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Forsythiaside prevents β-amyloid-induced hippocampal slice injury by upregulating 2-arachidonoylglycerol via cannabinoid receptor 1-dependent NF-κB pathway

Liqing Chen[a], Yan Yan[a], Tinggui Chen[a], Liwei Zhang[a], Xiaoxia Gao[a], Chenhui Du[b,**], Huizhi Du[a,*]

[a] Institute of Molecular Science, Shanxi University, Taiyuan, 030006, China
[b] School of Traditional Chinese Materia Medica, Shanxi University of Chinese Medicine, Taiyuan, 030619, China

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ABSTRACT

In the study, the neuroprotectivities of forsythiaside, a main constituent of *Forsythia suspensa* (Thunb.) Vahl (*F. suspensa*, Lianqiao in Chinese), were investigated in the hippocampal slices. Forsythiaside suppressed the overexpression of cyclooxygenase-2 (COX-2) and monoacylglycerol lipase (MAGL) proteins induced by β-amyloid ($A\beta_{25-35}$) to upregulate the levels of 2-arachidonoylglycerol (2-AG), an endogenous endocannabinoids. Then the inhibition of forsythiaside on COX-2 was deeply studied by the molecular docking. Forsythiaside prevented neuroinflammation and apoptosis from $A\beta_{25-35}$ insults, and this action appeared to be mediated via cannabinoid receptor 1 (CB1R)-dependent nuclear factor-κB (NF-κB) signaling pathways. More importantly, forsythiaside functionally improved $A\beta_{25-35}$-induced learning and memory deficits, which was indicated by long term potentiation (LTP). Taken together, forsythiaside may have therapeutic potential for Alzheimer's diseases (AD) by increasing the levels of 2-AG.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive deterioration of cognitive function and loss of memory in association with widespread neuronal death and neuroinflammation. The neuropathological hallmarks of AD are extracellular senile plaques composed of β-amyloid (Aβ) deposits (Selkoe, 1989), consequent intracellular neurofibrillary tangles and eventual cerebral atrophy, etc (Centonze et al., 2007). In recent years the amyloid cascade-neuroinflammation hypothesis was raised: Aβ deposits also trigger glial/microglia cells that start proinflammatory cytokines, e.g., interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), etc. and chemokines, thereby causing pronounced neuroinflammation, which even further enhances the production of Aβ and brain-damaging effects and ultimately leads to neurodegeneration (Akiyama et al., 2000; Karl et al., 2012; Wyss-Coray, 2006). Indeed, production of proinflammatory cytokines and chemokines and the activation of the complement cascade have been observed in AD patients (Koppel and Davies, 2008). The inflammation within the brain plays a pivotal role in AD progress, so the antiinflammation provides novel insights into potential AD therapeutics.

The endocannabinoid system consists of G-protein-coupled cannabinoid receptors (CBRs) that can be activated by endocannabinoids (eCBs) plus associated biochemical machinery (including precursors, synthetic and degradative enzymes, transporters) (Alger and Kim, 2011). CBRs comprise Itype (CB1R), expressed in largely pre-synaptic neurons in the brain (i.e., the highest levels in cerebral cortex, hippocampus, basal ganglia and cerebellum) and II type (CB2R), abundant in immune cells (e.g., macrophages and T cells) and also highly expressed by the activated microglia in the central nervous system (Freund et al., 2003). As one of eCBs, 2-arachidonoylglycerol (2-AG) is a full agonist for CBRs, is mainly produced from diacylglycerol by diacylglycerol lipase (DAGL) to be involved in a variety of physiological and pathological processes and is hydrolyzed to arachidonic acid (AA) by monoacylglycerol lipase (MAGL) (Nomura et al., 2011; Sugiura et al., 2006). Moreover, 2-AG is a substrate for cyclooxygenase-2 (COX-2), mainly expressed in the hippocampal regions (Ho et al., 1999) and is oxygenated by COX-2 to form new types of prostaglandins: prostaglandin glycerol esters (PGEs) and prostaglandin ethanolamides, etc (Kozak et al., 2000). And thus COX-2 and/or MAGL inhibition can elevate endogenous 2-AG level. 2-AG elevation robustly suppressed production and accumulation of Aβ, protected neurons from inflammatory stimuli

* Corresponding author.
** Corresponding author.
E-mail addresses: duchenxi_2001@163.com (C. Du), duhuizhi@sxu.edu.cn (H. Du).

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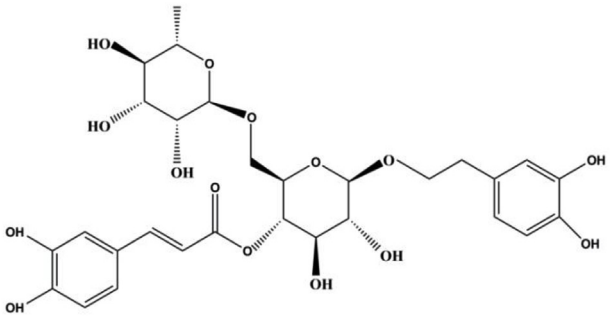

Fig. 1. Structure of forsythiaside.

and neurotoxicity, reduced cellular apoptosis, inhibited neurodegeneration, maintained integrity of hippocampal synaptic structure and function and improved long-term synaptic plasticity, spatial learning and memory in AD animals, although the mechanisms involved were not fully elucidated (Bedse et al., 2015; Chen et al., 2011, 2012; Diego et al., 2007; Fernandez-Ruiz et al., 2010; Janefjord et al., 2014; Karl et al., 2012; Koppel and Davies, 2008; Nomura et al., 2011; Turcotte et al., 2015).

Forsythia suspensa (Thunb.) Vahl (*F. suspensa*, Lianqiao in Chinese) fruits are well-known traditional Chinese herbal medicines that have been widely used in China, Korea and Japan to treat inflammation, based on its antibacterial, antiinflammatory and antioxidant activities for long time. *F. suspensa* is one of authentic Chinese medicinal materials in Shanxi Province. Forsythiaside (Figs. 1, 3 and 4- dihydroxy-β-phenethyl-O-α-L-rhamnopyranosyl- (1 → 6)-4-O-caffeoyl-β-D- glucopyranoside), a phenylethanoid glycoside, is a major component to play the bioactivities in *F. suspensa* (Chinese Pharmacopoeia Commission, 2015; Qu et al., 2008). Recent studies proved that forsythiaside showed neuroprotective effects on Aβ-induced cells by downregulating acetylcholinesterase (Yan et al., 2017) and had the ability of improving learning and memory in AD model mice (Kim et al., 2009, 2011; Wang et al., 2013). Based on previous statement, we hypothesized that forsythiaside had the ability to prevent AD by upregulating the signal of 2-AG. Among the Aβ fragments studied so far, as the shortest fragment of Aβ processed *in vivo* by brain proteases, $A\beta_{25\text{-}35}$ peptide represents significant levels of molecular aggregation and exhibits the same bioactivities with the full-length peptide (Frozza et al., 2009; Jo et al., 2011; Suh et al., 2008). Thus, this fragment is widely used in both *in vitro* and *in vivo* by neuroscience researches to establish the AD models. In the present work, we established $A\beta_{25\text{-}35}$-induced AD models in hippocampal slices and investigated the protection of forsythiaside against brain neuroinflammation and thus cognitive dysfunction by upregulating the signal of 2-AG.

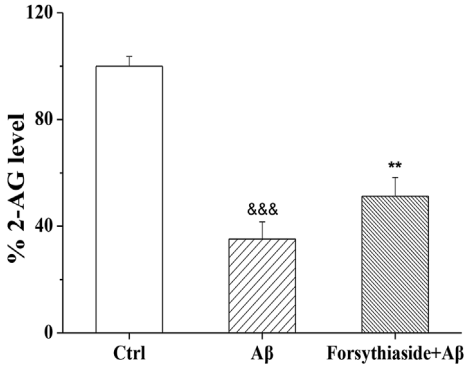

Fig. 2. Forsythiaside increased the 2-AG level in the slices ($n = 4$). Error bars indicate SEM. $^{\&\&\&}P < 0.001$, compared with control group; $^{**}P < 0.01$, compared with Aβ group.

2. Materials and methods

2.1. Preparation of organotypic hippoc ampal slices

ICR mice with 14- to 15-day-old were purchased from Experimental Animal Center of Shanxi Medical University (Grade II and Certificate No. of SCXK (JIN) 2015-0001). After mice were decapitated, the brains were dissected and kept in cold artificial cerebrospinal fluid (ACSF) containing 2.5 mM KCl, 108 mM NaCl, 45 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 12 mM glucose, and 0.5 mM ascorbic acid (Du et al., 2013). 400 μm thick slices were immediately cut in cold ACSF on a vibrating slicer (Leica VT1200S, German) and then were incubated in ACSF for about 1 h at RT. Slices were grouped and treated with drugs. ACSF was continuously bubbled with carbogen (5% CO_2 and 95% O_2). The protocol was approved by Ethics Committee of Scientific Research in Shanxi University (Approval Number: 2017FZS003). All experiments conformed to local and international guidelines on ethical use of animals and all efforts were made to minimize the number of animal used and suffering.

Slices from several mice were prepared, and then randomly divided into groups equally. 28 ± 2 Slices were used in each group. The slices in control group were incubated in ACSF. The slices in Aβ model group were treated with $A\beta_{25\text{-}35}$ of 5 μM for 2 h. After 0.5 h pretreated with forsythiaside at 80 μM, the slices that were treated with $A\beta_{25\text{-}35}$ of 5 μM for 2 h were referred as forsythiaside group. After pretreated with Rimonabant (SR1, a CB1R antagonist) at 3 μM for 0.5 h and then forsythiaside of 80 μM for 0.5 h, the slices that were treated with $A\beta_{25\text{–}35}$ of 5 μM for 2 h were referred as SR1 group. After 0.5 h pretreated with 2-AG at 3 μM, the slices that were treated with $A\beta_{25\text{-}35}$ of 5 μM for 2 h were referred as 2-AG group for Fig. 5 D1 and D2.

$A\beta_{25\text{-}35}$, with the purity of more than 98.0%, was purchased from Sangon Biotech (Shanghai, China). $A\beta_{25\text{-}35}$ was dissolved in purified water at the concentration of 1 mM and incubated at 37 °C for 4 d to obtain aggregated $A\beta_{25\text{-}35}$ and then stored at −20 °C before experiments. Forsythiaside, with the purity of 98.6%, was purchased from MustBio-Technology (Chengdu, China) and was made to the stock solution of 16 mM with purified water, then was kept at −20 °C before experiments. SR1 was purchased from Cayman (USA) and prepared as the stock solution of 0.11 mM at DMSO. To rule out potentially non-specific effects of the solvents, the same percentage of DMSO (v/v) was included in ACSF of the corresponding control group. 2-AG that was bought from Cayman (USA) was made the stock solution in acetonitrile and then stored at −80 °C before experiments. All other chemicals and regents were of analytical grade.

2.2. LC-MS/MS analysis for 2-AG

Endogenous 2-AG was quantified in mouse hippocampus tissues on Thermo Fisher Scientific Q Exactive LC-MS/MS (Thermo Fisher, USA) by a semi-quantitative analysis, based on the method of Daniel K. Nomura with moderate modifications (Nomura et al., 2011). Firstly the hippocampus tissues were weighed. The mixture solution of hexane and ethyl acetate (v/v = 1: 1) and phosphate buffer (pH = 7.4) were added to the EP tube containing the tissues in ice bath. The tissues were homogenized and then centrifuged at 4 °C. After the upper organic layer was dried with a gentle stream of nitrogen, the residue in the EP tube was re-dissolved in acetonitrile solution and centrifuged again. Finally 5 μL supernate was used for LC–MS/MS analysis using C18 column (2.1 × 75 mm, 2.5 μm), with 0.1% formic acid (A) and 100% acetonitrile (B) as a mobile phase. The flow rate of 200 μL/min was used for isocratic elution at 50% A: 50% B (v/v) for 7 min. The column temperature was set as 45 °C. The targeted LC-MS/MS analysis was performed in multiple reactions monitoring mode (MRM) using the following transitions: the electro-spray ionization (ESI) + MRM transition for 2-AG was 379.4.

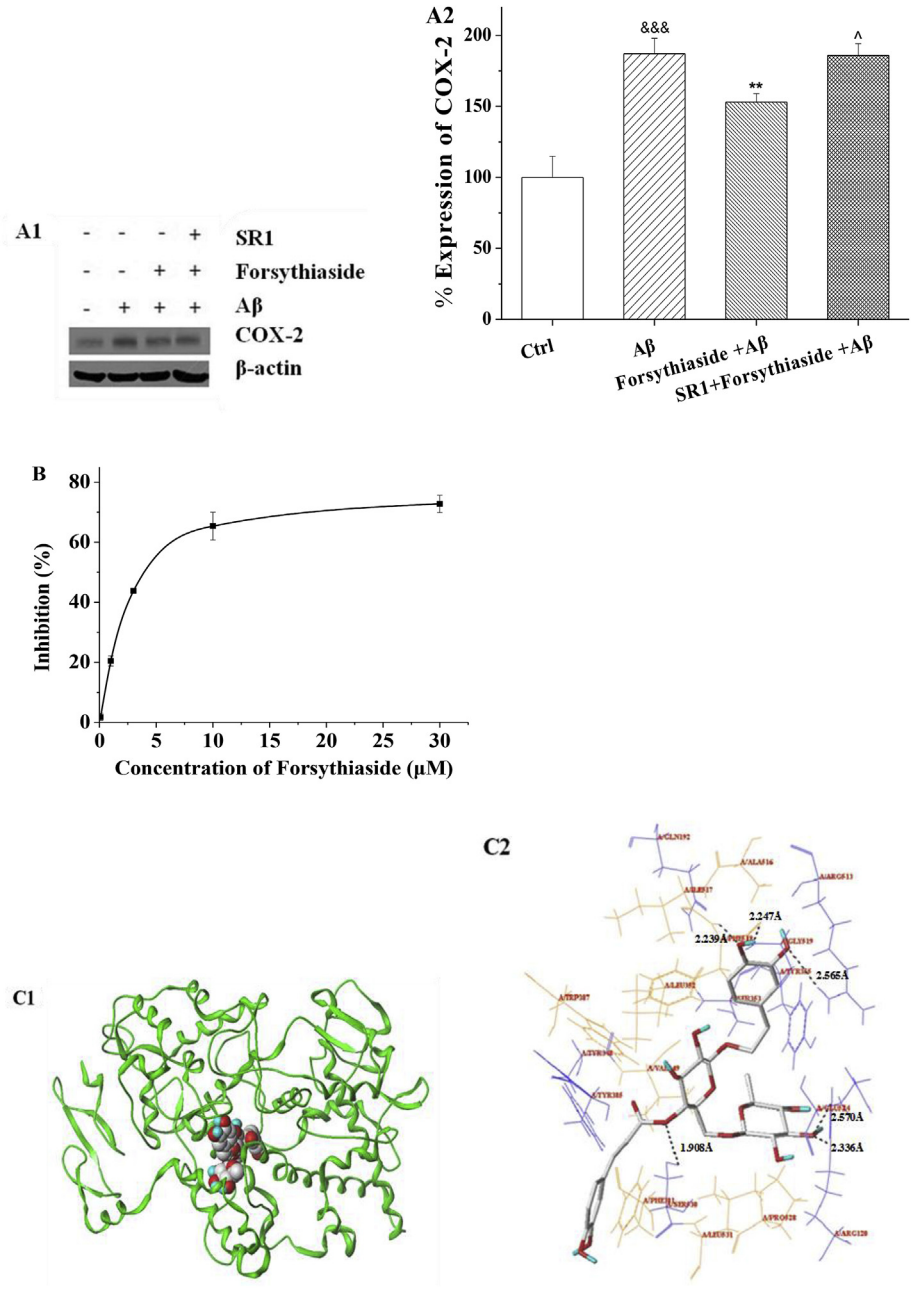

Fig. 3. Forsythiaside suppressed $A\beta_{25\text{-}35}$-induced COX-2 expression. (A1) Western blot analysis of COX-2 in hippocampal slices of mice under different conditions; (A2) Quantification of COX-2 expression (n = 5). +: with; -: without. $^{\&\&\&}P < 0.001$, compared with control group; $^{**}P < 0.01$, compared with Aβ group; $^{\wedge}0.01 < P < 0.05$, compared with forsythiaside group. (B) Inhibition curve of forsythiaside on COX-2, with n of 6. (C1) Superimposition of forsythiaside in the active site of COX-2. (C2) Black dotted lines indicated hydrogen bonds, whereas π-interactions, hydrophobic interactions, polar interactions or van der Waals interactions were not represented. Residues involved in these interactions were shown. The values are the bond distances.

2.3. COX-2 activity assay and measurement of TNF-α and PGE_2 level

COX-2 activity was tested using COX-2 Inhibitor Screening Kit (Beyotime, Shanghai, China) according to the manufacturer's protocols.

The contents of TNF-α in the incubating solution of hippocampal slices were measured according to the manufacturer's protocol for TNF-α kit (Xitang, Shanghai, China).

Hippocampal tissues were homogenized in PBS (pH = 7.4) and centrifuged at 13000 rpm for 10 min. The supernatants were collected and the contents of prostaglandin E_2 (PGE_2) were detected by the protocol of PGE_2 enzyme-linked immunosorbent assay Kit from Nanjing Jiancheng Bioengineering Institute.

2.4. Western blot analysis

Proteins were extracted from hippocampal tissues in ice-cold lysis buffer (RIPA: PMSF = 100:1) for 40 min, and homogenized by sonication at 4 °C for 30 s. The homogenates were centrifuged for 15 min at 4 °C and then quantified by BCA assay kit (Beyotime, China). After denatured at 100 °C for 5 min, proteins were run on a SDS-PAGE gel, and then were transferred to PVDF membranes (Merck, Germany). Following its blocking in 5% skim milk or 5% bovine serum albumin in 1 × TBST buffer (0.1% Tween-20 in PBS) at 4 °C overnight, the membranes were probed with rabbit monoclonal antibodies for β-actin, rabbit polyclonal antibodies for COX-2 (1:1000, Cell Signaling) and rabbit polyclonal antibodies for Bax (1:300), Bcl-2 (1:650) and active caspase-3 (1:500; Sangon); rabbit polyclonal antibodies for TNF-α, phospho—NF—κB, NF-κB (1:200; Bioss); or rabbit polyclonal antibodies for MAGL (1:500, Cayman) at 4 °C overnight, and HRP-conjugated goat anti-rabbit lgG (1:2000, Zhongshan, Beijing) for 1.5 h at RT. Finally the membranes were exposed instantly after adding freshly-made BeyoECL plus A and BeyoECL plus B. The films were scanned on a film scanner (M1005, HP), and the grayscale of bands were quantified by ImageJ software (National Institutes of Health). Band intensities were normalized to β-actin signal from the same lanes.

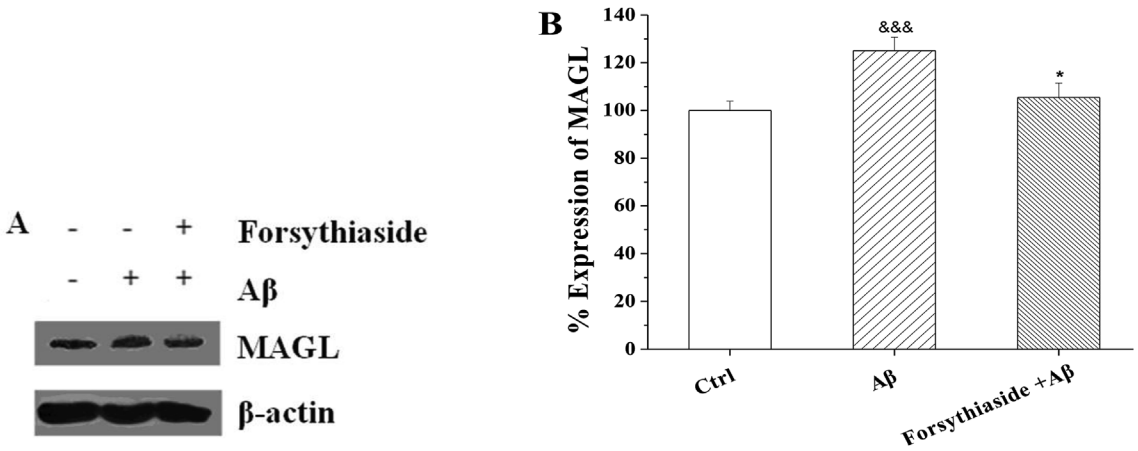

Fig. 4. Forsythiaside blocked $A\beta_{25\text{-}35}$-induced MAGL protein overexpression in hippocampal slices. (A) Western blot analysis of MAGL in hippocampus. (B) Quantifications of MAGL under different treatments ($n = 6$). +: with; -: without. $^{\&\&\&}P < 0.001$, compared with control group; * $0.01 < P < 0.05$, compared with Aβ group.

2.5. Electrophysiological recordings

The slices was transferred to a recording chamber and continuously perfused with ACSF bubbled with carbogen. The field excitatory post-synaptic potentials (fEPSPs) were recorded in response to stimulation of the hippocampus CA3 at a frequency of 100 Hz using an Axoclamp-700B patch-clamp amplifier (Molecular Devices, USA). Stimulating and recording pipettes of borosilicate glass were pulled with a P97 micro-pipette puller (Sutter Instruments, USA), filled with ACSF and placed at the middle one-third of the stratum radiatum of the CA1 region of the hippocampus. The data were analyzed in Digidata 1550 (Molecular Devices, USA).

2.6. Data analysis

Results were expressed as mean ± SEM. Comparisons between two groups were made using two-tailed t tests. Multiple comparisons were performed with one way ANOVA. Values of $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Forsythiaside increased the contents of endogenous 2-AG

2-AG is an endogenous lipid mediator involved in a variety of physiological, pharmacological and pathological processes. Firstly our aims were to track down the effects of forsythiaside on the 2-AG signal. The results showed that 5 μM $A\beta_{25\text{-}35}$ markedly decreased endogenous 2-AG levels to 35.21 ± 6.43% compared to control group (Ctrl, $P < 0.001$, $n = 4$) and forsythiaside at 80 μM moderately rescued this process (51.23 ± 7.04%, $P < 0.01$ vs. Aβ group, $n = 4$), as shown in Fig. 2. In a word, forsythiaside had the ability to elevate the endogenous 2-AG contents.

3.2. Forsythiaside upregulated 2-AG level by preventing $A\beta_{25\text{-}35}$-induced COX-2 elevation in a CB1R-dependent manner

Because COX-2 is a major metabolic way of 2-AG to prostaglandin and other inflammatory mediators, it is necessary to determine whether forsythiaside can upregulate 2-AG level by preventing COX-2. The expression levels of COX-2 proteins were first determined in hippocampus slices with or without forsythiaside. Fig. 3 A1 and A2 showed western blot bands of each group and the corresponding group data. The results indicated that $A\beta_{25\text{-}35}$ significantly enhanced COX-2 expression to 186.98 ± 10.86% ($P < 0.001$ vs. control group, $n = 5$) and pretreatment with forsythiaside attenuated the expression level to 153.02 ± 5.91% ($P < 0.01$ vs. Aβ group, $n = 5$). Additionally, this attenuation of COX-2 was reversed by SR1 at 3 μM (185.80 ± 8.33%, $P < 0.05$ vs. forsythiaside group, $n = 5$). This finding implied that forsythiaside prevented COX-2 overexpression caused by $A\beta_{25\text{-}35}$ via the CB1R.

We next investigated the inhibitory activities of forsythiaside on COX-2 enzyme using COX-2 inhibitor screening Kit. As shown in Fig. 3 B, the inhibition of forsythiaside on COX-2 activities increased with the concentration of forsythiaside, with IC_{50} value of 4.89 μM. The inhibition of forsythiaside at 30 μM on COX-2 activities reached at 72.78 ± 2.88%. This indicated that forsythiaside had substantial inhibitory effects against COX-2.

Molecular docking study was carried out to deeply explore the interactions of forsythiaside and COX-2 proteins with Autodock 4.2.6 program (Morris et al., 1996). In this respect, the pdb structure of COX-2, with the code of 5KIR, was retrieved from the RCSB PDB database. Then the COX-2 structure was optimized by deleting the water molecules and ligands and adding H (hydrogen) and then was saved. The structure of forsythiaside was optimized and saved with the parameters of method: Conj Grad, Initial Optimization: Simplex, Termination: Gradient (0.01 kcal/(mol*A)), Max Iterations: 1000, Force Field: Tripos, Charges: Gasteiger-Huckel, along with the others of default values. Finally, the molecular docking was conducted to investigate the potential binding mode of forsythiaside with the catalytic domain of COX-2. Flexible docking in Fig. 3 C1 and C2 revealed a binding pose (with the total score of 4.604) where the carboxylate group formed six hydrogen bonds (H-bonds) at the mouth of the COX active site including two with Arg 120 and two with Phe 518, another with Ser 530 and Arg 513, respectively. The values represented the distances of H-bonds.

3.3. Forsythiaside also decreased MAGL expression to upregulate 2-AG level

As an important hydrolyzing enzyme of 2-AG, MAGL enacted a major role of regulating the level of endogenous 2-AG (Dinh et al., 2002). So, we further investigated whether forsythiaside increased the levels of endogenous 2-AG by inhibiting MAGL. The data in Fig. 4 showed that $A\beta_{25\text{-}35}$ increased MAGL expression to 125.05 ± 5.66% ($P < 0.001$ vs. control group, $n = 6$) and forsythiaside effectively reversed this process at 105.48 ± 6.01% ($P < 0.05$ vs. Aβ group, $n = 6$). This finding implied that inhibition of MAGL expression was the other approach for forsythiaside to improve the content of 2-AG in the slices.

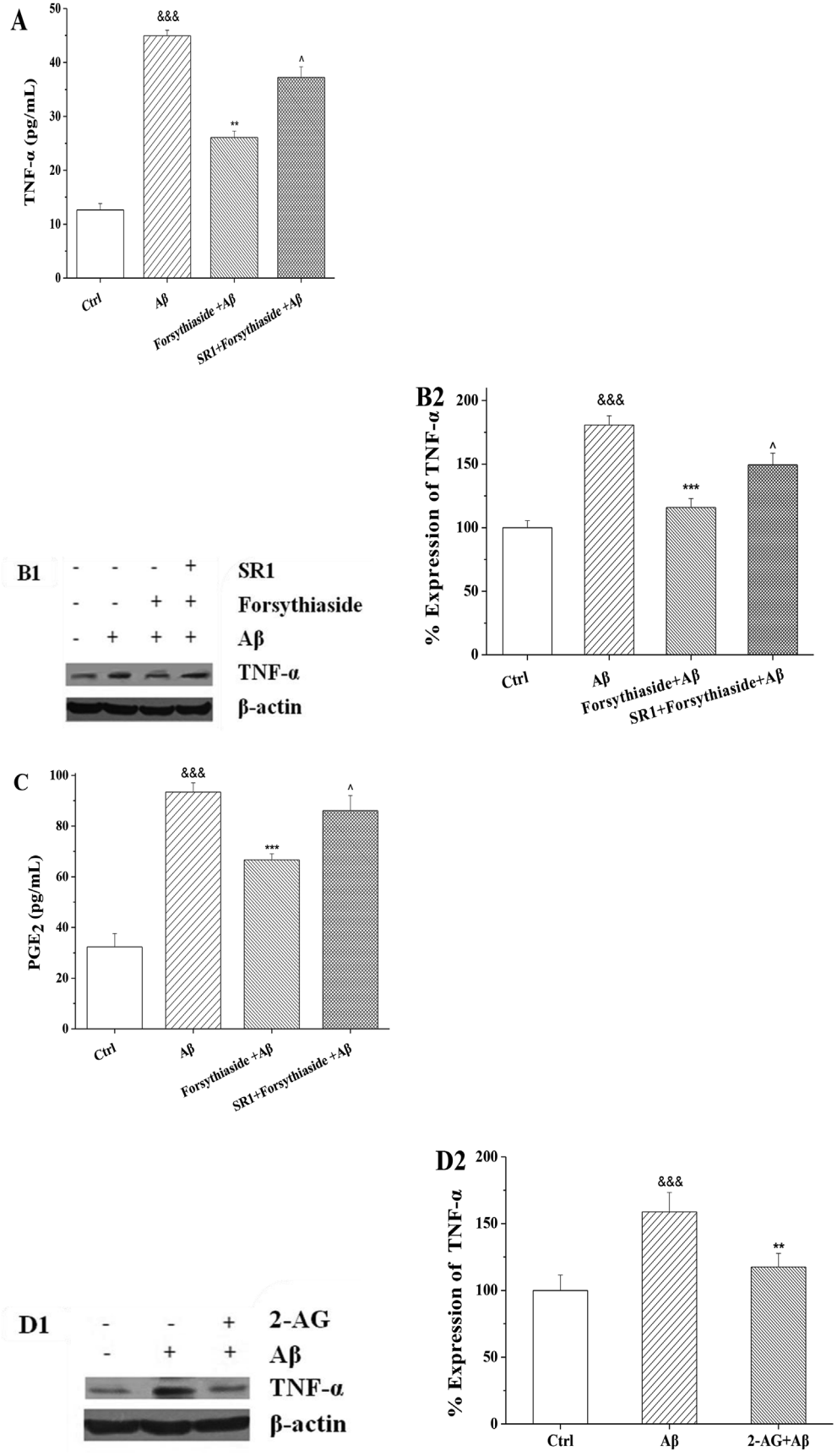

Fig. 5. Forsythiaside attenuated $A\beta_{25\text{-}35}$-induced inflammation factor production via CB1R. (A) TNF-α production in ACSF of each group ($n = 3$). (B1) Forsythiaside attenuated Aβ-induced TNF-α protein via CB1R. (B2) Quantifications of TNF-α protein under different treatments ($n = 5$). (C) PGE_2 production in each group ($n = 3$). (D1) Exogenous 2-AG decreased TNF-α protein overexpressed by Aβ. (D2) Quantifications of TNF-α protein in each group ($n = 4$). +: with; -: without. $^{\&\&\&}P < 0.001$, compared with control group; $^{***}P < 0.001$, $^{**}0.001 < P < 0.01$, compared with Aβ group; $^{\wedge}0.01 < P < 0.05$, compared with forsythiaside group.

3.4. Forsythiaside protected the slice inflammatory insults against $A\beta_{25\text{-}35}$ via CB1R

Because the neuroinflammation played a vital role in the progress of AD, the potential effects of forsythiaside on the inflammatory insults were determined in the hippocampal slices. As presented in Fig. 5A, the release levels of TNF-α, a representative inflammatory factor, were significantly higher in $A\beta_{25\text{-}35}$-treated slices than that in control slices (44.94 ± 1.04 vs. 12.62 ± 1.20 pg/mL, $P < 0.001$, $n = 3$). However, the excessive TNF-α production induced by $A\beta_{25\text{-}35}$ was significantly repressed by forsythiaside to 26.08 ± 1.20 pg/mL ($0.001 < P < 0.01$ vs. Aβ group, $n = 3$), and then this process were interestingly blocked by SR1, a CB1R antagonist (37.26 ± 1.97 pg/mL, $P < 0.05$ vs. forsythiaside group, $n = 3$).

As seen in Fig. 5 B1 and B2, the variation trend of expressions of TNF-α protein in hippocampus slices were consistent with that of the levels of TNF-α in ACSF. The expression of TNF-α protein in forsythiaside group returned to 115.93 ± 7.01% from 180.70 ± 7.31% in Aβ group ($P < 0.001$, $n = 5$). This process was also blocked by SR1 (149.33 ± 9.30%, $P < 0.05$ vs. forsythiaside group, $n = 5$).

Here, we further tested the levels of the other inflammatory factor PGE_2, one of 2-AG metabolites. It was found that the elevated PGE_2 levels by $A\beta_{25\text{-}35}$ treatment were statistically attenuated by pretreating the slices with forsythiaside (66.66 ± 2.40 vs. 93.41 ± 3.66 pg/mL, $P < 0.001$, $n = 3$). Then the attenuation was blocked by SR1 (86.04 ± 6.02 pg/mL vs. forsythiaside group, $P < 0.05$, $n = 3$), as presented in Fig. 5 C. The results suggested that forsythiaside prevented $A\beta_{25\text{-}35}$-induced neuroinflammation via the CB1R.

Additionally, we administrated 2-AG into ACSF to clarify whether a similar modulation could be obtained with exogenously applied 2-AG. As presented in Fig. 5D, TNF-α protein overexpressed by $A\beta_{25\text{-}35}$ was significantly decreased with exogenous 2-AG treatment (3 μM) by western blot assays (117.60 ± 7.95% vs. 158.86 ± 7.85%, $P < 0.001$, $n = 4$). Thus, the results similar to those obtained with endogenous elevation of 2-AG could be observed with exogenous 2-AG administration, which confirmed that forsythiaside prevented inflammatory responses and corresponding protein expressions by increasing endogenous 2-AG signal.

3.5. Forsythiaside changed active caspase-3 and Bax/Bcl-2 expressions triggered by $A\beta_{25\text{-}35}$

Activation of caspase-3 is a characteristic of apoptosis and the ultimate executor of apoptotic damage (Dickson, 2004). Data in Fig. 6 A1 and A2 demonstrated that the levels of caspase-3 proteins were significantly enhanced in $A\beta_{25\text{-}35}$-treated slices compared with the control (177.94 ± 15.35% vs. 100 ± 9.10%, $P < 0.001$, $n = 6$), then were reduced by forsythiaside preadministration (98.83 ± 11.18%, $P < 0.01$ vs. Aβ group, $n = 6$), and the attenuation was finally blocked by SR1 (139.04 ± 11.42%, $P < 0.01$ vs. forsythiaside group, $n = 6$).

To confirm again the amendatory effects of forsythiaside on the apoptosis, the expressions of other kinds of apoptosis-associated proteins including Bcl-2 and Bax were examined (Fig. 6 B). After incubation of the hippocampal slices with 5 μM $A\beta_{25\text{-}35}$, Bcl-2 expression decreased and Bax expression increased. The ratio of Bcl-2/Bax was also reduced compared to the control group. Forsythiaside pretreatment prevented $A\beta_{25\text{-}35}$-induced downregulation of Bcl-2 protein and upregulation of Bax protein, and almost reversed the ratio of Bcl-2/Bax protein to the control group. Finally, the phenomenon was partly blocked by SR1. These results reminder us that $A\beta_{25\text{–}35}$-stimulated apoptosis in hippocampal slices might be mediated by forsythiaside preadministration via CB1R.

3.6. Forsythiaside protected hippocampus slices via NF-κB pathway

Because NF-κB is a main transcription factor in the expression of inflammatory cytokines and apoptosis etc (Kulms and Schwarts, 2006), we next investigated whether NF-κB pathway participated in the neuroprotection processes induced by forsythiaside. $A\beta_{25\text{-}35}$, forsythiaside or SR1 treatment did not alter the levels of NF-κB compared with the control group ($P > 0.05$, $n = 4$, Fig. 7 A1 and A2). However, forsythiaside effectively attenuated $A\beta_{25\text{–}35}$-induced phosphorylation of NF-κB (p−NF−κB, 89.33 ± 7.41% vs. 163.50 ± 11.22%, $P < 0.001$, $n = 6$) and the action was blocked by SR1 (119.45 ± 4.03%, $P < 0.01$ vs. forsythiaside group, $n = 6$), as displayed in Fig. 7 B1 and B2. In conclusion forsythiaside prevented Aβ-induced hippocampal slice injury by upregulating 2-AG via CB1R-dependent NF-κB pathway.

3.7. Increment of 2-AG ameliorated impairments induced by Aβ in hippocampal long-term synaptic plasticity

Here, to provide functional evidence whether forsythiaside ameliorated $A\beta_{25\text{–}35}$-induced synaptic signaling impairments, fEPSPs were recorded by 100 Hz high-frequency stimulation (HFS) with 1 s duration in hippocampal slices. fEPSPs for each group were recorded in six slices from three mice. As illustrated in Fig. 8 A and B, the magnitude of long-term potentiation (LTP, 30–60 min after HSF) was 127.75 ± 5.35% in control slices ($n = 6$) but only 99.70 ± 6.82% in $A\beta_{25\text{–}35}$-treated slices ($P < 0.001$, $n = 6$). Pretreatment of forsythiaside blocked $A\beta_{25\text{–}35}$-induced decrease (135.13 ± 6.09%, $P < 0.001$ vs. Aβ group, $n = 6$), which disappeared by the pre-administration of SR1 (101.57 ± 5.71%, $P < 0.001$ vs. forsythiaside group, $n = 6$). These results confirmed that forsythiaside improved $A\beta_{25\text{–}35}$-stimulated excitatory synaptic transmission.

4. Discussion

AD is known to be associated with various inflammatory reactions and resulting neuronal degeneration that leads to dysfunction and loss of the synapses that are involved in learning and memory deficits (Tuppo and Arias, 2005; Wyss-Coray, 2006). In the development process of AD, Aβ promotes inflammation that in turn fastens and worsens AD. Recently, as it has the ability to modulate a range of aspects of AD pathology, the endocannabinoid system emerged as a novel potential therapeutic target to treat AD (Chen et al., 2011; Marchalant et al., 2008). Because 2-AG is more abundant than AEA (anandamide), the other endogenous CB1R agonist in the brain and behaves as a full agonist for CB1R, we are particularly concerned about the bioactivities of 2-AG. Besides involved in a variety of physiological processes of AD (Sugiura et al., 2006), 2-AG elevation robustly inhibited production and accumulation of Aβ, resulting inflammation, neurotoxicity, and cellular apoptosis and also improved spatial learning and memory in AD animals, whose mechanisms involved were extensively discovered in recent findings (Akiyama et al., 2000; Bedse et al., 2015; Chen et al., 2011, 2012; Fernandez-Ruiz et al., 2010; Janefjord et al., 2014; Karl et al., 2012; Koppel and Davies, 2008; Nomura et al., 2011; Turcotte et al., 2015). To clarify our hypothesis that the neuroprotection of forsythiaside against Aβ-induced neuroinflammatory insults were achieved through upregulating 2-AG signal, the effects of forsythiaside on 2-AG hydrolyzing ways and corresponding neuroprotective mechanisms were investigated in the paper. In the study, we firstly confirmed that forsythiaside could rescue the drop of 2-AG level caused by $A\beta_{25\text{-}35}$.

Upregulation of COX-2 expression has been discovered in the frontal cortex neuron and hippocampal pyramidal layer in AD patients and its expression levels may correlate with the amount of Aβ plaques (Ho et al., 1999; Pasinetti and Aisen, 1998). Some epidemiologic studies also found that selective COX-2 inhibitors may reduce AD risk and alleviate dementia aggravation, and thus, may be a therapeutic target for AD (Pasinetti and Aisen, 1998). Here, forsythiaside strongly inhibited COX-2 activity with IC_{50} of 4.89 μM by forming six H-bonds at the mouth of the COX-2 active site and moderately suppressed the over-

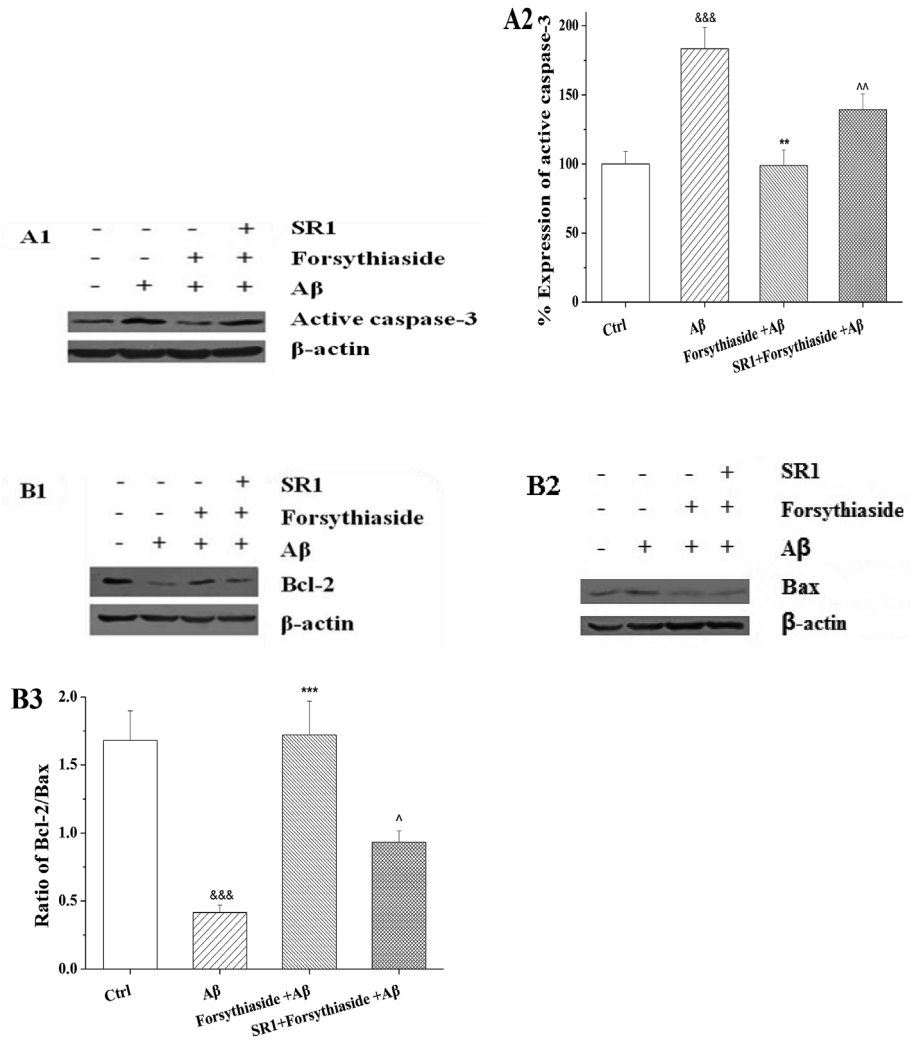

Fig. 6. Forsythiaside relieved $A\beta_{25\text{-}35}$-induced apoptosis via CB1R. (A1) Western blot analysis of active caspase-3 proteins. (A2) Quantifications of active caspase-3 proteins under different treatments. (B1) Western blot analysis of Bcl-2. (B2) Western blot analysis of Bax. (B3) Ratio of Bcl-2/Bax proteins. +: with; -: without. $^{\&\&\&}P < 0.001$, compared with control group; $^{***}P < 0.001$, $^{**}P < 0.01$, compared with Aβ group; $^{\wedge\wedge}\ 0.001 < P < 0.01$ and $^{\wedge}\ P < 0.05$, compared with forsythiaside group; $n = 6$.

expression of COX-2 proteins induced by $A\beta_{25\text{-}35}$. Due to 2-AG-produced suppressions of COX-2 expression in response to proinflammatory (Du et al., 2011), forsythiaside may directly upregulate the levels of 2-AG that inhibited over-expression of COX-2 induced by inflammation/$A\beta_{25\text{-}35}$, which should be deeply investigated. Moreover, inhibition of MAGL, the enzyme hydrolyzing 2-AG, may be a new and potentially safer way to suppress the proinflammatory cascades that underlie neurodegenerative disorders (Aso and Ferrer, 2014; Chen et al., 2012). In current study, $A\beta_{25\text{-}35}$ increased MAGL expression to a certain extent, while forsythiaside pulled the over-expression nearly to the normal level, which suggested MAGL may be the other target of forsythiaside, so we should deeply study the inhibitory effect of forsythiaside on MAGL activity at chemical level and in the cells, hippocampal slices, even *in vivo* mice or knockout mice. However, it was fact that forsythiaside moderately rescued the drop of 2-AG level resulted from $A\beta_{25\text{-}35}$ insult in the paper. Because 2-AG contents may be influenced by a lot of factors in such a complex organism, for example, 2-AG synthesis enzyme (including PLC/DAGL and lysophosphatidate phosphatase), and at lesser extent 2-AG is also metabolized by fatty acid amide hydrolase (FAAH), serine hydrolase α/β hydrolase 6 (ABDH6) and serine hydrolase α/β hydrolase 12 (ABDH12) (Sugiura et al., 2006), we need to thoroughly discover the roles of these factors on 2-AG production regulated by forsythiaside.

In addition, Aβ triggers inflammatory responses (PGE_2 and TNF-α production, etc), reactive oxygen species (ROS) and resulting pro-apoptotic cascades that together causes the progressively cognitive abnormality (Kayed et al., 2003; Tamagno et al., 2006). The neuroprotective effects of 2-AG (including anti-inflammation and anti-apoptosis, etc) has been extensively investigated, CB1R-dependent inhibition of proinflmmatory cytokines and COX-2 was the probable mechanisms and NF-κB was one of related pathways (Chen et al., 2011; Panikashvili et al., 2001; Suh et al., 2008; Zhang and Chen, 2008). We also demonstrated that NF-κB phosphorylation were likely involved in the 2-AG elevation-induced neuroprotective effects (including anti-inflammation and anti-apoptosis, etc) against Aβ toxicity via CB1R dependent mechanism therein 2-AG was increased by forsythiaside pretreatment.

Brain cognitive function deficit is one of the important inflammatory responses to brain injuries, that is, Aβ might modulate NF-κB phosphorylation/activity and thus lead to LTP impairment by binding or activating TNF-α receptor and triggering the production of TNF-α (Samidurai et al., 2018; Suh et al., 2008). Endocannabinoid modulation of both GABAergic and glutamatergic synaptic transmission and plasticity has been largely investigated. For example, 2-AG could inhibit Aβ-induced decrease of LTP, namely, improve cognitive dysfunction (Bedse et al., 2015; Hughes and Herron, 2018; Sugiura et al.,

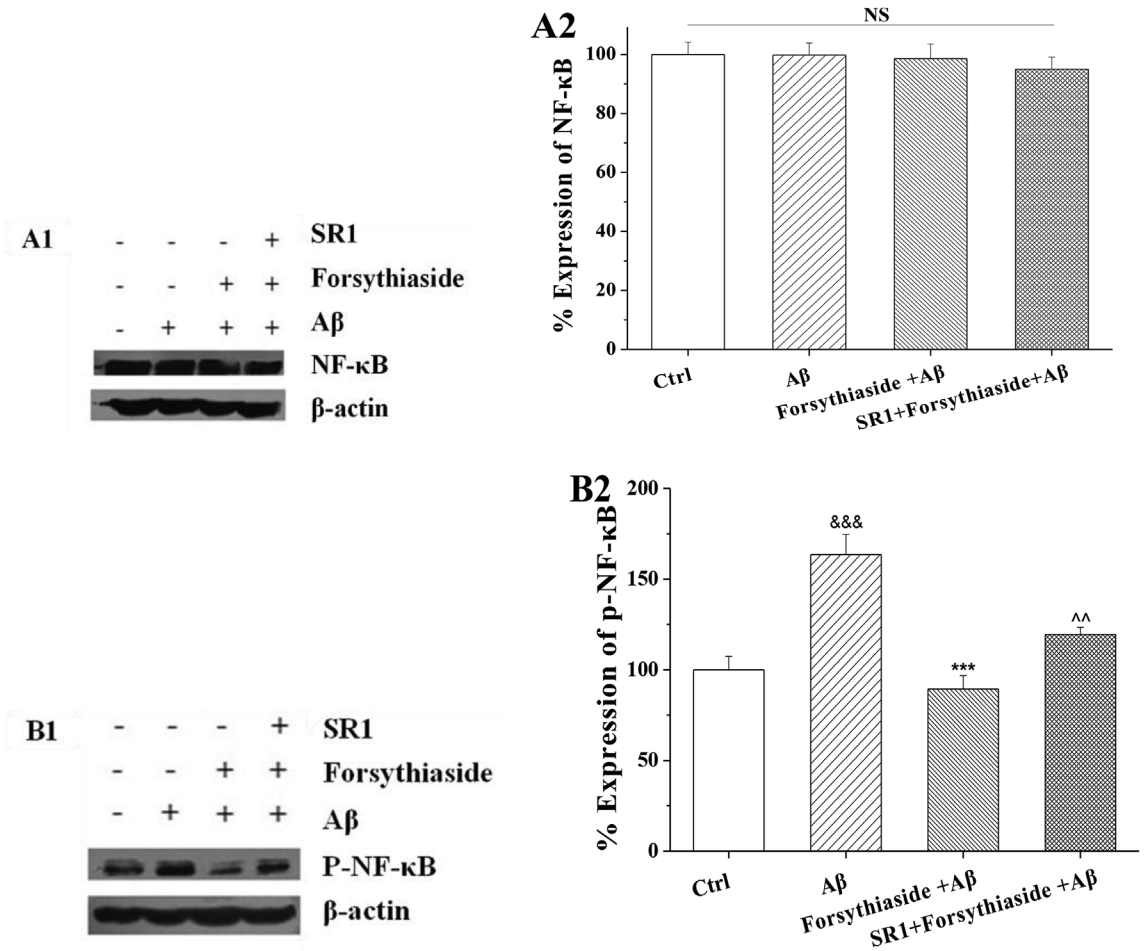

Fig. 7. The effects of forsythiaside on NF-κB and p—NF—κB proteins. (A1) Western blot analysis of NF-κB. (A2) Quantifications of NF-κB proteins under different treatments ($n = 4$). (B1) Western blot analysis of phosphorylation of NF-κB. (B2) Quantifications of p—NF—κB proteins under different treatments ($n = 6$). +: with; -: without. NS, no significant; $^{\&\&\&}P < 0.001$, compared with control group; $^{***}P < 0.001$, compared with Aβ group; $^{\wedge\wedge}P < 0.01$, compared with forsythiaside group.

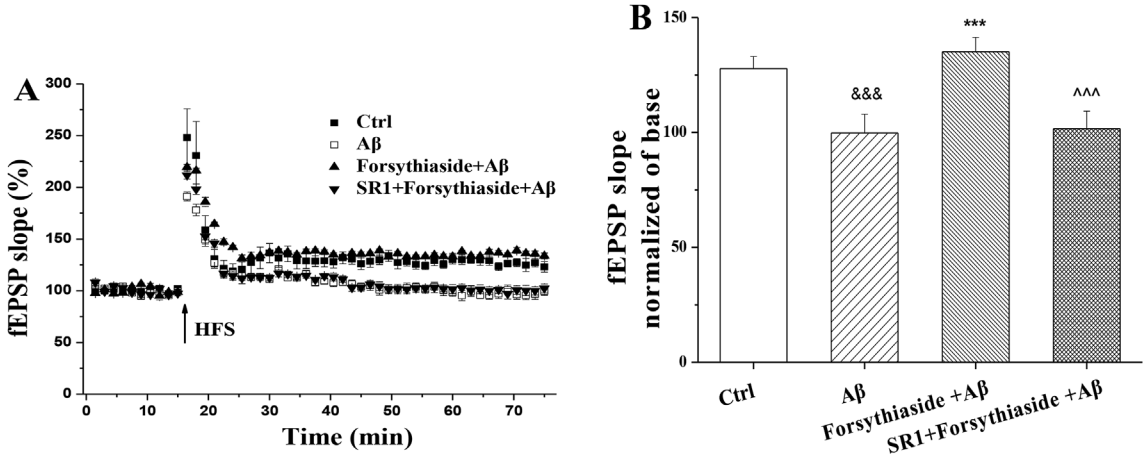

Fig. 8. Forsythiaside improved $A\beta_{25\text{-}35}$-reduced LTP. (A) Time courses of fEPSP slopes. (B) Mean values of the changes of fEPSP slop averaged from 30 to 60 min after HSF ($n = 6$ slices/3 animals for each group). $^{\&\&\&}P < 0.001$, compared with control group; $^{***}P < 0.001$, compared with Aβ group; $^{\wedge\wedge\wedge}P < 0.001$, compared with forsythiaside group.

2006). These effects have been due to inhibition of glutamate transmission, reduction of calcium influx and subsequent inhibition of noxious cascades, such as TNF-α generation and oxidative stress (Ramírez et al., 2005). In the present study, we provided the first evidence that forsythiaside improved Aβ-induced LTP by upregulating 2-AG.

A massive loss of neurons, activation of astrocytes and proliferation and rapid invasion of microglial cells concomitantly occur in AD (Meda et al., 1995). Cannabinoids may prevent AD pathology by blockade of microglial activation (Ramírez et al., 2005). The previous studies revealed that the expression of CB2R was much higher than that of CB1R on activated microglial cells, indicating a predominant function of the

CB2R on inflammation and subsequent responses (Walter et al., 2003). Even in recent years it was in quantity found that CB2R is also expressed in nervous system as well as in immune system. As prominent one among the known proinflammatory stimuli in the nervous system, prostaglandins are also produced by COX-1 and COX-2 in neurons and glial cells. Though organotypic hippocampal slices are suitable to specifically analyze the neuroprotective effects of drugs, just like used in the current paper, the roles of CB2R and COX-1, glial, microglial and astrocyte cells were still unclear and need to be in depth explored even in transgenic mouse model of AD.

Due to the complexity of AD and the poor availability of animal models that are really relevant to the human disease, the investigation about the role of the eCB system in AD are not enough much and even controversial and the mechanisms involved are not fully elucidated (Altamura et al., 2015; Bisogno and Marzo, 2008; Karl et al., 2012; Van der Stelt et al., 2006). Because the eCB system is intrinsic protective system able to release eCBs on demand in response to different stimuli (Marsicano et al., 2003), other eCBs, AEA, may either prevented Aβ neurotoxicity (Iuvone et al., 2004; Milton, 2002). We should focus on the neuroprotective effects of other eCBs, except for 2-AG.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

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