Genistein Alleviates β -Amyloid-Induced Inflammatory Damage Through Regulating Toll-Like Receptor 4/Nuclear Factor κ B

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ABSTRACT Genistein (GEN), a major soybean isoflavone (SIF), might possess neuroprotective properties through its anti-inflammatory activity. We hypothesized that GEN could prevent the inflammatory damage detected in C6 cells induced by β -amyloid peptides 25–35 (A β 25–35). Accordingly, we evaluated the inflammatory damage induced by A β 25–35 and the protective effect of GEN against A β 25–35 in C6 cells. In our study, the C6 glial cells (rats glioma cell lines) were preincubated with or without GEN for 2 h following incubation with A β 25–35 for another 24 h. Then, methylthiazolyl tetrazolium (MTT) assay was used to assess the cell viability. Immunofluorescence staining was used to identify the C6 cells. Inflammatory factors tumor necrosis factor (TNF)- α and interleukin (IL)-1 β were analyzed by using enzyme-linked immunosorbent assay (ELISA). Western blot analysis and reverse transcription-polymerase chain reaction analysis were performed to assess the expression of Toll-like receptors 4 (TLR4), inhibitor of kappaB-alpha (I κ B- α). The current results showed that GEN could alleviate A β 25–35-induced cell apoptosis and prevent A β 25–35-induced TNF- α and IL-1 β release from C6 cells. In addition, GEN prevented A β 25–35-induced upregulation of the gene and protein expression of TLR4, and GEN significantly upregulated the expression of I κ B- α in C6 cells damaged by A β 25–35. These results suggest that GEN can alleviate the inflammatory stress caused by A β 25–35 treatment, which might be associated with the neuroprotective effect of GEN regulating the TLR4/NF κ B signaling pathway.

KEY WORDS: • *beta-amyloid peptides* • *genistein* • *inflammatory factors* • *neuroprotection*

INTRODUCTION

ALZHEIMER'S DISEASE (AD) IS A progressive neurodegenerative disease characterized by gradual advancement of memory loss. The definitive diagnosis of AD is based on the presence of extracellular amyloid plaques, which comprised neurotoxic β amyloid peptides (A β). Inflammation contributes to the pathogenesis of some neurodegenerative diseases such as AD, since amyloid plaques are infiltrated by reactive microglia and astrocytes.¹

Studies have shown that inflammatory processes are part of the brain pathology in AD.^{2,3} A β might function as an immune stimulus for glial cell activation in the brain of AD patient. Once activated by A β , these glial cells can produce a series of proinflammatory factors, such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , which have been implicated in the development of AD.⁴ Moreover, the release of these factors enhances A β -induced glial activation and creates a continuous cycle of inflammatory reaction. Although the exact relationship between the glial cells and

AD remains unclear, it is clear that inflammation is an early and critical event driving the disease process.

Some studies have suggested that Toll-like receptors (TLRs), which are produced by glial cells, promoted inflammatory disorders.⁵ In AD mice, the activation of TLR4 was required for clearance of A β .⁶ Furthermore, it was reported that the nuclear factor κ B (NF κ B) signaling pathway can be activated by TLR4.⁷ Thus, the TLR4/NF κ B signaling pathway might play a major role in the neuroinflammation induced by A β .

Many phytochemicals are known to exert anti-inflammatory properties.^{8,9} Genistein (GEN) is a major bioactive isoflavone obtained primarily from soybeans.¹⁰ It has been demonstrated that GEN has numerous activities, such as inhibition of protein tyrosine kinase, antioxidation, anti-apoptosis, and deregulation of mitochondrial membrane pore permeability.^{11–13} GEN modulates signaling pathways not only by triggering cell survival and apoptotic responses but also through reversing endogenous inflammatory stress,^{14–16} especially in the model of nervous system disease such as multiple sclerosis.¹⁷

Thus far, there has been little research exploring the mechanism of GEN's action on neuroinflammation. It was reported that GEN has anti-inflammatory effects on human brain microvascular endothelial cells and BV-2

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cells,^{18,19} but it remains less clear whether GEN has similar neuroprotective effects against A β -induced neuroinflammation.

Thus, in this study, we investigated the neuroprotective effects of GEN against A β -induced neuroinflammation and whether GEN could regulate the TLR4/NF κ B signaling pathway in the C6 cell culture system.

MATERIALS AND METHODS

Materials

GEN purchased from Sigma Chemical Co. A β 25-35 (Sigma) was aggregated in an incubator for 3 days, dissolved in deionized distilled water at a concentration of 625 μ M, and then kept at -20°C freezer until used.

Cell line and culture conditions

C6 cells (rat glioma cell line) were maintained in the DMEM, which was supplemented with 10% fetal bovine serum and antibiotics, at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells were seeded at an appropriate density (1×10^6 cells/mL) in a culture dish. The culture medium was changed every day.

Immunofluorescent staining

Immunofluorescent staining was performed to verify the C6 cells as astrocytes. Briefly, C6 cells cultured on glass coverslips were plated into 24-well plates for 24 h and then treated with indicated reagents for 1 h. Upon the completion of drug treatment, glass coverslips were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at room temperature for 30 min. After washing, coverslips were blocked with 10% goat serum for 1 h and then incubated with the rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) overnight at 4°C , followed with incubation with rhodamine-conjugated rabbit IgG for 1 h. After washing, coverslips were incubated with 4', 6-diamidino-2-phenylindole (DAPI) at room temperature for 10 min. Finally, coverslips were washed with PBS and mounted with aqueous mounting media. Fluorescence signals were analyzed by fluoview microscopy (OLYMPUS).

Cell viability

Cell viability was determined by methylthiazolyl tetrazolium (MTT) assay. Briefly, the C6 cells were pre-incubated with or without GEN (12.5, 25, 50, and 100 μ M) for 24 h and after replacing the medium with 20 μ L MTT solution (5 mg/mL), cells were incubated for another 4 h at 37°C , then the absorbance was recorded at 570 nm by microtiter plate reader (Tecan).

Measurement of inflammatory factor in cell supernatant

Supernatants of C6 cells were collected to measure the levels of IL-1 β and TNF- α by immunoassay using a com-

mercially available enzyme-linked immunosorbent assay (ELISA) kit (Groundwork Biotechnology Diagnosticate Ltd.). Cell samples were added in duplicate to microtiter wells and assayed according to routine procedures. The resulting color reaction was measured at 450 nm with an ELISA reader (Infinite M200; TECAN).

Reverse transcription-polymerase chain reaction analysis

Total RNA was purified using TRIzol (Invitrogen). A reverse transcriptase kit (A3500; Applied Promega) was used for reverse transcription. The mRNA expression of TLR4, I κ B- α , and β -actin (used as an invariant control) was measured. The forward and reverse primer sequences are shown in Table 1. Amplification products were electrophoresed on a 2.0% agarose gel. Then, FluorChem FC2 software (Alpha Innotech) was used to analyze the gray value of the mRNA expression in each group and the ratio of the TLR4 and I κ B- α mRNA against β -actin mRNA was calculated and control was set as 1.

Western blot analysis

Cells (1×10^7 cells) in different treatment groups were collected and washed with PBS, whereas the cell nucleus preparation was carried out using the nucleoprotein extraction kit (Thermo Biotechnology). After centrifugation, sedimented cells were lysed by vigorous shaking in the RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.4], 1 mM DTT, and protease inhibitors) for 40 min at 4°C and then centrifuged again at 16,000 g for 20 min. The supernatant was separated and collected for protein analysis. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology). Protein samples (50 μ g) were loaded and separated by a 10% (TLR4) or 12% (I κ B- α) SDS-acrylamide gel electrophoresis and wet transferred to polyvinylidene fluoride blots at 60 V for 2 h. The membrane was blocked by a fresh blocking buffer (Tris-buffered saline, containing 5% skim milk powder) at room temperature for 1 h. Immunoblots were performed with appropriate antibodies. A primary antibody (diluted in 1:1000 with TBST and 1% nonfat dry milk) for anti-TLR4 (Santa Cruz Biotechnology) and anti-I κ B- α (Cell Signaling Biotechnology) was incubated with membrane for 12 h at 4°C . β -actin (Santa Cruz Biotechnology) was used as housekeeping in cells in our study. The proper secondary antibodies were

TABLE 1. THE FORWARD AND REVERSE PRIMER SEQUENCES OF TLR4 AND I κ B- α

Name of gene	Forward primer sequence	Reverse primer sequence	Annealing temperature ($^{\circ}\text{C}$)
TLR4	5'-GAATGAGGACT GGGTGAGAAA-3'	5'-TCTGCTAAGAA GGCGATACAA-3'	53
I κ B- α	5'-ATTGCTGAGGCA CTTCTGAAAGC-3'	5'-CACTTCAACAGG AGCGAGACCAG-3'	58

incubated for 1 h at room temperature. The blots were washed again thrice with the TBST buffer, and transferred proteins were visualized by an alkaline phosphatase reaction kit according to the manufacturer's instructions. Then, FluorChem FC₂ software (Alpha Innotech) was used to analyze the gray value of the protein expression in each group and the ratio of the TLR4, I κ B- α protein expression against β -actin protein expression was calculated and control was set as 1.

Statistical analysis

Data are presented as mean $\pm$ SE and analyzed using SPSS 11.5 (SPSS, Inc.) by one-way analysis of variance as appropriate. The least significant difference *post hoc* test was used to test differences between means and $P < .05$ was considered to be statistically significant.

RESULTS

Cell morphology and cell identification

As shown in Figure 1A and B, the C6 cells were characteristically star-shaped glial cells under a microscope. GFAP labeling was used to identify astroglial cells. Figure 1C–E showed that C6 cells, which were stained with GFAP and DAPI staining, had the normal nucleus and the features of astroglial cells.

Cell viability in C6 cells

Viability of C6 cells was determined by MTT assay and the results are presented in Figure 2A and B. A β 25-35-treated C6 cells had a significantly lower cell viability than the control group. However, the cell viability in GEN (25, 50, 100 μ M)-pretreated groups was much higher than the only A β 25-35-treated group in C6 cells.

TNF- α and IL-1 β levels

The levels of inflammatory factors (TNF- α and IL-1 β) in C6 cells were measured and the results are shown in Table 2. A β 25-35 significantly increased the levels of TNF- α and IL-1 β compared with the control groups, however, GEN decreased the levels of TNF- α and IL-1 β induced by A β 25-35.

TLR4 expression in C6 cells

A β 25-35 significantly upregulated the mRNA and protein expression of TLR4 compared with the control group (Fig. 3). GEN pretreatment prevented the upregulation of the gene and protein induced by A β 25-35.

I κ B- α expression in C6 cells

GEN significantly upregulated the expressions of both I κ B mRNA and protein levels in C6 cells treated by A β 25-35. GEN reversed the I κ B- α expression induced by A β 25-35 in C6 cells (Fig. 4).

FIG. 1. (A) The morphological shapes of C6 cells in microscope. (B) The glial fibrillary acidic protein (GFAP) staining of C6 cells. (C) The 4', 6-diamidino-2-phenylindole (DAPI) staining of C6 cells. The cell nucleus was stained with blue. (D) The GFAP staining of C6 cells. The cells were stained with green. (E) The whole C6 cells merged (C and D). Color images available online at www.liebertpub.com/jmf

DISCUSSION

Soybeans and foods containing soy isoflavones are believed to prevent a variety of human chronic diseases, including neurological disorders.^{20,21} GEN, a predominant soy isoflavone, has been shown to modulate signaling pathways that elevate endogenous anti-inflammatory responses in astrocytes by activating peroxisome proliferator-activated receptors.²² GEN also has the ability to suppress oxidative stress signaling pathway as well as to regulate the activity of several defense genes in inflammation, such as nitric oxide synthase and glutathione peroxidase. In our previous studies, we have demonstrated that soybean isoflavone alleviates

TABLE 2. THE PROTECTIVE EFFECT OF GENISTEIN ON THE LEVELS OF IL-1 β AND TNF- α IN C6 CELLS

Group	IL-1 β (pg/mL)	TNF- α (pg/mL)
Control	254.48 $\pm$ 10.68	749.51 $\pm$ 26.22
A β 25-35	288.99 $\pm$ 13.39*	857.92 $\pm$ 31.42*
GEN+A β 25-35	257.52 $\pm$ 5.49 $^\#$	740.54 $\pm$ 28.78 $^\#$
GEN	244.14 $\pm$ 9.34 $^\#$	693.41 $\pm$ 63.80 $^\#$

IL-1 β and TNF- α level of C6 cell from untreated cells (control group); cells exposed to 25 μ M A β 25-35 (A β 25-35 Group); cells exposed to 50 μ M GEN 2 h before 25 μ M A β 25-35 was added (GEN 50 μ M+A β 25-35 group); cells exposed to 50 μ M GEN (GEN 50 μ M). All the data are shown with mean $\pm$ SE. Mean values were significantly different from those of the control group: * P < .05; mean values were significantly different from those of the A β 25-35 group: $^\#P$ < .05.

IL, interleukin; TNF, tumor necrosis factor.

FIG. 4. The IκB-α expression [protein expression in (A) and mRNA expression in (B)] in C6 cells damaged by Aβ25-35 and the protective effect of GEN. IκB-α expression of C6 cell from untreated cells (control group); cells exposed to 25 μM Aβ25-35 (Aβ25-35 group); cells exposed to 50 μM GEN, 2 h before 25 μM Aβ25-35 was added (GEN 50 μM + Aβ25-35 group); cells exposed to 50 μM GEN (GEN 50 μM). All the data are shown with mean ± SE. Mean values were significantly different from those of the control group: **P* < .05; mean values were significantly different from those of the Aβ25-35 group: †*P* < .05.

β-amyloid 1–42-induced inflammatory response to improve learning and memory ability in rats;²³ in this study, we further investigated the neuroprotective effects of GEN against Aβ-induced neuroinflammation and whether GEN could regulate the TLR4/NFκB signaling pathway in the C6 cell culture system. We first used the MTT assay to determine the lowest dose at which Aβ25-35 damaged C6 cells and 20 μM Aβ25-35 was used. In addition, the cell viability in the 50 μM GEN-treated group was significantly increased compared to that of controls, which suggests that GEN could promote cell proliferation in C6 cells treated with Aβ25-35. Therefore, the 50 μM GEN was chosen to investigate the neuroprotective effect of GEN against Aβ25-35 Aβ-induced neuroinflammation in C6 cells.

The results of this study indicated that GEN protected C6 cells by suppressing the inflammatory response induced by Aβ25-35, as evidenced by the decreased TNF-α, the down-regulated TLR4, and upregulated IκB-α. The inflammation in the brain is mediated predominantly by two types of glial cells, astrocytes and microglia.²⁴ The activation of glial cells produces a wide spectrum of proinflammatory factors,

including TNF-α and IL-1β, both of which can be induced by Aβ.²⁵ Therefore, we selected these two proinflammatory cytokines as biomarkers to investigate whether GEN has the anti-inflammatory effects and to elucidate the underlying mechanisms. The results showed that GEN decreased the release of TNF-α and IL-1β induced by Aβ25-35 in C6 cells.

The activation of NFκB and TLR4 can occur as a result of TNF-α activation, through stimulation of ubiquitin degradation of IκB.^{26–28} NFκB and TLR4 seem to be key factors to modulate the release of TNF-α and IL-1β. By inducing the production of proinflammatory cytokines and cell adhesion molecules in immune cells, TLRs may indirectly damage neurons in conditions such as ischemic stroke and multiple sclerosis.²⁹ The TLR4 gene is a member of the TLR family, which emerged as a candidate susceptibility gene in neuroinflammation for AD.³⁰ In the brains of AD patients, high levels of TLR4 are expressed in areas surrounding the Aβ plaques in activated glial cells. The close association between Aβ plaques and glial cells has led to their suspected involvement in plaque formation.³¹

Since TLR4 was shown to play an important role in mediating the activation of proinflammatory cytokines in various types of cultured cells through multiple mechanisms, we attempted to examine the possible involvement of GEN in the TLR4 expression both in the mRNA and protein level in Aβ25-35-treated C6 cells. GEN pretreatment reversed the upregulated effects of the genes and proteins induced by Aβ25-35. Therefore, it was inferred that TLR4 might be the key target for GEN to inhibit the release of TNF-α and IL-1β in C6 cells.

The activation of TLR4 induces the phosphorylation of inhibitor of kappa-B (IκB) and dissociates IκB from NFκB. NFκB translocates into the nucleus, activates and regulates the transcription of genes related to inflammatory responses.^{32,33} The two major forms of IκB proteins (IκB-α and IκB-β) degrade in cells following cellular stimulation, including inflammatory cytokines and oxidants.^{34,35} The expression of mRNA and protein of IκB-α in C6 cells was measured to investigate whether IκB-α expression was involved in the GEN's neuroprotective effect against Aβ25-35 in C6 cells. The current results showed that GEN significantly upregulated the expression of IκB-α both in the protein and mRNA level in C6 cells treated with Aβ25-35. GEN has the potential to reverse the expression of IκB-α when the C6 cells were treated with Aβ25-35, which indicated that IκB-α expression was involved in the GEN's protective effect against neuroinflammation induced by Aβ25-35. Our data suggest that a mechanism of inhibition of NFκB regulation by GEN involves blocking the cleavage of IκB-α.

Our results showed that GEN was able to reduce NFκB levels through a novel regulatory mechanism involving the generation of a cleaved form of IκB-α. We further demonstrated that the decrease in NFκB correlated with the downregulation of the protein expression of TLR4. GEN prevents the neuroinflammation induced by Aβ25-35 through the TLR4/NFκB signaling pathway in C6 cells.

In summary, GEN's neuroprotective effect against A β 25-35 in C6 cells could be related to the regulation of TLR4/NF κ B signaling pathway, which suppresses the inflammatory response in C6 cells induced by A β 25-35.

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AUTHOR DISCLOSURE STATEMENT

Everyone in our work has approved the final version of the manuscript. No competing financial interests exist.

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