Bioorganic & Medicinal Chemistry 19 (2011) 4021–4027

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Neuroprotective effects of linarin through activation of the PI3K/Akt pathway in amyloid-β-induced neuronal cell death

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ARTICLE INFO

Article history:
Received 8 April 2011
Revised 12 May 2011
Accepted 13 May 2011
Available online 19 May 2011

Keywords:
Linarin
Flavonoid
Aβ
Neuroprotection
Acetylcholinesterase
Akt
PC12

ABSTRACT

Linarin, a natural occurring flavanol glycoside derived from *Mentha arvensis* and *Buddleja davidii* is known to have anti-acetylcholinesterase effects. The present study intended to explore the neuroprotective effects of linarin against $A\beta_{25-35}$-induced neurotoxicity with cultured rat pheochromocytoma cells (PC12 cells) and the possible mechanisms involved. For this purpose, PC12 cells were cultured and exposed to 30 μM $A\beta_{25-35}$ in the absence or presence of linarin (0.1, 1.0 and 10 μM). In addition, the potential contribution of the PI3K/Akt neuroprotective pathway in linarin-mediated protection against $A\beta_{25-35}$-induced neurotoxicity was also investigated. The results showed that linarin dose-dependently increased cell viability and reduced the number of apoptotic cells as measured by MTT assay, Annexin-V/PI staining, JC-1 staining and caspase-3 activity assay. Linarin could also inhibit acetylcholinesterase activity induced by $A\beta_{25-35}$ in PC12 cells. Further study revealed that linarin induced the phosphorylation of Akt dose-dependently. Treatment of PC12 cells with the PI3K inhibitor LY294002 attenuated the protective effects of linarin. Furthermore, linarin also stimulated phosphorylation of glycogen synthase kinase-3β (GSK-3β), a downstream target of PI3K/Akt. Moreover, the expression of the anti-apoptotic protein Bcl-2 was also increased by linarin treatment. These results suggest that linarin prevents $A\beta_{25-35}$-induced neurotoxicity through the activation of PI3K/Akt, which subsequently inhibits GSK-3β and up-regulates Bcl-2. These findings raise the possibility that linarin may be a potent therapeutic compound against Alzheimer's disease acting through both acetylcholinesterase inhibition and neuroprotection.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of senile plaques composed of β-amyloid peptide (Aβ) strongly associated with the loss of cognition and memory. The most dramatic abnormalities noted in AD brains are associated with the cholinergic system.[1,2] Acetylcholinesterase (AChE) plays a key role in the regulation of the cholinergic system by terminating signaling at cholinergic synapses, and hence, inhibition of AChE has emerged as one of the most promising strategies for the treatment of AD.[3] To date, most of the drugs available in the market for the treatment of AD are AChE inhibitors. There is an interest in finding new naturally occurring AChE inhibitors with fewer side effects since some of the chemically synthesized AChE inhibitors tend to cause severe side effects such as nausea, vomiting, bradycardia, anorexia and sweating.

Linarin (acacetin-7-*O*-β-D-rutinoside, Fig. 1) is a naturally occurring selective acetylcholinesterase (AChE) inhibitor isolated from the *Mentha arvensis*[4] and *Buddleja davidii*.[5] Structure–activity relationships have shown a strict requirement of certain flavonoid structures for inducing AChE inhibition, since certain compounds with similar structures to linarin (such as acacetin, apigenin-7-*O*-glucoside, roifolin, rutin, hesperidin, etc.) do not show obvious activity.[5] However, there has been no assessment of AChE inhibition or the potential neuroprotective effects of linarin in a neuronal injury model. In the present study, we

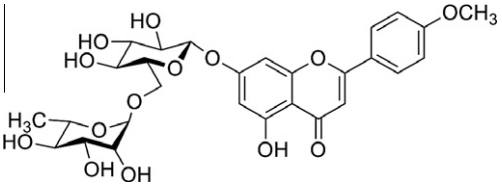

Figure 1. Chemical structural of linarin.

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doi:10.1016/j.bmc.2011.05.021

explored the effects of linarin as an AChE inhibitor and for its neuroprotective effects against $A\beta_{25-35}$-induced neurotoxicity in PC12 cells and evaluated the underlying mechanisms of neuroprotection, to investigate its potential utility as a treatment for halting the progression of AD.

2. Material and methods

2.1. Reagents

Linarin was obtained from the Department of Natural Products Chemistry of Shandong University (Jinan, China) and its purity was >98%. $A\beta_{25-35}$ and Akt inhibitor (LY294002) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were from Invitrogen/Gibco (Carlsbad, CA, USA). Antibodies to Akt, p-Akt (Ser473), GSK-3β, p-GSK-3β (Ser9) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies to Bcl-2, GAPDH and β-actin, anti-mouse-horseradish peroxide (HRP) IgG, anti-rabbit-HRP-IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The JC-1 probe and the caspase-3 activity assay kit were provided by Beyotime Institute of Biotechnology (Haimen, China).

2.2. Peptide preparation

$A\beta_{25-35,}$ which is the most toxic peptide fragment derived from the amyloid precursor protein, was dissolved in deionized distilled water at a concentration of 1 mM and incubated at 37 °C for 72 h to induce its aggregation.[6]

2.3. Cell culture and drug treatments

PC12 cells were obtained from the Department of Neurobiology, Shandong University. The cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C with 5% CO_2, and the medium was changed every other day.

PC12 cells were cultured in serum-free medium for 12 h prior to drug treatment. Linarin was added at indicated concentrations 1 h prior to $A\beta_{25-35}$ treatment. In experiments involving Akt kinase inhibition, LY294002 (20 μM) was added to the medium 1 h prior to linarin.

2.4. Cell viability assay

PC12 cells were seeded in 96-well plates at 0.5×10^4 cells/well and assessed for responses to $A\beta_{25-35}$ and linarin by MTT assay. Briefly, the cells were pretreated with vehicle alone or linarin (0.1, 1.0 and 10 μM) for 1 h, and then were exposed to 30 μM pre-aggregated $A\beta_{25-35}$ for 24 h in the continued presence of vehicle or linarin. After incubations, cells were treated with 5 mg/ml MTT for 4 h at 37 °C, then the medium was carefully removed. The formazan crystals used for the MTT assay were dissolved in 150 μl of DMSO and the absorbance was measured at 570 nm on a plate reader.

2.5. AChE activity assay

The assay for AChE activity was performed according to the methods developed by Ellman et al.[7] PC12 cells were pretreated with linarin for 1 h and then treated with 30 μM $A\beta_{25-35}$ for 24 h to stimulate AChE. After incubation, the cells were extracted by High ionic strength buffer (10 mM $NaHPO_4$, pH 7.0–8.0, 1 M NaCl, 10% Triton X-100, 1 mM EDTA) and the cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C, the supernatant were collected for the measurement of AChE activity.

2.6. Apoptosis assay

Apoptosis was detected with an Annexin V-FITC/PI double staining Kit (KeyGEN, Nanjing, China). At the end of incubation, cells were washed in cold PBS then centrifuged twice at 2000 rpm for 5 min and resuspended in 500 μl of binding buffer. FITC-labeled Annexin V (5 μl) and propidium iodide (PI, 5 μl) were then added to cells, after which they were incubated in the dark at room temperature for 15 min according to manufacturer's instruction. Cell apoptosis was measured using a FACScan flow cytometer (Becton Dickinson, USA). Annexin V-positive, PI-negative cells were scored as early apoptotic cells, and cells double-stained with both Annexin V and PI were considered late apoptotic cells. Control cells were negative for both stains.

2.7. Flow cytometric analysis of mitochondrial membrane potential using JC-1

Mitochondrial membrane potential was analyzed using an aggregate-forming lipophilic dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzamidazolocarbocyanin iodide). Cells in different treatments were labeled with JC-1 at 37 °C for 20 min. Afterward, cells were collected by centrifugation at 600×*g* for 5 min and pellets were gently resuspended in 0.5 ml PBS and analyzed by flow cytometry. Green and red fluorescence were analyzed on the FL1 (525 nm BP) and FL2 (575 nm BP) channels, respectively. The mitochondrial membrane potential value was expressed as the ratio of green/red fluorescence.

2.8. Caspase-3 activity assay

Caspase-3 activity was measured by cleavage of the chromogenic caspase substrate, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp *p*-nitroanilide). Approximately 50 μg of total protein was added to a reaction buffer containing Ac-DEVD-pNA (2 mM) and incubated for 2 h at 37 °C, after which the absorbance of the yellow pNA cleaved from its corresponding precursor was measured spectrometrically at 405 nm. The specific caspase activity, normalized to total protein concentrations of cell lysates, was then expressed as the fold increase over baseline caspase activity as determined from control cells cultured in DMEM with 10% FBS or vehicle treated control cells.

2.9. Western blot analysis

Western blotting was performed by using standard methods. Briefly, cells were exposed to lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine and 1 mM AEBSF, 50 mmol/l sodium fluoride, 1 mmol/L sodium orthovanadate), and after incubation on ice for 20 min, cells were collected, vortexed, and centrifuged at 14,000×ce:italic>g for 10 min at 4 °C. The supernatants were utilized for protein analysis and Western blots. Total protein concentration was determined by BCA method (Beyotime, Haimen, China). Protein samples (20 or 30 μg) were separated by SDS–PAGE on 12% Tris–Glycine gels and transferred to nitrocellulose. Equivalent sample loading was confirmed by Ponceau S staining and immunoblotting for β-actin or GAPDH served as an internal control. Membranes were blocked in 5% milk–TBS and incubated overnight at 4 °C in primary antibody. Antibodies used included Akt (1:1000), pSer493–Akt (1:1000), GSK-3β (1:1000), pSer9–GSK-3β (1:1000), Bcl-2 (1:200) and β-actin (1:1000). Signals from

HRP-conjugated-secondary-antibodies were visualized by an enhanced chemiluminescence detection kit (Pierce, Rockford IL, USA). Data within a linear range were quantified using Image Quant software (GE Amersham, Piscataway, NJ, USA).

2.10. Statistical analysis

Independent sample *t*-tests and one way ANOVA with Tukey–Kramer posthoc analyzes were performed as appropriate to the data using InStat software (Graph Pad, San Diego, CA). Data were considered significant at $P < 0.05$. All results were confirmed from a minimum of three independent experiments on replicate samples. Data represent mean ± standard deviation (SD).

3. Results

3.1. Linarin reduces $A\beta_{25-35}$-induced toxicity of PC12 cells

Aβ-induced apoptotic neuronal cell death is thought to be a critical event in the pathogenesis of AD.[8] To assess the impact of linarin on Aβ-induced apoptotic cell death, we first used an MTT assay to assess the dose response to $A\beta_{25-35}$ of PC12 cell viability. In preliminary studies, $A\beta_{25-35}$ was applied to cells at various concentrations (10, 20, 30, 40 μM) and viability was assessed 24 h later. As shown in Figure 2A, $A\beta_{25-35}$ induced a loss of PC12 cell viability in a dose-dependent manner. Exposure of PC12 cells to the highest dose of 40 μM $A\beta_{25-35}$ for 24 h resulted in survival of only 59.24% of cells when compared to sister cultures treated with vehicle alone.

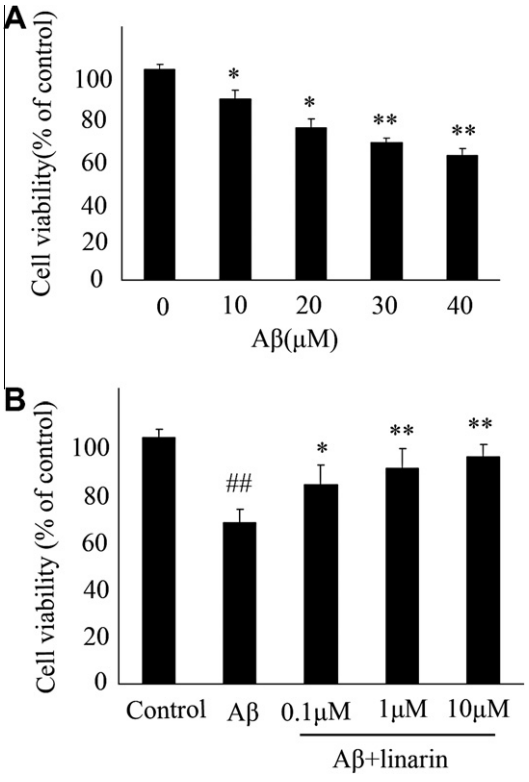

Figure 2. Protective effects of linarin on cell viability against $A\beta_{25-35}$-induced cytotoxicity in PC12 cells: (A) Effects of $A\beta_{25-35}$ on cell viability. PC12 cells were treated with the indicated concentrations (10–40 μM) of $A\beta_{25-35}$. (B) The effects of linarin on the viability of PC12 cells were measured by MTT assay after treatment of 24 h. All data are presented as the mean ± S.D. from triplicate independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ versus $A\beta_{25-35}$ treatment group. $^{\#\#}p < 0.01$ versus control.

Having established the toxicity of the highest dose of $A\beta_{25-35}$, we next evaluated the impact of a lower concentration at a range in which linarin-mediated protection could be fully assessed. For these studies, we investigated the effect of linarin on cell death in PC12 cells treated with 30 μM $A\beta_{25-35}$. To determine the optimal dose of linarin, we incubated PC12 cells with linarin at a range of 0.1–10 μM for 1 h prior to 30 μM $A\beta_{25-35}$. We found that linarin was able to block $A\beta_{25-35}$-induced cell death in a dose-dependent manner (Fig. 2B).

3.2. Linarin inhibits AChE activity in PC12 cells

We also confirmed that linarin inhibited AChE activity in neuronal PC12 cells. As shown in Figure 3, exposure of PC12 cells to 30 μM $A\beta_{25-35}$ resulted in a significant increase in AChE activity. After treatment with linarin, AChE activity was significantly decreased. Therefore, the data showed that linarin could inhibit AChE activity in PC12 cells.

3.3. Linarin-mediated PC12 protection occurs by inhibiting apoptosis induced by $A\beta_{25-35}$

To examine whether or not the $A\beta_{25-35}$-induced cell death of PC12 cells in our studies occurred by an apoptotic-like mechanism, we utilized flow cytometric analysis to measure the relative amounts of Annexin V and propidium iodide (PI) stained cells. Externalization of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane is a distinct phenomenon associated with early apoptosis. This method is well-established for measuring apoptosis for many model systems.[9] As Annexin V possesses a high affinity towards phosphatidyl serine, apoptotic cells can easily be detected using fluorescently labeled Annexin V. On the other hand, PI is often used to detect necrotic cells due to its ability to permeate damaged cell membranes. By measuring both Annexin V and PI, the relative impact of a toxin on necrosis and apoptosis can then be determined. As shown in Figure 4, 30 μM $A\beta_{25-35}$ significantly increased both early and late apoptotic death in PC12 cells, with the total apoptotic effect reaching ~28% by 24 h. However, pretreatment with linarin (0.1, 1.0, 10 μM) for 1 h prior to $A\beta_{25-35}$ exposure dose-dependently decreased the apoptotic rate to 20.52%, 11.57% and 7.32%, respectively (Fig. 4). Therefore, the data showed that linarin could protect PC12 cells from $A\beta_{25-35}$-induced apoptosis in a dose-dependent manner.

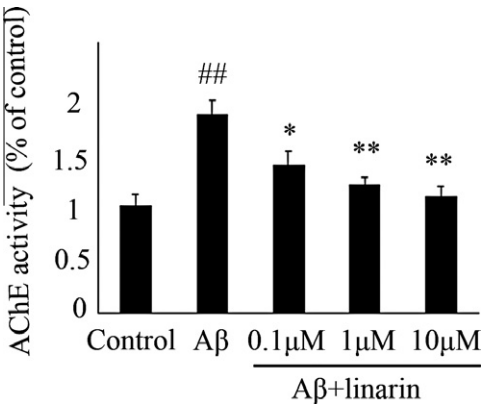

Figure 3. AChE inhibitory activity of linarin in $A\beta_{25-35}$-treated PC12 cells. The AChE inhibition effects of linarin in PC12 cells were measured by Ellman assay. Results represented mean ± S.D of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus $A\beta_{25-35}$ treatment group. $^{\#\#}p < 0.01$ versus control.

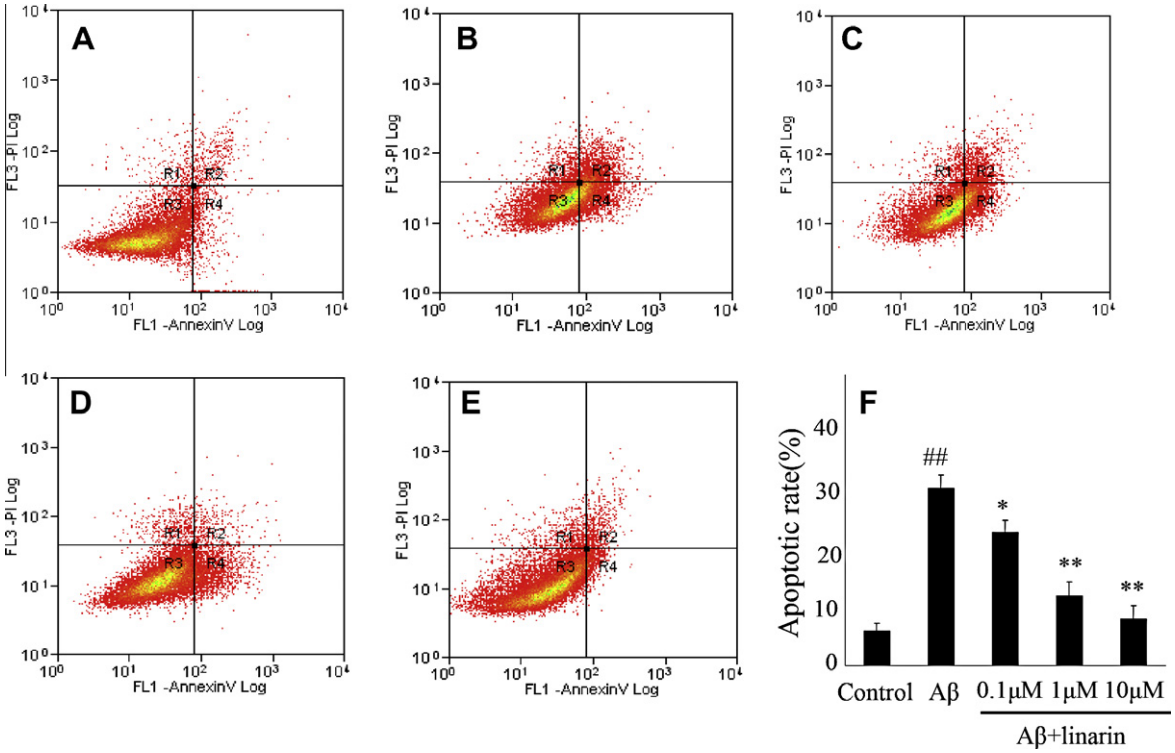

Figure 4. Protective effects of linarin on $A\beta_{25-35}$-induced apoptosis. PC12 cells were pretreated with linarin for 1 h, and then treated with 30 μM $A\beta_{25-35}$ for 24 h. Cell apoptosis were measured by Annexin-V/PI staining: (A) Control; (B) $A\beta_{25-35}$ 30 μM; (C,D,E) linarin 0.1, 1.0, 10 μM; The results were integrated into Figure 4F. All data are presented as the mean ± S.D. from triplicate independent experiments. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control.

3.4. Linarin restores mitochondrial membrane potentials in $A\beta_{25-35}$-treated PC12 cells

Increasing evidence suggests that altered mitochondrial function is linked to apoptosis and that a decrease in mitochondrial transmembrane potential is associated with mitochondrial dysfunction.[10] To assess this in our cells we used the mitochondrial probe JC-1 to measure changes in mitochondrial transmembrane potential. As shown in Figure 5, exposure of PC12 cells to 30 μM $A\beta_{25-35}$ for 24 h significantly decreased mitochondrial membrane potentials. However, a 1 h pre-incubation of cells with linarin was able to attenuate mitochondrial membrane potential depolarization induced by $A\beta_{25-35}$, raising the possibility that linarin may act at least in part by protecting mitochondrial function. To assess for this, we next measured caspase-3 induction in response to $A\beta_{25-35}$ in cells treated with or without linarin.

3.5. Linarin suppresses the activation of caspase-3 induced by $A\beta_{25-35}$

Caspase-3 is a key downstream effector of the cysteine protease family, that is involved in both the mitochondrial apoptotic pathway and the death receptor pathway.[11] Consequently, we investigated the ability of linarin to modulate caspase-3 activation.

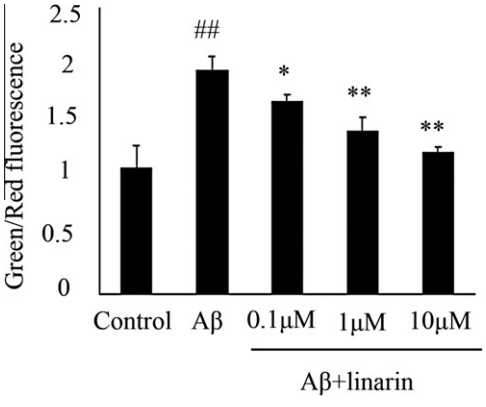

Figure 5. Protective effects of linarin on $A\beta_{25-35}$-induced mitochondrial membrane potential depolarization in PC12 cells. Cell were pretreated with linarin for 1 h, and then treated with 30 μM $A\beta_{25-35}$ for 24 h. Mitochondrial membrane potential was then determined with the lipophilic dye JC-1. All data are presented as the mean ± S.D. from triplicate independent experiments. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control.

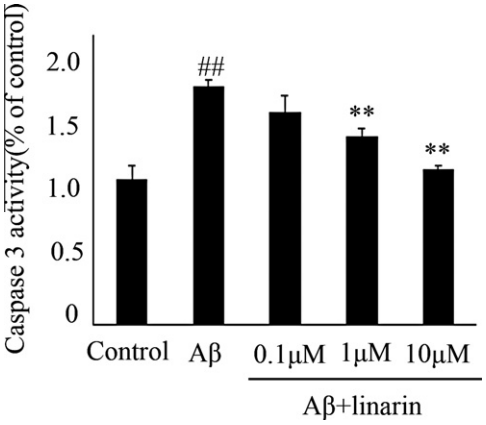

Figure 6. Protective effects of linarin against $A\beta_{25-35}$-induced activation of caspase-3 in PC12 cells. Cells were pretreated with linarin for 1 h, and then treated with 30 μM $A\beta_{25-35}$ for 24 h. All data are presented as the mean ± S.D. from triplicate independent experiments. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control.

PC12 cells treated with 30 μM $A\beta_{25-35}$ for 24 h showed a significant increase in the activity of caspase-3 (Fig. 6). However, after a 1 h pretreatment of cells with linarin prior to $A\beta_{25-35}$ treatment for 24 h, caspase-3 activity was significantly reduced, occurring in a dose-dependent manner.

3.6. The neuroprotective effect of linarin involves the PI3K pathway

A major pathway that blocks caspase-3 activation is associated with the kinase PI3K and its downstream effector kinase, Akt.[12] To explore potential intracellular signaling mechanisms responsible for the protective effects of linarin against $A\beta_{25-35}$-induced PC12 cell death, we assessed for changes in PI3K/Akt activation, which can be measured on Western blots using phosphorylation state specific antibodies. As shown in Figure 7A, $A\beta_{25-35}$ (30 μM) significantly reduced Akt phosphorylation, indicative of the pro-apoptotic effects of $A\beta_{25-35}$ noted in our cells. However, pretreatment with linarin upregulated Akt phosphorylation, with higher doses producing a larger effect, suggesting that linarin stimulates PI3K/Akt signaling in PC12 cells. To further assess the contribution of Akt, the PI3K inhibitor LY294002 (20 μM) was used. Our results demonstrated that PI3K inhibition 1 h before linarin blocked the phosphorylation of Akt in our cells (Fig. 7B), while total Akt levels did not change. These data strongly suggest that linarin-mediated protection against $A\beta_{25-35}$-induced apoptosis involves the activation of the PI3K/Akt pathway. Interaction between the PI3K and GSK signaling is also known to affect cell viability.[13] To explore the impact of Akt and GSK-3β in our model system we also evaluated the effects of linarin on GSK-3β.

3.7. Linarin downregulates GSK-3β activity through PI3K/Akt

Increased GSK-3β activity has been implicated in neuronal cell death and the inactivation of GSK-3β can result in the prevention or abatement in apoptotic injury in neurons.[14] Activation of GSK-3β is easily measured via assessing decreases in serine 9 phosphorylation (PSer9). Ser9 phosphorylation is mediated by multiple mechanisms including factors that inhibit the PI3K/Akt pathway.[15,16] Therefore, we next investigated whether linarin enhanced PSer9 on GSK-3β and if this occurred through activation of PI3K/Akt. As shown in Figure 8, $A\beta_{25-35}$ (30 μM) treatment resulted in a marked decrease in the level of PSer9 on GSK-3β. Conversely, pretreatment with linarin sustained normal levels of PSer9, indicative of GSK-3β inhibition and a reduced tendency toward apoptosis. Additionally, blockade of PI3K/Akt activity by LY294002 pretreatment also blocked GSK-3β PSer9 increases that were conferred by linarin treatment (Fig. 8). We therefore postulate that PSer9-associated-GSK-3β inhibition in response to linarin may present a downstream mechanism by which linarin protects neuronal cells against $A\beta_{25-35}$ induced apoptosis. A key neuroprotective molecule, that is upregulated in response to PI3K/Akt is the anti-apoptotic protein Bcl-2.[17] As linarin was able to activate Akt, we presumed that linarin may have also contributed to the up-regulation of Bcl-2 to mediate neuroprotective effects.

3.8. Linarin stimulates Bcl-2 expression by effects on PI3K/Akt

Considering that Bcl-2 is a major anti-apoptotic protein and an important downstream substrate of Akt, we also tested the effects of linarin on Bcl-2 protein levels. It was reported that Akt activation enhances the expression of Bcl-2.[17] As demonstrated in Figure 9, cells treated with linarin exhibit an increase in Bcl-2 levels as measured by Western blot and this effect on Bcl-2 increases was attenuated by LY294002, indicating that the up-regulation of Bcl-2 by linarin involves the PI3K/Akt pathway.

4. Discussion

The purpose of this study was to investigate neuroprotective effects of linarin against Aβ neurotoxicity and to explore the signaling pathways involved. As the PI3K/Akt pathway is a major contributor to neuroprotection, we assessed whether linarin stimulated the activity of PI3K/Akt. First we demonstrated that cell viability was significantly decreased in response to $A\beta_{25-35}$ by pretreatment with linarin, which increased cell viability in a concentration-dependent manner (Fig. 2). In addition, Annexin-V and JC-1 staining, which are well-established measures of apoptotic cell death and mitochondrial damage, were both decreased after treatment with linarin (Figs. 4 and 5). These findings suggest that

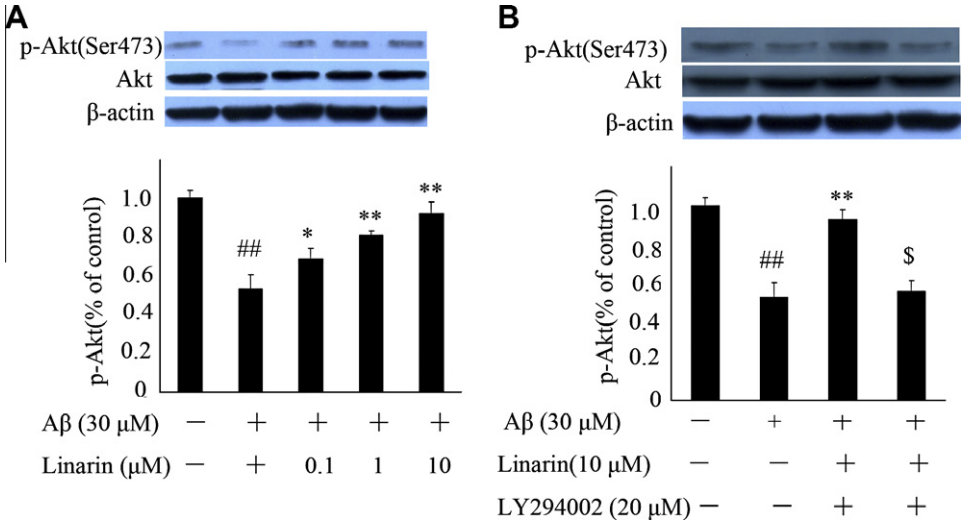

Figure 7. Involvement of PI3K/Akt in the protective effects of linarin on $A\beta_{25-35}$-induced apoptosis. Western blot analysis of PC12 cells for activated phospho-Akt. (A) Linarin activated Akt in a dose-dependent manner in $A\beta_{25-35}$-induced PC12 cells. Cells were pretreated with linarin for 1 h, and then treated with 30 μM $A\beta_{25-35}$ for 24 h. (B) PI3K/Akt pathway inhibitor LY294002 inhibited linarin-mediated activation of Akt. PC12 cells were pretreated with 20 μM LY294002 for 1 h, and then treated with 10 μM linarin for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h. Densitometry values are mean ± S.D. from triplicate independent experiments. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control, $^{\$}p$ <0.01 versus 10 μM linarin treatment group.

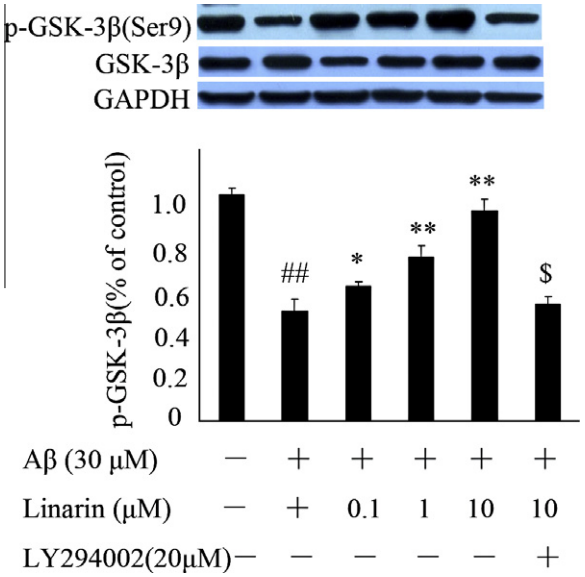

Figure 8. Linarin inhibit GSK-3β by activating PI3K/Akt pathway in PC12 cells after exposure to $A\beta_{25-35}$. Cells were treated with 30 μM $A\beta_{25-35}$ or $A\beta_{25-35}$ combined with linarin plus 20 μM LY294002. The blots presented are representatives of three independent experiments with similar results. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control, $^{\$}p$ <0.01 versus 10 μM linarin treatment group.

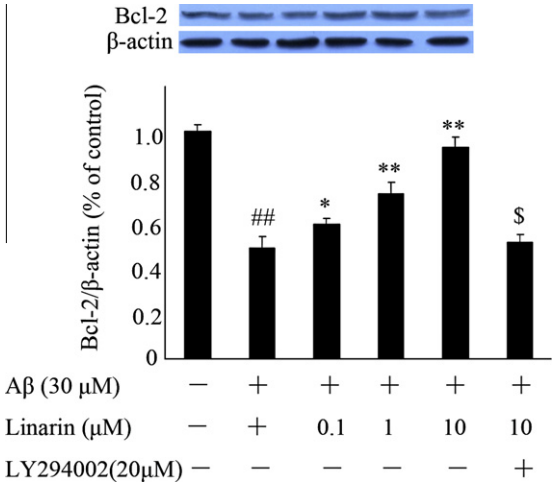

Figure 9. Linarin increased Bcl-2 protein expression in PC12 cells after exposure to $A\beta_{25-35}$. Cells were treated with 30 μM $A\beta_{25-35}$ or $A\beta_{25-35}$ combined with linarin plus 20 μM LY294002. The blots presented are representatives of three independent experiments with similar results. $^{*}p$ <0.05; $^{**}p$ <0.01 versus $A\beta_{25-35}$ treatment group, $^{\#\#}p$ <0.01 versus control, $^{\$}p$ <0.01 versus 10 μM linarin treatment group.

linarin reduced cell death associated with Aβ toxicity. Further study revealed that the protective effects of linarin were mediated by neuroprotective phosphorylation of both Akt and GSK-3β (Figs. 7 and 8).

It has been reported that currently available AChE inhibitors exert neuroprotective effects,[18,19] but that the neuroprotective effects of those AChE inhibitors were mediated through mechanisms unrelated to inhibition of the catalytic activity of AChE. In contrast, our study illustrated that linarin protected PC12 cells against $A\beta_{25-35}$-induced neurotoxicity in addition to its AChE inhibiting effect. Thus, it is of great interest to investigate the mechanism of action responsible for potentially unique neuroprotective effects of linarin.

The PI3K signaling pathway plays a central role in neuronal survival.[20,21] The activation of PI3K has previously been shown to prevent Aβ-induced neuronal death.[22,23] Therefore, we examined the role of the PI3K pathway in the neuroprotective effects of linarin. The finding that linarin increased Akt phosphorylation and the neuroprotective effects of linarin were attenuated by a PI3K inhibitor strongly support a role for this signaling pathway in linarin-mediated neuroprotection. Although our studies did not demonstrate exactly how linarin activates the PI3K pathway, it is likely by interaction with insulin-like-growth factor receptors, which bind IGF to simulate neuroprotective signaling which opens the door for future investigations.[24]

Recently, GSK-3β, a regulator of the Wnt/β-catenin pathway, has been the focus of many studies exploring the pathogenic mechanisms underlying AD.[25,26] GSK-3β is a multifunctional enzyme that affects a diverse range of biological and cellular activities. Specifically, GSK-3β is a downstream target of the PI3K pathway and is also one of the major tau kinases.[27] Activation of GSK-3β is associated with neuronal death and the formation of paired helical filaments by phosphorylation of tau protein at multiple sites both in vitro and in vivo.[28,29] Upon activation of the PI3K/Akt pathway, GSK-3β can be phosphorylated at Ser9 which leads to its inactivation, resulting in decreased tau phosphorylation. Here, we investigated the phosphorylation of GSK-3β in PC12 cells after $A\beta_{25-35}$ toxicity. $A\beta_{25-35}$-associated activation of GSK-3β was significantly suppressed by pretreatment of cells with linarin, indicating that the regulatory effect of linarin on GSK-3β inhibition may contribute to linarin-mediated neuroprotection. Future studies to investigate the role of linarin on tau phosphorylation and GSK-3β activity are also planned.

It is well known that the anti-apoptotic protein Bcl-2 plays a central role in regulating programmed cell death and is associated with various neurodegenerative disorders including AD.[30] Recent reports show that Bcl-2 is downstream of the PI3K/Akt cascade and that up-regulation of Bcl-2 is a main reason for cell survival,[31,32] which is thought to be associated with mitochondrial maintenance. In the present study, we found that treatment with linarin increased Bcl-2 protein levels. Furthermore, a specific PI3K inhibitor prevented the up-regulation of Bcl-2 by linarin. Taken together our findings strongly support a protective role for linarin acting via increasing Bcl-2 levels secondary to activation of PI3K, and that this activation also prevented mitochondrial damage.

In conclusion, these studies provide the first evidence that a small naturally-derived molecule, linarin has robust neuroprotective effects and can potently attenuate Aβ-mediated neurotoxicity, likely by activation of the PI3K/Akt pro-survival pathway. Further studies are needed to elucidate the full spectrum of cellular mechanisms underlying the neuroprotective effects of linarin, which may ultimately contribute as a novel therapy for the treatment of AD.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81001616), Science and Technology Development Project of Shandong Province (No. 2010GSF10223), the Independent Innovation Foundation of Shandong University (No. 2009TS110), the Foundation for Excellent Young and Middle-Aged Scientists of Shandong Province (No. BS2010YY036), and start up funding from the Department of Neurology, University of Pittsburgh, School of Medicine (R.G.P.).

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