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ARTICLE

Alpha-linolenic acid regulates amyloid precursor protein processing by mitogen-activated protein kinase pathway and neuronal apoptosis in amyloid beta-induced SH-SY5Y neuronal cells

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Abstract Alpha-linolenic acid (ALA), which is an omega-3 fatty acid from plant oils, has been reported to have beneficial effects on human brain health. However, the protective effect of ALA and its mechanism of action against amyloid beta (Aβ)-mediated neurotoxicity, neuronal apoptosis and amyloid precursor protein (APP) processing are unclear. To investigate the neuroprotective effect of ALA, we treated $A\beta_{25\text{-}35}$-induced SH-SY5Y cells with ALA (1, 2.5, 5 and 25 μg/mL). In our results, $A\beta_{25\text{-}35}$-induced neuronal cell loss was observed, whereas ALA significantly increased the cell viability and decreased lactate dehydrogenase release. In addition, over-production of reactive oxygen species caused by $A\beta_{25\text{-}35}$ was attenuated by treatment with ALA, and these inhibitory activities were mediated by regulation of the mitogen-activated protein kinase signaling pathway. Furthermore, our data shows that $A\beta_{25\text{-}35}$ cause an increase in protein expression of APP-C-terminal fragment β, β-site APP-cleaving enzyme and presenilin-1 in SH-SY5Y cells, while ALA significantly down-regulated the expression of those amyloidogenic APP processing-related proteins. In addition, we confirmed that ALA enhanced α-secretase activity by up-regulating the protein levels of A distintegrin and metalloprotease 10 and tumor necrosis factor-α-converting enzyme, indicating that ALA could promote non-amyloidogenic signaling pathways. ALA also significantly attenuated $A\beta_{25\text{-}35}$-induced neuronal apoptosis by up-regulation of the Bcl-2/Bax ratio. These findings suggest that ALA may be a beneficial agent for promoting prevention of Alzheimer's disease.

Keywords Alpha-linolenic acid · Alzheimer's disease · Amyloid beta · Neuronal apoptosis · Neuronal cell

Introduction

Alzheimer's disease (AD) is characterized by abnormal accumulation of neurofibrillary tangles and senile plaques, resulting in neuronal dysfunction and death. The most important component of these plaques is amyloid beta (Aβ), which is produced by amyloid precursor protein (APP) [1]. It was previously demonstrated that generation of Aβ is mediated by two pathways: the amyloidogenic and non-amyloidogenic pathways. Cleavage of APP by β- and γ-secretase leads to generation of amyloidogenic toxic Aβ peptide. In an alternative pathway, APP proteolysis by α-secretase generates soluble N-terminal fragment (sAPPα), which is non-toxic and neuroprotective with autocrine activity [2–4]. Thus, regulation of APP processing toward the non-amyloidogenic pathway is a therapeutic target for AD.

Accumulation of Aβ could substantially increase the production of reactive oxygen species (ROS), thus stimulating oxidative stress and neuronal apoptosis via modulation of the mitogen-activated protein kinase (MAPK)

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signaling pathway [5, 6]. The MAPK family is composed of extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK) and p38. The pathway of activated MAPK has been implicated in neuronal cell death in the brain of AD patients [7]. In addition, MAPK also plays a critical role in APP metabolism and Aβ production [8]. It was demonstrated that MAPK promotes amyloidogenic/β-APP-cleavage versus non-amyloidogenic/α-APP-cleavage. Moreover, oxidative stress stimulates β- and γ-secretase with activation of JNK and c-jun, thus promoting Aβ production [9].

Numerous studies have suggested that dietary *n*-3 polyunsaturated fatty acids (PUFAs) exert a beneficial effect on cognitive improvement via promotion of neuron survival [10–13]. However, marine-derived eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are associated with toxicological effect or methyl mercury poisoning; thereby, a plant-derived PUFA, alpha-linolenic acid (ALA), is considered as the best alternative source [14]. Because ALA is an essential fatty acid, ALA must be obtained through dietary intake. In general, source of ALA includes green leafy vegetables and some nuts. In particular, ALA content is high in seed oils such as flaxseed (known as linseed), rapeseed, perilla and chia oil. Among the oils, perilla oil consistently contains the highest proportion of ALA, 54–64% [15]. Flaxseed oil also contains over 50% of ALA (average 53% of total fatty acids) [16]. Rapeseed oil and walnut oil contribute to the common dietary sources of ALA (10 and 9%, respectively) [17]. Administration of dietary ALA reduces neuronal cell death and improves cognitive function during natural aging with no side effects, suggesting that ALA has nutraceutical potential for treatment/prevention of neurodegenerative diseases [18–21]. Consistent with these reports, we previously reported that administration of perilla oil and its major fatty acids, ALA, improved cognitive decline in an $A\beta_{25\text{-}35}$-injected AD mouse model, having a comparable effect to that of DHA [22]. Although increasing evidences for ALA's effect on human health has been demonstrated, the underlying mechanisms by which ALA might influence neuronal cells against Aβ remain to be established.

The current study describes the neuroprotective effect of ALA on $A\beta_{25\text{-}35}$-induced oxidative stress, neurotoxicity and neuronal apoptosis. Additionally, we investigated the modulation of APP processing in SH-SY5Y cells after ALA treatment.

Materials and methods

Preparation of sample

Perilla frutescens var. *japonica* oil was contributed by the Southern Area Crop Science, Rural Development Administration (Miryang, Republic of Korea). The fatty acid composition of perilla oil used in this study was ALA (515.20 mg/g), linoleic acid (141.16 mg/g), oleic acid (147.05 mg/g), stearic acid (23.08 mg/g) and palmitic acid (52.12 mg/g) [23]. ALA was obtained from perilla oil after treatment of urea with cooling by high-yield methods as described in previous study [24]. Before use, ALA was freshly prepared as a stock solution in dimethyl sulfoxide (DMSO), and final concentration adjusted by DMSO did not affect in cell viability. The ALA was stored in a refrigerator at 4 °C with nitrogen gas for stability.

Instruments and reagents

$A\beta_{25\text{-}35}$, dichlorofluorescin diacetate (DCF-DA) and DMSO were supplied from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin/streptomycin and fetal bovine serum (FBS), was purchased from WelGENE (Daegu, Korea). Radioimmunoprecipitation assay (RIPA) buffer and protein marker were purchased from Elpis Biotech (Daejeon, Korea). In addition, 30% bis-acrylamide solution was purchased from Bio-Rad (Hercules, CA, USA). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Billerica, MA, USA).

Cell culture

SH-SY5Y human neuroblastoma cells were obtained from the KCLB (Korean Cell Line Bank, Seoul, Korea). The cells were maintained in a humidified atmosphere incubator at 37 °C, 5% CO_2 and DMEM containing 1% penicillin/streptomycin and 10% FBS. Cell culture media were replaced with 0.05% trypsin–EDTA in phosphate-buffered saline (PBS).

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay

After reaching confluent growth, the cells were seeded (5×10^4 cells/mL) into a 96-well plate and incubated overnight. After that, each concentration of ALA (1, 2.5, 5, and 25 μg/mL) was administered to cells for 2 h and $A\beta_{25\text{-}35}$ (25 μM) was added. After incubation for 24 h, cell viability was determined by a previously described method, using the

MTS solution (Promega, Madison, WI, USA) according to the manufacturer's instruction, and the absorbance of each well was read at 490 nm [25].

Lactate dehydrogenase (LDH) release assay

According to the manufacturer's protocol, the LDH release was assessed by a LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan). SH-SY5Y cells were plated at a density of 5×10^4 cells/mL into a 96-well plate and incubated overnight. Cells were treated with ALA (1, 2.5, 5 and 25 μg/mL) for 2 h, and $A\beta_{25\text{-}35}$ (25 μM) was added. After incubation for 24 h, the supernatant (100 μL) and reaction mix (100 μL) were added to a 96-well plate and incubated for 30 min at room temperature. All samples were measured for absorbance at 490 nm using a microplate reader [26].

Measurement of ROS production

ROS scavenging property of cells after ALA treatment was measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA). SH-SY5Y cells were seeded at a density of 5×10^4 cells/mL into a 96-well plate and incubated overnight. Cells were treated with ALA (5–25 μg/mL) for 2 h, and $A\beta_{25\text{-}35}$ (25 μM) was added. After 24 h, the cells were incubated with 80 μM DCFH-DA at 37 °C for 30 min, and then, florescence was monitored for 60 min, at wavelengths of 480 nm for excitation and 535 nm for emission, using a florescence plate reader (BMG LABTECH, Ortenberg, Germany) [27].

Hoechst 33342 staining

SH-SY5Y cells were seeded on 8-well chamber slides and treated with ALA for 2 h. Cells were exposed to 25 μM $A\beta_{25\text{-}35}$ for 24 h. Media were removed, and cells were washed with PBS (pH 7.4). The cells were next fixed with 4% paraformaldehyde for 10 min. Fixed cells were washed with PBS and treated with Hoechst 33342 (NucBlue® Live ReadyProbes® Reagent, Life Technologies, Grand Island, NY, USA) for 20 min. The cells were observed using a fluorescence microscope (Olympus BX50, Tokyo, Japan).

Western blotting

SH-SY5Y cells were lysed according to the manufacturer's instructions using RIPA buffer containing 1 × protease inhibitor cocktail (Sigma Co., St Louis, MO, USA). Proteins were separated by electrophoresis in 10–13% SDS-PAGE and blotted onto PVDF membranes. The membrane was blocked with 5% skim milk solution for 1 h at room temperature and then washed with PBS-T. The membrane was incubated overnight at 4 °C with primary antibody [ERK, pERK1/2, JNK and pJNK (1:1000, Cell Signaling, Beverly, MA, USA); β-site APP-cleaving enzyme (BACE; 1:1000, Cell Signaling); presenilin-1 (PS1; 1:1000, Cell Signaling); anti-APP, C-terminal (1:1000; Sigma); a disintegrin and metalloprotease 10 (ADAM10; 1:200, Santa Cruz, CA, USA); tumor necrosis factor-α-converting enzyme (TACE; 1:200, Santa Cruz); Bcl-2 (1:200, Santa Cruz); Bax (1:200, Santa Cruz); and β-actin (1:200, Santa Cruz)]. After washing, the membrane was incubated with the appropriate HRP-conjugated secondary antibodies. Western bands were visualized using a chemiluminescent imaging system (Davinci Chemi, Seoul, Korea).

Statistical analysis

Results are expressed as mean ± SD. Statistical significance was determined by Student's *t* test analysis using the program IBM SPSS version 23. Significance was set at $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{*}P < 0.05$ and $^{**}P < 0.01$. $^{\#}$Significantly different from the control group; *significantly different from the $A\beta_{25\text{-}35}$-treated vehicle group.

Results

Effect of ALA on cell viability in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

To examine the effect of ALA on SH-SY5Y cells induced by $A\beta_{25\text{-}35}$, the MTS assay was carried out. No significant effect on cell proliferation was observed when cells were

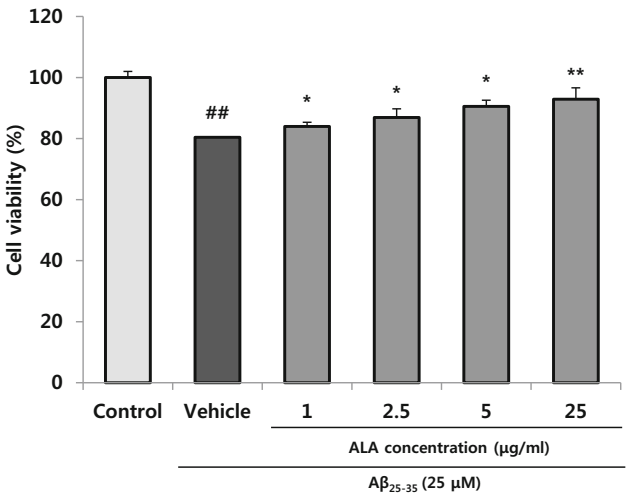

Fig. 1 Effect of ALA on cytotoxicity of $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis

treated with ALA alone for 24 h (data not shown). Figure 1 shows that $A\beta_{25\text{-}35}$, which is a neurotoxic Aβ peptide, significantly decreased the viability of SH-SY5Y cells (80.40%). However, when cells were exposed to various concentrations of ALA (1, 2.5, 5 and 25 μg/mL) prior to treatment with $A\beta_{25\text{-}35}$, ALA enhanced SH-SY5Y cell survival rate from 1 to 25 μg/mL, showing 83.98, 86.90, 90.53 and 92.89%, respectively.

Effect of ALA on LDH release in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

The protective efficacy of ALA against $A\beta_{25\text{-}35}$-induced neuronal cell damage was evaluated by the changes in LDH levels. Figure 2 shows that $A\beta_{25\text{-}35}$ significantly increased the LDH activity (14.23%) compared to the control group (4.17%). However, ALA pretreatment reduced $A\beta_{25\text{-}35}$-mediated LDH release, treatments of 5 and 25 μg/mL, in particular, exerted protective effects against $A\beta_{25\text{-}35}$-induced cell damage, inhibiting LDH release from 14.23 to 4.86 and 1.69%, respectively.

Effect of ALA on ROS production in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

We investigated the inhibitory effect of ALA on ROS production in $A\beta_{25\text{-}35}$-treated SH-SY5Y cells. Figure 3 shows that $A\beta_{25\text{-}35}$ significantly increased ROS levels compared to that in the control group. However, ALA treatment inhibited ROS production. These findings suggest that ALA was able to attenuate oxidative stress by inhibiting ROS production, demonstrating its anti-oxidative effect in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells.

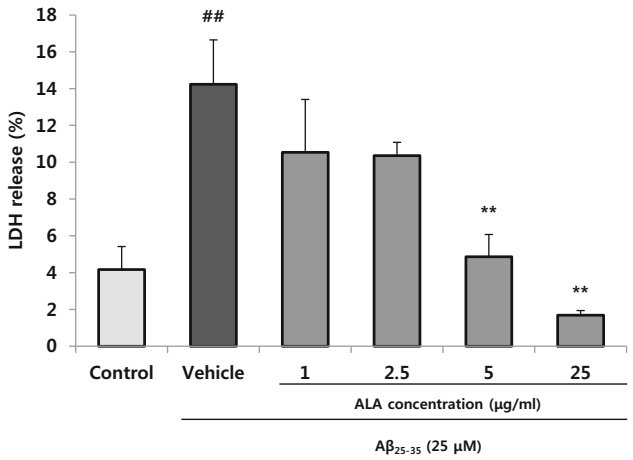

Fig. 2 Effect of ALA on LDH release of $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis

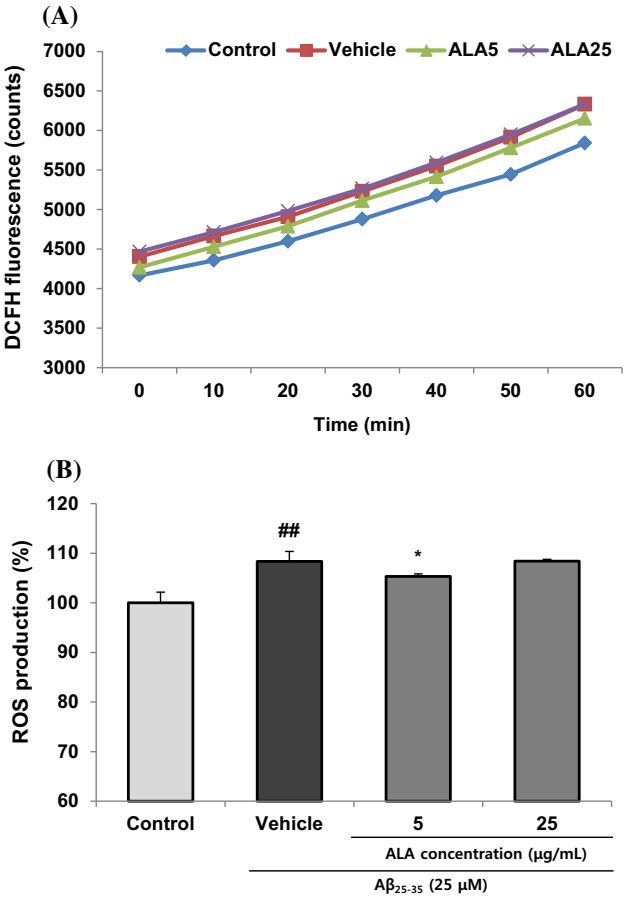

Fig. 3 Effect of ALA on ROS production in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. (**A**) Time course of change in intensity of ROS fluorescence with ALA treatment. (**B**) The production of ROS treated with ALA. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$ compared to the vehicle group by Student's *t* test analysis

Effect of ALA on ERK1/2 and JNK signaling pathway in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

To examine the molecular mechanism involving MAPK activity after ALA treatment, western blot studies were performed. As shown in Fig. 4, protein levels of pERK1/2 and pJNK were induced by $A\beta_{25\text{-}35}$, indicating that ERK1/2 and JNK MAPK signaling is associated with cell death in the presence of $A\beta_{25\text{-}35}$. However, ALA significantly inhibited phosphorylation of ERK1/2 and JNK. These results indicated that ALA might protect $A\beta_{25\text{-}35}$-induced ROS via modulation of the ERK1/2 and JNK signaling pathway.

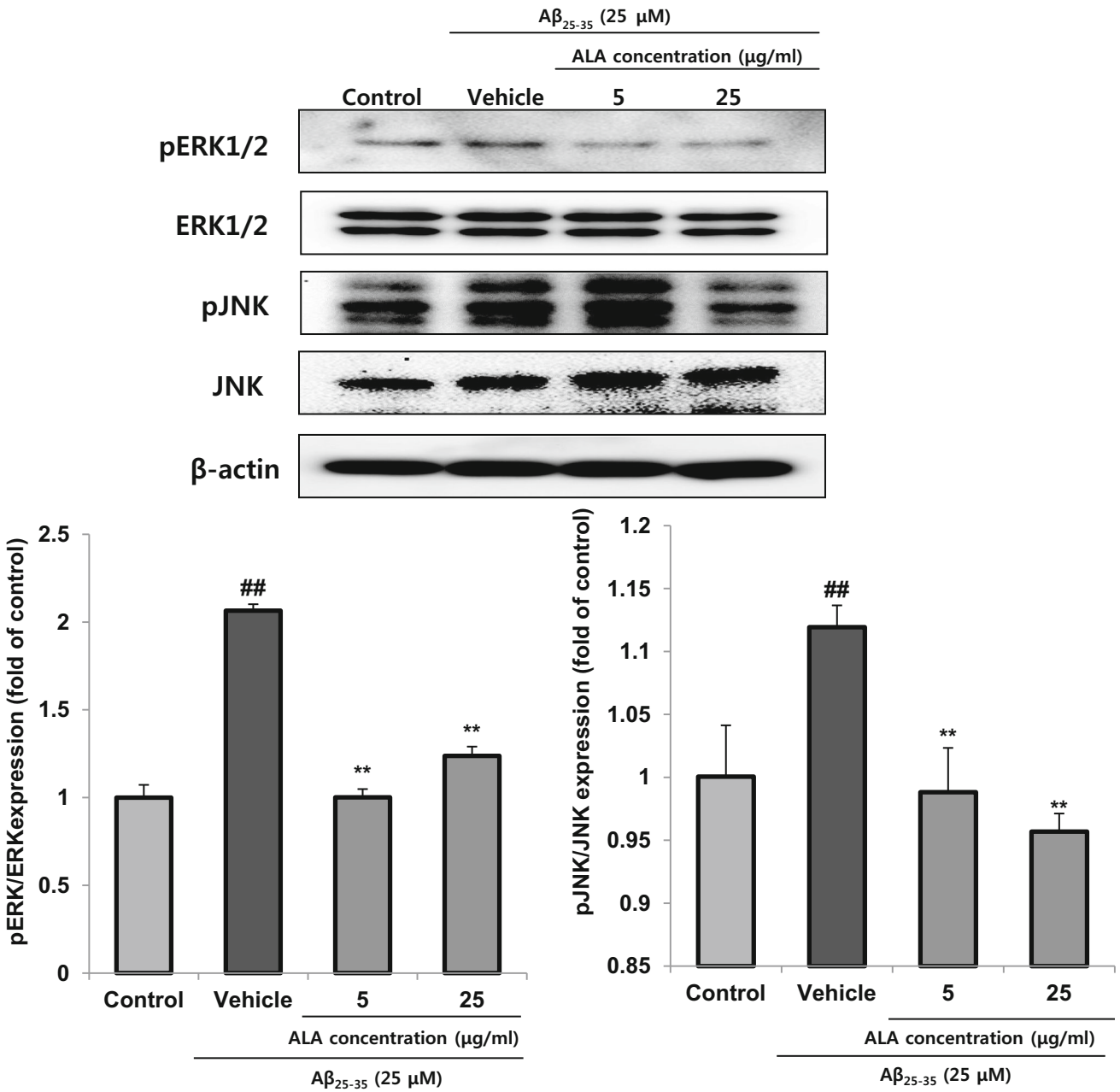

Fig. 4 Effect of ALA on the protein levels of pERK1/2, ERK1/2, pJNK and JNK in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis. β-actin was used as a loading control

Effect of ALA on APP processing in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

The effect of ALA on processing of APP in SH-SY5Y cells was observed by western blotting. Our results show that SH-SY5Y cells treated with $A\beta_{25\text{-}35}$ resulted in an obvious increase in C-terminal fragment β (CTFβ), BACE and PS-1 expression. Although the levels of APP did not show a significant increase, it showed an increasing trend after $A\beta_{25\text{-}35}$ treatment (Fig. 5). However, ALA (especially at a concentration of 25 μg/mL) significantly inhibited those proteins compared to the vehicle group, indicating that ALA might reduce the production of Aβ by down-regulation of amyloidogenic APP processing. Meanwhile, compared to the $A\beta_{25\text{-}35}$-treated group, treatment with ALA induced the non-amyloidogenic pathway by up-regulating the expression of ADAM10 and TACE, which are components of α-secretase (Fig. 6). These findings suggest that stimulating the non-amyloidogenic pathway in response to ALA may provide a neuroprotective effect against Aβ.

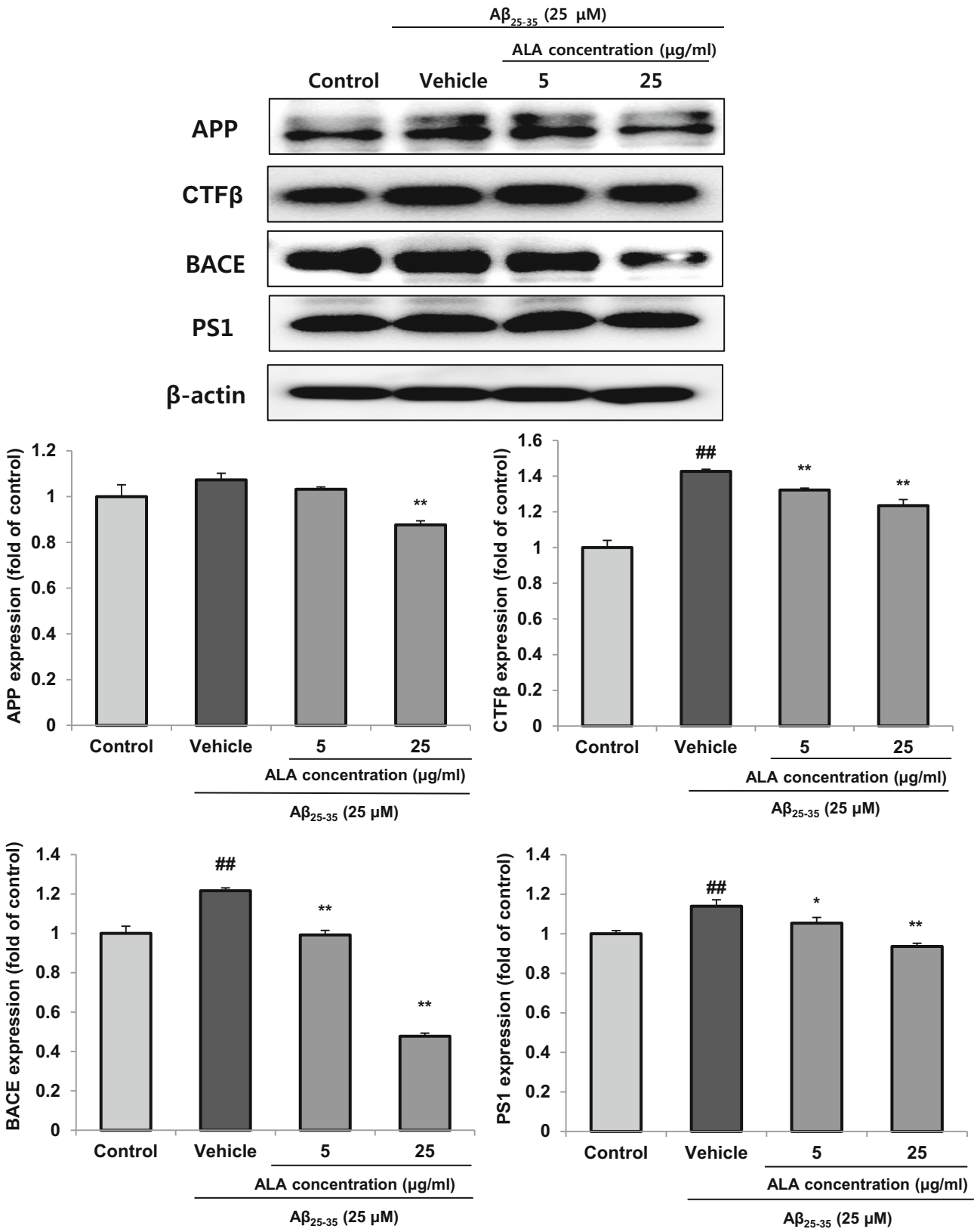

Fig. 5 Effect of ALA on amyloidogenic pathway-related protein expressions in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis. β-actin was used as a loading control

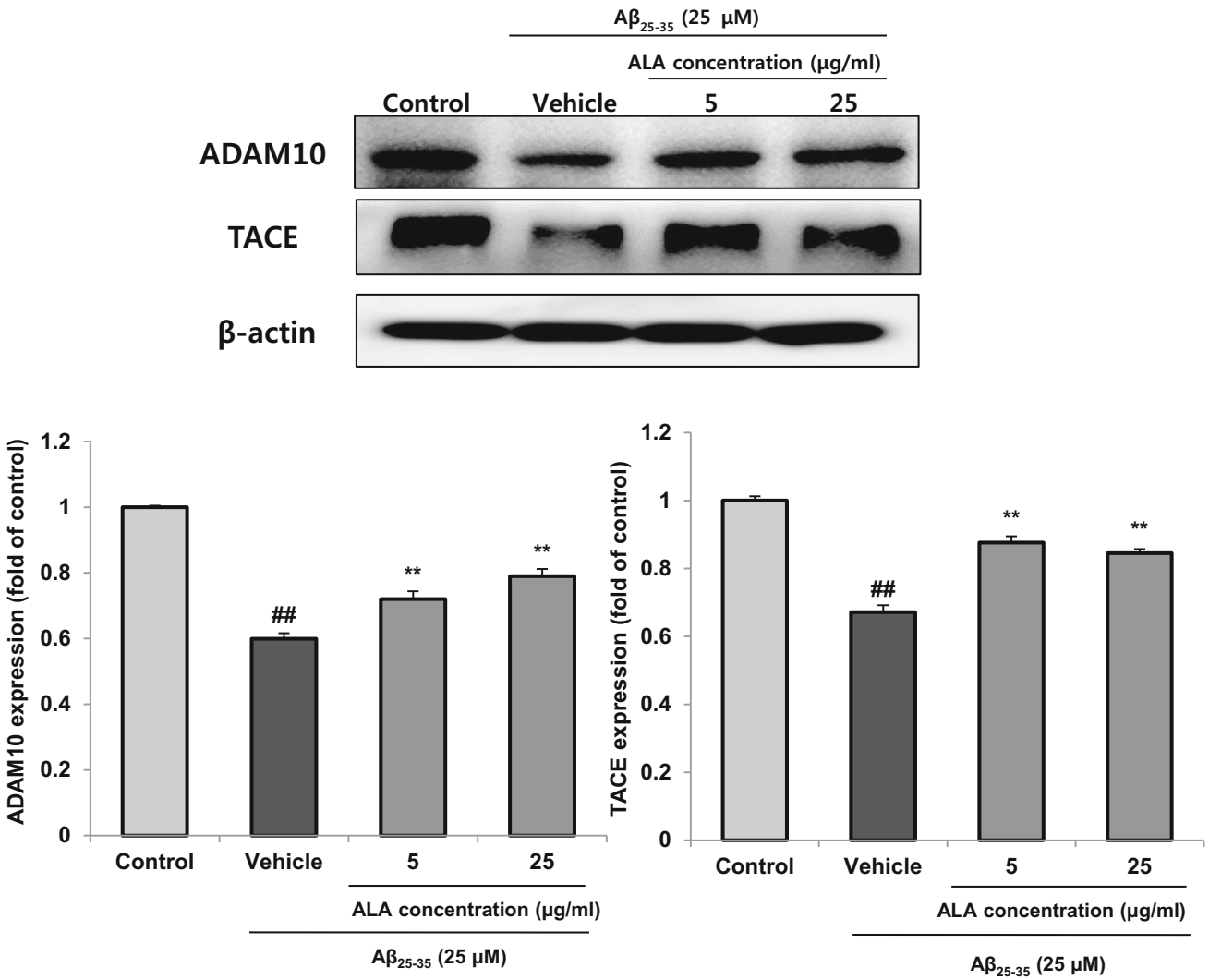

Fig. 6 Effect of ALA on non-amyloidogenic pathway-related protein expression in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values present the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis. β-actin was used as a loading control

Effect of ALA on neuronal apoptosis in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells

To elucidate whether ALA has a neuroprotective effect against $A\beta_{25\text{-}35}$-induced apoptosis, we performed Hoechst 33342 staining. Figure 7(A) and (B) show the number of apoptotic cells. A significant increase in the number of fragmented nuclein stained with Hoechst 33342 was observed after $A\beta_{25\text{-}35}$ treatment, with the ratio of apoptotic cells increasing from 5.38 to 20.13%. This ratio decreased to 9.20% after treatment of ALA (25 μg/mL). To further corroborate a change in apoptosis-related protein levels, Bax and Bcl-2 expressions were assessed by western blotting. As shown in Fig. 7(C), we observed a decrease in the expression of Bcl-2/Bax ratio in $A\beta_{25\text{-}35}$-treated cells, as compared to that in non-treated cells. However, ALA treatment at 5 and 25 μg/mL resulted in increased expression of Bcl-2 and decreased expression of the Bax protein. These results suggest that changes in condensed nuclear and apoptotic bodies induced by $A\beta_{25\text{-}35}$ were significantly attenuated via up-regulation of Bcl-2 and down-regulation of Bax in the presence of ALA.

Discussion

A number of studies have shown that accumulation of Aβ in the brain is strongly associated with oxidative stress, leading to neuronal cell death [28, 29]. Aβ induces the generation of free radicals and was found to have a neurotoxic effect [30]. Free radicals can promote protein cross-linking to form Aβ aggregates. The vicious cycle involved

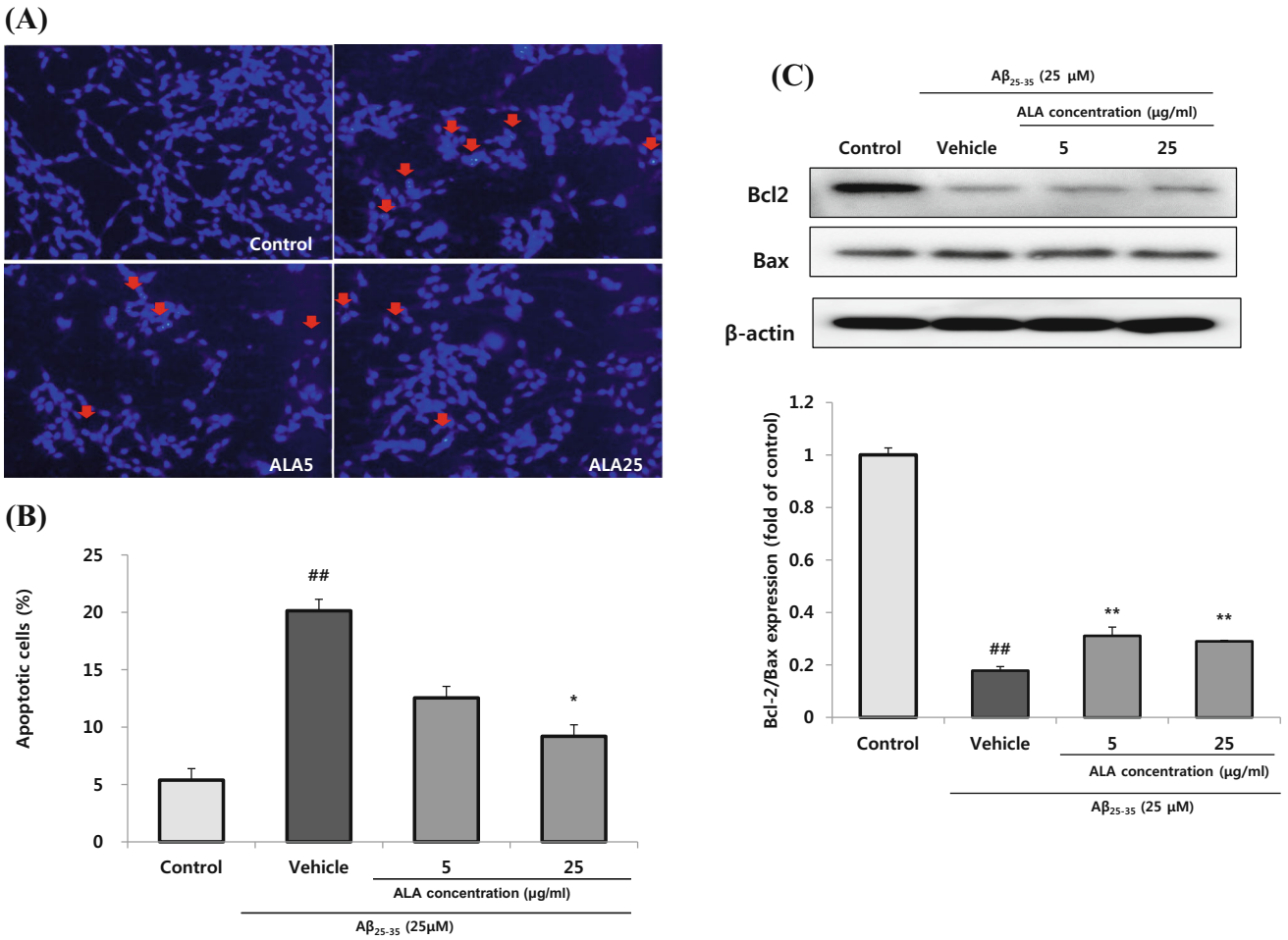

Fig. 7 Effect of ALA on neuronal apoptosis in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. (**A**) The nuclear morphology of cells was determined by Hoechst 33342 staining. Representative images of nuclei stained with Hoechst 33342. The apoptotic bodies are indicated with arrows. (**B**) Quantification of the nuclei demonstrating apoptotic bodies was observed under a fluorescence microscope. Magnification was ×400. (**C**) Effects of ALA on Bcl-2 and Bax protein expressions in $A\beta_{25\text{-}35}$-induced SH-SY5Y cells. Values represent the mean ± SD. $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle group by Student's *t* test analysis. β-actin was used as a loading control

in this abnormal APP processing and Aβ metabolism may be further reinforced by oxidative stress [31–33]. Recent studies have suggested that a possible strategy for AD prevention/treatment is the use of natural products [34–36]. Using natural sources is usually considered to be safer and have fewer side effects than chemically synthesized drugs. Dietary supplementation of *n*-3 PUFA, especially DHA, diminished Aβ-induced neuronal toxicity and the amyloid plaque burden in vivo [37]. However, it remains uncertain whether ALA has a neuroprotective effect against Aβ and the mechanism underlying this effect.

The SH-SY5Y human neuroblastoma cells extensively have been used to evaluate the changes of neurotransmitter release, neuronal apoptosis and neurodegeneration, so as to mimic responses of neurons in experimental neuroscience research [38]. AD relates to high concentrations of the Aβ, which causes apoptosis in neuronal cells [39]. Aβ-induced neuronal damage in the SH-SY5Y cells is contributed to molecular signaling mechanisms that might be involved in the degeneration process of AD. The Aβ initiates the APP processing, leading to activation of mitochondrial and extramitochondrial apoptotic pathway [40]. In the present study, we used SH-SY5Y neuronal cells treated with Aβ to examine the role of ALA in regulating APP processing and neuronal apoptosis against Aβ.

We have previously demonstrated that administration of perilla oil, which contains high levels of ALA, ameliorated cognitive decline in an $A\beta_{25\text{-}35}$-injected mouse model [22]. In addition, treatment with perilla oil and ALA protected hydrogen peroxide-induced neuronal apoptosis through inactivation of caspase-9 and PARP and also up-regulated levels of Bcl-2 relative to Bax (not published). In this study, the protective effect and possible mechanism of ALA on $A\beta_{25\text{-}35}$-medicated oxidative stress, neuronal

apoptosis and APP processing were examined. $A\beta_{25-35}$ treatment markedly decreased cell viability; however, ALA treatment significantly attenuated cell loss in SH-SY5Y induced by $A\beta_{25-35}$. This protective effect of ALA was also confirmed by LDH assay, as shown by significant inhibition of LDH release at ALA concentrations of 5 and 25 μg/mL.

ROS production caused by Aβ is known to be strongly associated with the senile plaques deposit in the brain of AD, resulting in neuronal cell death [41]. In agreement with this notion, we observed that treatment of $A\beta_{25-35}$ induced oxidative stress by generating ROS. In contrast, ALA scavenged the ROS to alleviate oxidative stress in cells induced with $A\beta_{25-35}$. During the progression of AD, the neuronal apoptosis response to ROS correlates with activation of MAPK signaling [42]. We further investigated whether ALA protects oxidative stress and neurotoxicity by modulating the MAPK pathway. Our results showed that $A\beta_{25-35}$ significantly increased phosphorylation of ERK1/2 and JNK, indicating that $A\beta_{25-35}$ induces neurotoxicity by activation of MAPK signaling. However, the activation of these kinases was significantly down-regulated by ALA, suggesting that ALA is involved in the protective effect against $A\beta_{25-35}$-mediated ROS production and neurotoxicity through the ERK1/2 and JNK signaling pathway. In agreement with our findings, ALA also inhibited phosphorylation of MAPKs in lipopolysaccharide-induced RAW 264.7 cells [43]. Lee et al. [44] reported that inhibition of the ERK kinase pathway could enhance α-secretase, but inhibit β- and γ-secretase activity, thereby inhibiting Aβ formation. In addition, activation of JNK is involved in APP metabolism. Consistent with a role in JNK signaling, a previous study has demonstrated that inhibition of JNK activity is correlated with prevention of amyloidogenic cleavage of APP and reduction in Aβ production [45]. We speculate that inhibition of pERK1/2 and pJNK may participate in APP processing.

APP undergoes two major metabolic pathways: the amyloidogenic and non-amyloidogenic pathways. Under normal physiological conditions, APP is mainly cleaved by α-secretase and then secretes sAPPα, which is a non-toxic Aβ. However, APP cleaved by β- and γ-secretases generates neurotoxic Aβ [46]. Therefore, regulation of these enzymes (α, β and γ) is a prominent target for treatment of AD. Recently, Amin et al. [47] demonstrated that treatment of Aβ significantly elevated the protein expression levels of AD markers like APP and BACE in SH-SY5Y neuronal cells. Our results revealed that treatment with $A\beta_{25-35}$ significantly increased CTFβ, BACE and PS1 protein expression levels, whereas ALA suppressed those levels in SH-SY5Y cells. This indicates that Aβ-driven amyloidogenesis was blocked by ALA treatment. In contrast, ALA treatment increased expression of ADAM10 and TACE, which are considered to be the most likely candidates for α-secretase, thereby inhibiting Aβ production through induction of the non-amyloidogenic pathway. It has been shown that altered APP processing in SH-SY5Y cells exposed to chronic hypoxia resulted in decreased sAPPα levels together with down-regulation of ADAM10 and TACE protein expression [48]. Taken together, our findings suggest that enhancement of ADAM10 and TACE activity by ALA treatment supports the non-amyloidogenic route of APP metabolism.

The Aβ treatment may result in neuronal cell death. In addition, neuronal apoptosis might be responsible for the accumulation of Aβ, which would lead to increased apoptosis in neighboring neurons. Aβ-induced neuronal cell death is associated with down-regulation of anti-apoptotic Bcl-2 and with up-regulation of pro-apoptotic Bax protein expressions [49, 50]. In the present study, ALA treatment prevented SH-SY5Y cells from $A\beta_{25-35}$-induced neurotoxicity, as shown by a decline in apoptotic cell bodies in Hoechst 33342 staining. We also found that $A\beta_{25-35}$ treatment resulted in down-regulation of Bcl-2 as well as up-regulation of Bax expression, whereas treatment with ALA up-regulated the level of Bcl-2/Bax protein expression. These results demonstrated that ALA could attenuate $A\beta_{25-35}$-induced neuronal apoptosis by preventing amyloidogenic APP processing.

Since a bioconversion of ALA to DHA is very limited, dietary intake of ALA has been little focused on brain health compared to supplementation with EPA/DHA. However, plant-derived ALA represents more sustainable sources of omega-3 fatty acids and exerts identical effects as DHA in various physiological processes [51]. There are evidences that ALA is a potent neuroprotective agent against ischemia in animal models and ALA plays a protective role from stroke risk [52, 53]. In addition, ALA protected neuronal death against glutamate toxicity [54]. Furthermore, it has been indicated that dietary ALA-enriched perilla oil and EPA/DHA-enriched fish oil exert similar physiological activity [55]. Our previous study also demonstrated that ALA administration improved cognitive ability through APP processing in Aβ-injected AD mice model, comparable to DHA [56], suggesting that ALA may independently or directly contribute to brain health.

In summary, our findings suggest that ALA from perilla oil increased cell viability and decreased LDH release against $A\beta_{25-35}$ treatment. In addition, ALA attenuated $A\beta_{25-35}$-induced ROS production via regulation of MAPK signaling as indicated by down-regulation of ERK1/2 and JNK phosphorylation. These functions are associated with inhibition of the amyloidogenic pathway and promotion of the non-amyloidogenic pathway, leading to the reduction of Aβ deposits. Moreover, ALA treatment prevented neuronal apoptosis through the up-regulation of Bcl-2/Bax

protein expression ratio. Therefore, ALA may serve as a useful therapeutic agent for prevention of AD progression.

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