Bee Venom Phospholipase A2 reduces Tau phosphorylation through inhibition of GSK3 β expression

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Abstract

Background

Alzheimer's disease (AD) is characterized by neuronal cell death and neuroinflammation. Neurofibrillary tangle (NFTs) is one of the neuropathological hallmarks of AD. Also our previous study indicated that bee venom leads to neuroprotective effects in a lipopolysaccharide (LPS)-induced AD mouse model. Thus, in this study we investigated whether that phospholipase A2 (PLA2) reduces tau phosphorylation and neuroinflammation, and thus ameliorates AD development.

Results

To validate pathological activities in *in vivo*, we examined the inhibitory effect of bvPLA2 on memory loss and tau phosphorylation as well as neuroinflammation by subcutaneous injection of bvPLA2 (0.5 mg/kg) to Tg2576 mice. For *in vitro* study, we examined the effect of bvPLA2 on cell death, tau pathology and neuroinflammation by treatment of bvPLA2 in LPS-activated PC12 cells. Our study showed that bvPLA2 mitigated memory impairment and spatial memory in Tg2576 mice. Agreed with the memory improvement, tau level and phosphorylation of tau were decreased by bvPLA2 treatment. Expression level of pro-inflammatory cytokines and inflammation-related proteins were also decreased in the brain of bvPLA2-treated Tg2576 mice.

Conclusions

Consideration of reduced tau level and phosphorylation of tau, GSK3 β phosphorylation was studied. Phosphorylated GSK3 β on Ser9 was significantly increased by treatment of bvPLA2, but a phosphorylated GSK3 β on Tyr216 was significantly decreased in the Tg2576 mice brains. These data thus indicate that bvPLA2 prevents memory impairment through reduction of tau phosphorylation.

Background

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease [1]. An estimated 6.2 million Americans age 65 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 [2]. 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, and it is recognized that none of these drugs can facilitate a full recovery from AD—they can only temporarily relieve symptoms [3]. 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) in addition to the extracellular plaques of amyloid β protein ($A\beta$) peptides [4]. NFTs are primarily comprised of aggregated, microtubule-associated protein tau [5]. Tau, a natively unfolded cytosolic protein, helps in microtubule stabilization and axonal transport [6]. The hyperphosphorylation of tau results in the failure of its microtubule-binding function, which leads to an accumulation in the neurons and causes cell death [7]. Thus, a major hypothesis explaining the pathogenesis of AD is the tau hypothesis [8]. 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 [9]. Upon encountering aggregates and damaged neurons, microglia can increase phagocytosis of extracellular tau oligomers and release inflammatory cytokines [10]. Overactivation of microglia can also lead to memory loss and neuroinflammation in AD [9]. It was also found that patients with symptomatic AD pathology showed activation of microglia accompanied with a higher accumulation of tau [11]. 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 [12]. Overexpression of GSK3 β in adult Tet-OFF GSK3 β transgenic mice induces tau phosphorylation, neurodegeneration, and learning deficits [13]. Lithium, a direct [14] and indirect [15, 16] inhibitor of GSK3, has been shown to suppress tau phosphorylation, enhance tau–microtubule binding, and promote microtubule assembly [15]. Previous studies [16] have reported that treating mice with a GSK3 β -specific inhibitor significantly attenuates phosphorylation of tau protein and prevents the tau-induced neurodegeneration. These data indicate that GSK3 β could be a significant contributing kinase for tau phosphorylation and thus the progression of AD. In the present study, we investigated whether bvPLA2 could reduce tau phosphorylation through activation of GSK3 β 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 [17]. Abnormal membrane phospholipid metabolism has been documented in the AD brain, and reduced phospholipase A 2 (PLA 2) activity correlates with a higher number of senile plaques and neurofibrillary tangles, greater brain atrophy and greater clinical severity of dementia [8, 9]. It is also noteworthy that a Japanese individual with neuroaxonal dystrophy, an event causing AD that was associated with a novel compound heterozygous mutation in a splicing site of the PLA2G6 gene [18]. It was also reported that inhibition of PLA2 increases tau phosphorylation at Ser214 in embryonic rat hippocampal neurons [19]. Increased iPLA2 activity and levels of phosphorylated GSK3B in platelets are associated with donepezil treatment in Alzheimer's disease patients [20].

Bee venom is composed of various peptides and enzymes including phospholipase A2 (PLA2) [21]. Our previous study found that bee venom offers neuroprotective effects in a lipopolysaccharide (LPS)-induced AD mouse model [19], and other researchers have also reported that bee venom has therapeutic effects against other neurological diseases such as Parkinson's disease [20]. Moreover recent our study

showed that bee venom derived PLA2 has anti-inflammatory and anti-amyloidogenic effects in a Tg2576 mouse model [22]. Therefore, we investigated the possible protective effect of bvPLA2 on Tau pathology and memory dysfunction in a Tg2576 AD mouse model.

Results

bvPLA2 improves cognitive function

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 group showed shorter escape latency and distance than the Tg2576 control group. On the final day, the bvPLA2-treated Tg2576 group showed an escape latency and distance of approximately 19.38 s and 1522.24 cm, in contrast to the Tg2576 control group's 29.66 s and 2311.65 cm (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 target for memory reconsolidation. The time spent in the quadrant zone increased in the bvPLA2-treated Tg2576 group (21.26%) compared to the Tg2576 control group (10.2%) (Fig. 1D). The water maze and probe tests showed that the Tg2576 control group spent more time finding the platform than the bvPLA2-treated 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 group showed an increased discrimination index (61.56%) compared to the Tg2576 control 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

The phosphorylated tau protein is considered a central mediator of AD pathogenesis. To measure the level of total tau and p-tau (serine 396), ELISA was performed in the Tg2576 mice brains. Total tau levels were higher approximately up to 1937 pg/mg in the Tg2576 control group and bvPLA2-treated Tg2576 group decreased to 459 mg/pg. Also, P-tau (Serine 396) levels were approximately up to 6.215 pg/ μ g in the Tg2576 control group and decreased to 2.94 pg/ μ g in the bvPLA2-treated Tg2576 group (Fig. 2A). To confirm the protein expression levels of phosphorylated tau and total tau, immunohistochemistry and Western blot were performed in the mice brains. The bvPLA2-treated Tg2576 group showed lowered expression of phosphorylated tau and total tau compared to the Tg2576 control group (Fig. 2B). The number of T-tau and s396 of P-tau reactive cell number was also lowered in bvPLA2-treated Tg2576 group (Fig. 2C and D). Even though the memory improving effect of donepezil was similar to that of bvPLA2, inhibitory effect of donepezil on tau level and tau phosphorylation was not significant (Fig. 2A-D).

Inhibitory effect of bvPLA2 targeting on GSK3 β

To determine which signaling pathway is involved in the reduced tau phosphorylation by bvPLA2, the involvement of the GSK3 β signaling pathway, which is highly related to tau phosphorylation, was investigated by Western blotting in the Tg2576 mice brains. The expression level of p-GSK3 β (Tyr216) was significantly decreased in bvPLA2-treated Tg2576 brains compared to Tg2576 brains, whereas the Ser9 was upregulated by bvPLA2 treatment (Fig. 3A). Immunohistochemistry was performed to confirm the expression level of Ser9 and Tyr216 of p-GSK3 β in the brains. The number of Ser9 of GSK3 β reactive cells was not much difference bvPLA2-treated group compared to that in Tg2576 control group (Fig. 3B). However, the number of Tyr216 of GSK3 β were reduced in the bvPLA2-treated group (Fig. 3C). Similar to the effects on tau level and phosphorylate tau (Fig. 3A-C). We next investigated the protein-protein interaction by docking experiment by using ZDOCK and Pull down assay. According to the in silico analysis results, PLA2 was judged to bind to the substrate junction site of GSK3 β . Among many amino acids, Tyr216 of GSK3 β showed 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 by immunoblotting with anti-GSK3 β antibody. GSK3 β protein level was higher in bvPLA2-Sepharose 6B beads, suggesting that bvPLA2 could directly bind to GSK3 β (Fig. 3E).

Inhibitory effect of bvPLA2 on neuroinflammation in Tg2576 mice brains

Along with the accumulation of A β , neuroinflammation and proinflammation cytokines are key factors of AD. Microglia and astrocytes are major sources of cytokines in AD, which contribute to neuroinflammation. 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. Additionally, GFAP (an astrocyte marker protein) and IBA-1 (a microglial marker protein) showed higher levels in the Tg2576 control group than the bvPLA2-treated Tg2576 group. Immunohistochemistry was performed to confirm the expression level of activated astrocyte cells via GFAP staining in the brain (Fig. 4A). To measure pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in the brains, ELISA was performed in the Tg2576 mice brains. The cytokine levels in the brains of the Tg2576 control group were higher than those of the bvPLA2-treated Tg2576 group (Fig. 4B). To confirm pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in the mice brains, qRT-PCR was performed. The mRNA levels of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in the brains of the bvPLA2-treated Tg2576 group were lower than those in the Tg2576 control group (Fig. 4C). Anti-inflammatory effects of donepezil were significant except the IL-6 level (Fig. 4A-4C).

Inhibitory effect of bvPLA2 on LPS induced cell viability, neurite outgrowth, cytokine level and GSK3 β expression in PC12 cell

We previously found that LPS induced AD model was suitable for the study of AD mechanism since LPS could damage neuronal function and plasticity, and thus memory dysfunction [23, 24]. Neurite outgrowth is closely associated to neuronal functions, thus the effect of bvPLA2 (0.01, 0.1, 1 $\mu\text{g/mL}$) on LPS (1 $\mu\text{g/mL}$) inhibited cell survival in PC12 cells was performed by neurite outgrowth and cell viability tests. The neurite outgrowth was decreased by treatment of LPS in PC12 cells. However, bvPLA2 prevented LPS-induced decrease of neurite outgrowth (Fig. 5A). bvPLA2 also recovered LPS-inhibited all growth (Fig. 5B). Next, to investigate inhibitory effect of bvPLA2 on LPS induced cytokine level, qRT-PCR and ELISA were processed. The mRNA levels of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) on the LPS (1 $\mu\text{g/mL}$) induced PC12 cells were higher than those in the control group. However bvPLA2 treated (0.01, 0.1, 1 $\mu\text{g/mL}$) PC12 cells were lowered than those in LPS treated PC12 cells (Fig. 5C). To confirm inhibitory effect of bvPLA2 on LPS induced cytokine level, ELISA was proceeded. The cytokine levels in bvPLA2 treated (0.01, 0.1, 1 $\mu\text{g/mL}$) PC12 cells were lowered compare to LPS (1 $\mu\text{g/mL}$) induced PC12 cells (Fig. 5D). We also investigated the tau phosphorylation and GSK3 β signaling pathway in PC12 cells after treatment with LPS (1 $\mu\text{g/mL}$) and bvPLA2 (0.01, 0.1, 1 $\mu\text{g/mL}$). The expression level of T-tau, p-tau and Tyr216 of GSK3 β was increased by treatment of LPS, but it was decreased by treating bvPLA2. However, the expression level of Ser9 of GSK3 β was increased by treating bvPLA2 (Fig. 5E).

Inhibitory effect of bvPLA2 on A β deposition

It is widely known that the deposition of A β drives AD pathogenesis. To measure A β_{1-42} and A β_{1-40} levels, ELISA was performed in the Tg2576 mice brains. The A β_{1-42} level in the brains of the Tg2576 control group was higher than that of the bvPLA2-treated Tg2576 group (Supplementary Fig. S1A). However, there were no significant differences in the A β_{1-40} levels of the Tg2576 control group and the bvPLA2-treated Tg2576 group (Supplementary Fig. S1B). We performed a β -secretase activity assay in the brains to determine how bvPLA2 reduces A β . The β -secretase activity also significantly decreased by treatment of bvPLA2 (Supplementary Fig. S1C). Western blot was performed to confirm the levels of BACE1, and C99 involved with A β production using mice brains. The expression of BACE1, and C99 was decreased in the brains of the bvPLA2-treated Tg2576 group compared to the Tg2576 control group (Supplementary Fig. S1D).

Discussion

There are many hypotheses regarding the progression of AD. Among those hypotheses, A β and tau pathology are the most representative and are two major extra- and intracellular hallmarks of AD. Intracellular NFTs of hyperphosphorylated tau have pathological functions that lead to neurodegeneration [25]. Furthermore, accumulating evidence has shown a relationship between AD and tau pathology [26, 27]. Many clinical studies have shown that tau proteins are potential biomarkers for AD [28]. The latest research showed that tau is a more significant factor than A β in the treatment of AD [29]. Moreover, other research suggests that tau-targeting treatment has a stronger correlation to cognitive improvement than A β -targeting treatment [30]. Our present research shows that bvPLA2 prevents memory impairment Tg2576 in AD mice. Tg2576 mice overexpress a mutant form of APP because of their

Swedish mutation, and this form is associated with amyloidogenesis. In the present study, we found that more interestingly, not only amyloidogenic effect but also elevated level of tau phosphorylation were seen in Tg2576 mice brain. Tau phosphorylation were reduced by injections of bvPLA2 but not by donepezil. Even though the molecular mechanisms are not clear, these results indicated that bvPLA2 could improve memory functions not only by reducing amyloidogenic effect but also preventing tau phosphorylation.

Much research and review evidence shows that GSK3 β phosphorylates serine and threonine residues of tau in paired helical filaments [31]. Moreover, increased activity of GSK3 β could phosphorylate tau molecules abnormally in AD [32]. Thus, we investigated whether the inhibitory effect of bvPLA2 in tau phosphorylation could be associated with GSK3 β expression. Both our *in vivo* and *in vitro* studies showed the elevated level of GSK3 β in Tg2576 mice and LPS treated PC12 cells were decreased by treating with bvPLA2, and these inhibitory effects were associated with the downregulation of tau phosphorylation. Therefore, these results indicate that bvPLA2 on tau phosphorylation could be associated with the inhibition of GSK3 β expression. Despite these results, the mechanisms by which GSK3 β regulates tau phosphorylation are not fully known. It is well known that the phosphorylation of Tyr216 in GSK3 β increases GSK3 β activity, whereas the phosphorylation of Ser9 inactivates GSK3 β activity [33]. It is also recognized that increasing levels of Ser9 decreases tau phosphorylation. Our Western blot results show that treatment with bvPLA2 increased the level of Ser9 but decreased Tyr216, and the changes were associated with tau phosphorylation in both wild-type and Tg2576 mice as well as LPS-treated PC12 cells. Clinical research has also demonstrated that in patients with AD, Ser9 is reduced noticeably, which activates GSK3 β activity [34]. Both *in vivo* and *in vitro* research also showed that GSK3 β inhibitors such as lithium and SB415286 slightly increased Ser9 phosphorylation of GSK3 β in mouse brains and in CHO-K1 cells [33]. Moreover, PC12 cells treated with NGF increased GSK3-mediated tau phosphorylation, while GSK3 inhibitors lithium, alsterpaullone, and valproate reduced the phosphorylation of tau [35]. These data combined with our data suggest that the inhibition of Ser9 phosphorylation of GSK3 β could be significantly associated with tau phosphorylation, and thus the effect of bvPLA2 on Ser9 may be significant for the inhibition of tau phosphorylation. Phosphorylation of Tyr216 in GSK3 β increased the phosphorylation of tau [36]. Furthermore, GSK3 β activation was performed through Tyr216 phosphorylation and resulted in tau phosphorylation in SH-SY5Y cells [36]. Other research also shows that upregulation of p-GSK3 β (Tyr216) was also found in the R6/2 (Huntington's disease) mice hippocampus. In addition, the increased expression of GSK3 β in the astrocytes of R6/2 mice appeared to be the main driver of Tau phosphorylation.[37]. GSK3 β could be inactivated through phosphorylation in serine 9 and activated by phosphorylation in tyrosine 216. Overexpression or overactivity of GSK3 β increases tau phosphorylation, leading to axonal transport alterations, hippocampal neurodegeneration [38]. But it is not known how GSK3 β becomes to phosphorylate tau abnormally in AD brains. Similar results were found in PC12 cells treated by LPS with bvPLA2. These data thus indicate that treatment with bvPLA2 increases GSK3 β activity by enhancing phosphorylation of Ser9 GSK3 β as well as decreasing Tyr216 GSK3 β , and thus reduces tau phosphorylation.

Phospholipase A2 derived from bee venom (bvPLA2) is a main component of bee venom [39]. 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 [40]. Several clinical application studies have been done. Intraperitoneal injection of bvPLA2 (0.2 mg/kg) has reduced high-fat-diet-induced obesity in mice. Intratracheal injection of bvPLA2 (10 µg/kg body weight) has prevented ovalbumin-induced allergic asthma in mice [41]. In addition, subcutaneous injection of bvPLA2 (80 ng/ear) has alleviated atopic dermatitis-like symptoms [42]. Moreover, bvPLA2 has been shown to mitigate neuroinflammation in AD [42–44]. 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. Repeated-dose toxicity tests with rats for 13 weeks showed a no-observed-adverse-effect level (NOAEL) of 1.875 mg/kg/day. A beagle was used as a non-rodent model and was tested for repeated-dose toxicity for 4 weeks, and the NOAEL was shown to be 0.6 mg/kg/day. Thus, the dose we used for the AD mice model was about 1/8–1/26 of NOAEL. Therefore, the bvPLA2 dose used in the present study showed a reasonable therapeutic index, thus indicating that bvPLA2 could be useful for the treatment of AD. It is widely known that donepezil is a promising candidate against AD, but it has several side effects. Our study demonstrated that bvPLA2 mitigates Tau phosphorylation by phosphorylating GSK3β on Serine9 and interfering phosphorylation on Tyrosine216 in Tg2576 mice and PC12 cells. Therefore, bvPLA2 has potential as a therapeutic candidate in the treatment of AD.

Conclusions

Our study demonstrated that bvPLA2 mitigates Tau phosphorylation by phosphorylating GSK3 beta on Serine9 and interfering phosphorylation on Tyrosine216 in Tg2576 mice and PC12 cells. Therefore, bvPLA2 has potential as a therapeutic candidate in AD characterized by tau pathology.

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. Tg2576 mice were randomly divided into five groups with 10 mice in each group: the control group and the bvPLA2 (1 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.

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 [45]. 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 22–25°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), (San Diego, 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 cryostat (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°C, a denaturation step of 30 seconds at 94°C, an annealing step of 30 seconds at 60°C and a extension step of a minute at 72°C 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-1 β , Forward primer: 5'-CCTTCCAGGATGAGGACATGA-3', Reverse primer: 5'- TGAGTCACAGAGGATGGGCTC-3';

IL-6, Forward primer: 5'-GAGGATAACCACTCCCAACAGACC-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-1 β 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-1 β : #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 $\times$ g for 15 minutes at 4°C. In brief, 50 μ L 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-1 β 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-1 β 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 GSK3 β and bvPLA2 was performed using ZDOCK (New Drug Development Center, Osong Medical Innovation Foundation, Cheongju, Korea). Only one monomer of the GSK3 β was used in the docking experiments and conditioned using ZDOCK. Base sequence of GSK3 β [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 GSK3 β (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 μ g/mL streptomycin) in 10% CO₂. For NGF treatment, 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 $\pm$ 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.

Abbreviations

AD
Alzheimer's disease
NFTs
Neurofibrillary tangles
bvPLA2
bee venom phospholipase A2
BACE1
 β -secretase 1
GSK3 beta
Glycogen synthase kinase 3 beta
IL
interleukin
TNF- α
tumor necrosis factor α
GFAP
glial fibrillary acidic protein

Declarations

Ethics approval and consent to participate

The experimental protocols were carried out 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 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 was automatically maintained at 21~25°C and relative humidity of 45~65% with controlled 12hours light-dark cycle illuminating from 06:00 a.m. to 06:00 p.m. Food and water were available ad libitum. They were fed a pellet diet consisting of crude protein 20.5 %, crude fat 3.5 %, crude fiber 8.0 %, crude ash 8.0 %, calcium 0.5 %, phosphorus 0.5 % 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 by the Chungbuk National University Animal Care Committee.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Competing interests

The authors declare that they have no competing interests.

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Authors' 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, DYH, SBH, JY, DJS 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.

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Figures

Figure 1. bvPLA2 improves cognitive function in AD

Figure 1

bvPLA2 improves cognitive function in AD.

A timeline shows administration of bvPLA2 and validate cognitive function in Tg2576 mice (a). To validate effect of bvPLA2 on cognitive fuction, we executed the water maze test (b, c), the probe test (d), and the Object Location Memory Task (e). Memory and learning ability in Tg2576 were evaluated by the escape latencies (b, sec) and escape distance (c, cm) for 6 days, and after Water maze test, measured time spent in target quadrant (d, %) by probe test for 1 day. Each value is mean $\pm$ S.E.M. from 5 mice. #, Significantly different from WT group ($p < 0.05$). *, Significantly different from Tg2576 control group ($p < 0.05$).

Figure 2. Inhibitory effect of bvPLA2 on tau phosphorylation

Figure 2

Inhibitory effect of bvPLA2 on tau phosphorylation.

To measure the level of total tau, ELISA was performed in the mice brain (a). Western blot was assessed to validate protein level of total tau and phosphorylation tau (b). Each value is mean ± S.E.M. from 5 mice. #, Significantly different from WT group (p < 0.05). Immunostaining of total tau and phosphorylation tau in the hippocampus were performed in 20 μm-thick sections of mice brain (c) and (d).

Figure 3. Inhibitory effect of bvPLA2 targeting on GSK3β

Figure 3

Inhibitory effect of bvPLA2 targeting on GSK3β.

Western blot was performed to verify protein level of p-GSK3β (Ser9), p-GSK3β (Tyr216) (a). Immunostaining of p-GSK3β (Ser9) (b) and p-GSK3β (Tyr216) (c) in the hippocampus were performed in 20 μm-thick sections of mice brain and (c). bvPLA2 directly binds to major 10 Amino acids on GSK3β . And Tyr216 is one of greatest effect on binding (d). Pull-down assay with GSK3β was performed to determine whether bvPLA2 binds to GSK3β (e).

Figure 4. Effects of bvPLA2 on neuroinflammation in AD mice model brain

Figure 4

Effects of bvPLA2 on neuroinflammation in AD mice model brain.

Immunostaining of GFAP in the hippocampus were performed in 20 μ m-thick sections of mice brain (a). ELISA was assessed to validate pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in the mice brain hippocampus site (b). The mRNA expression level of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) in the mice brain hippocampus site were assessed by qRT-PCR (c). Each value is mean $\pm$ S.E.M. from 6 mice. Each value is mean $\pm$ S.E.M. from 5 mice. #, Significantly different from WT group ($p < 0.05$). *, Significantly different from Tg2576 control group ($p < 0.05$).

Figure 5. Inhibitory effect of bvPLA2 on LPS induced cytokine level and GSK3β interaction in PC12 cell

Figure 5

Inhibitory effect of bvPLA2 on LPS induced cytokine level and GSK3β interaction in PC12 cell.

The effect of bvPLA2 (0.01, 0.1, 1 μg/mL) on LPS (1 μg/ml) induced PC12 cells were performed with neurite outgrowth (a). Cell viability tests assay was performed by treating bvPLA2 (0.01, 0.1, 1 μg/mL) on LPS (1 μg/ml) on PC12 cells (b). ELISA was performed to validate pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in PC12 cells (c). qRT-PCT was performed to measure the mRNA expression level 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 representative for three different experiments. Each value is mean ± S.E.M. from 3 samples. #, Significantly different from control group (p < 0.05). *, Significantly different from LPS group (p < 0.05).

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