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Original Article

Liquiritigenin inhibits $A\beta_{25-35}$-induced neurotoxicity and secretion of $A\beta_{1-40}$ in rat hippocampal neurons

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Aim: To examine whether liquiritigenin, a newly found agonist of selective estrogen receptor-β, has neuroprotective activity against β-amyloid peptide (Aβ) in rat hippocampal neurons.
Methods: Primary cultures of rat hippocampal neurons were pretreated with liquiritigenin (0.02, 0.2, and 2 μmol/L) prior to $A\beta_{25-35}$ exposure. Following treatment, viability of the cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis and by a lactate dehydrogenase activity-based cytotoxicity assay. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and levels of reactive oxygen species (ROS), as well as apoptotic rates, were determined. Our studies were extended in tests of whether liquiritigenin treatment could inhibit the secretion of $A\beta_{1-40}$ as measured using an ELISA method. In order to analyze which genes may be involved, we used a microarray assay to compare gene expression patterns. Finally, the levels of specific proteins related to neurotrophy and neurodenegeration were detected by Western blotting.
Results: Pretreated neurons with liquiritigenin in the presence of $A\beta_{25-35}$ increased cell viability in a concentration-dependent manner. Liquiritigenin treatment also attenuated $A\beta_{25-35}$-induced increases in $[Ca^{2+}]_i$ and ROS level and decreased the apoptotic rate of neurons. Some genes, including B-cell lymphoma/leukemia-2 (Bcl-2), neurotrophin 3 (Ntf-3) and amyloid β (A4) precursor protein-binding, family B, member 1 (Apbb-1) were regulated by liquiritigenin; similar results were shown at the protein level by Western blotting.
Conclusion: Our results demonstrate that liquiritigenin exhibits neuroprotective effects against $A\beta_{25-35}$-induced neurotoxicity and that it can decrease the secretion of $A\beta_{1-40}$. Therefore, liquiritigenin may be useful for further study as a prodrug for treatment of Alzheimer's disease.

Keywords: liquiritigenin; selective ERβ agonist; neuroprotection; Aβ secretion

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Introduction

In addition to its classic function as a sex hormone, estrogen plays key regulatory roles in various biological pathways. It has outstanding neuroprotective and neurotrophic activities and has been linked to neurodegenerative diseases, including Alzheimer's disease (AD)[1, 2]. A large body of work has shown that estrogen can block β-amyloid peptide (Aβ)-induced neuronal cell death and influence Aβ secretion[3–7]. However, long-term compliance with estrogen administration is estimated to be no more than 15%–40% because of undesirable side effects[8–10]. As a result, several phytoestrogens with fewer side effects and potential neuroprotective effects have been developed as alternative treatment strategies[11, 12].

Liquiritigenin (7,4′-dihydroxyflavanone, Figure 1) is a flavonoid extracted from *Glycyrrhizae* radix that exhibits life-enhancing properties and is frequently used in traditional Oriental medicine to treat injury or swelling and for detoxification. Our interest in liquiritigenin developed as a result of the following observations. First, liquiritigenin was shown to be a highly selective agonist of estrogen receptor-β (ERβ)[13], an observation that is consistent with our previous findings (unpublished data). ERβ is expressed in brain centers related to learning and memory and is less likely than ERα to be related to sexual function[14, 15]. Second, liquiritigenin has been shown in several studies to exert cytoprotective effects

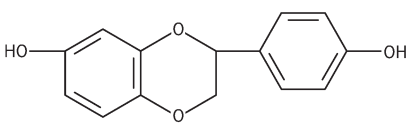

Figure 1. Chemical structure of liquiritigenin.

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in vitro and *in vivo*[16, 17]; recent studies demonstrate that liquiritigenin exerts anti-inflammatory effects through the NF-κB pathway[18]. Third, we previously observed that liquiritigenin did not induce proliferation of MCF-7 and T47D breast cancer cells (data not shown), which is consistent with other studies[13]. Finally, pharmacokinetic studies from our laboratory have demonstrated that liquiritigenin exhibits good intestinal absorption and blood-brain barrier permeability[19]. These characteristics suggest that liquiritigenin may have a therapeutic function in the brain.

Materials and methods

Cell culture

Primary rat hippocampal neurons were obtained from newborn Wistar rats (postnatal day 0, obtained from the Academy of Military Medical Science, grade SPF, certificate number: SCXK(Jing)2005-0013) as previously described[12] with minor modifications. Procedures were carried out in accordance with the Institutional Animal Care Guidelines of the Chinese Society of Laboratory Animals Science. Briefly, after being dissected from the brains of the rats, hippocampi were treated with 0.25% trypsin for no more than 30 min at 37 °C and were dissociated by repeated passage through a series of fire-polished, constricted Pasteur pipettes. Neurons were seeded into poly-*L*-lysine (100 μg/mL)-coated plates and grown in serum-free DMEM-F12 medium supplemented with 2% B27 (Sigma, USA). This method yielded cell cultures consisting of more than 90% neurons. Cell cultures were routinely observed by phase-contrast inverted microscopy and were maintained for 7 d before treatment.

Aβ is a 39−43 amino acid peptide that is derived from amyloid precursor protein. The toxicity of $A\beta_{25-35}$ is very similar to that of $A\beta_{1-42}$. Both peptides are easily amyloidogenic, so $A\beta_{25-35}$ was used in the present study. Seven-day-old neurons were pretreated with or without 0.02, 0.2, or 2 μmol/L liquiritigenin (synthesized in our institute; purity >95% by HPLC) alone for 24 h; then 10 μmol/L $A\beta_{25-35}$ (Sigma, USA), the concentration of which was chosen as previously described[20], was added together with the liquiritigenin for 72 h. To produce neurotoxicity, $A\beta_{25-35}$ (diluted in saline) was incubated at 37 °C for 7 d to make fibril formation before use.

Cell viability detection

Seven-day-old neurons were plated into 96-well plates (5×10^4 cells/well) and treated with liquiritigenin for 24 h and $A\beta_{25-35}$ for 72 h. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described[21]. Absorbance at 540 nm was measured using a microplate reader (ThermoLab, USA). The viability of vehicle-treated control groups (not exposed to $A\beta_{25-35}$) was defined as 100%.

Lactate dehydrogenase activity-based cytotoxicity assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme expressed in most cells, including neurons. When the plasma membrane is damaged, LDH is rapidly released into the cell culture supernatant. In the context of this study, we used LDH release as a biochemical index of cytotoxicity. Culture supernatant 100 μL was collected and LDH activity was measured using LDH detection kits (BIOSINO, China) according to the manufacturer's instructions. Colorimetric absorbance at 570 nm was measured using an automatic chemistry analyzer (ELx800, Italy). LDH activity in each group was calculated as percentage of the LDH activity of cells that had been exposed to $A\beta_{25-35}$ alone, which was defined as 100%.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the Ca^{2+}-sensitive fluorochrome Fluo-3/acetoxymethyl ester (Fluo-3/AM, Biotium, USA). Fluorescence intensity was analyzed using a fluorescence spectrophotometer (FOLARstar, BMGLABTech, German) with excitation and emission wavelengths of 488 nm and 526 nm; $[Ca^{2+}]_i$ was calculated according to the described method[22].

Measurement of intracellular reactive oxygen species

The level of intracellular reactive oxygen species (ROS) was determined using 2,7-dichlorodihydro fluorescent diacetate (DCFH-DA, Beyotime, China), following the manufacturer's instructions. The fluorescence intensity of cells was measured with a fluorescence spectrophotometer, with excitation and emission wavelengths of 490 nm and 520 nm, respectively.

Hoechst 33342 staining

The morphology of nuclear chromatin was assessed by staining with the fluorescent dye Hoechst 33342 as described[11]. Briefly, cells were fixed with 3.7% paraformaldehyde (*v/v*) and then stained with Hoechst 33342 (10 μg/mL) for 10 min at room temperature. After washing, each field of cells was analyzed using a fluorescence microscope (Zeiss, Germany) and recordings were made using a CCD camera (Apogee). DNA-fragmented cells stained with Hoechst 33342 take on a bright blue.

Flow cytometric detection of apoptotic cells

For detection of apoptotic rate, Annxin V-FITC/PI (Jingmei Biotech, China) staining was performed following the manufacturer's instructions. Cells that could be stained with Annxin V but not with PI were defined as apoptotic. Apoptotic rate was measured with a flow cytometer (FCM, Becton Dickinson, USA) using CellQuest software.

Treatment of cells with liquiritigenin to detect soluble $A\beta_{1-40}$

Seven-day-old neurons were cultured in 96-well dishes and treated separately in the presence of 0.02, 0.2, 2 μmol/L liquiritigenin or vehicle for 24 h. For each treatment, three wells containing the same number of cells were used. The levels of soluble $A\beta_{1-40}$ (the predominant form of Aβ peptide) in the conditioned medium were assayed using the sensitive sandwich ELISA detection kit, following the manufacturer's instructions (Uscnlife, USA). Levels of $A\beta_{1-40}$ were expressed in pg/mL as deduced from the appropriate standard curve

run in parallel with the assay.

Microarray assay

In order to understand which genes may be influenced by exposure of neurons to liquiritigenin, a microarray technique was used to explore gene expression profiles. Seven-day-old cells were seeded into 6-well plates at a density of 2×10^6/well and were treated with/without 2 μmol/L liquiritigenin for 24 h. The Oligo GEArray Rat Neurogenesis and Neural Stem Cell Microarray (ORN-404, Superarray, USA), which includes 263 genes, was chosen because it contains genes related to both neurotrophy and neuroprotection. After treatment, cells were lysed using 1 mL TRIzol (Sigma, USA) and immediately delivered to the manufacturer. The entire microarray procedure, including RNA preparation and reverse transcription PCR, was performed by the manufacturer. The fold change was calculated for each gene as the ratio of liquiritigenin/control. In this experiment, only the high dose of 2 μmol/L liquiritigenin was chosen and the assay was not repeated because of its costliness.

Western blotting

In light of results from microarray and similar studies[23–25], we selected four representative proteins, including B-cell lymphoma/leukemia-2 (Bcl-2), Bcl-2-associated X protein (Bax), neurotrophin 3 (Ntf-3) and amyloid β (A4) precursor protein-binding, family B, member 1 (Apbb-1), for further analysis to validate whether liquiritigenin affects related protein expression. For detection of Bcl-2, Bax and Ntf-3, seven-day-old rat hippocampal neurons were seeded into 6-well plates at a density of 2×10^6 cells/well. After an overnight incubation, cells were treated with/without liquiritigenin (0.02, 0.2, or 2 μmol/L) for 24 h, followed by addition of 10 μmol/L $A\beta_{25-35}$ and further incubation with liquiritigenin for 72 h. For targeted detection of Apbb-1, cells were treated with liquiritigenin (0.02, 0.2, or 2 μmol/L) alone for 24 h, then lysed with 4 °C cell lysis buffer (Beyotime, China) as recommended by the manufacturer. SDS-PAGE and Western blotting were performed according to standard protocols [22] using 40 μg protein per lane. Primary antibodies were as follows: Bcl-2 (Beyotime, China, 1:500 dilution), Bax (Beyotime, China, 1:500 dilution), Ntf-3 (Chemicon, USA, 1:1000 dilution), Apbb-1 (Bioss, China, 1:200 dilution), β-tubulin (Walterson, China, 1:1000 dilution, as internal control) and secondary antibody (goat anti-rabbit or goat anti-mouse IgG-HRP, Zhongshan, China, 1:2000 dilution). The grey levels were presented as the ratio against β-tubulin and were analyzed using the software Scion Image for Windows (http:// www.scioncorp.com).

Statistical analysis

All data, except those of microarray assay, are expressed as mean±SEM; assays were repeated in at least three independent experiments (in the context, "*n*" represents the number of repetitions), each performed in triplicate. Data comparisons among groups were assessed by one-way ANOVA. Between two groups, Dunnett's test was used; $P<0.05$ was assumed to indicate statistical significance.

Results

Liquiritigenin protects primary rat hippocampal neurons against $A\beta_{25-35}$-induced cytotoxicity

As shown in Figure 2A, $A\beta_{25-35}$ reduced cell viability to 69.5%±3.8% ($P<0.01$ *vs* control, $n=3$) of control; when cells were pre-incubated with 0.02, 0.2, or 2 μmol/L liquiritigenin, cell viability was 73.5%±1.3% ($P>0.05$ *vs* $A\beta_{25-35}$, $n=3$), 79.5%±2.0% ($P<0.01$ *vs* $A\beta_{25-35}$, $n=3$) and 83.4%±1.6% ($P<0.01$ *vs* $A\beta_{25-35}$, $n=3$), respectively, compared with control.

Liquiritigenin reduces cell membrane permeability

Our results (Figure 2B) show that $A\beta_{25-35}$ increased 4.3-fold of LDH leakage compared with control ($P<0.01$ *vs* control, $n=3$); when cells were treated with liquiritigenin, LDH leakage decreased to 80.3%±2.7% (0.2 μmol/L, $P<0.01$ *vs* $A\beta_{25-35}$, $n=3$) and 70.1%±1.1% (2 μmol/L, $P<0.01$ *vs* $A\beta_{25-35}$, $n=3$) that of cells treated with $A\beta_{25-35}$ alone. However, 0.02 μmol/L liquiritigenin had no effect on cell permeability.

Liquiritigenin inhibits $A\beta_{25-35}$-induced increases in Ca^{2+} influx

The Ca^{2+}-sensitive fluorescent probe Fluo-3/AM was used to monitor alterations in $[Ca^{2+}]_i$ by spectrofluorometry. As illustrated in Figure 2C, cells that were incubated with $A\beta_{25-35}$ alone had a much higher $[Ca^{2+}]_i$ than control cells (1468.6±184.7 nmol/L *vs* 601.0±65.6 nmol/L, $P<0.01$, $n=3$); $[Ca^{2+}]_i$ was significantly decreased by pretreatment with 0.2 μmol/L (1014.3±142.1 nmol/L, $P<0.05$ *vs* $A\beta_{25-35}$, $n=3$) or 2 μmol/L (748.6±34.3 nmol/L, $P<0.01$ *vs* $A\beta_{25-35}$, $n=3$) liquiritigenin, but not by 0.02 μmol/L liquiritigenin.

Liquiritigenin decreases $A\beta_{25-35}$-induced upregulation of cellular ROS

DCFH-DA, the fluorescence of which increases along with intracellular ROS, was used for detection of cellular ROS. As shown in Figure 2D, $A\beta_{25-35}$ increased 3.0-fold of intracellular ROS level compared with control ($P<0.01$ *vs* control, $n=3$); when 0.2 or 2 μmol/L liquiritigenin was added before $A\beta_{25-35}$, intracellular ROS levels decreased to 48.2%±2.8% ($P<0.01$ *vs* $A\beta_{25-35}$, $n=3$) and 41.3%±5.9% ($P<0.01$ *vs* $A\beta_{25-35}$, $n=3$), respectively, of ROS levels in cells treated with $A\beta_{25-35}$ alone.

Liquiritigenin protects neurons against $A\beta_{25-35}$-induced apoptosis

Nuclear morphology was evaluated using the membrane-permeable blue dye Hoechst 33342. As shown in Figure 3A, the content of DNA-fragmented cells increased after $A\beta_{25-35}$ treatment. There were fewer DNA-fragmented cells after incubation of the neurons with 0.2 or 2 μmol/L liquiritigenin.

Annexin V-FITC/PI staining gave similar results; Figure 3B shows typical histograms from FCM. As shown in Figure 3C, the apoptotic rate of cells treated with 10 μmol/L $A\beta_{25-35}$ alone was much higher than control (31.27%±1.76% *vs* 1.92%±0.36%, $P<0.01$, $n=4$). Pretreatment with 0.2 or 2 μmol/L liquiritigenin decreased the apoptotic rate significantly; it decreased to 21.94%±0.90% and 13.95%±1.79%, respectively, of cells treated

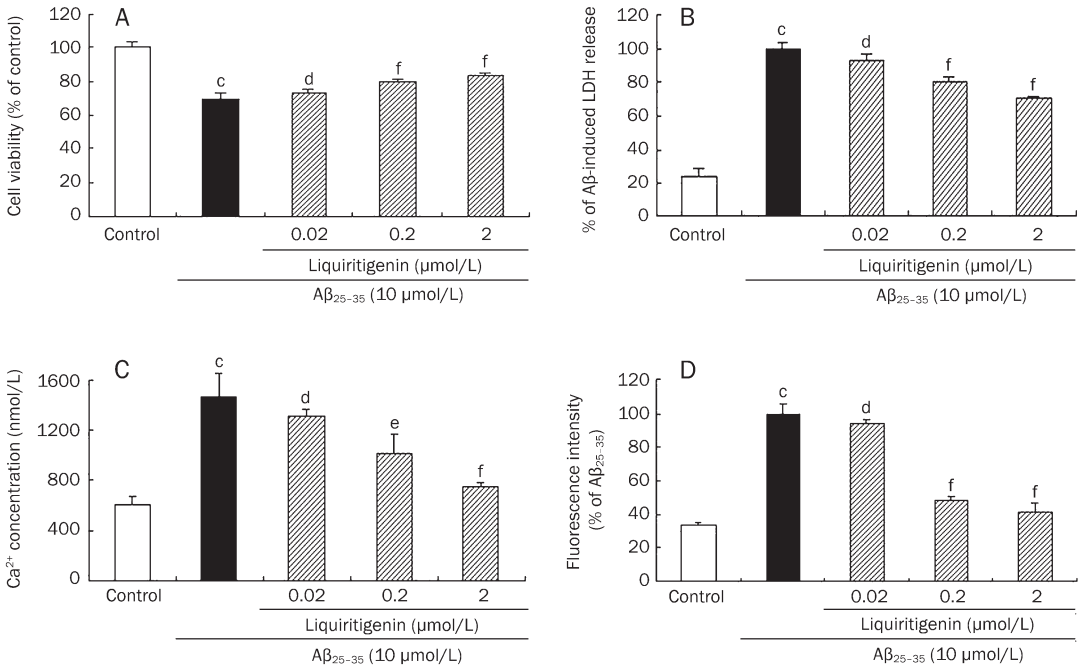


Figure 2. Liquiritigenin exhibits neuroprotective activity against $A\beta_{25-35}$-induced damage in primary rat hippocampal neuron cells. Cells were pretreated with various concentrations of liquiritigenin for 24 h prior to insult by 10 μmol/L $A\beta_{25-35}$ for another 72 h. (A) Liquiritigenin increases cell viability as determined by MTT assay. (B) LDH leakage into the supernatant was reduced by pretreating cells with liquiritigenin. (C) Liquiritigenin inhibits $A\beta_{25-35}$-induced up-regulation of $[Ca^{2+}]_i$ detected with Fluo-3/AM fluorescent dye, the intensity of which increases in parallel with concentration of cytoplasmic free Ca^{2+}. (D) Effect of liquiritigenin on $A\beta_{25-35}$-induced increase of intracellular ROS level measured by DCFH-DA fluorescent dye, the intensity of which increases with cytosolic ROS level. Bars represent mean±SEM (n=3). [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* control; [d]P>0.05, [e]P<0.05, [f]P<0.01 *vs* $A\beta_{25-35}$ alone.

with $A\beta_{25-35}$ alone (P<0.01 *vs* $A\beta_{25-35}$, n=4).

Liquiritigenin regulates secretion of soluble $A\beta_{1-40}$

Hippocampal neurons were treated with liquiritigenin and the concentration of $A\beta_{1-40}$ in the supernatant was measured using ELISA. Figure 4 shows that incubation of neurons with 0.2 or 2 μmol/L liquiritigenin decreased $A\beta_{1-40}$ levels significantly (0.2 μmol/L: 73.5±5.0 pg/mL, 2 μmol/L: 65.2±2.9 pg/mL *vs* control: 138.5±21.8 pg/mL, P<0.01, n=5). However, 0.02 liquiritigenin did not show any effects.

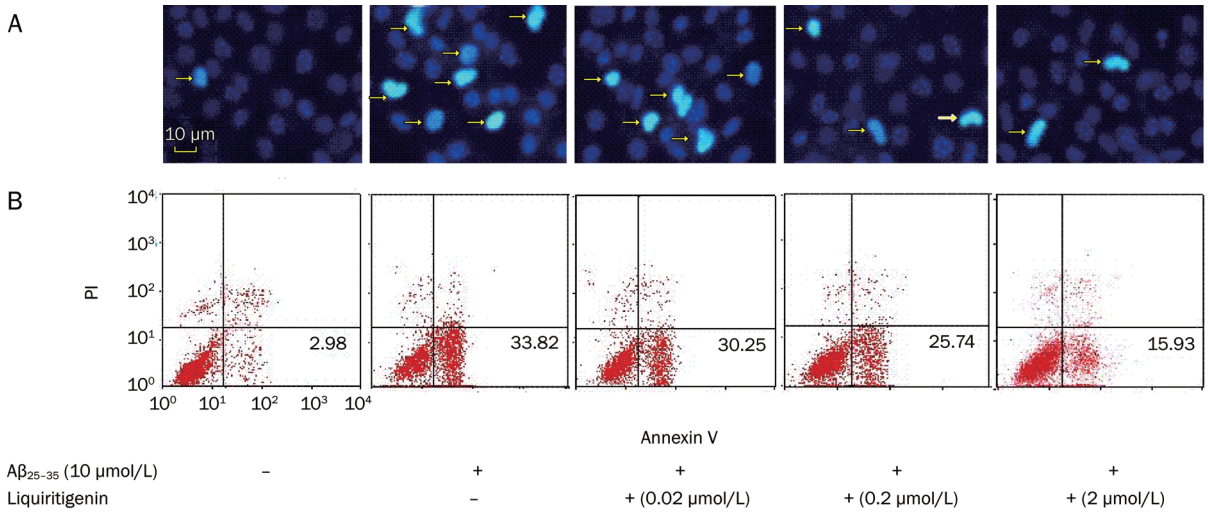
Effects of liquiritigenin on some genes involved in neuroprotection and neurotrophy

Using the rat neurogenesis and neural stem cell microarray assay, we could determine differential gene expression in liquiritigenin-treated and untreated cells through a simple side-by-side hybridization experiment. If the fold change is greater than 1, the result is reported as a fold up-regulation. If the fold change is less than 1, the negative inverse of the result is reported as a fold down-regulation. Figure 5A shows the results of the microarray assay; from it, we can see that some genes were up-regulated whereas others were down-regulated.

Figure 5B shows quantitative analysis of the microarray assay, which indicating that the expression level of Bcl-2, the most important anti-apoptotic gene, was increased by 6.25-fold (n=1), while the expression level of Bax was similar to that of control (n=1). Ntf-3 and Ntf-5 were up-regulated by 7.65 and 5.43-fold, respectively (n=1). Furthermore, the expression of Apbb-1, which is related to amyloidosis, was sharply decreased by 13.2-fold (n=1) after liquiritigenin treatment.

Changes in related protein expression

The proteins Bcl-2, Bax, Ntf-3 and Apbb-1 were chosen for further analysis. Figure 5C shows a representative result of Western blotting. From the quantitative analysis (Figure 5D) we can see that $A\beta_{25-35}$ treatment reduced Bcl-2 expression significantly compared with control (0.28±0.02 *vs* 0.66±0.03, P<0.01, n=3); when 0.2 μmol/L or 2 μmol/L liquiritigenin was added, the ratio of protein expression increased markedly (0.2 μmol/L: 0.35±0.01, P<0.05 *vs* $A\beta_{25-35}$; 2 μmol/L: 0.49±0.03, P<0.01 *vs* $A\beta_{25-35}$, n=3). There was no significant difference in Bax expression among the groups. Ntf-3 expression in cells treated with $A\beta_{25-35}$ alone was reduced to less than half of control (0.23±0.04 *vs* 0.60±0.03, P<0.01, n=3); it could be up-regulated by liquiritigenin treatment in a dose-dependent manner (0.2 μmol/L: 0.33±0.02, P<0.05 *vs* $A\beta_{25-35}$; 2 μmol/L: 0.46±0.04, P<0.05 *vs* $A\beta_{25-35}$, n=3). The expression of Apbb-1 was reduced by 0.2 μmol/L (0.37±0.04, P<0.01 *vs* control, n=3) and 2 μmol/L liquiritigenin (0.31±0.04, P<0.01 *vs* control, n=3), respectively, compared with control, but not by 0.02 μmol/L liquiritigenin (0.48±0.06, P>0.05 *vs* control, n=3).



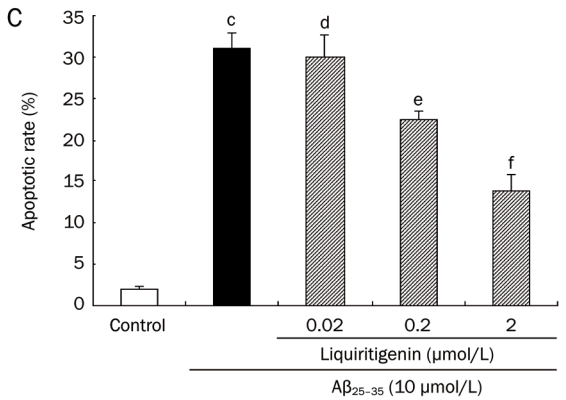


Figure 3. Liquiritigenin protects neuron cells against $A\beta_{25-35}$-induced apoptosis. (A) Nuclei were stained with fluorescent dye Hoechst 33342 for assessment of apoptosis; arrowheads indicate apoptotic cells. Magnification: ×200. (B) Representative FCM histograms stained by Annexin V-FITC/PI. Annexin V^+ and PI^- cells were designed as apoptotic. (C) Apoptotic rate of cells, determined by FCM. Bars represent mean±SEM (n=4). [a]P>0.05, [b]P<0.05, [c]P<0.01 *vs* control; [d]P>0.05, [e]P<0.05, [f]P<0.01 *vs* $A\beta_{25-35}$ alone.

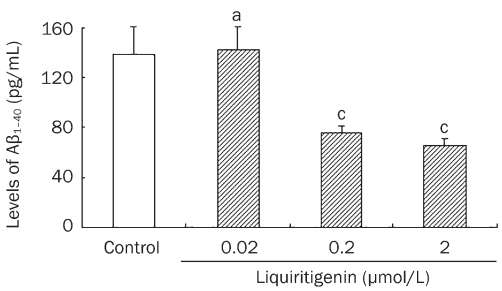


Figure 4. Liquiritigenin reduces the release of $A\beta_{1-40}$ into the medium in primary rat hippocampal neuron cells. Cells were cultured in the presence of liquiritigenin (0, 0.02, 0.2, 2 μmol/L) for 24 h and were assayed for $A\beta_{1-40}$ with ELISA (mean±SEM, n=5). [a]P>0.05, [c]P<0.01 *vs* control.

Discussion

In patients with AD, brain Aβ aggregates into clumps called oligomers that can accumulate and form deposits called amyloid plaques, which are thought to be a major pathologic mechanism of AD. Aβ-induced neurotoxicity has been attributed in various studies to Ca^{2+} influx, generation of ROS, induction of apoptosis, and other causes. The present study confirms that $A\beta_{25-35}$ can cause neural cell death, an increase of Ca^{2+} influx and generation of ROS, as previously evidenced in other studies[20]. In addition, we have shown that $A\beta_{25-35}$ treatment can decrease the expression of Ntf-3 protein, an important neurotrophic factor; such a decrease in Ntf-3 protein may also contribute to the neuronal death induced by $A\beta_{25-35}$.

The present study provides evidence that liquiritigenin at a dosage in the range of 0.2–2 μmol/L can prevent $A\beta_{25-35}$-induced injury to rat hippocampal neurons, as shown by cell viability measurements using MTT and LDH detection assays. In a previous study, Kim *et al* showed that liquiritigenin exerted anti-inflammatory effects against lipopolysaccharide in Raw264.7 cells at a concentration ranging from 3 to 30 μmol/L[18]. We reckon that the differences in effective dose observed in the two studies may be due to the use of different cells and/or different toxic factors.

It has been reported that ROS generation is a consequence of Ca^{2+} accumulation[26]. However, in many experiments, free radicals are responsible for the increase in $[Ca^{2+}]_i$. ROS-induced membrane damage causes further Ca^{2+} influx, and resultant accentuated Ca^{2+} influx will in turn induce the generation of further ROS [27]. In the present study, $A\beta_{25-35}$ elicited significant $[Ca^{2+}]_i$ increase and ROS accumulation, both of which contributed to the excitatory neurotoxicity of $A\beta_{25-35}$, as previously reported[20]. Both $[Ca^{2+}]_i$ increase and ROS accu-

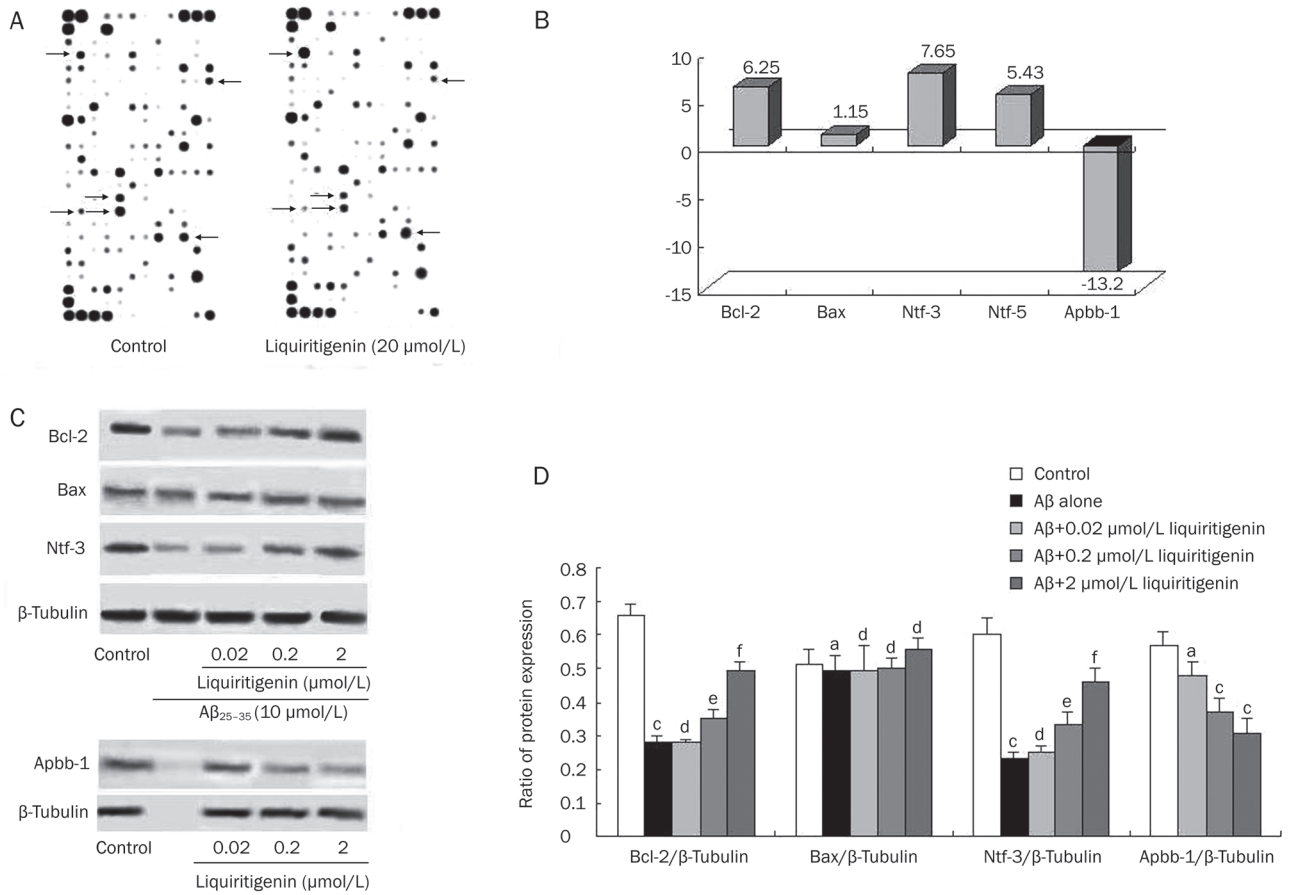


Figure 5. Effect of liquiritigenin on expression of genes Bcl-2, Bax, Ntf-3, Ntf-5, Apbb-1 and relative protein expression in rat primary neurons. (A) Figures of microarray assay. Each marking represents a type of gene; arrowheads indicate some genes that were regulated by 2 μmol/L liquiritigenin treatment. (B) Changes in gene expression pattern determined by microarray assay. The fold change was calculated for each gene as the ratio of liquiritigenin/control (n=1). (C) Representative photos of Western blots. For detection of Bcl-2, Bax, and Ntf-3, cells were pretreated with various concentrations of liquiritigenin for 24 h prior to insult by 10 μmol/L $A\beta_{25-35}$ for 72 h. For examination of Apbb-1 levels, cells were treated with liquiritigenin alone. (D) Quantitative densitometric analysis. Ratio is expressed as percentage of grey level of β-tubulin (mean±SEM, n=3). [a]P>0.05, [b]P<0.05,[c] P<0.01 *vs* control; [d]P>0.05, [e]P<0.05, [f]P<0.01 *vs* $A\beta_{25-35}$ alone.

mulation were blocked by liquiritigenin to some extent. However, we have not clarified whether liquiritigenin suppresses ROS generation through the inhibition of $[Ca^{2+}]_i$ increase, or conversely, whether decreases in ROS generation prevents increases in $[Ca^{2+}]_i$. Elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading and/or increases in cellular levels of ROS is still a matter for further research.

In the present study, cultured hippocampal neurons exposed to $A\beta_{25-35}$ for more than 72 h showed increased chromatin condensation, a typical feature of apoptotic cell death. Bcl-2 family proteins are a critical regulatory factor in cellular response to apoptosis through mitochondrial pathways. In most instances, anti-apoptotic and pro-apoptotic factors in the Bcl-2 family have synergistic effects and play alternating roles. In the present study, it was shown that liquiritigenin exerted its anti-apoptotic role in the presence of $A\beta_{25-35}$ at least in part through increasing the expression of Bcl-2, suggesting that liquiritigeinin could regulate mitochondrial function and thereby inhibit neuronal apoptosis. With regard to the underlying cellular mechanism, we previously observed that an ER antagonist, ICI 182 780, could partially block the anti-apoptotic effect of liquiritigenin in our culture system (unpublished data). Since liquiritigenin has a 20-fold higher affinity for ERβ than for ERα[13], it is likely that its neuroprotective ability is mediated by ERβ. Meanwhile, the partial blocking effect of ICI 182 780 also suggests that the protective effect may be mediated by multiple pathways, including that of mitogen-activated protein kinase (MAPK)[3]. The results of the microarray assay and Western blotting in the present study indicate that liquiritigenin probably has some neurotrophic actions, including causing an increase in the expression of Ntf-3 at both the genetic and protein levels. Since we observed that $A\beta_{25-35}$ treatment inhibited the expression of Ntf-3, whereas liquiritigenin treatment could attenuate this effect, the action of liquiritigenin on Ntf-3 may account to some extent for its neuroprotective effect. This deduction is consistent with previous findings showing that Ntf-3 protects primary cortical

neurons against Aβ toxicity by limiting caspase-8, caspase-9 and caspase-3 cleavage and that it can also induce an up-regulation of neuronal apoptosis inhibitory protein-1 expression in neurons, thereby promoting the inhibition of Aβ-induced neuronal apoptosis[28].

Targeting generation and initial formation of amyloid assemblies is a preferred approach for therapeutic intervention in amyloidosis, and studies have reported that estrogen could be used for this purpose[4, 6, 7]. Most Aβ is composed of a peptide designated $Aβ_{40}$, $Aβ_{1–40}$, or, in some cases, $Aβ_{x–40}$. Because $Aβ_{1–40}$ is a main form of Aβ, our demonstration that liquiritigenin inhibits its secretion is meaningful. The sensitive sandwich ELISA was used in this experiment because the expression level of $Aβ_{1–40}$ was so low that it could not be detected using Western blotting or other methods. Herein, we have also shown that Apbb-1, a peptide that forms the extracellular amyloid fibrils of Alzheimer senile plaques, was down-regulated by liquiritigenin. This finding could explain why liquiritigenin inhibited the accumulation of Aβ, but more research is needed to verify this hypothesis.

In pioneer studies in the field of AD treatment using SERMs, Carroll *et al* found that both PPT (a selective ERα agonist) and DPN (a selective ERβ agonist) could reduce Aβ accumulation in the brain in 3×Tg-AD mice[29]. The present research reports that a newly found ERβ agonist, liquiritigenin, showed satisfactory effects against $Aβ_{25–35}$-induced insults in primary neurons. Liquiritigenin may therefore serve as a named NeuroSERM[30, 31], in other words, an estrogen alternative that selectively targets and activates estrogen mechanisms of action in the brain while avoiding activation of estrogen receptors peripheral to the brain, particularly in reproductive organs. Furthermore, our results are consistent with the finding that the expression of the ERβ gene is positively correlated with increased levels of learning and memory ability in some mouse models of AD, while ERα shows almost no relationship to learning and memory improvements[32].

Our data confirm the potential of NeuroSERMs in protecting against AD neuropathology and support continued development and investigation in this field. An important qualification is that we have examined the effects of liquiritigenin only *in vitro*; follow-up studies that address the application of liquiritigenin *in vivo* and its potential therapeutic value are needed. From a signaling standpoint, there is a significant need for targeted research to better elucidate the genomic/non-genomic signaling pathways through which liquiritigenin exerts its effects. Future research should be aimed at rapidly providing answers to these key questions.

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Author contribution

Qiu-jun LU and Li-bo ZOU designed the research; Rui-ting LIU performed the research; Qiu-jun LU contributed new reagents and analytic tools; Rui-ting LIU analyzed data; Rui-ting LIU wrote the paper; Qiu-jun LU and Li-bo ZOU revised the paper.

Abbreviations

Alzheimer's disease (AD); β-amyloid peptide (Aβ); estrogen receptor- β (ERβ); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT); lactate dehydrogenase (LDH); intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$); reactive oxygen species (ROS); flow cytometer (FCM); B-cell lymphoma/leukemia-2 (Bcl-2), Bcl-2-associated X protein (Bax); neurotrophin (Ntf) and amyloid β (A4) precursor protein-binding, family B, member 1 (Apbb-1).

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