**Gamma-linolenic acid ameliorates Aβ-induced neuroinflammation through NF-κB and MAPK signalling pathways**

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**A B S T R A C T**

Beta-amyloid (Aβ) are known to form senile plaques causing neuroinflammation, which was accepted as the major pathological mechanism in Alzheimer’s disease (AD). To elucidate the molecular mechanism of gamma-linolenic acid (GLA) on neuroprotective actions in inflammation, the effect of GLA on Aβ-stimulated in PC12 cells was investigated. Pre-treatment of GLA significantly decreased Aβ-stimulated cytotoxicity through the reduction of ROS and downregulation of caspase-3, thereby attenuating apopotic morphological alteration. GLA inhibited the production of proinflammatory cytokines including TNF-α and PGE2 and further blocked NF-κB subunit p65 activation by suppressing IkB-α degradation. Mechanistic studies revealed that the inhibitory effect of GLA was accompanied by reducing expression of ERK1/2 and JNK activity but not by p38 MAPK. In conclusion, given that GLA prevents the Aβ damage via NF-κB signaling pathway, all of which may provide an exciting view of the potential application of GLA as a future research for AD.

**1. Introduction**

Alzheimer’s disease (AD) is the most common form of senile dementia of unknown origin characterized by progressive memory and cognitive impairment. Despite of its distinct histopathological hallmark, characterized by neuronal degeneration, synaptic loss, presence of senile plaques and neurofibrillary tangles, its etiology is ambiguous (Esposito et al., 2006). However, it is widely accepted that β-amyloid (Aβ) peptides, the main constituent of senile plaques, play a central role in AD pathogenesis (Huang & Mucke, 2012). Aggregates of Aβ, generated from β- and γ-secretase cleavage of amyloid precursor protein, have been a neurotoxic event that plays a critical role in the development and progress of AD including oxidative stress, inflammation, aberrant synaptic plasticity and memory impairment (Chopra, Misra, & Kuhad, 2011; Sendrowski, Sobaniec, Stasiak-Barmuta, Sobaniec, & Popko, 2015). Aβ could induce an excessive generation of reactive oxygen species (ROS), which is indexed by the formation of membrane lipids peroxidation, mitochondrial damage and DNA fragmentation and thus may cause neuronal dysfunction, dementia, and further lead to death (Zhang et al., 2013).

Though the mechanisms through which Aβ exerts its toxicity have not yet been completely revealed, it appears that neuroinflammation is one of the critical feature in AD pathogenesis. The deposition of Aβ has been shown to activate neuroinflammatory responses by inducing the expression of inflammatory cytokines and mediators via nuclear factor κB (NF-κB) activation (Zhao et al., 2013). When NF-κB activated, elevates IkB-α phosphorylation and degradation allowing the translocation of NF-κB into the nucleus and binds to NF-κB binding sites in the promoter regions of inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2) and nitric oxide (NO) (Shih, Wang, & Yang, 2015). In addition, the activation of NF-κB is mediated by a wide diversity of upstream protein kinases such as mitogen-activated protein kinases (MAPKs). MAPK family members including extracellular protein regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. MAPKs have been proposed to be important signaling components linking extracellular stimuli to cellular responses. In different cell models including SH-SY5Y cells, C6 glial cells, PC12 cells and BV2 microglial cells, MAPK members are activated by Aβ and this activation is usually associated with cell death (Bing et al., 2014; Lei et al., 2014; Raha et al., 2016; Youn, Lee, Jeong, Ho, & Jun, 2016).

GLA is found mostly in the plant seed oils of evening primrose, borage, black current and hemp. GLA, a member of n-6 polyunsaturated fatty acids (PUFA), can be produced in variety of human tissues from linoleic acid (LA) by δ-6-desaturase. However, the lack of δ-6-desaturase activity has been shown in various physiologic state, including nutrient deficiency, diabetes and aging. Supplement with GLA to offset the decreased δ-6-desaturase activity may attenuate cancer, osteoporosis, cardiovascular disease, diabetic neuropathy and inflammatory diseases (Das, 2007; Kapoor & Huang, 2006).
Dietary LA is metabolized by Δ-6 desaturase to form GLA, which is rapidly elongated to DGLA then further converted to arachidonic acid (AA) by Δ-5-desaturase. LA competes with α-linolenic acid (n-3 PUFA) for Δ-6 desaturase and thereby eventually inhibits formation of anti-inflammatory EPA, of which dietary GLA does not. Furthermore, dietary GLA increases the content of DGLA, the immediate precursor of anti-inflammatory 5-LOX metabolites including prostaglandins, leukotrienes, and platelet-activating factor (Fan & Chapkin, 1998).

Chang et al., reported that GLA is more potent than LA in inhibition of LPS-induced NF-κB transcriptional activity and inflammatory events in RAW264.7 macrophages (Chang et al., 2010). In addition, GLA had anti-inflammatory property by impeding palmitic acid-induced the activation of the ERK, p38 MAPK and PKC-θ and transcriptional activity of NF-κB in C2C12 myotubes (Chen et al., 2015). GLA induced tumor regression and preserved the surrounding normal brain tissue in animal glioma model, and three open-label clinical studies showed that intratumoral injection of GLA was effective in reducing the size of glioma without any significant side effects (Bakshi, Mukherjee, Bakshi, Banerji, & Das, 2003; Das, Prasad, & Reddy, 1995; Naidu, Das, & Kishana, 1992). Furthermore, several long-term studies have demonstrated that up to 2.8 g of GLA daily is well tolerated for treatment of rheumatoid arthritis without any major side effect (Leventhal, Boyce, & Zurier, 1993; Zurier et al., 1996). According to clinical studies of GLA considering a variety of conditions, oral doses of GLA in trials have ranged from 0.5 to 3 g per day.

Although previous reports indicated that GLA has anti-inflammatory activities, the molecular mechanism underlying its effect in Aβ-induced neuroinflammation have not yet been clearly explored. Our previous study demonstrated that GLA not only suppressed recombinant human BACE1 activity but also non-competitively bound and restrained human BACE1 in silico docking model system (Youn et al., 2014), which evokes our interest in GLA and Aβ-induced inflammatory signaling pathway. Therefore, the present study investigated the protective effect of GLA against Aβ cytotoxicity and explored its possible underlying mechanisms.

2. Materials and methods

2.1. Cell culture and peptide preparation

Rat pheochromocytoma (PC12) cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in RPMI1640 medium supplemented with penicillin (100 U/mL), 5% FBS, and 10% horse serum and incubated at 37 °C in a 5% CO2 incubator. PC12 cells have been widely used as a cellular model in the studies of neurotoxicity, apoptosis, and brain damage (Lv et al., 2017; Zurier et al., 1996). According to clinical studies of GLA concentration, the immediate precursor of dietary LA is metabolized by Δ-6 desaturase and thereby eventually inhibits formation of anti-inflammatory EPA, of which dietary GLA does not. Furthermore, dietary GLA increases the content of DGLA, the immediate precursor of anti-inflammatory 5-LOX metabolites including prostaglandins, leukotrienes, and platelet-activating factor (Fan & Chapkin, 1998).

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2.2. Measurement of cell viability and ROS level

Cell viability was assessed by using MTT assay. Briefly, PC12 cells in 96-well plates were cultured at a density of 1 × 105 cells per well for 24 h and then pre-incubated with or without GLA (10, 50 and 100 μM) for 1 h following incubation with 50 μM Aβ25–35 for another 24 h. After adding the medium with 20 μL MTT solution (final concentration, 1 mg/mL), the cells were incubated at 37 °C for 4 h. The supernatants were aspirated off and formazan crystals were dissolved with DMSO. The absorbance was recorded at 570 nm by using an ELX808 plate reader (Biotek, Winonski, VT, USA).

ROS level was determined by using CM-H2DCFDA (In Vitrogen, Carlsbad, CA, USA). PC12 cells were loaded with 10 μM CM-H2DCFDA at 37 °C for 30 min in the dark and then washed twice with Hank’s balanced salt solution (HBSS), and finally, the fluorescence intensity was measured at the excitation wavelength of 485 nm and the emission wavelength of 530 nm using a fluorescence spectrophotometer (FLX800, Biotek, Winonski, VT, USA).

2.3. Determination of apoptotic cells and caspase-3 activity

PC12 cells were fixed with formaldehyde (4% in PBS) and then stained with Hoechst 33342, fluorescent dye, for 20 min (Molecular Probes, Eugene, OR, USA). The morphology change was observed with fluorescence microscope (Olympus, Tokyo, Japan). The activation of caspase-3 was determined using colorimetric protease assay kit (BioVision, Palo Alto, CA, USA). The protein contents of the cell extracts were incubated with DEVD-pNA and the production of pNA was measured using a plate reader at 405 nm.

2.4. Measurement of PGE2 and NO release

PGE2 levels were determined by ELISA kit (R&D, Minneapolis, MN, USA) and NO concentration were determined using colorimetric reaction with Griess reagent (Sigma-Aldrich). The medium and primary antibody solution were added to a 96-well plate pre-coated with goat anti-mouse IgG and then PGE2 conjugate was added. After 1 h reaction, any unbound antibody-enzyme reagent was washed and removed. Substrate solution was added and the intensity (405 nm) was detected using ELX 808 microplate reader.

To measure NO level, cell culture media were mixed with equal volume of Griess reagent in a 96-well plate. After 10 min incubation at room temperature, nitrite production was calculated from the absorbance of the mixture at 550 nm using standard curve of NaN3O2.

2.5. Flow cytometry analysis

For assessment of cell cycle and apoptosis, flow cytometry was analyzed using the MUSE™ cell analyzer (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. To determine the effect on cell cycle, cultured cells were washed with PBS and fixed with 70% ethanol for at least 3 h at –20 °C. After washing, cell cycle reagent cells were added and incubate for 30 min at RT in the dark. To detect apoptosis, cells were mixed with the Muse™ Annexin V and Dead Cell reagent, and then incubated for 20 min at RT in the dark.

2.6. Western blotting

Proteins obtained from cell lysates were separated by SDS-PAGE and then transferred to a PVDF membrane (Millipore). After blocking with in PBST buffer containing 5% nonfat dry milk for 1 h, the membrane was incubated overnight at 4 °C with primary antibodies and then incubated with corresponding secondary antibodies at RT for 1 h. Transferred proteins were visualized by an ECL reaction kit (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). To determine the levels of primary antibody such as iNOS, COX-2, p65, IkBα and MAPKs was prepared as previously described (Youn et al., 2016).

2.7. Statistical analysis

All data were expressed as the means ± SD of three independent experiments. Statistical comparisons of differences between groups were conducted using the Student’s t test, considering ‘p < .05, "p < .01 and ""p < .001 as significant differences.
3. Results

3.1. GLA protected Aβ_{25-35}-induced cell death and intracellular accumulation of ROS

The neuroprotective effects of GLA were evaluated by measuring the viability of PC12 cells incubated with Aβ_{25-35} in the presence or absence of GLA using MTT assay and FACS. Toxicity assays showed that GLA is not toxic to cell viability at the concentration up to 100 µM, presenting an average survival rate of 100% (data not shown). As shown in Fig. 1A, treatment with 50 µM Aβ_{25-35} alone induced about 40% cell death when compared with the control (p < .001). However, pretreatment of GLA restored cell viability in a dose dependent manner with 75.68 ± 1.40, 85.26 ± 1.38 and 97.86 ± 2.25% at 25, 50 and 100 µM respectively.
100 μM (p < .001), suggesting that GLA protects PC12 cells against Aβ25–35-induced cell death.

To determine whether Aβ25–35-induced neurotoxicity was attributable to cell-cycle arrest, flow cytometric analysis was performed. As shown in Fig. 1B and C, flow cytometry showed a significant decrease in the percentage of cells in G0/G1 (p < .05) at 100 μM GLA followed by a significant S increase (p < .05) in comparison to control cells. At 100 μM concentration, GLA almost restored a G0/G1 cell cycle arrest.

Oxidative stress is an early event in the development and progression of AD. GLA was further tested for its ability to antagonize Aβ25–35 induced cell damage by elevated ROS levels. As shown in Fig. 1D and E, staining intensity and larger numbers of bright fluorescent particles in cells were visibly enhanced by Aβ25–35 treatment, indicating the presence of intracellular ROS. When the cells were incubated with different concentrations of GLA (25, 50 and 100 μM) in the presence of Aβ25–35, the ROS levels significantly decreased to 81.18 ± 9.09% (p < .01), 74.85 ± 2.18% (p < .001) and 66.99 ± 3.49% (p < .001), respectively.

3.2. GLA blocked Aβ25–35-induced apoptosis and caspase-3 activation

In order to confirm the protective effect of GLA against Aβ25–35-induced PC12 cells death, the nuclear morphological change in the apoptotic cells was investigated by using Hoechst 33342 staining (Fig. 2A). In the control group, the nuclei of PC12 cells were round and apoptotic cells was investigated by using Hoechst 33342 staining with a significant S increase of iNOS and COX-2 (364.90 ± 20.96 and 354.20 ± 26.42, p < .001). However, GLA significantly reduced Aβ25–35-stimulated iNOS protein production in a dose dependent response. Similar to its inhibitory effect on iNOS expression, GLA also effectively suppressed Aβ25–35-induced activation of COX-2 in PC12 cells. In particular, GLA at 100 μM blocked iNOS expression under the basal level, comparable to the untreated control (91.17 ± 15.88, p < .001).

3.4. GLA repressed Aβ25–35-mediated NF-κB, IκB-α and MAPK expression

As shown in Fig. 4A, Aβ25–35 significantly increased the levels of p65 by 300.06 ± 2.37% as compared with the control group (p < .001). However, GLA rapidly suppressed the expression of p65 in a dose-dependent manner. In particular, the highest concentration of GLA showed almost complete inhibition of p65 phosphorylation to 100.13 ± 8.51% (p < .001). In parallel with the inhibitory effect of GLA on phosphorylation of p65, Aβ25–35-induced IκB-α degradation was obviously blocked by GLA (Fig. 4B).

As presented in Fig. 4, the phosphorylation levels of p38, ERK1/2, and JNK were significantly increased after Aβ25–35 treatment (p < .001). It was observed that GLA decreased Aβ25–35-induced phosphorylation of ERK1/2 and JNK in a dose-dependent manner, whereas it had no effect on the expression level of p38 in Aβ25–35-stimulated PC12 cells. In particular, the phosphorylation of ERK was markedly suppressed almost to its basal level when treated with 100 μM (102.02 ± 11.43%, p < .001). These data suggested that GLA regulated inflammatory reactions by inhibiting ERK and JNK signaling pathways. One mechanism by which fatty acids may exert its anti-inflammatory effect is by up-regulation of MAPKs and here we show that GLA is capable of decreasing ERK and JNK phosphorylation.

4. Discussion

Since substantial evidences showed the major role of oxidative stress in the pathophysiology of AD, the effect of GLA on Aβ25–35-induced intracellular ROS production was investigated. In the present study, GLA protected PC12 cells against Aβ25–35 by decreasing ROS generation, restoring cell cycle arrest, and increasing cell viability. In addition, GLA blocked caspase-3 activation, suggesting that GLA could exert a protective role at the execution phase of apoptosis. GLA in bovine oil improved Aβ25–35-induced memory impairment in male rats through increasing ROS scavenging effect, suggesting that antioxidant property of GLA was implicated in its attenuation of oxidative damage in the hippocampal tissue and cognitive dysfunction (Ghabremanitamadon et al., 2014). Interestingly, selective tumoricidal action of GLA by increasing both ROS and lipid peroxide production as well as altering in energy metabolism was confirmed by several studies in human lung, breast, and prostatic cells (Das, 1991) (see Fig. 5).

The neurotoxic effect of Aβ has been shown to involve multiple intracellular signaling pathways such as phosphorylation cascades leading to activation of NF-κB and MAPKs (Lonpré, Garneau, Christen, & Ramassamy, 2006). The present data showed that GLA decreased NF-κB activation in Aβ25–35-induced insult. Consistent with our result, it has been reported that GLA reduced LPS-stimulated NF-κB production in RAW264.7 macrophage and primary goat mammary gland epithelial cells (Cao et al., 2016; Chang et al., 2010). GLA attenuated glycination-induced memory loss by inhibiting the generation of advanced glycation end products (AGEs), which mediates NF-κB pathway activation in Sprague-Dawley rats (Khan, Haider, Mahmood, Rooome, & Abbas, 2017). In agreement with the observation made in RAW264.7
macrophages, our data demonstrated that GLA suppressed both ERK and JNK activation (Chang et al., 2010). However, in C2C12 murine skeletal muscle cell line, GLA reduced palmitic acid-induced phosphorylation of ERK and p38 (Chen et al., 2015).

Several studies have indicated that BACE1 promoter transactivation and activity are regulated by the transcription factor NF-κB, suggesting that the inhibition of NF-κB pathway leads to suppression of BACE1 activity (He et al., 2011). For instance, (-)-epigallocatechin-3-gallate, a compound from green tea modified cognitive function and β- and γ-secretase activity through inhibition of NF-κB pathway in presenilin 2 mutant mice (Lee et al., 2009). In our previous study, GLA inhibited BACE1 activity indicating that the compound might regulate BACE1

Fig. 2. Effect of GLA on Aβ25–35 induced apoptosis and caspase-3 activation in PC12 cell (A) Morphological apoptosis was determined by Hoechst 33342 staining under fluorescence microscopy (magnification × 400). (B) Histogram representing level of cellular apoptosis. (C) Distribution of early and late apoptotic cells analyzed by flow cytometry. (D) Histogram representing percentage of early and late apoptotic cells. (E) Caspase-3 activity was measured with the colorimetric caspase-3 assay kit. *p < .05, **p < .01, and ***p < .001 vs. the group treated with Aβ25–35 alone.
activation by way of blocking NF-κB signaling pathway (Youn et al., 2014).

GLA is major component of membrane phospholipids where they increase both integrity and fluidity of cellular membrane. Studies in animals clearly showed that GLA display neuroprotective property and exert beneficial effects on the cognitive function with aging. Horrobin (1997) revealed that GLA improved impaired nerve function in diabetic rats through functional changes in neuronal membranes, alterations in nerve prostaglandin synthesis and regulation in nerve blood flow (Horrobin, 1997). Also, GLA supplementation of 260 mg/day enhanced nerve conduction velocity by altering membrane composition and restoring Na⁺, K⁺, and ATPase activity in streptozotocin-diabetic rats (Coste et al., 1999). Furthermore, Kavanagh et al. have shown that GLA treatment increased anti-inflammatory cytokines in hippocampus of

Fig. 3. Effect of GLA on Aβ25-35 induced cytokines, iNOS and COX-2 expression in PC12 cells. Representative western blots of (A) NO, (B) PGE2, (C) TNF-α, (D) iNOS and COX-2 expression. ***p < .001 compared to control group. *p < .05, **p < .01 and ***p < .001 compared to Aβ25-35 alone.
LPS-treated rats, suggesting that the results may be linked with fatty acid-induced upregulation of peroxisome proliferator-activated receptor gamma (PPAR-γ) possessing anti-inflammatory property (Kavanagh, Lonergan, & Lynch, 2004).

Even though the important role of GLA in neuroprotection has been discovered, the mechanism by which GLA enter the brain are still not fully understood. The neuroprotective agents must move across the blood–brain barrier (BBB) to attain a critical therapeutic concentration within central nervous system. Current study regarding the uptake of fatty acid demonstrated both transport protein facilitated movement and passive diffusion of fatty acids across the BBB may require (Mitchell, On, Del Bigio, Miller, & Hatch, 2011). The chemical structure of fatty acids such as the degree of saturation, and the chain length affects the rate of transport, with short to medium chain saturated fatty acids (SFAs) penetrating the microvessel monolayer easier than longer ones, while unsaturated fatty acids accumulated to a higher degree than SFA of similar chain length in basolateral medium. In transport, furthermore, fatty acid transport proteins (FATPs) including FATP 1–6, fatty acid translocase (FAT)/CD36, intracellular fatty acid binding proteins 1–9 (FABP), plasma membrane FABP, etc. were identified as responsible for fatty acid uptake (Guest, Garg, Bilgin, & Grant, 2013).

Although further research into the mechanism of GLA transport across the BBB is required, it is still meaningful that the present study offered a novel evidence which indicated that GLA, a natural product, exerted a protective effect on PC12 cells in Aβ25–35-induced PC12 cells of neurotoxic damage. In addition, GLA reduced the production of proinflammatory mediators and cytokines by inactivating their corresponding protein, which was connected with inhibition of ERK and JNK MAPK and NF-κB signaling cascades. In conclusion, this study provides a basis for elucidating a therapeutic potential of GLA in preventing and/

Fig. 4. Effect of GLA on Aβ25–35 induced NF-κB and MAPKs activation in PC12 cells. (A) Representative western blots of p65 and IκBα expression. (B) Representative western blots of MAPKs (p38, ERK 1/2 and JNK) phosphorylation. ###p < .001 compared to control group. *p < .05, **p < .01 and ***p < .001 compared to Aβ25–35 alone.
Fig. 5. Schematic diagram of anti-inflammatory pathway of GLA on Δ5-35 induced NF-κB and MAPK signalling pathway.

or treating degenerative disorders of the brain as well as a possibility for the use as a functional food, nutraceutical or dietary supplement.

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References


