP; 1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Tau phosphorylation at Serine 396 (P-Tau s396; 1:500; Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), Total tau (T-Tau; 1:50; Santa Cruz Biotechnology, Inc.,Santa Cruz, CA, USA), Glycogen synthase kinase-3 beta phosphorylation at Serine 9 and Tyrosine 216 (GSK3β (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) After incubation with the primary antibodies, brain sections were washed three times in PBS for 10 minutes each. After washing, brain sections were incubated for 1-2 hours at room temperature with the biotinylated goat anti-rabbit, goat anti-mouse, or donkey anti-goat IgG-horseradish peroxidase (HRP) secondary antibodies (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Brain sections were washed three times in PBS for 10 minutes each and visualized by a chromogen diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) reaction for up to 10 minutes. Finally, brain sections were dehydrated in ethanol, cleared in xylene, mounted with Permount (Fisher Scientific, Hampton, NH, USA), and evaluated on a light microscope (Microscope Axio Imager. A2; Carl Zeiss, Oberkochen, Germany; × 50 and × 200).

Western blot analysis

Western blotting was performed as described. To detect target proteins, specific antibodies against iNOS, IBA-1, GFAP and BACE1 (1:1000; Abcam, Inc., Cambridge, UK), P-tau (PHF-13) (1:500; Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), T-Tau (D-8), P-Tau (PHF-6), p-GSK3β and β-actin (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used. The blots were then incubated with the corresponding conjugated secondary antibodies such as anti-mouse, anti-rabbit and anti-goat purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Immunoreactive proteins were detected with an enhanced chemiluminescence Western blotting detection system.

Measurement cytokine level

The pro-inflammatory and anti-inflammatory cytokines level was measured by quantitative reverse transcription polymerase chain reaction(qRT-PCR). Total RNA was extracted using RiboEX (Geneall biotechnology, Seoul, Korea) from hippocampus tissue and cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) for custom-designed primers and β-actin was used for house-keeping control using HiPi Real-Time PCR SYBR green master mix (ELPIS biotech, Daejeon, Korea). Cycling conditions consisted of a initial denaturation step of 3 minutes at 94℃, a denaturation step of 30 seconds at 94℃, an annealing step of 30 seconds at 60℃ and a extension step of a minute at 72℃ followed by 40 cycles. The values obtained for the target gene expression were normalized to β-actin and quantified relative to the expression in control samples.

Each sample was run with the following primer pairs :

β-actin, Forward primer: 5'-GGCTGTATTCCCCTCCATCG-3', Reverse primer: 5'-CCAGTTGGTAACAATGCCATGT-3';

TNF-α, Forward primer: 5'-TCTTCTCATTCCTGCTTGTGG-3', Reverse primer: 5'-CACTTGGTGGTTTGCTACGA-3';

IL-1β, Forward primer: 5'-CCTTCCAGGATGAGGACATGA-3', Reverse primer: 5'-TGAGTCACAGAGGATGGGCTC-3';

IL-6, Forward primer: 5'-GAGGATACCACTCCCAACAGACC-3', Reverse primer: 5'-AAGTGCATCATCGTTGTTCATACA-3'.

Along with quantitative reverse transcription polymerase chain reaction(qRT-PCR), enzyme-linked immune-sorbent assay (ELISA) was used to measure pro-inflammatory and anti-inflammatory cytokine level. Lysates of brain tissue were obtained through a protein extraction buffer containing protease inhibitor. TNF-α, IL-1β and IL-6 levels were assessed in each region utilizing a commercially available enzyme-linked immune-sorbent assay (ELISA) from LABISKOMA (Seoul, Korea, TNF-α: #K1331186, #K0331196, IL-1β: #K1331231, # K0331212, IL-6: #K0331230, # K0331212). Protein was extracted from brain tissues using a protein extraction buffer (PRO-PREP; Intron Biotechnology, Kyungki-do, Korea), incubated on ice for 1 hour, and centrifuged at 13,000×g for 15 minutes at 4 °C. In brief, 50 μL of sample was added into a precoated plate and incubated for 2 hours at 37 °C. After removing any unbound substances, a biotin-conjugated antibody specific for TNF-α, IL-1β and IL-6 was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was then added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount TNF-α, IL-1β and IL-6 bound in the initial step. Following the addition of stop solution the optical density was measured at 450nm in a Molecular Devices VersaMax.

Docking experiment

Docking studies between GSK3β and bvPLA2 was performed using ZDOCK (New Drug Development Center, Osong Medical Innovation Foundation, Cheongju, Korea). Only one monomer of the GSK3β was used in the docking experiments and conditioned using ZDOCK. Base sequence of GSK3β [Gene ID: 2932] and bvPLA2 [Gene ID: 406141] were retrieved from National Center for Biotechnology Information. Molecular graphics for the best binding model were generated using sillico analysis results.

Pull-down assay

bvPLA2 was conjugated with Epoxy-activated Sepharose 6B (GE Healthcare Korea, Seoul, Korea). Briefly, bvPLA2 (1 mg) was dissolved in 1 mL of coupling buffer (0.1 M NaHCO3 and 0.5 M NaCl, pH 11.0). The Epoxy-activated Sepharose 6B beads (0.1 g) were swelled and washed in 1 mM HCl on a sintered glass filter, then washed with the coupling buffer. Epoxy-activated Sepharose 6B beads were added to the bvPLA2-containing coupling buffer and rotated at 4 °C overnight. The control unconjugated Sepharose 6B beads were prepared as described above in the absence of bvPLA2. After washing, unoccupied binding sites were blocked with a blocking buffer (0.1 M Tris-HCl, pH 8.0) at room temperature for 3 h. The bvPLA2-conjugated Sepharose 6B was washed with three cycles of alternating pH wash buffers (buffer 1: 0.1 M acetate and 0.5 M NaCl, pH 4.0; buffer 2: 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0). bvPLA2-conjugated beads were then equilibrated with a binding buffer (0.05 M Tris-HCl and 0.15 M NaCl, pH 7.5). The PC12 cell lysate (3 mg of protein) was mixed with bvPLA2-conjugated Sepharose 6B or unconjugated Sepharose 6B and incubated at 4 °C overnight. The beads were then washed three times with TBST. The bound proteins were eluted with sodium dodecyl sulfate (SDS) loading buffer and were separated using SDS/polyacrylamide gel electrophoresis, followed by immunoblotting with antibodies against GSK3β (1:500, Santa Cruz Biotechnology, Dallas, TX, USA).

Analysis of cell growth and neurite outgrowth

PC12 cells (pheochromocytoma cells derived from the adrenal gland of Rattus norvegicus) (ATCC, Manassas, VA), were grown in 75-cm2 culture flasks at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (4.5 g/L glucose, L-glutamine, without pyruvate), supplemented with 10% bovine calf serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in 10% CO2. For NGF treatment,

Yoo SS, et al., J Biotechnol Biomed 2023
DOI:10.26502/jbb.2642-91280084

PC12 cells were treated with 100 ng/mL of NGF (Sigma-Aldrich, St. Louis, MO) dissolved in complete media for three consecutive days. Control cells without NGF were also grown under the same conditions. For quantitative assessment of neurite outgrowth, PC12 cells were only treated with NGF for 2 days instead of 3, given that the density of neurite outgrowth does not allow for proper tracing of neurites belonging to a specific cell body.

Statistical analysis

The data were analyzed using the GraphPad Prism software (Version 4.03; GraphPad software, Inc., San Diego, CA, USA). Data are presented as mean ± S.E.M. The differences in all data were assessed by one-way analysis of variance (ANOVA). When the P value in the student's t-test indicated statistical significance, the differences were assessed by the Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

Results and discussion

bvPLA2 improves cognitive function

bvPLA2 is a main component of bee venom [26]. bvPLA2 belongs to the group III secretory PLA2 (sPLA2), which has an inflammatory effect by catalyzing hydrolysis on ester bonds at position sn-2 of membrane phospholipids, which release many types of acids, such as arachidonic acid and lysophosphatidic acid [27]. Several clinical application studies have been performed. Intraperitoneal injection of bvPLA2 (0.2 mg/kg) has reduced high-fat-diet-induced obesity in mice [28]. Intratracheal injection of bvPLA2 (10 µg/kg body weight) has prevented ovalbumin-induced allergic asthma in mice [29]. In addition, subcutaneous injection of bvPLA2 (80 ng/ear) has alleviated atopic dermatitis-like symptoms [30]. Moreover, bvPLA2 has been shown to mitigate neuroinflammation in AD [30-32]. In our study, subcutaneous injection of bvPLA2 (0.5 mg/kg once a week for 4 weeks) mitigated AD symptoms such as a lack of spatial learning and memory deficits. To investigate the inhibitory effect of bvPLA2 on memory loss in the Tg2576 AD-related mouse model, mice were subcutaneously administered bvPLA2 (0.5 mg/kg) once a week for 4 weeks. After 4 weeks, the Morris water maze, probe, and object location memory tests were performed sequentially to validate spatial learning ability and memory (Fig. 1A). The bvPLA2-treated Tg2576 mouse group showed shorter escape latency and distance than the Tg2576 control group. On the final day, the bvPLA2-treated Tg2576 mouse group showed an escape latency and distance of approximately 19.38 s and 1522.24 cm, respectively, in contrast to the Tg2576 control group's escape latency and distance of 29.66 s and 2311.65 cm, respectively (Fig. 1B and C). The day after the water maze test (day 7), a probe test was performed to validate the time spent in the quadrant zone of the target for memory reconsolidation. The time spent in the quadrant zone increased in the bvPLA2-treated Tg2576 mouse group (21.26%) compared with the Tg2576 mouse control group (10.2%) (Fig. 1D). The water maze and probe tests showed that the Tg2576 mouse control group spent more

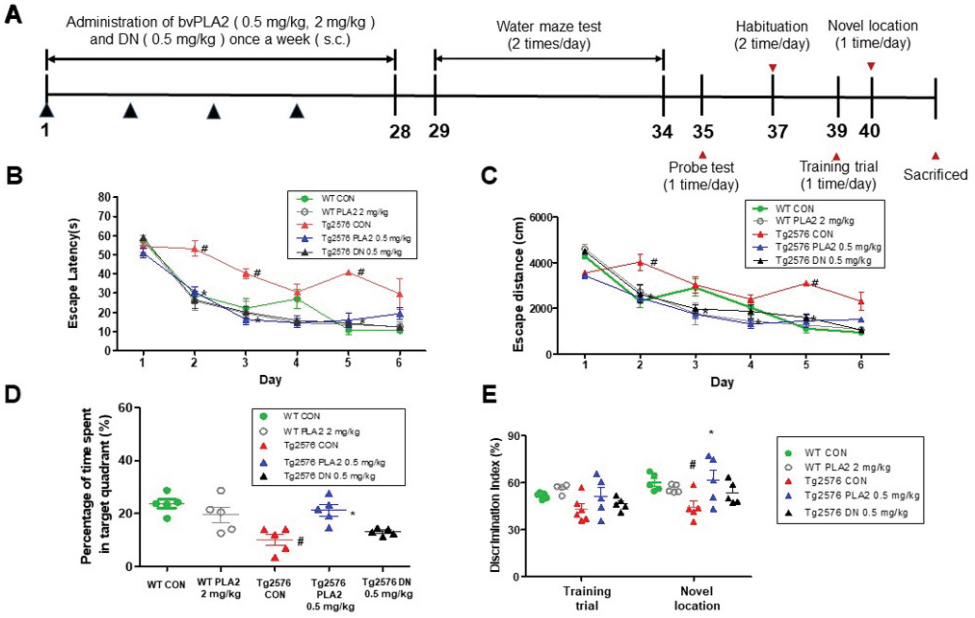

Figure 1: bvPLA2 improves cognitive function in AD. A timeline shows administration of bvPLA2 and validation of cognitive function in Tg2576 mice (a). To validate the effects of bvPLA2 on cognitive function, we performed the water maze test (b, c), probe test (d), and object location memory task (e). Memory and learning ability in Tg2576 mice were evaluated by the escape latencies (b, sec) and escape distance (c, cm) for 6 days, and after the water maze test, the time spent in the target quadrant was measured (d, %) the probe test for 1 day. Each value is reported as the mean ± S.E.M. from 5 mice. #, Significantly different from the WT group ($p < 0.05$). *, Significantly different from the Tg2576 mouse control group ($p < 0.05$).

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Yoo SS, et al., J Biotechnol Biomed 2023
DOI:10.26502/jbb.2642-91280084

time finding the platform than the bvPLA2-treated Tg2576 mouse group did. Next, we performed an object location memory test to validate short-term and spatial memory. In the training trial, no significant differences in the discrimination index (%) were observed; however, in the test trial (novel location), the bvPLA2-treated Tg2576 mouse group showed an increased discrimination index (61.56%) compared with the Tg2576 control mouse group (44.6%) (Fig. 1E). The memorial-improving effect of bvPLA2 is comparable to the effect of donepezil. Therefore, our results indicated that bvPLA2 inhibits memory defects in Tg2576 mice.

Inhibitory effect of bvPLA2 on tau phosphorylation

Phosphorylated tau protein is considered a central mediator of AD pathogenesis. To measure the total levels of tau and p-tau (serine 396), ELISA was performed on the brains of Tg2576 mice. Total tau levels were higher at approximately 1937 pg/mg in the Tg2576 mouse control group than in the bvPLA2-treated Tg2576 mouse group, in which these levels decreased to 459 mg/pg. Furthermore, the p-tau (Ser396) levels were approximately 6.215 pg/μg in the Tg2576 mouse control group and decreased to 2.94 pg/μg in the bvPLA2-treated Tg2576 mouse group (Fig. 2A). To confirm the protein expression levels of phosphorylated tau and total tau, immunohistochemistry and Western blot analyses were performed in the mice brains. The bvPLA2-treated Tg2576 mouse group showed decreased expression of phosphorylated tau and total tau compared with the Tg2576 mouse control group (Fig. 2B). The number of T-tau and p-tau (Ser396) reactive cells was also decreased in bvPLA2-treated Tg2576 mouse group (Fig.2C and D). Although the memory-improving effect of donepezil was similar to that of bvPLA2, the inhibitory effect of donepezil on tau levels and tau phosphorylation was not significant (Fig2A-D).

Inhibitory effect of bvPLA2 targeting on GSK3β

Much research and review evidence shows that GSK3β phosphorylates the serine and threonine residues of tau in paired helical filaments [33]. Moreover, increased activity of GSK3β could phosphorylate tau molecules abnormally in AD [34]. Also, It is well known that the phosphorylation of Tyr216 in GSK3β increases GSK3β activity, whereas the phosphorylation of Ser9 inactivates GSK3β activity [35]. It is also recognized that increasing levels of Ser9 phosphorylation decrease tau phosphorylation. To determine which signaling pathway is involved in the reduced tau phosphorylation by bvPLA2, the involvement of the GSK3β signaling pathway, which is highly related to tau phosphorylation, was investigated by Western blotting analysis in the brains of Tg2576 mice. The expression level of p-GSK3β (Tyr216) was significantly decreased in the brains of bvPLA2-treated Tg2576 mice compared with the brains of control Tg2576 mice, whereas Ser9 was upregulated by bvPLA2 treatment (Fig. 3A). Immunohistochemistry was performed to confirm

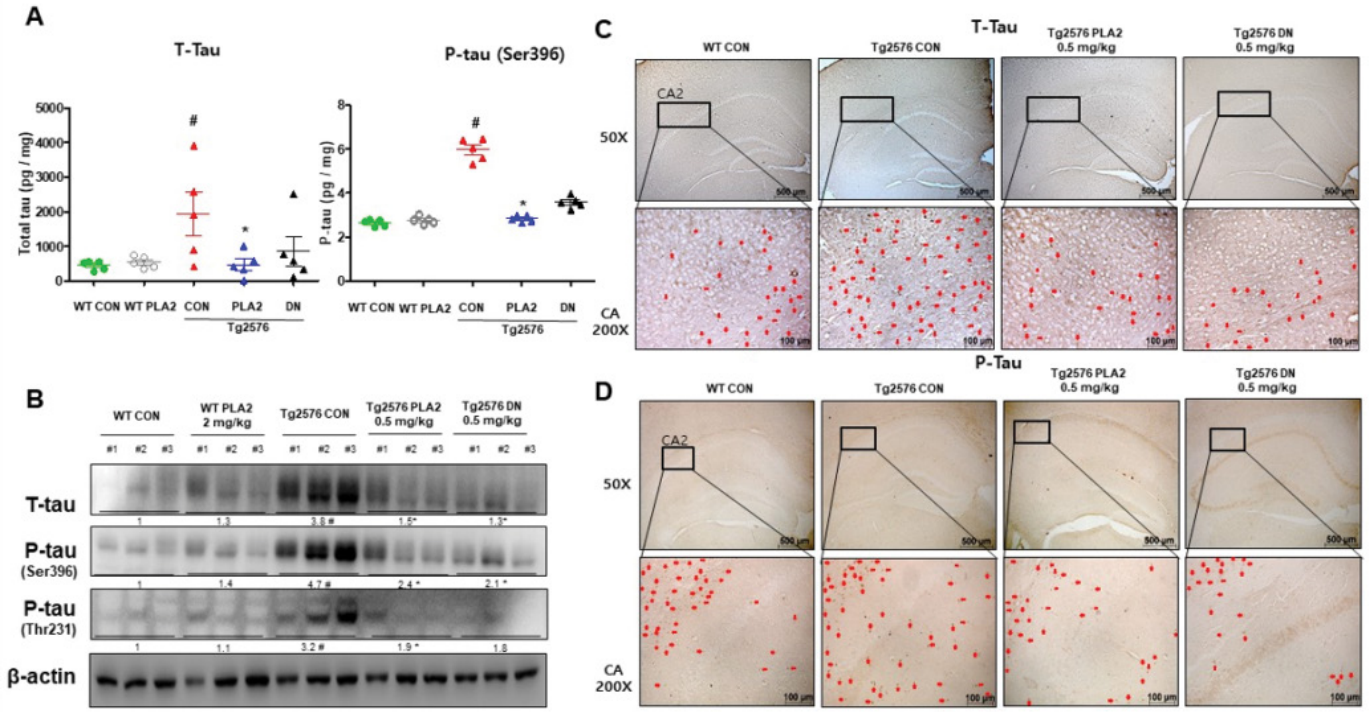

Figure 2: Inhibitory effect of bvPLA2 on tau phosphorylation. To measure the total tau level, ELISA was performed in the brains of mice (a). Western blot analysis was performed to validate the protein levels of total tau and phosphorylated tau (b). Each value is reported as the mean±S.E.M. from 5 mice. #, Significantly different from the WT group ($p<0.05$). Immunostaining of total tau and phosphorylated tau in the hippocampus were performed in 20 μm-thick sections of the brains of mice (c) and (d).

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Yoo SS, et al., J Biotechnol Biomed 2023
DOI:10.26502/jbb.2642-91280084

the expression levels of Ser9 and Tyr216 of p-GSK3β in the brains. The number of Ser9 of GSK3β reactive cells was not considerably different in the bvPLA2-treated and control Tg2576 mouse groups (Fig. 3B). However, the number of Tyr216 of GSK3β was reduced in the bvPLA2-treated group (Fig. 3C). Similar to the effects on tau and phosphorylated tau levels (Fig.3A-C). We next investigated protein-protein interactions with a docking experiment by using ZDOCK and pull-down assay. According to the in sillico analysis results, PLA2 was determined to bind to the substrate junction site of GSK3β. Among many amino acids, Tyr216 of GSK3β showed a great binding effect with PLA2 (Fig. 3D). We incubated whole cell lysate from PC12 cells in Sepharose 6B beads and bvPLA2-conjugated Sepharose 6B beads and then detected them by immunoblotting with anti-GSK3β antibody. The protein level of GSK3β was higher in bvPLA2-Sepharose 6B beads, suggesting that bvPLA2 could directly bind to GSK3β (Fig. 3E).

Inhibitory effect of bvPLA2 on neuroinflammation in the brains of Tg2576 mice

Along with the accumulation of Aβ, neuroinflammation and proinflammation cytokines are key factors associated with AD. Microglia and astrocytes are major sources of cytokines that contribute to neuroinflammation in AD. Western blotting was performed to validate the expression levels of neuroinflammation-related proteins in the mouse brain. The Tg2576 control group showed higher expression levels of inflammation-related proteins (iNOS and COX-2) than the bvPLA2-treated Tg2576 group. In addition, there were higher levels of GFAP (an astrocyte marker protein) and IBA-1 (a microglial marker protein) in the Tg2576 mouse control group than in the bvPLA2-treated Tg2576 mouse group. Immunohistochemistry was performed to confirm the expression levels of activated astrocyte cells via GFAP staining in the brain (Fig. 4A). To measure pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the brains, ELISA was performed using the brains of Tg2576 mice. The cytokine levels in the brains of Tg2576 mice in the control group were higher than those of Tg2576 mice in the bvPLA2-treated group (Fig. 4B). To confirm the presence of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the brains of mice, qRT-PCR was performed. The mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the brains of Tg2576 mice in the bvPLA2-treated group were lower than those in the brains of Tg2576 mice in the control group (Fig. 4C). The anti-inflammatory effects of donepezil were significant except for the IL-6 level (Fig.4A-4C).

Inhibitory effect of bvPLA2 on cell viability, neurite outgrowth, cytokine levels, and GSK3β expression in LPS-treated PC12 cells

We previously found that an LPS-induced AD model was suitable for the study of AD mechanisms since LPS

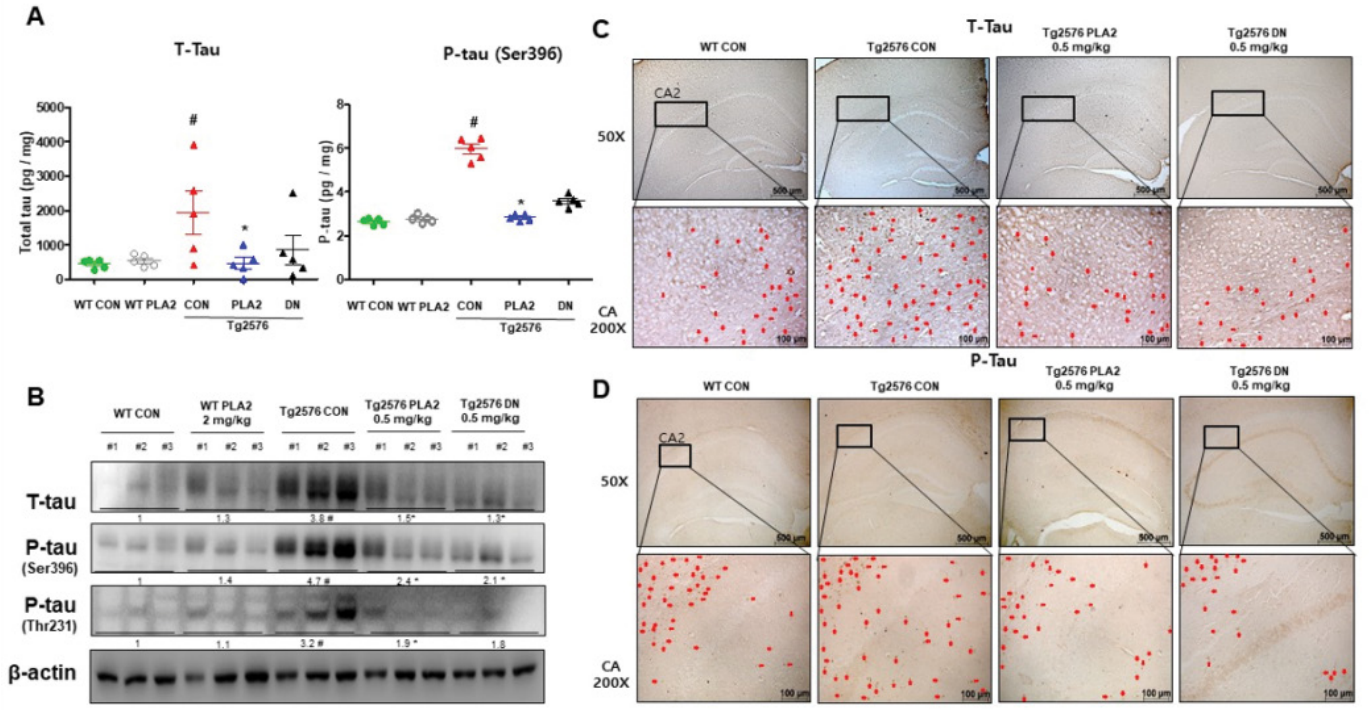

Figure 3: Inhibitory effect of bvPLA2 targeting on GSK3β. Western blot analysis was performed to verify the protein levels of p-GSK3β (Ser9), and p-GSK3β (Tyr216) (a). Immunostaining for p-GSK3β (Ser9) (b) and p-GSK3β (Tyr216) (c) in the hippocampus was performed in 20 μm-thick sections of the brains of mice (c). bvPLA2 directly binds to 10 major amino acids on GSK3β . Tyr216 showed one of greatest binding effect (d). A pull-down assay with GSK3β was performed to determine whether bvPLA2 binds to GSK3β (e).

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DOI:10.26502/jbb.2642-91280084

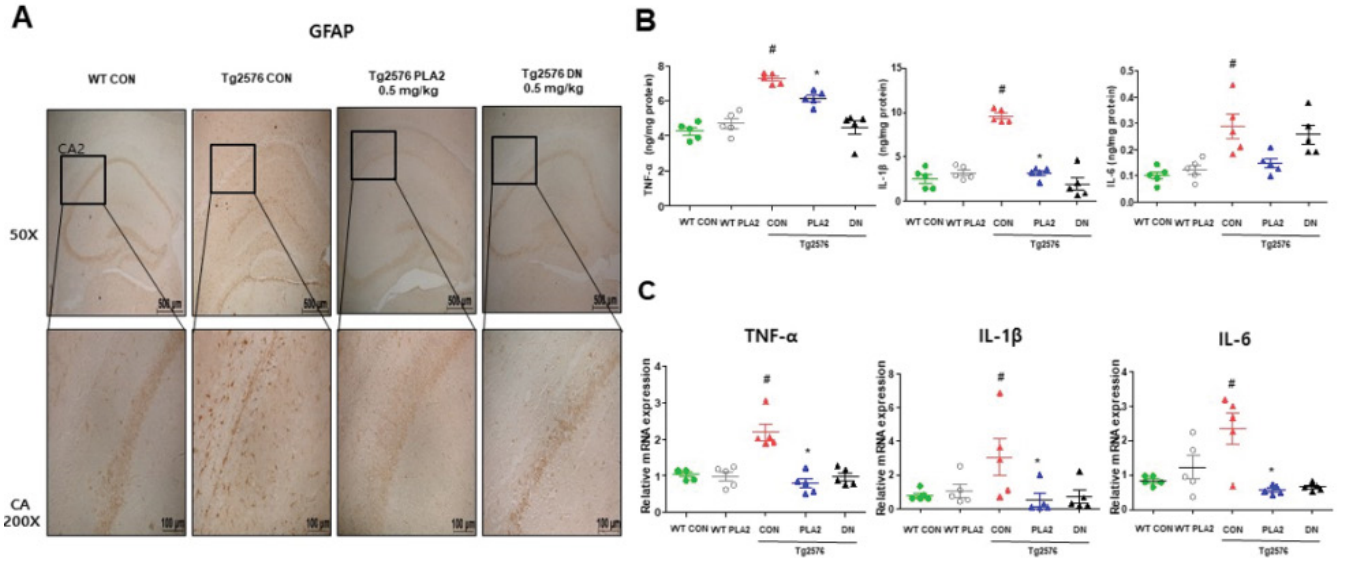

Figure 4: Effects of bvPLA2 on neuroinflammation in a mouse model of AD. Immunostaining for GFAP in the hippocampus was performed in 20 μm-thick sections of the brains of mice (a). ELISA was performed to validate the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the hippocampi of the brains of mice (b). The mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the hippocampi of the brains of mice were assessed by qRT-PCR (c). Each value is reported as the mean ± S.E.M. from 5 mice. Each value is reported as the mean ± S.E.M. from 5 mice. #, Significantly different from the WT group ($p < 0.05$). *, Significantly different from the Tg2576 mouse control group ($p < 0.05$).

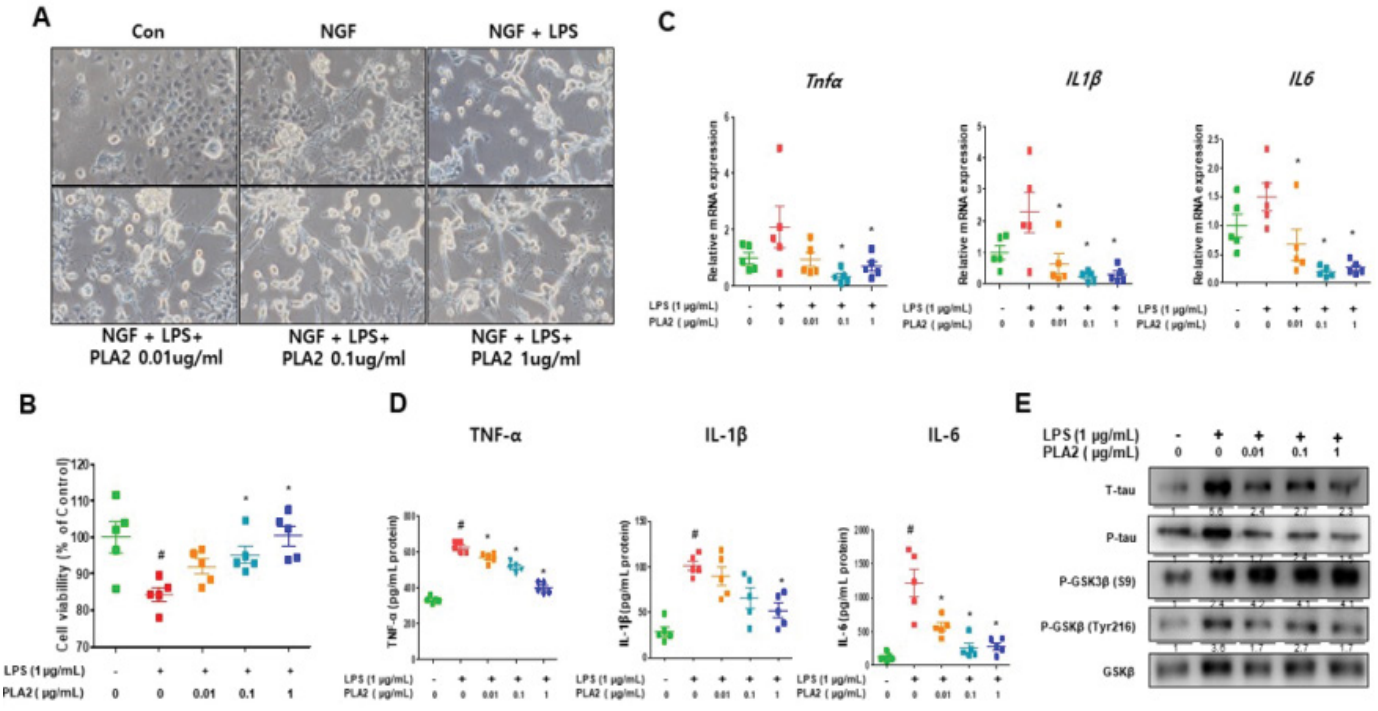

Figure 5: Inhibitory effect of bvPLA2 on LPS induced cytokine levels and GSK3β interactions in PC12 cells. The effects of bvPLA2 (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL) on LPS (1 μg/ml)-treated PC12 cells were evaluated by neurite outgrowth (a). A cell viability assay was performed by treatment with bvPLA2 (0.01, 0.1, 1 μg/mL) of LPS (1 μg/ml)-treated PC12 cells (b). ELISA was performed to validate the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in PC12 cells (c). qRT-PCT was performed to measure the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in PC12 cells (d). The expression of T-tau, P-tau, P-GSK3β (S9), P-GSK3β (Tyr216) and actin were detected by Western blotting in PC12 cells treated with bvPLA2 (e). Each data point is representative of three different experiments. Each value is reported as the mean ± S.E.M. from 5 samples. #, Significantly different from control the group ($p < 0.05$). *, Significantly different from the LPS group ($p < 0.05$).

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cam damage neuronal function and plasticity and thus cause memory dysfunction [36, 37]. Neurite outgrowth is closely associated with neuronal functions; thus, the effect of bvPLA2 (0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) on LPS (1 μg/ml)-treated PC12 cells was assessed using neurite outgrowth and cell viability tests. Neurite outgrowth was decreased by treatment of PC12 cells with LPS. However, bvPLA2 prevented LPS-induced decrease in neurite outgrowth (Fig 5A). Furthermore, bvPLA2 also recovered LPS- inhibited neurite outgrowth (Fig. 5B). Next, to investigate inhibitory effect on bvPLA2 on LPS-induced cytokine levels, qRT-PCR and ELISA were performed. The mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the LPS (1 μg/ml)-treated PC12 cells were higher than those in the control group. However, bvPLA2 treatment (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL) of PC12 cells led to decrease levels compared with those in LPS-treated PC12 cells (Fig. 5C). To confirm inhibitory effect of bvPLA2 on LPS-induced cytokine levels, ELISA was performed. The cytokine levels in bvPLA2 treated (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL) PC12 cells were decreased compare with those in LPS (1 μg/ml)-treated PC12 cells (Fig. 5D). We also investigated the tau phosphorylation and GSK3β signaling pathway in PC12 cells after treatment with LPS (1 μg/mL) and bvPLA2 (0.01 μg/mL, 0.1 μg/mL, 1 μg/mL). The expression levels of T-tau, p-tau and Tyr216 of GSK3β was increased by treatment with LPS, but they were decreased by treatment with bvPLA2. However, the expression level of Ser9 of GSK3β was increased by treatment with bvPLA2 (Fig. 5E).

Inhibitory effect of bvPLA2 on Aβ deposition

It is widely known that the deposition of Aβ drives AD pathogenesis. To measure Aβ1-42 and Aβ1-40 levels, ELISA was performed on the brains of Tg2576 mice. The Aβ1-42 level in the brains of Tg2576 mice in the control group was higher than that in the brains of Tg2576 mice in the bvPLA2-treated group (Supplementary Fig 1A). However, there were no significant differences in the Aβ1-40 levels in the Tg2576 mouse control group and the bvPLA2-treated Tg2576 mouse group (Fig. Supplementary 1B). We performed a β-secretase activity assay of the brains to determine how bvPLA2 reduces Aβ. The β-secretase activity was also significantly decreased by treatment with bvPLA2(Fig. Supplementary 1C). Western blot analysis was performed to confirm the levels of BACE1, and C99 associated with Aβ production using mice brains. The expression of BACE1, and C99 was decreased in the brains of Tg2576 mice in the bvPLA2-treated group compared with those in the Tg2576 mouse control group (Supplementary Fig. 1D).

Conclusion

Subcutaneous injection of bvPLA2 (0.5 mg/kg once a week for 4 weeks) mitigated AD symptoms such as a lack of spatial learning and memory deficits in Tg2576 mice. Moreover, our in vivo and in vitro studies showed that the elevated levels of GSK3β in Tg2576 mice and LPS-treated PC12 cells were decreased by treatment with bvPLA2, and these inhibitory effects were associated with the downregulation of tau phosphorylation. Docking experiments also show that bvPLA2 has a high binding affinity for Tyr216, one of the important phosphorylation sites of GSK3β. Therefore, our study demonstrates that bvPLA2 mitigates tau phosphorylation by phosphorylating GSK3β on Ser9 and interfering with phosphorylation on Tyr216 in Tg2576 mice and PC12 cells. Moreover, it was confirmed that not only tau phosphorylation but also accumulation of Aβ, neuroinflammation and proinflammation cytokines, which are key factors of AD, were inhibited by treatment of bvPLA2 in Tg2576 mice and LPS-treated PC12 cells. Based on the above results, bvPLA2 has potential as a therapeutic candidate in the treatment of AD.

Ethicals approval

The experimental protocols were conducted according to the guidelines for animal experiments of the Faculty of Disease Animal Model Research Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) as well as the Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Research Center at Chungbuk National University, Korea (CBNUA-1452-20-01). All efforts were made to minimize animal suffering and to reduce the number of animals used. All mice were housed in cages that were automatically maintained at 21-25°C and relative humidity of 45-65% with a controlled 12-hour light-dark cycle of illumination from 06:00 a.m. to 06:00 p.m. Food and water were available ad libitum. They were fed a pelleted diet consisting of 20.5 % crude protein, 3.5 % crude fat, 8.0 % crude fiber, 8.0 % crude ash, 0.5 % calcium, and 0.5 % phosphorus per 100 g of the diet (obtained from Daehan Biolink, Chungcheongbuk-do, Korea). During this study, all efforts were made to minimize the number of animals used and their pain and discomfort. All studies were approved by and performed according to the ethical guidelines of the Chungbuk National University Animal Care Committee.

Author contributions

SSY conducted most of the experiments, performed data analysis, generated most of the experimental mice and was the primary writer of the manuscript. IJY, HJH, YJK, YWH, SBH, JY, PHP, HIR, ESP, WKL and DYC provided advice throughout the project. JTH supervised the entire project and had a major role in experimental design, data interpretation, and writing the manuscript. All authors read and approved the final manuscript

Conflicts of interest

The authors have no conflicts of interest to declare.

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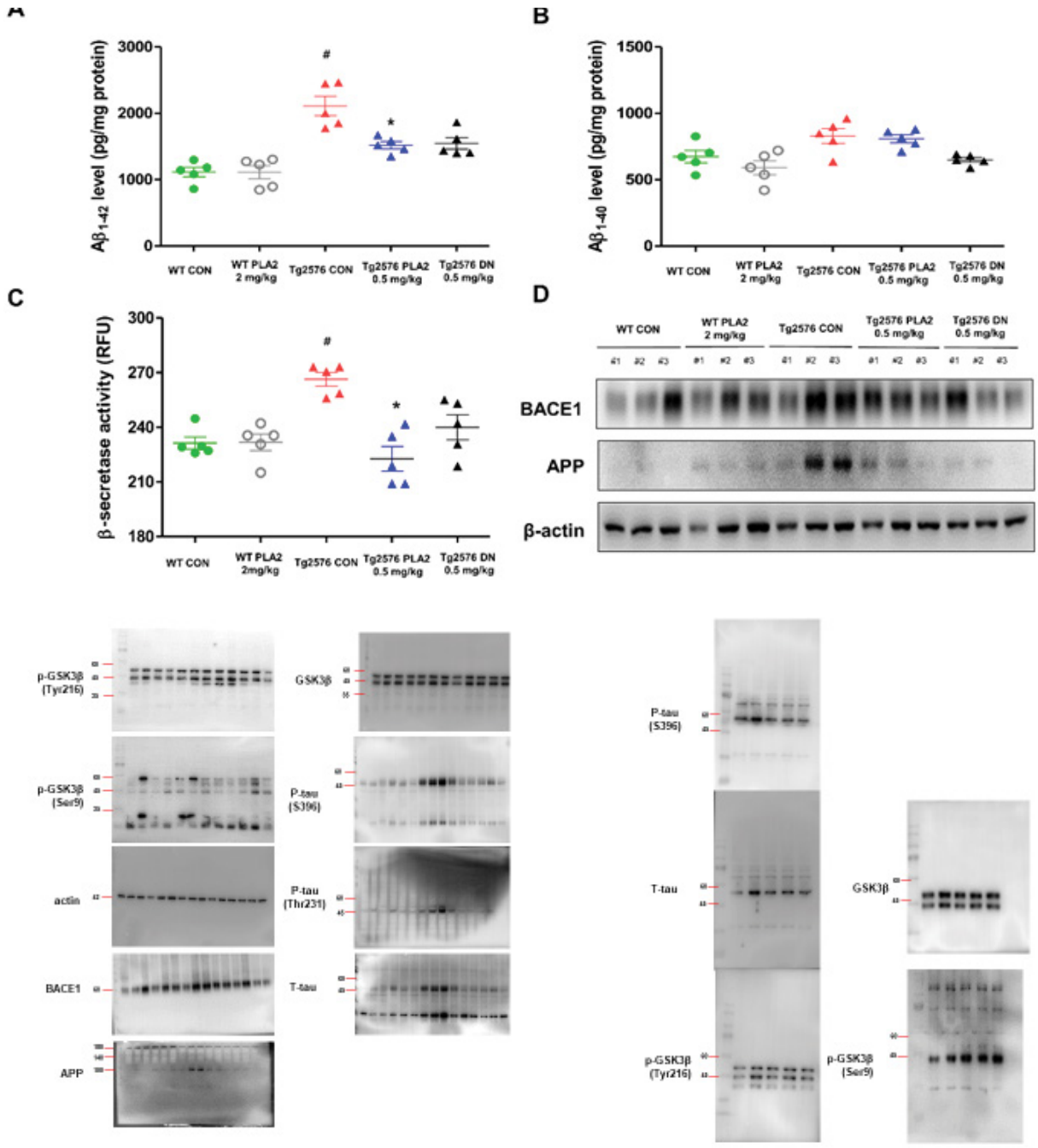
SUPPLEMENTARY FILES

Supplementary Figure S1: Inhibitory effect of bvPLA2 on accumulation of Aβ. The levels of Aβ1–42 ($F(4, 20) = 17.81$, $p<0.0001$, a) and Aβ1–40 ($F(4, 20) = 5.743$, $p=0.0030$, b) in the brains of Tg2576 mice were evaluated by ELISA. The activity of β-secretase in the brains of mice was investigated by using β-secretase activity assay kit ($F(4, 20) = 10.13$, $p=0.0001$, c). The protein level of APP and BACE1 were assessed by Western blot analysis (d). Main effects were determined by one-way ANOVA followed by Bonferroni's multiple post hoc test. Each value is reported as the mean±S.E.M. from 5 mice. #, Significantly different from the WT group ($p<0.05$). *, Significantly different from the Tg2576 mouse control group ($p<0.05$).



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