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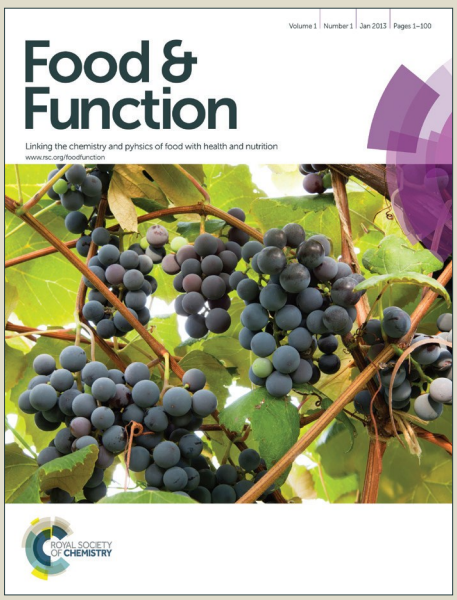

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ARTICLE TYPE

Neuroprotective effect of loganin against $A\beta_{25-35}$-induced injury *via* NF-κB-dependent signaling pathway in PC12 cells

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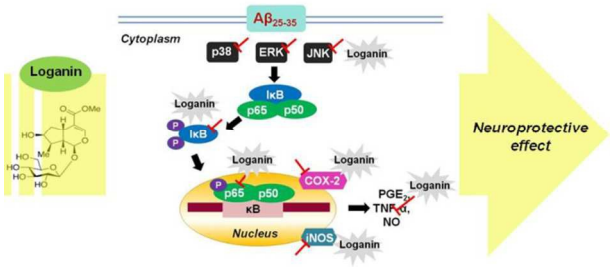
Table of contents entry

$A\beta_{25-35}$-induced neurotoxicity was ameliorated by the inhibition of the NF-κB dependent signaling pathway.



Amyloid-beta protein (Aβ), the main constituent of senile plaques is believed to play a pivotal role in the pathogenesis of Alzheimer's disease (AD). AD is closely associated with inflammatory reactions which are considered as responses to Aβ deposition. The present study investigated the effect of loganin on $A\beta_{25-35}$-induced inflammatory damage and their underlying molecular mechanism of neuroprotective action. Loganin predominantly prevented $A\beta_{25-35}$-stimulated cell death through suppressing ROS generation, and attenuating apoptosis by inhibiting caspase-3 activity and regulating cell cycle. Furthermore, loganin suppressed the level of TNF-α and protein expression of iNOS and COX-2 in $A\beta_{25-35}$-injured PC12 cells. These inhibitions appeared to correlate with the suppression of NF-κB activation by loganin, as pre-treating cells with loganin blocked the translocation of NF-κB into the nuclear compartment and degradation of the inhibitory subunit IκB. Loganin substantially inhibited phosphorylation of MAPKs including ERK1/2, p38 and JNK, which are closely related to regulation of NF-κB activation. Taken together, the results implied that loganin attenuated neuroinflammatory responses through the inactivation of NF-κB by NF-κB dependent inflammatory pathways and phosphorylation of MAPK in $A\beta_{25-35}$-induced PC12 cells.

Introduction

Alzheimer's disease (AD) is the most common age associated form of chronic neurodegenerative disorder characterized by progressive loss of neurons and synapses.[1] Extracellular amyloid plaque and intracellular neurofibrillary tangle are major pathological hallmarks in AD.[2] Even though the pathoetiology of AD still remains obscure, aggregates of Aβ, the main constituent of amyloid plaques, is believed to play a causative role in the pathogenesis of AD.

Aβ is generated from a sequential proteolytic cleavage of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase in the amyloidogenic pathway.[3] Increasing evidence demonstrated that Aβ triggers a cascade of events such as neurotoxicity, oxidative injury, and inflammatory response that contribute to the progression of AD.[4,5] In addition, reactive oxygen species (ROS) have been revealed to cause the formation of Aβ fibrils, which in turn accelerates oxidative stress, inflammatory responses and more Aβ accumulation leading to ultimate cell death.

Compelling evidences have supported that inflammation is associated with AD, and deposition of Aβ has shown to activate neuroinflammatory responses by indcucing the expression of inflammatory cytokines, chemokines and mediators through nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) signaling pathways.[6] NF-κB, a heterodimer complex with p65 and p50 subunits, is a vital transcription factor that regulates gene expression involved in immune system and inflammatory responses. In normal physiological conditions, NF-κB forms a cytoplasmic complex with its inhibitor IκBα as an inactive form.[7] When exposed to stimuli such as Aβ, ROS, and

inflammatory cytokines, IκBα undergoes phosphorylation and degradation resulting in free NF-κB to translocate to the nucleus and bind to its target genes to initiate transcription of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and proinflammatory cytokines such as TNF-α.[8] Activation of the NF-κB signaling pathway can be modulated by MAPKs such as c-Jun *N*-terminal kinase (JNK), extracellular-signal regulated kinase 1/2 (ERK1/2), and p38, which phosphorylate downstream transcription factors that promote expression of proinflammatory cytokines and mediator genes including interleukin, TNF-α, iNOS, and COX-2.[9]

Series of direct or indirect damages by Aβ neurotoxicity cause neuronal dysfunction, neuronal death and dementia where neuroinflammation plays a critical role. Therefore, neuroinflammation is recently recognized as an essential feature in AD pathology and a potential target in treatment and/or prevention of AD.[10]

Corni fructus (CF), the red fruit of *Cornus officinalis* Sieb. et Zucc, has been used both as food and medicinal herb for its antineoplastic, analagesic, diuretic, and kidney and liver protective properties in China, Japan, and Korea.[11,12] Loganin, a major iridoid glycoside of CF, has a variety of biological effects such as antidiabetic, immune regulatory, hypoglycemic, hepatoprotective, and cognitive enhancing activity.[13-16] In our previous study, loganin was isolated from the EtOAc fraction of CF ethanol extract and its specific and selective inhibitory activity against BACE1 was observed.[17] In spite of many previous studies, the mechanism that loganin suppresses $A\beta_{25-35}$-induced neurotoxicity through inflammatory signaling pathway has not been studied so far. Therefore, the aim of the present study was to determine the protective property of loganin against $A\beta_{25-35}$-stimulated neuroinflammatory injury and their underlying molecular mechanism of neuroprotective action.

Experimental

Materials

Loganin used in this study was isolated in our previous study.[17] RPMI 1640 Medium, phosphate buffered saline (PBS), donor equine serum, trypsin 0.25% solution, and penicillin were obtained from Hyclone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS) was obtained by PAA Laboratories (Linz, Austria). HBSS, N2 supplement, and RPMI 1640 phenol red free medium were obtained from Gibco BRL (Grand Island, NY, USA). CM-H_2DCFDA and Hoechst 33342 dye were purchased from Molecular Probes (Eugene, OR, USA). Caspase-3/CPP32 colorimetric assay kit was obtained from BioVision (Pato Alto, CA, USA).

$A\beta_{25-35}$, MTT, and resveratrol, used as a positive control in the experiments, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies against iNOS, COX-2, TNF-α, β-actin, monoclonal antibodies, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phospho-JNK, phospho-ERK, phospho-p38, phospho-IκB-α and phospho-p65 monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Cell culture and peptides

PC12 cells are sensitive to toxicity of stimuli including H_2O_2, Aβ, and etc, have been extensively utilized as *in vitro* neuronal model system in Aβ studies.[18-20] PC12 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in RPMI1640 medium supplemented with 10% donor equine serum, 5% FBS and 100 U/mL penicillin at 37℃ in humidified 95% air 5% CO_2 incubator. All cells were cultured in collagen-coated culture dishes. $A\beta_{25-35}$, the most toxic peptide fragment derived from APP, was dissolved in DMSO at the concentration of 1 μM and stored at -20℃. The stock solution was diluted to desirable concentrations immediately before use. In all experiments, the final DMSO concentration in each sample was less than 0.01% that did not affect cell growth and death.

MTT assay

Cell viability was determined using conventional MTT reduction assay. Briefly, PC12 cells were pre-incubated in 96 well plates at a density of 1×10^5 cells/100 μL for 24 h. After 24 h incubation to allow cells to adhere, the medium was changed to serum-free N2 defined medium and were pre-incubated with different concentrations of loganin. After incubating for 1 h, cells were treated with 50 μM $A\beta_{25-35}$ for 24 h. Then, cells were incubated with MTT solution for 3 h. Supernatants were aspirated off and formazan crystals were dissolved with 100 μL DMSO. The values of absorbance at 570 nm were measured using a microplate reader (ELX808, Biotek, Winooski, VT, USA). Results were expressed as the percentage (%) of MTT reduction, assuming that the absorbance of control cells was 100%.

Measurement of intracellular ROS accumulation

The intercellular ROS production was measured by the oxidative conversion of cell permeable 2',7'-dichlorofluorescein diacetate to fluorescent dichlorofluorescein. In brief, cells were seeded on 96 well plates with 1×10^5 cells per well and cultured 24 h for stabilization. After treatment with 50 μM $A\beta_{25-35}$ at 37℃ for 24 h in the presence or absence of loganin, the medium was switched to HBSS with 10 μM CM-H_2DCFDA for 30 min. Then CM-H_2DCFDA dye in medium was removed carefully and 100 μL HBSS was added. The ROS-associated fluorescence intensity was determined with fluorescence spectrophotometer (FLX800, Winooski, VT, USA) with emission and excitation wavelength at 528 and 485 nm, respectively.

Hoechst 33342 staining assay

Hoechst 33342, a fluorescent stain for labeling DNA, was used to determine the characteristic features of apoptotic cells. Briefly, PC12 cells were plated at a density of 1×10^6 cells on coverslips in 6 well plates. After 24 h exposure to 50 μM $A\beta_{25-35}$, cells were fixed in 4% formaldehyde for 20 min at RT and then stained with Hoechst 33342 dye at the concentration of 1 μg/mL in PBS for 20 min at RT. Hoechst 33342 stained images were obtained using a fluorescence photomicroscope (Olympus, Tokyo, Japan). Data were expressed as a ratio of apoptotic cells to total cells.

Measurement of caspase-3 activity

PC12 cells were plated at a density of 3×10^6 cells in 6 well plates. The protein concentration was confirmed by BCA assay. Activity of caspase-3 was measured using the cleavage of colorimetric substrate DEVD-*p*NA. Each protein sample was dissolved with reaction buffer, incubated with 4 μM DEVD-*p*NA substrate at 37 ℃ for 1 h, and samples were analyzed in a plate reader at 405 nm.

Flow cytometry analysis

PC12 cells were seeded at 6 well plates and exposed to 50 μM $A\beta_{25-35}$ with various concentrations of loganin for 24 h. After treatments, cells were washed with 1X PBS and resuspended in 1X PBS for flow cytometry analysis. Cell viability was analyzed by the Muse™ count kit using Muse™ cell analyzer from Millipore (Billerica, MA, USA). Annexin V and Dead Cell Assay was performed following manufacturer's instruction. Briefly, after the indicated treatments, the cells were incubated with Annexin V and Dead Cell Reagent and the results for dead, late apoptotic, early apoptotic, and live cells were counted. Cell cycle was determined by Muse™ cell cycle reagent following manufacturer's protocol.

Western blot analysis

PC12 cells were treated with various concentrations of the samples with $A\beta_{25-35}$, and then cells were washed twice with 1X PBS and harvested using a cell scraper. The cell were resuspended by lysis buffer on incubated on ice for 1 h. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4℃. The supernatant was divided and the amount of protein was examined by BCA assay. The samples were loaded by 10% SDS-polyacrylamide gel, and transferred to PVDF membranes with a semidry transfer system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% nonfat milk in 1X PBST for overnight at 4℃, washed with 1X PBST, and was incubated with primary antibodies overnight at 4℃ (TNF-α, iNOS and COX-2 for 30 h, phospho-p65 and phospho-IκB-α for 4 h and phospho-ERK1/2, phospho-JNK, and phospho-p38 for 1 h).

After hybridization with primary antibody, membrane was washed seven times with 1X PBST, the incubated with secondary antibody for 1 h at 4℃ and washed seven times with 1X PBST. Final detection was performed with Western Blotting Luminol reagents (Santa Cruz, CA, USA). Goat polyclonal antibodies against the active forms of COX-2 (1:1000) and TNF-α (1:1000), rabbit monoclonal antibodies against the active forms of phospho-IκB-α (1:1000) and phospho-p65 (1:1000) and rabbit polyclonal antibodies against active forms of iNOS (1:1000), phospho-ERK1/2 (1:1000), phospho-JNK (1:1000), and phospho-p38 (1:1000) were used in this study.

Immunocytochemistry

PC12 cells grown in 6 well plate were fixed with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 10 min at room temperature. After several washes with PBS, cells were permeabilized with Triton X-100 solution in PBS for 20 min and incubated with primary antibodies (anti-NF-κB, 1:200) for overnight at 4℃. Cells were then washed with PBS, incubated with FITC-conjugated goat anti-rabbit IgG secondary antibodies in 2 % FBS for 1 h at 37℃ (1:250) and stained with Hoechst 33342. Immunofluorescence was visualised using a Olympus photomicroscope (Tokyo, Japan). Images were captured with the software NIS-Elements F 2.0 (Nikon, Tokyo, Japan).

Statistical analysis

All experiments were performed in triplicate. Data of each experiment expressed as mean± SD. The values were compared with the control using analysis of variance followed by unpaired Student's test. Differences were considered to be statistically significant at $p<0.001$, $p<0.01$, and $p<0.05$.

Results

Protective effect of loganin against $A\beta_{25-35}$-induced cell death in PC12 cells

The chemical structure of loganin is presented in Fig. 1. As estimated by manual method of MTT, the viability of cells incubated with 50 μM $A\beta_{25-35}$ for 24 h significantly reduced to 64.97±1.53% (Fig. 2A, $p<0.001$). However, when cells were pretreated with loganin, cell death caused by Aβ significantly decreased in a dose-dependent manner. In particular, pretreatment of loganin at 10 and 50 μM strongly increased PC12 cell survival to 80.64±5.36% and 90.82±3.93%, respectively ($p<0.01$ and $p<0.001$). Loganin itself did not exhibit any apparent cytotoxicity even at the highest concentration (50 μM, data not shown).

Next, cell viability was also determined by automated FACS method using double DNA intercalating fluorescent dyes. The 50 μM $A\beta_{25-35}$ treatment of PC12 cells was found to decrease the viability to 56.80±1.35% ($p<0.001$) and this substantially increased to 94.77±2.69%, 97.71±1.56%, and 99.02±0.99% by pre-treating with loganin at 1, 10 and 50 μM, respectively (Fig. 2B, $p<0.01$, $p<0.01$ and $p<0.001$, respectively). Compared to the results of MTT assay, loganin at all tested concentrations exhibited better survival effect of PC12 cells when measured by FACS. Both results revealed that loganin significantly suppressed $A\beta_{25-35}$-induced cell death in PC12 cells.

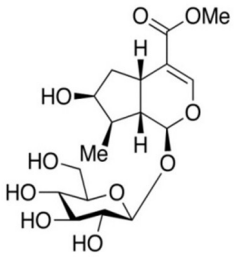


Fig. 1. Structure of loganin isolated from *Corni fructus*

(A)

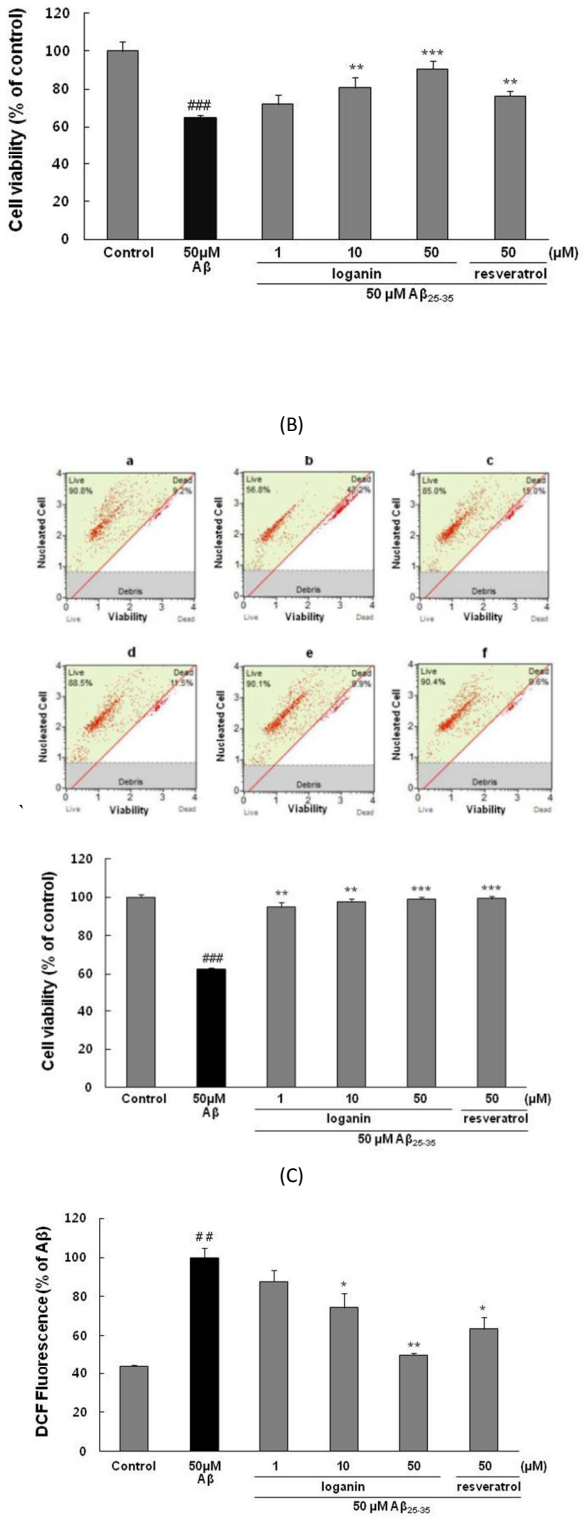


Fig. 2. Protective effect of loganin against $A\beta_{25\text{-}35}$-induced cell death. For determining cell viability, PC12 cells were pretreated with loganin for 1 h and further treated with 50 μM with $A\beta_{25\text{-}35}$ for 24h. (A) Cell viability assessed by MTT reduction. The group treated with $A\beta_{25\text{-}35}$ alone. (B) Cell viability evaluated by flow cytometry. (a) Control (b) 50 μM $A\beta_{25\text{-}35}$ (c) 50 μM $A\beta_{25\text{-}35}$ +1 μM loganin (d) 50 μM $A\beta_{25\text{-}35}$+10 μM loganin (e) 50 μM $A\beta_{25\text{-}35}$ +50 μM loganin (f) 50 μM $A\beta_{25\text{-}35}$+50 μM resveratrol. (C) Inhibitory effect of loganin against $A\beta_{25\text{-}35}$-induced intracellular ROS accumulation. PC 12 cells were pretreated with loganin for 1 h and then treated with 50 μM with $A\beta_{25\text{-}35}$ for 24h. Intracellular ROS production was measured using CM-H_2DCFDA fluorescent dye. $^{###}p<0.001$ and $^{##}p<0.01$ *vs.* control group. $^{***}p<0.001$, $^{**}p<0.01$, and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25\text{-}35}$ alone

Inhibitory effect of loganin against $A\beta_{25\text{-}35}$-induced intracellular ROS accumulation in PC12 cells

50 μM $A\beta_{25\text{-}35}$ treatment dramatically boosted ROS level ($p<0.01$) in PC12 cells. The suppression of ROS generation was sensitive to loganin, which evidently reduced to 73.72±8.39% and 51.90±0.96% at concentrations of 10 and 50 μM, respectively ($p<0.05$, and $p<0.01$, respectively) (Fig. 2C). Intracellular oxidative stress by $A\beta_{25\text{-}35}$ treatment was alleviated by loganin treatment, indicating that loganin suppressed $A\beta_{25\text{-}35}$-induced damages at least in part through the inhibition of ROS generation.

Inhibitory effect of loganin against $A\beta_{25\text{-}35}$-induced apoptosis in PC12 cells

To evaluate whether $A\beta_{25\text{-}35}$–stimulated cell death resulted from apoptosis or not, both flow cytometry analysis and Hoechst 33342 staining assay were performed. As presented in Fig. 3A, $A\beta_{25\text{-}35}$ treatment significantly increased the late apoptotic percentage. The percentage of late apoptosis in the control group was 9.01±0.89%, whereas $A\beta_{25\text{-}35}$ treatment resulted in significant increase of late apoptosis to 37.45±3.48% ($p<0.001$). However, pretreatment of PC12 cells with loganin at 1, 10 and 50 μM significantly decreased the percentage of late apoptosis to 21.04±2.08%, 12.90±1.03% and 10.27±0.99%, respectively in a dose dependent manner ($p<0.01$, $p<0.01$ and $p<0.001$, respectively).

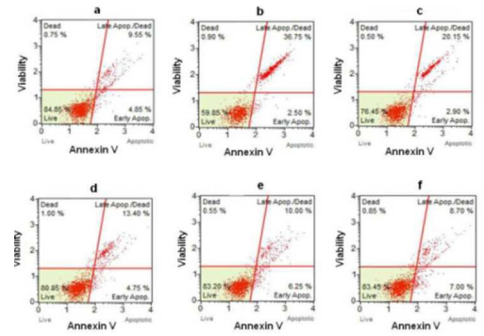
Furthermore, apoptotic morphological changes were observed with Hoechst 33342 staining. While the control had round blue nuclei of viable cells (Fig. 3Ba), chromatin condensation and nuclear fragmentation were detected after $A\beta_{25\text{-}35}$ treatment (Fig. 3Bb). These distinguished morphological manifestations of apoptosis were gradually alleviated after pre-incubation with loganin (Fig. 3Bc, 3Bd and 3Be). Especially 50 μM loganin showed the strongest anti-apoptotic effect and the cell damage from $A\beta_{25\text{-}35}$ was recovered to that of the control cells. The proportion of apoptotic cells was calculated in Fig. 3C. 11.08±0.16% of apoptotic cells in total population of control increased to 39.18±1.16% with the exposure to 50 μM $A\beta_{25\text{-}35}$ ($p<0.001$). Pre-incubation with loganin at 10 and 50 μM dose dependently inhibited $A\beta_{25\text{-}35}$-stimulated apoptosis by 33.89±1.82 % ($p<0.01$) and 29.11±0.78% ($p<0.001$), respectively.

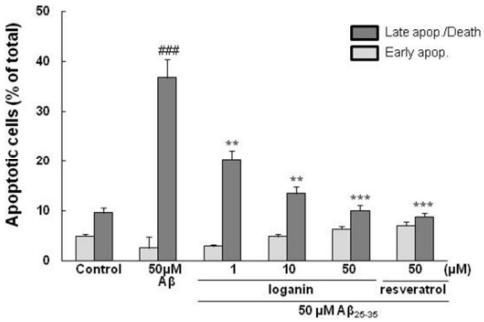
Protective effect of loganin against $A\beta_{25\text{-}35}$-induced caspase-3 activation

The initiation and execution of apoptosis is involved in activation of a family of caspases. As presented in Fig. 3D, caspase-3 was activated about 1.64 fold by 50 μM of $A\beta_{25\text{-}35}$ treatment ($p<0.001$), while pretreatment of loganin significantly attenuated $A\beta_{25\text{-}35}$-induced caspase-3 activation. 50 μM of loganin almost completely blocked caspase-3 activation to the basal level (1.02 fold, $p<0.001$). In addition, the inhibitory effect of loganin at 50 μM was similar to that of resveratrol, a positive control (1.05 fold) without significant difference. Above results indicated that $A\beta_{25\text{-}35}$-stimulated apoptosis might be mediated, at least in part,

by caspase-3 and the protective property of loganin might be attributed to suppression of the caspase cascade and oxidative stress.

(A)





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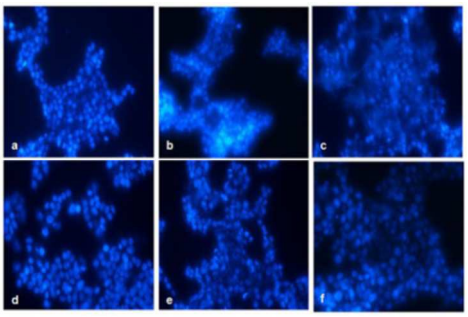

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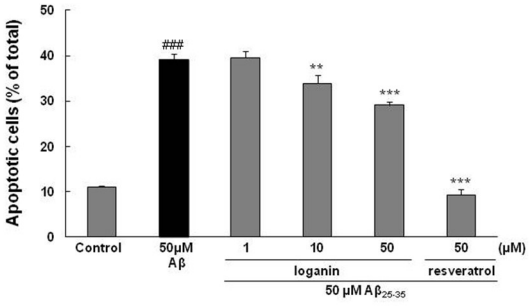

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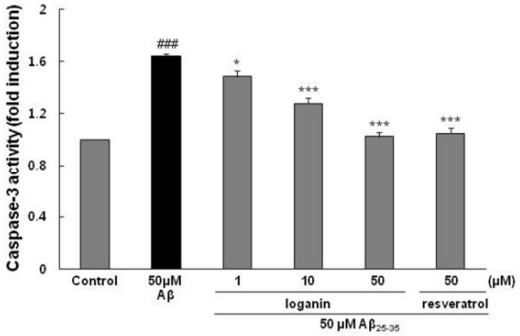
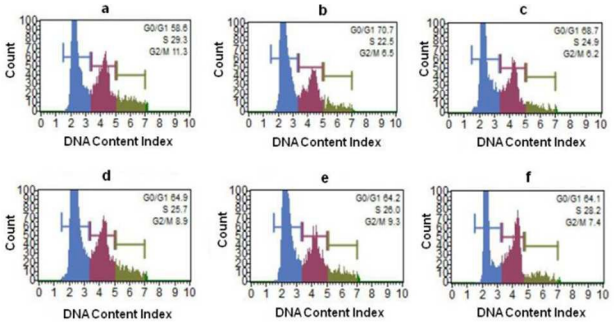


Fig. 3. Protective effect of loganin on $A\beta_{25-35}$ induced PC12 cell apoptosis. Cells were pretreated with loganin for 1 h followed by $A\beta_{25-35}$ 50 μM incubation for 24 h (A) Flow cytometric analysis of apoptosis using Annexin V staining PC12 cells treated with loganin. (B) Morphological apoptosis was determined by Hoechst 33342 staining under fluorescence microscopy (magnification × 400). (a) Control (b) 50 μM $A\beta_{25-35}$ (c) 50 μM $A\beta_{25-35}$ +1 μM loganin (d) 50 μM $A\beta_{25-35}$+10 μM loganin (e) 50 μM $A\beta_{25-35}$ +50 μM loganin (f) 50 μM $A\beta_{25-35}$+50 μM resveratrol. (C) Histogram showing the percentage of apoptotic cells in total cell population after different treatments. (D) Protective effect of loganin against $A\beta_{25-35}$-induced caspase-3 activation. Caspase-3 activity was measured with the colorimetric caspase-3 assay kit. $^{###}p<0.001$ *vs.* control group. $^{***}p<0.001$, $^{**}p<0.01$, and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25-35}$ alone.

Protective effect of loganin on $A\beta_{25-35}$-induce cell cycle arrest in PC12 cells

The distribution of cells in various phases of the cell cycle was investigated by flow cytometry. Figure 4 indicated that $A\beta_{25-35}$ exposure significantly increased an arrest in G0/G1 phase from 57.6±1.41% to 76.5±0.99% ($p<0.01$) with a concomitant reduction at DNA synthesis S phase (Aβ *vs* control, 16.8±0.57% *vs* 28±1.84%, $p<0.05$) and G2/M (Aβ *vs* control, 6.7±0.42% *vs* 14.4±3.25%). $A\beta_{25-35}$ delayed the progression of cells through the cell cycle with the cells accumulating in the G1 phase. Loganin, however, significantly alleviated cell cycle disruption stimulated by $A\beta_{25-35}$ and almost restored the percentage of cells in G0/G1 and S phases to control values. Arresting cell cycle at specific checkpoints allows delay for later event until earlier one is completed which provides time to repair damages for cell integrity.[21,22] However, apoptosis is initiated if damage is too far to be repaired.

(A)



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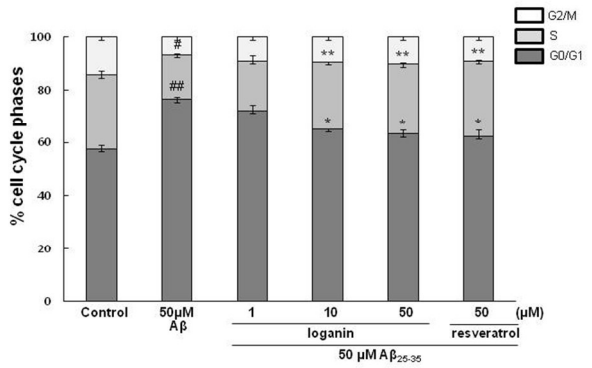

Fig. 4. Cell cycle analysis of PC12 cells treated with loganin. Cell cycle profile of PC12 cells were pretreated with loganin for 1 h and further treated with 50 μM of $A\beta_{25-35}$ for 24 h with Muse™ cell analyzer (Millipore). (a) Control (b) 50 μM $A\beta_{25-35}$ (c) 50 μM $A\beta_{25-35}$+1 μM loganin (d) 50 μM $A\beta_{25-35}$+10 μM loganin (e) 50 μM $A\beta_{25-35}$+50 μM loganin (f) 50 μM $A\beta_{25-35}$+50 μM resveratrol. $^{\#\#}p<0.01$, and $^{\#}p<0.05$ *vs.* control group. $^{**}p<0.01$, and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25-35}$ alone.

Protective effect of loganin against TNF-α expression in $A\beta_{25-35}$- injured PC12 cells

In order to examine the effect of loganin on pro-inflammatory cytokine level, the expression of TNF-α was evaluated. The cells were pretreated for 1 h with various concentrations of loganin and subsequently stimulated by $A\beta_{25-35}$ for 30 h to assess TNF-α expression. As shown in Fig. 5, TNF-α level was significantly increased to 287±2.31% in response to $A\beta_{25-35}$ ($p<0.001$). However, loganin pretreatment at 10 and 50 μM significantly inhibited TNF-α production to 126.27±14.92% and 106.87± 10.97%, respectively ($p<0.01$).

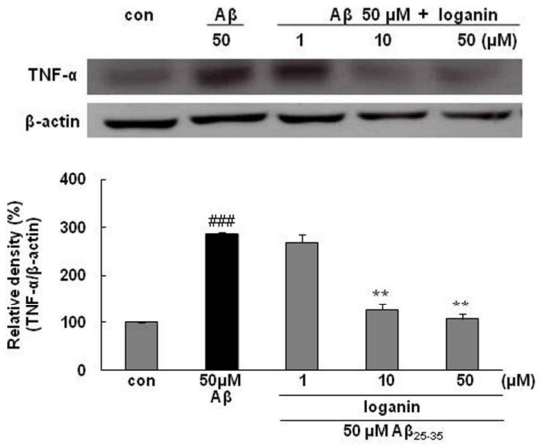

Fig. 5. Effects of loganin on $A\beta_{25-35}$-induced TNF-α expression in PC 12 cells. The total lysates of the proteins were subjected to Western blot analysis, as described in Experimental. The ratio of immunointensity between the TNF-α and β-actin was calculated. $^{\#\#\#}p<0.001$ *vs.* control group. $^{**}p<0.01$ *vs.* the group treated with $A\beta_{25-35}$ alone.

Protective effect of loganin against iNOS and COX-2 expression in $A\beta_{25-35}$-induced PC12 cells

In addition to TNF-α, we determined whether loganin modulate pro-inflammatory mediators such as iNOS and COX-2. Treatment of $A\beta_{25-35}$ alone led to marked increase in iNOS and COX-2 expression in PC 12 cells ($p<0.001$ and $p<0.01$, respectively) and loganin displayed dose-dependent ability to downregulate the $A\beta_{25-35}$–stimulated iNOS and COX-2 expression (Fig. 6). Loganin alone exhibited no effect on iNOS and COX-2 expression (data not shown). These results clearly demonstrated that loganin might be effective in reducing the production of iNOS and COX-2 enzymes responsible for the synthesis of NO and PGE_2, respectively.

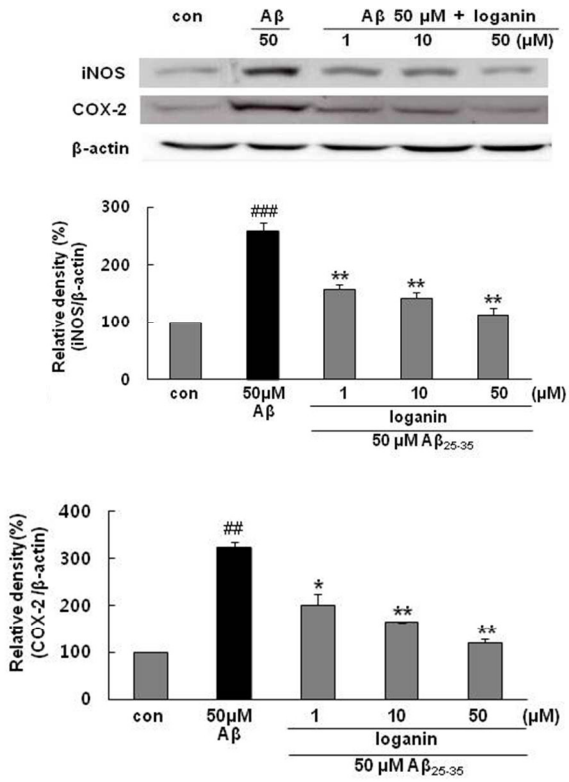

Fig. 6. Effects of loganin on $A\beta_{25-35}$-induced iNOS and COX-2 expression in PC12 cells. The total lysates of the proteins were subjected to Western blot analysis, as described in Materials and Methods. The ratio of immunointensity between the COX-2/iNOS and β-actin was calculated. Each bar represents mean±SD from three independent experiments. $^{\#\#\#}p<0.001$ and $^{\#\#}p<0.01$ *vs.* control group. $^{**}p<0.01$ and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25-35}$ alone.

Protective effect of loganin against $A\beta_{25-35}$-induced NF-κB and IκB-α phosphorylation in PC12 cells

The modulating effect of loganin on the expression of transcription factors such as NF-κB and its inhibitor IκB-α was evaluated by Western blot. As illustrated in Fig. 7(A), $A\beta_{25-35}$ markedly enhanced phosphorylation of NF-κB subunit p65 and IκB-α ($p<0.001$ and $p<0.01$, respectively). Loganin significantly reduced $A\beta_{25-35}$-induced p65 activation in dose dependent response. Furthermore, loganin significantly suppressed the phosphorylation and degradation of IκB-α stimulated by $A\beta_{25-35}$. Loganin at 10 μM almost blocked IκB-α phosphorylation (101.44±12.52%, $p<0.001$). These results suggest that loganin might repress the $A\beta_{25-35}$-induced NF-κB upregulation, resulting in the prevention of the overexpression of inflammatory

molecules. $A\beta_{25-35}$-stimulated nuclear translocations of NF-κB were visualized in Fig. 7(B). When cells were treated with loganin, $A\beta_{25-35}$-activated translocation of NF-κB was rarely detected in nuclei. Loganin markedly attenuated the translocation of NF-κB.

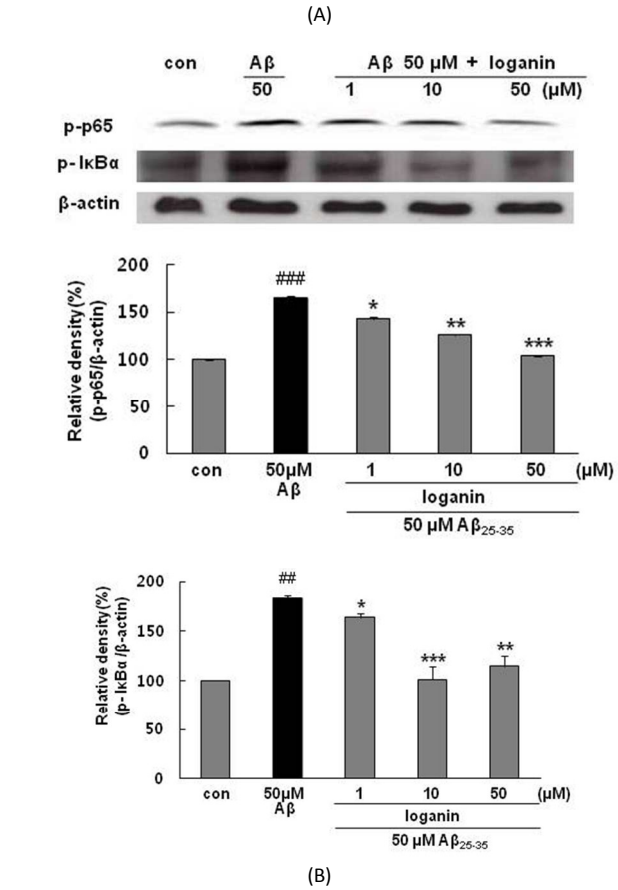

Fig. 7. Effects of loganin on $A\beta_{25-35}$-induced NF-κB and IκBα expression in PC 12 cells. (A) Protein expressions of NF-κB and IκBα in PC12 cells incubated with loganin with or without $A\beta_{25-35}$ for 24 h. The total lysates of the proteins were subjected to Western blot analysis, as described in Materials and Methods. The ratio of immunointensity between p-p65/IκBα and β-actin was calculated. $^{###}p<0.001$ and $^{##}p<0.01$ *vs.* control group. $^{***}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25-35}$ alone. (B) FITC-conjugate goat anti-NF-kB/p65 (a-e), Hoechst 33342 (f-j) and merged immunofluorescences (k-o) were visualized by microscopy (magnification × 40).

Protective effect of loganin against $A\beta_{25-35}$-induced phosphorylation of MAPKs in PC12 cells

The effect of loganin on the $A\beta_{25-35}$-induced phosphorylation of p-38, ERK and JNK was investigated to confirm whether the downregulation of NF-κB by loganin is mediated through MAPKs. Phosphorylation of p38, ERK1/2, and JNK were significantly elevated when treated with $A\beta_{25-35}$ alone (Fig. 8, $p<0.01$, $p<0.001$, and $p<0.001$, respectively). Loganin significantly suppressed the phosphorylation of p38, ERK1/2, and JNK in $A\beta_{25-35}$-stimulated PC12 cells in dose-dependent manner. In particular, loganin significantly inhibited p38, ERK1/2 and JNK activation to basal level almost comparable to the control at 50 μM. Interestingly, loganin potently inhibited JNK even at the concentration of 1 μM, suggesting that the suppression of NF-κB by loganin may occur through the modulation of MAPK activation predominantly *via* JNK.

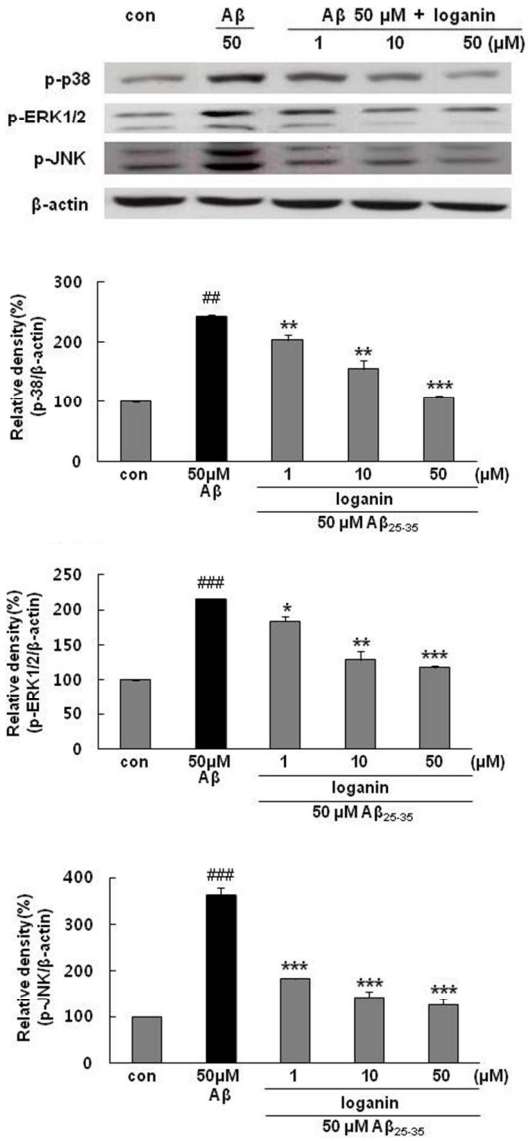

Fig. 8. Effects of loganin against $A\beta_{25-35}$-induced phosphorylation of MAPKs in PC12 cells. The total lysates of the proteins were subjected to Western blot analysis, as described in Materials and Methods. $^{###}p<0.001$ and $^{##}p<0.01$ *vs.* control group. $^{***}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$ *vs.* the group treated with $A\beta_{25-35}$ alone.

Discussion

It has been proposed that Aβ generated from the amyloidogenic proteolytic process of APP is the main player in the pathogenesis of AD. Aβ accumulation takes place at early stage of AD cascade of events in cellular dysfunction through neuroinflammation.[23] Therefore, the suppression of Aβ-induced neuroinflammatory toxicity might be one of the critical strategies in AD prevention.

$A\beta_{25-35}$ used in the present study is considered to be short but strong toxic peptide fragment possessing neurotoxic effects comparable to $A\beta_{1-40/1-42}$. It has been proposed that $A\beta_{25-35}$ represents the main functional domain in the full length molecule of Aβ, which reveals that it is suitable for determining if loganin affords protection against Aβ-induced damage.[24] Multiple lines of evidence have implicated the neurotoxicity of Aβ can be mediated by ROS, which may contribute to increase apoptosis in AD.[25-27] Apoptosis, a fundamental process of cell death, is associated with a series of biochemical changes including activation of caspases.[28] Both oxidized Aβ plaques and activated caspase family including caspase-3 that mediates mitochondrion-initiated apoptosis were detected in AD patients.[29]

AD is known to be associated with various inflammatory reactions considered as responses to Aβ deposition. NF-κB signaling cascade has been demonstrated to play a key role in generation and regulation of proinflammatory mediators including COX-2, iNOS and pro-inflammatory cytokines in the inflammatory process of AD.[30] Activation of NF-κB signaling cascade is associated with the activations of MAPKs, which consequently phosphorylate and activate other kinases or stimulate downstream transcription factors resulting in the alteration of the target gene expression. In the present study, the preaggregated Aβ caused a strong inflammatory reaction characterized by upregulated COX-2 and iNOS through ERK, JNK, p38 MAPK activation, which were suppressed by the treatment of loganin. These results suggest that the protective effects of loganin against Aβ-induced injury may be due to the inhibition of the phosphorylation of JNK, p38, and ERK 1/2 MAPKs. Therefore, for the maximum neuroprotective effects, blockade of NF-κB which is the rate-limiting step in the inflammatory cascade has to be achieved instead of targeting each individual factor in multifactorial disorder such as AD.[31]

Loganin, a major iridoid glycoside of CF, has also been found in *Flos lonicerae, Fruit cornus, and Strychonos nux vomica*. Loganin has shown to possess various biological properties such as plasma glucose lowering, immune regulating, and kidney protecting effects.[11,16] Loganin also exhibited protective effects against hepatic injury and other diabetic complications.[32] Several studies proved the effectiveness of irioid glycosides of CF (ICF) on dementia. ICF ameliorated the hyperphosphorylation of tau by increasing the activity of PP2A, the major protein phosphatase in the brain that removes phosphate residues from tau, thus preventing tau from assembly into paired helical filaments and neurofibrillary tangles.[33,34] Intragastric administration of ICF was demonstrated to augment the expression of growth-related protein and nerve growth factor and to diminish neuronal loss in the medial septum of rats with bilateral fornix/fimbria transaction.[35]

Learning and memory deficits are the early clinical manifestations of AD.[36] Regarding AD researches, administration of loganin ameliorated scopolamine-induced memory deficits in mice.[13] Kwon et al.[14] demonstrated that H_2O_2-induced damage was attenuated by loganin treatment, which resulted from suppressing apoptosis and ROS accumulation in SH-SY5Y cells. Recent study by Babry et al. (2013) showed that administration of loganin improves spatial learning and memory in diabetic rats.[37] Furthermore, loganin exhibited neuroprotective effect by inhibiting apoptosis in nonvascular injury to brain and decreasing cerebral infarct size in cerebral ischemia rats.[38] Furthermore, anti-neuroinflammatory property of loganin has been demonstrated as a useful therapeutic applicant in other diseases such as type 2 diabetes, stroke and cerebral infarction.[39-41]

Conclusion

The present study demonstrated that natural occurring loganin inhibited $A\beta_{25-35}$ stimulated neurotoxicity through reducing ROS generation, restoring cell cycle, inhibiting apoptosis and suppressing caspase-3 activity. Inflammatory cell damage mediated by $A\beta_{25-35}$ was significantly restored by loganin treatment. Specifically, anti-neuroinflammatory effect of loganin might have exerted through downregulating p38, ERK1/2, and JNK pathways resulting in blocking NF-κB pathway and thereby suppressing expressions of inflammatory target proteins including COX-2, iNOS and TNF-α, which finally induced the blockage of inflammation cascade.

Taken together, our novel data suggests that $A\beta_{25-35}$-stimulated neuroinflammation might be crucial target to alleviate neuronal damages, which not only provides a scientific basis underlying the prevention of AD but also present a positive insight in the prevention of AD through anti-neuroinflammation. Loganin could be a useful agent in AD prevention by inhibiting cell death related to the Aβ-induced neuroinflammation. Further relevance of our findings with primary neuron cells and *in vivo* clinical situations remains to be investigated for confirmation.

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Table of contents entry

$A\beta_{25-35}$-induced neurotoxicity was ameliorated by the inhibition of the NF-κB dependent signaling pathway.