

Ginkgolide B-induced AMPK pathway activation protects astrocytes by regulating endoplasmic reticulum stress, oxidative stress and energy metabolism induced by A β ₁₋₄₂

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Abstract. Ginkgolide B (GB), the diterpenoid lactone compound isolated from the extracts of *Ginkgo biloba* leaves, significantly improves cognitive impairment, but its potential pharmacological effect on astrocytes induced by β -amyloid (A β)₁₋₄₂ remains to be elucidated. The present study aimed to investigate the protective effect and mechanism of GB on astrocytes with A β ₁₋₄₂-induced apoptosis in Alzheimer's disease (AD). Astrocytes obtained from Sprague Dawley rats were randomly divided into control, A β , GB and GB + compound C groups. Cell viability and apoptosis were analyzed using Cell Counting Kit-8 and flow cytometry assays, respectively. Protein and mRNA expression levels were analyzed using western blotting and reverse transcription-quantitative PCR, respectively. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), reactive oxygen species (ROS) and ATP were determined using the corresponding commercial kits. The findings revealed that GB attenuated A β ₁₋₄₂-induced apoptosis and the 5' adenosine monophosphate-activated protein kinase (AMPK) inhibitor compound C reversed the protective effects of GB. In addition, GB reversed A β ₁₋₄₂-induced oxidative damage and energy metabolism disorders, including decreases in the levels of SOD, GSH-Px and ATP and increased the

levels of MDA and ROS in astrocytes, while compound C reversed the anti-oxidative effect and the involvement of GB in maintaining energy metabolism in astrocytes. Finally, GB decreased the expression levels of the endoplasmic reticulum stress (ERS) proteins and the apoptotic protein CHOP and increased both mRNA and protein expression of the components of the energy metabolism-related AMPK/peroxisome proliferator-activated receptor γ coactivator 1 α /peroxisome proliferator-activated receptor α and anti-oxidation-related nuclear respiratory factor 2/heme oxygenase 1/NAD(P)H dehydrogenase (quinone 1) pathways and downregulated the expression of β -secretase 1. However, compound C could antagonize these effects. In conclusion, the findings demonstrated that GB protected against A β ₁₋₄₂-induced apoptosis by inhibiting ERS, oxidative stress, energy metabolism disorders and A β ₁₋₄₂ production probably by activating AMPK signaling pathways. The findings provided an innovative insight into the treatment using GB as a therapeutic in A β ₁₋₄₂-related AD.

Introduction

Ginkgolide B (GB) is one of the main components of the extracts of *Ginkgo biloba* leaves and the pharmacodynamic component with the strongest activity and the highest specificity of the *Ginkgo* diterpenoid lactones (1). GB has been found to possess notable effects on the central nervous system, such as improving the cognitive function of patients with Alzheimer's disease (AD) and promoting the neuroprotective effects on acute hypoxic/ischemic injury (2,3). The high purity of GB enables it to pass through the blood-brain barrier and be easily absorbed, which is beneficial in the treatment of neurodegenerative diseases (4,5), including AD, which is a destructive central nervous system lesion characterized by declining learning, memory loss and cognitive dysfunction (6,7). At present, the pathogenesis of AD remains to be elucidated, but it is well known that the abnormal metabolism and deposition of β -amyloid (A β) in the brain tissues contribute to AD (8). Activated astrocytes are found around A β deposition (9,10) and are the most abundant type of cells in the central nervous system. A previous study indicates that astrocytes induce A β degradation and clearance and the degradation of A β mediated

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by astrocytes is impeded in the early stage of AD (11). Thus, astrocytes have regulatory roles in the onset of AD (12). GB has been found to have clinical effects on neuroprotection (13), but the protective effect of GB on astrocytes in AD and its potential molecular mechanism remain to be elucidated.

A β is the main factor leading to AD cognitive dysfunction and neurodegeneration because the excessive generation and aggregation of A β results in a series of pathological and physiological changes, including endoplasmic reticulum (ER) stress (ERS), oxidative stress (OS), inflammatory response, energy metabolism disorder, tau hyperphosphorylation, synaptic degeneration, cell dysfunction and even apoptosis, causing abnormal learning, memory, cognition and behavior (14,15). The aforementioned pathological processes interact with each other, stimulating the onset and progression of AD.

ER, which takes part in protein synthesis, post-translational modification and correct folding of proteins, is necessary to maintain the normal function of cells (16). ER dysfunction or loss of integrity causes ERS, which is key to neurodegenerative diseases (17,18). Previous studies on patients with AD and the brain tissues of animal models demonstrate that A β seriously disturbs the functions of ER, leading to excessive generation of A β , ERS activation (19,20) and finally cell dysfunction and apoptosis (21). A previous study showed that mitochondrial damage was important in the pathogenesis of AD because mitochondria supplied energy, exchanged information, antagonized OS and provided energy for various cellular activities (22). The overaggregation of A β influences the energy metabolism of mitochondria, decreases the generation of ATP and produces numerous oxygen radicals in the mitochondria to weaken the ability of cells to provoke oxidation and cause OS (23), resulting in apoptosis (24). Meanwhile, oxides and lipid peroxides increase the levels of the key amylase β secretase 1 (BACE1) to cause the excessive generation of A β (25). The two interact with each other to cause malignancy, mitochondrial dysfunction and serious oxidative injury, thus exacerbating the toxic responses of A β (26). Astrocytes are important in neurodegenerative diseases and preventing the abnormal changes in astrocytes may help treat the diseases (27,28). Therefore, the following hypothesis was proposed in the present study: GB might antagonize the neurotoxicity of A β , prevent ERS and OS, protect the normal metabolism of astrocytes and interrupt the excessive generation of A β , thus protecting astrocytes and preventing the progression of AD.

Materials and methods

Reagents. GB was purchased from National Institutes for Food and Drug Control China, with 98% purity ascertained by high-performance liquid chromatography. A β peptide fragments (A β ₁₋₄₂; cat. no. SCP0038) was purchased from Sigma-Aldrich (Merck KGaA). Cell counting kit 8 (CCK-8; cat. no. C0039), annexin V-FITC apoptosis detection kit (cat. no. C1062), total superoxide dismutase assay kit (SOD; cat. no. S0109), lipid peroxidation malondialdehyde assay kit (MDA; cat. no. S0131), glutathione peroxidase assay kit (GSH-Px; cat. no. S0056), reactive oxygen species assay kit (ROS; cat. no. S0033), ATP assay kit (cat. no. S0131S), IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. A0208; 1:10,000), BCA Protein Assay kit

(cat. no. P0012S) and BSA (cat. no. ST023) were purchased from Beyotime Institute of Biotechnology. Minibest universal RNA extraction kit (cat. no. 9767), PrimeScript™ RT reagent kit with gDNA eraser (perfect real time; cat. no. RR047) and TB Green® Premix Ex Taq™ (Tli RNase H Plus; cat. no. RR420) were purchased from Takara Bio Inc. DMEM/F12 medium (cat. no. C11330500BT) and fetal bovine serum (cat. no. 10099141C) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Clarity Western ECL substrate was purchased from Bio-Rad Laboratories, Inc. Compound C, antibodies against β -actin (cat. no. 4970; rabbit; 1:1,000), β -secretase (BACE1; cat. no. 5606; rabbit; 1:1,000), β -tubulin (cat. no. 6181; rabbit; 1:1,000), protein kinase RNA-like endoplasmic reticulum kinase (PERK; Phospho Thr980; cat. no. 3179; rabbit; 1:1,000), PERK (cat. no. 3192; rabbit; 1:1,000), eukaryotic translation initiation factor 2 subunit α (eIF2 α ; PhosphoSer51; cat. no. 5199; rabbit; 1:1,000), eIF2 α (cat. no. 9079; rabbit; 1:1,000), 5' adenosine monophosphate-activated protein kinase (AMPK; Phospho Thr172; cat. no. 2531; rabbit; 1:1,000), AMPK (cat. no. 2532; rabbit; 1:1,000), heme oxygenase 1 (HO-1; cat. no. 82206; rabbit; 1:1,000) were purchased from Cell Signaling Technology, Inc., inositol-requiring enzyme 1 α (IRE1 α ; cat. no. 37073; rabbit; 1:1,000), proliferator-activated receptor γ coactivator 1 α (PGC-1 α ; cat. no. 54481; rabbit; 1:1,000), C/EBP-homologous protein (CHOP; cat. no. 179823; rabbit; 1:1,000), 78 kDa glucose-regulated protein (GRP78; cat. no. 108613; rabbit; 1:1,000), activating transcription factor 6 (ATF6; cat. no. 203119; rabbit; 1:1,000), nuclear factor erythroid 2-related factor 2 (Nrf2; cat. no. 137550; rabbit; 1:1,000), NAD(P)H dehydrogenase (quinone 1) (NQO1; cat. no. 28947; rabbit; 1:1,000) and peroxisome proliferator-activated receptor α (PPAR α ; cat. no. 215270; rabbit; 1:1,000) were obtained from Abcam. All primers were purchased from Sangon Biotech Co., Ltd.

Animals. A total of 3 pregnant Sprague Dawley (SD) rats (age, 2 months; 2-3 weeks pregnancy; weight, 260-270 g) were purchased from Experimental Animal Center, Yangzhou University (Yangzhou, China). The rats were accommodated in an animal room with a 12-h light/dark cycle and *ad libitum* access to food and water at a temperature and humidity of 22 \pm 1°C and 50 \pm 10%, respectively. The pregnant rats were fed with basic diet until postpartum. All experiments were carried out following the guidelines and protocols approved by the Ethics Committee for the Use of Experimental Animals of Jiangsu Kanion Pharmaceutical Co. Ltd. and the State Key Laboratory of New Pharmaceutical Process for Traditional Chinese Medicine (Lianyungang, China; approval no. 2019012). The 1-2-day-old rats and postpartum SD rats were sacrifice with 5% isoflurane, followed by cervical dislocation for the confirmation of mortality.

Astrocyte cultures. The cerebral cortices of 1-2-day-old rats were digested in 0.25% trypsin solution at 37°C for 30 min and the same volume of DMEM/F12 containing 10% fetal bovine serum was added to stop digestion. The cortices were repeatedly blown with a straw until the tissue mass disappeared completely and the liquid was turbid. The suspension was filtered through a 200-mesh sterile screen and the filtrate

was collected and centrifuged (300 x g) for 5 min at 20–25°C. The cells were then resuspended in DMEM/F12 [10% (v/v) fetal bovine serum] and incubated at 37°C in the presence of 5% CO₂ and 90% relative humidity. At 90% confluence of the cells, microglial cells and oligodendrocytes were removed from astrocyte cultures by shaking (200 rpm) overnight at 37°C (29).

Glial fibrillary acidic protein (GFAP) is a marker of astrocytes, which can be identified by GFAP immunofluorescence staining. The identification of purified astrocytes is presented in Fig. S1 (magnification, x100). Following staining with GFAP, green fluorescence was exhibited under a fluorescence microscope. Following staining with DAPI, blue fluorescence was exhibited under a fluorescence microscope. The cell morphology after fused cells stained by GFAP or DAPI and the purity of astrocytes were >95% (Fig. S1). Astrocytes with purity >95% were used in subsequent experiments.

Drug treatments. The optimal concentration and time of action of GB and A β ₁₋₄₂ were determined based on a previous study (30) and the pre-experimental results of their influence on astrocyte activity (Fig. S2). As demonstrated in Fig. S2, the cells treated with a concentration of 10 μ M A β ₁₋₄₂ in DMEM/F12 for 24 h as an A β ₁₋₄₂-damage model and the cell vitality treated with the concentration of 20 and 40 μ M GB in DMEM/F12 for 24 h showed no difference compared with the normal group although the cell activity was significantly improved in 80 μ M GB. In addition, the cell viability of 100 and 200 μ M GB groups was lower compared with the 20, 40 and 80 μ M GB groups. The concentration of compound C, an effective and reversible AMPK inhibitor, was determined based on the protein expression of AMPK, as demonstrated in Fig. S2. The preliminary results showed that 10 μ M compound C in DMEM/F12 for 24 h inhibited AMPK phosphorylation compared with the normal group.

Subsequently, 24 h after of seeding, the medium was replaced by a fresh medium containing the drugs. The astrocytes were randomly divided into the following groups: Control (without any treatment in DMEM/F12), A β (treated with the final concentration of 10 μ M A β ₁₋₄₂ in DMEM/F12 for 24 h), GB (treated with the final concentration of 10 μ M A β ₁₋₄₂ and 20, 40 or 80 μ M GB in DMEM/F12 for 24 h) and 80 μ M GB + compound C (treated with the final concentration of 10 μ M A β ₁₋₄₂, 80 μ M GB and 10 μ M compound C in DMEM/F12 for 24 h).

CCK-8 assay. The cell viability was evaluated using the CCK-8 assay. In brief, 100 μ l of astrocytes were plated into 96-well plates (1x10⁴ cells per well) and treated by groups, with three repeats per group. Then, 10 μ l of CCK-8 solution was added to each well and the plate was further incubated at 37°C for 2 h. The optical density at 450 nm was determined using a microplate reader (Molecular Devices, LLC).

Annexin V/PI assay. The early and late apoptosis of astrocytes was detected by flow cytometry. Astrocytes (~2 ml) at the concentration of 1x10⁵/ml were seeded into 6-well plates and treated by groups. Then, the astrocytes were washed twice with PBS, digested with 0.25% trypsin without EDTA, collected in the centrifuge tube, centrifuged (300 x g) for 5 min at 20–25°C,

and washed three times with PBS. The supernatant was discarded and 195 μ l of the binding buffer was added to resuspend the astrocytes. Subsequently, 5 μ l of Annexin V/FITC and 10 μ l of PI were added to the astrocytes in the dark, followed by detection using flow cytometry (ACEA NovoCyte; ACEA Bioscience, Inc.; Agilent Technologies, Inc.) and NovoExpress 1.2.1 software (ACEA Biosciences, Inc.; Agilent Technologies, Inc.). **SOD, MDA, GSH-Px, ROS and ATP assay.** The cells were collected, washed once or twice with PBS, precipitated, resuspended, and mixed with PBS. They were ruptured ultrasonically, and the homogenate was used for determining the SOD and GSH-Px activities and MDA, ROS and ATP content using commercially available kits (SOD assay kit with NBT; GSH-Px assay kit, colorimetric method; MDA assay kit, thiobarbituric acid method; ROS assay kit with DCFH-DA; and ATP assay kit, respectively) following the manufacturer's protocols.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. In total, 2 ml suspension containing astrocytes (1x10⁵ cell/ml) was inoculated into the 6-well plates and treated with the indicated treatments, with three replicates per treatment. Total RNA was extracted from cells using the Minibest universal RNA extraction kit, followed by reverse transcription to synthesize cDNA using PrimeScript RT Master Mix. The reverse transcription was conducted at 37°C for 15 min and 85°C for 5 sec. According to the manufacturer's protocols, the gene transcripts were quantified using the RT-qPCR reaction system (20 μ l) with TB Green and PCR was carried out under the following conditions: Initial denaturation at 95°C for 10 min; followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 95°C for 15 sec; and a final extension at 60°C for 1 min. The following primer pairs were used for qPCR: ACTB forward, 5'-TGTCACCAACTGGACGATA-3' and ACTB reverse, 5'-GGGGTGTGTAAGGTCTCAA-3'; CHOP forward, 5'-CCTCGCTCTCCAGATCCAGTCAG-3' and CHOP reverse, 5'-TCTCCTGCTCCTTCTCCTTCATGC-3'; Nrf2 forward, 5'-TGACTCCGGCATTCACTGA-3' and Nrf2 reverse, 5'-GTGGGTCTCCGTAATGGAAGA-3'; HO-1 forward, 5'-TATCGTGCTCGCATGAACTCTG-3' and HO-1 reverse, 5'-GTTGAGCAGGAAAGCGGTCTTAG-3'; NQO1 forward, 5'-AGAAGCGTCTGGAGACTGTCTGG-3' and NQO1 reverse, 5'-GATCTGGTTGTCGGCTGGAATGG-3'; AMPK forward, 5'-ATGATGAGGTGGTGGAGCAGAGG-3' and AMPK reverse, 5'-GTTCTCGGCTGTGCTGGAATCG-3'; PGC1 α forward, 5'-TTGAAGAGCGCCGTGTGAT-3' and PGC1 α reverse, 5'-AAACTTCAAAGCGGTCTCTCA-3'; PPAR α forward, 5'-TGACTTGCCATATTTATAGCTGTCA-3' and PPAR α reverse, 5'-GATGTCCTCGATGGGCTTCA-3'. All RT-qPCR experiments were repeated three times and the relative expression levels were quantified using the 2^{- $\Delta\Delta$ C_q} method (31).

Western blotting. The total protein was extracted from astrocytes with ice-cold RIPA buffer and the protein content was estimated using a BCA protein assay kit. The total protein (30 μ g) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene fluoride membrane for 2 h in an ice-cold buffer. The membranes were blocked in 5% BSA for 1 h

at room temperature, incubated overnight at 4°C with the corresponding primary antibodies, washed, incubated with horse radish peroxidase-conjugated secondary antibody for 1 h at room temperature and developed using an enhanced chemiluminescence kit. Gray-scale scanning and quantification were performed using Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data were presented as mean \pm standard deviation from ≥ 3 independent experiments. Statistical analysis permitted normalization of the $2^{-\Delta\Delta Cq}$ of RT-qPCR and the value of protein gray of western blotting in the A β group and the cell activity of CCK8 in control group. Statistical analyses were performed by one-way ANOVA test evaluating significant differences between treatments using SPSS v17.0 statistical software (SPSS, Inc.). The comparison between the two groups was conducted by LSD method with homogeneous variances and Tamhane method with inhomogeneous variances. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of GB on A β_{1-42} -induced cell viability in astrocytes. The activity of astrocytes is demonstrated in Fig. 1A. The activity of astrocytes clearly decreased in the A β group compared with the control group ($P < 0.01$), while the activity was higher in the 20, 40 and 80 μM GB groups compared with the A β group in a concentration-dependent manner ($P < 0.05$; $P < 0.01$). However, the cell viability was significantly lower in the 80 μM GB + compound C group compared with the 80 μM GB group ($P < 0.01$). The results demonstrated that A β_{1-42} decreased astrocyte viability and caused cellular damage, while GB enhanced astrocyte viability and protected the cells. In addition, the effects of GB on A β_{1-42} -induced decreased astrocyte viability were inhibited by the AMPK inhibitor compound C.

Effect of GB on A β_{1-42} -induced cell apoptosis in astrocytes. Apoptosis detection was performed on each group of astrocytes using flow cytometry and the results are demonstrated in Fig. 1B and C. Compared with the control group, A β_{1-42} treatment clearly increased the apoptotic rate of astrocytes ($P < 0.01$). The number of apoptotic astrocytes was significantly lower in the 20, 40 and 80 μM GB groups than in the A β group ($P < 0.05$, $P < 0.01$), while the apoptotic rate was significantly higher in the 80 μM GB + compound C group than in the 80 μM GB group ($P < 0.01$). Hence, GB effectively reduced the A β_{1-42} -induced apoptosis of astrocytes and compound C inhibited this effect.

Effect of GB on A β_{1-42} -induced OS-related factors in astrocytes. As demonstrated in Fig. 2A-D, A β_{1-42} clearly decreased the activities of SOD and GSH-Px and distinctly increased the MDA content and ROS levels in astrocytes compared with the control group ($P < 0.05$; $P < 0.01$). The activities of SOD and GSH-Px visibly increased ($P < 0.01$) while the MDA content and ROS levels significantly decreased ($P < 0.05$, $P < 0.01$) in the 40 and 80 μM GB groups compared with the A β group. The activities of SOD and GSH-Px significantly decreased ($P < 0.05$; $P < 0.01$) and the MDA content and ROS level significantly

increased ($P < 0.05$; $P < 0.01$) in the 80 μM GB + compound C group compared with the 80 μM GB group. Thus, the results showed that GB could attenuate the A β_{1-42} -induced decrease in antioxidants in astrocytes; however, compound C could provoke this effect.

Effect of GB on A β_{1-42} -induced levels of ATP in astrocytes. As demonstrated in Fig. 2E, A β_{1-42} clearly decreased the content of ATP in astrocytes compared with the control group ($P < 0.01$), whereas the content of ATP significantly increased ($P < 0.01$) following 80 μM GB treatment compared with the A β treatment. However, the content of ATP was significantly lower in the 80 μM GB + compound C group compared with the 80 μM GB group ($P < 0.01$). Thus, GB could enhance the ATP level of astrocytes following treatment with A β_{1-42} and compound C could prevent this effect.

Effect of GB on expressions of ERS signal molecule in astrocytes. The data presented in Fig. 3A and B indicate that the protein expression levels of CHOP, GRP78, phosphorylated (p)-PERK, p-eIf2 α and ATF6 significantly increased in the A β group compared with the control group ($P < 0.05$, $P < 0.01$). In addition, 20, 40 and 80 μM GB notably repressed the upregulation of CHOP, GRP78 and p-PERK; 40 and 80 μM GB significantly inhibited the upregulation of p-eIf2 α and IRE1 α ; and 20 and 80 μM GB notably repressed the upregulation of ATF6 ($P < 0.05$; $P < 0.01$) compared with A β alone. However, 80 μM GB + compound C treatment significantly increased the protein expression levels of CHOP and ERS marker proteins ($P < 0.05$; $P < 0.01$) compared with 80 μM GB treatment (Fig. 4A and B). The experimental results proved that GB protected astrocytes from A β_{1-42} -induced apoptosis via inhibiting ERS, while AMPK inhibitor compound C could prevent this effect. Therefore, the mechanism of GB inhibiting ERS may be closely related to the AMPK signaling pathway.

Effect of GB on the expression of related proteins and genes by Nrf2 pathway. As demonstrated in Fig. 5A and B, the protein expression levels of Nrf2 and NQO1 were significantly lower in the A β group compared with the control group ($P < 0.05$). However, the expression levels of Nrf2 and HO-1 proteins in the 80 μM GB group and the levels of NQO1 protein in the 20, 40 and 80 μM GB groups were significantly higher compared with the A β group ($P < 0.05$). As demonstrated in Fig. 6, the expression levels of Nrf2, HO-1 and NQO1 genes were significantly higher in the 40 and 80 μM GB groups compared with the A β group ($P < 0.05$; $P < 0.01$). However, the mRNA and protein expression levels of Nrf2, HO-1 and NQO1 were significantly lower in the 80 μM GB + compound C group compared with the 80 μM GB group ($P < 0.05$; Figs. 6 and 7A and B). The results indicated that GB could reduce A β_{1-42} -induced OS by activating the Nrf2-HO-1-NQO1 pathway, while compound C prevented this effect. Therefore, the mechanism of GB inhibiting OS may be closely related to the AMPK signaling pathway.

Effect of GB on the expression of related genes and proteins by AMPK pathway. As demonstrated in Fig. 8A and B, A β_{1-42} clearly decreased the protein expression levels of p-AMPK and PPAR α and increased the expression level of BACE1 protein

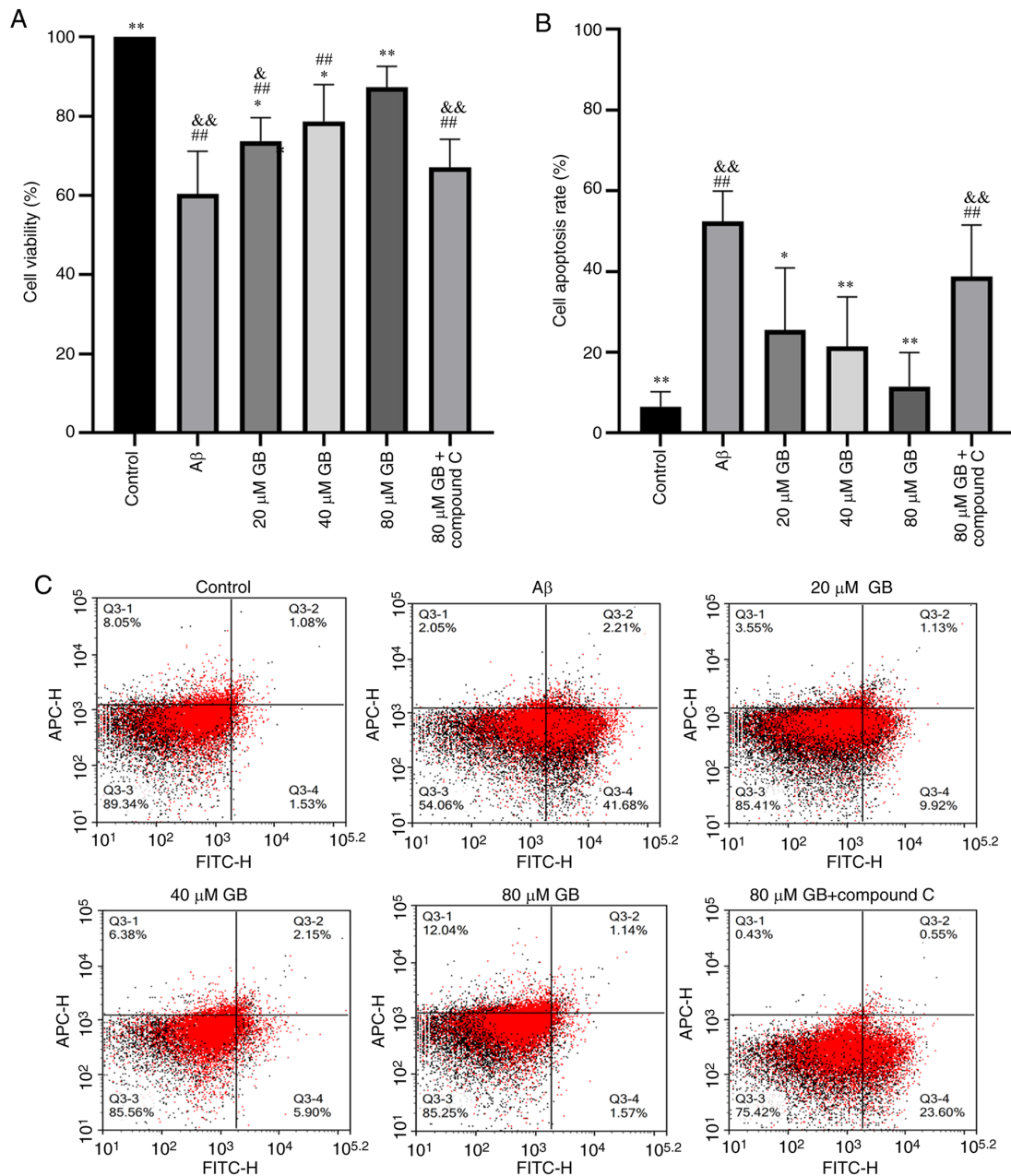


Figure 1. Effect of GB on cell viability and apoptosis are affected by A β_{1-42} in astrocytes. (A) Astrocyte viability was detected using CCK-8 assay. (B and C) Apoptosis detection was performed on each group of astrocytes by flow cytometry. All the astrocytes were randomly divided into control group (without any treatment in DMEM/F12), A β group (treated with the final concentration of 10 μ M A β_{1-42} in DMEM/F12 for 24 h), GB group (treated with the final concentration of 10 μ M A β_{1-42} and 20, 40 or 80 μ M GB in DMEM/F12 for 24 h) and 80 μ M GB + compound C group (treated with the final concentration of 10 μ M A β_{1-42} , 80 μ M GB and 10 μ M compound C in DMEM/F12 for 24 h). ##P<0.01 vs. control group, *P<0.05 and **P<0.01 vs. A β group, &P<0.05 and &&P<0.01 vs. 80 μ M GB group. GB, ginkgolide B; A β , β -amyloid.

compared with the control group (P<0.05; P<0.01). In addition, the expression levels of p-AMPK, PGC1 α and PPAR α proteins in the 40 and 80 μ M GB groups and the expression level of PPAR α proteins in the 20 μ M GB group significantly increased (P<0.05; P<0.01), while the expression level of BACE1 protein in the 40 and 80 μ M GB groups significantly decreased (P<0.05; P<0.01). As demonstrated in Fig. 9, the expression level of the AMPK gene in astrocytes significantly decreased and the expression level of the BACE1 gene significantly increased after treatment with A β_{1-42} compared with that in the control group (P<0.05). In contrast, the gene and protein expression levels of p-AMPK/AMPK, PGC1 α and PPAR α

were significantly lower and the gene and protein expression level of BACE1 were significantly higher in the A β and 80 μ M GB + compound C group compared with the 80 μ M GB group (P<0.05; P<0.01; Figs. 9, 10A and B). The results showed that GB protected astrocytes from A β_{1-42} -induced apoptosis by regulating energy metabolism, while compound C could prevent this effect.

Discussion

The A β protein cascade hypothesis remains the dominant theory of AD pathogenesis; it hypothesizes that the main

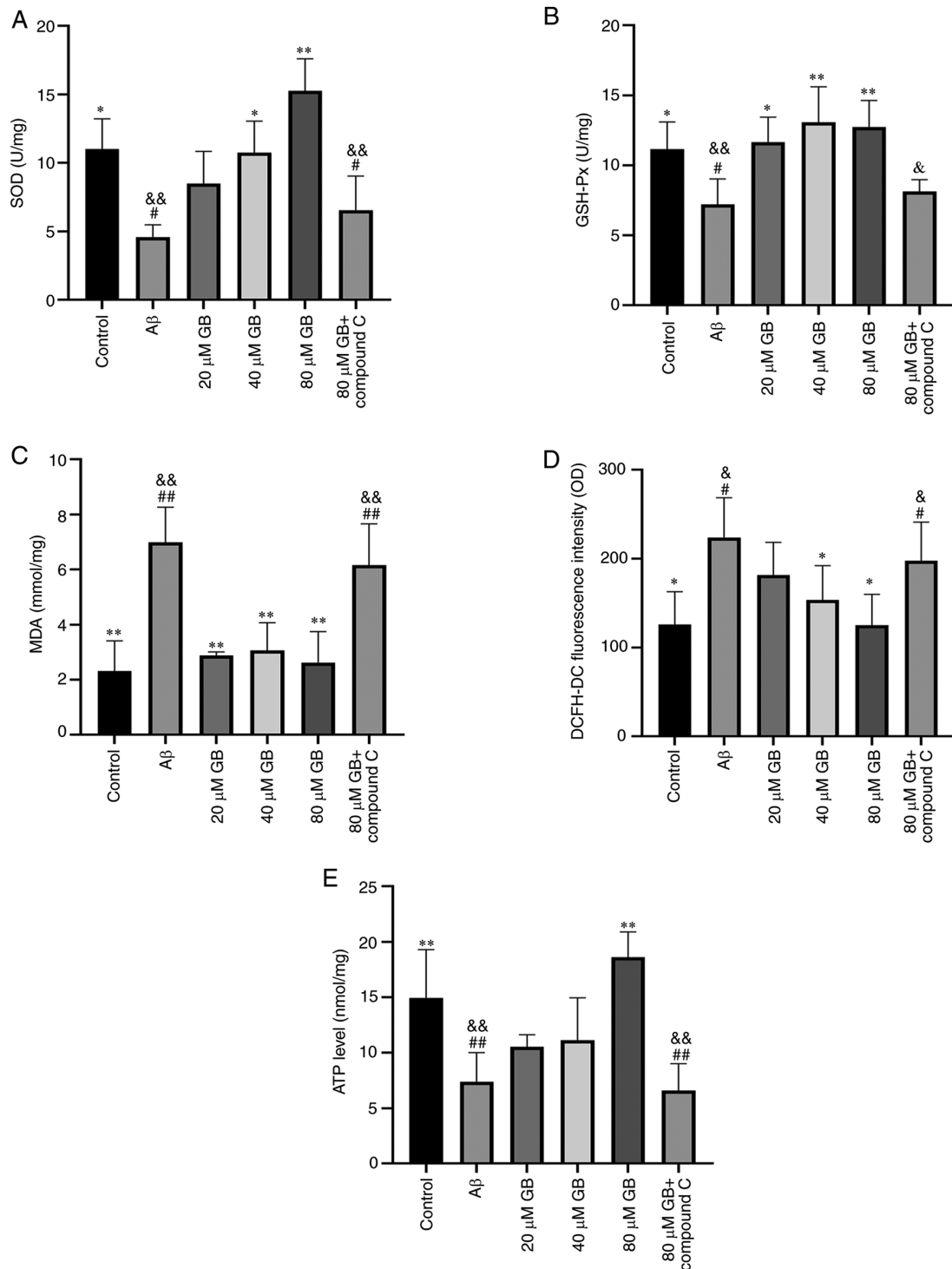


Figure 2. Effect of GB on A β_{1-42} -induced levels of OS-related factors and ATP in astrocytes. (A) GB increased the activity of SOD in A β_{1-42} treated astrocytes and compound C decreased this effect. (B) GB increased the activity of GSH-Px in A β_{1-42} treated astrocytes and compound C decreased this effect. (C) GB decreased the activity of MDA in A β_{1-42} treated astrocytes and compound C decreased this effect. (D) GB decreased ROS level in A β_{1-42} treated astrocytes and compound C decreased this effect. (E) GB increased the content of ATP in A β_{1-42} treated astrocytes and compound C decreased this effect. #P<0.05 and ##P<0.01 vs. control group, *P<0.05 and **P<0.01 vs. A β group, &P<0.05 and &&P<0.01 vs. 80 μ M GB group. GB, ginkgolide B; A β , β -amyloid; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

component of amyloid plaque A β is excessively generated to cause neuronal death, synaptic loss, hyperphosphorylated tau protein and declining cognitive function (32,33). A β_{1-42} soluble oligomers are the major form in A β and they aggregate more easily and are more toxic. They surround necrotic synapses and activated astrocytes (34,35), the major components

of the central nervous system and closely related to the maintenance and normal operation of brain functions. The abnormal functions and degeneration of these synapses and activated astrocytes can induce neurodegenerative diseases. Physiologically, astrocytes can take up and internalize the A β from cells and degrade them to protect neurons (36,37).

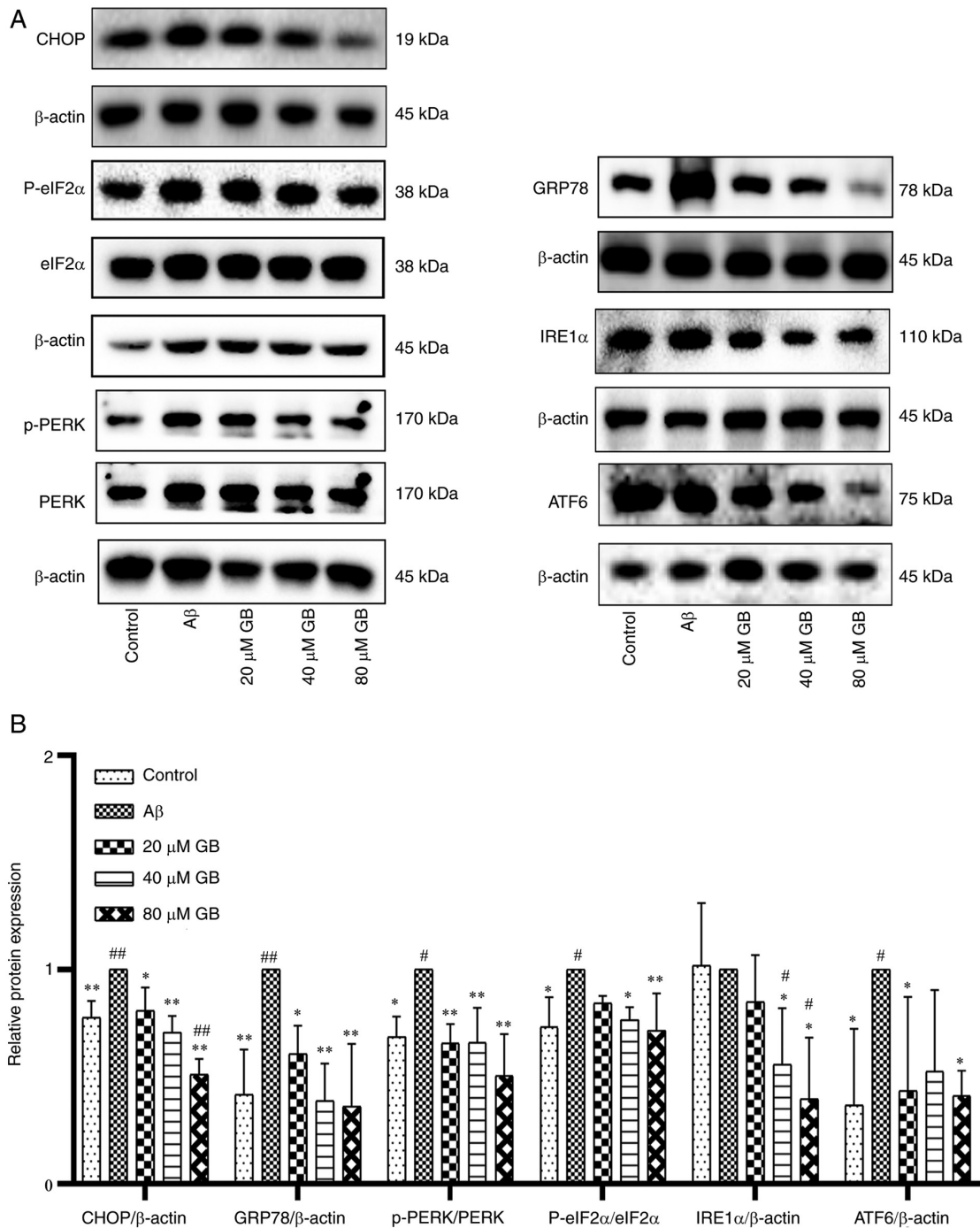


Figure 3. Effect of GB on expressions of ERS signal molecule in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of ERS following treatment with 10 μ M A β_{1-42} with different GB concentration (20, 40 and 80 μ M GB) or treated with 10 μ M A β_{1-42} for 24 h. *P<0.05 and **P<0.01 vs. control group, #P<0.05 and ##P<0.01 vs. A β group. GB, ginkgolide B; ERS, endoplasmic reticulum stress; A β , β -amyloid; p-, phosphorylated; GRP78, binding immunoglobulin protein; PERK, protein kinase R like endoplasmic reticulum kinase; eIF2 α , eukaryotic translation initiation factor 2 subunit 1; IRE1 α , inositol-requiring enzyme 1 α ; ATF6, activating transcription factor 6.

However, pathologically, A β causes the overactivation of astrocytes, leading to ERS, OS, inflammatory response, and finally apoptosis (19,38). The present study found that A β_{1-42} (10 μ M) caused astrocyte apoptosis and GB (20, 40 and 80 μ M) relieved astrocyte apoptosis caused by A β_{1-42} effectively, implying that GB could protect astrocytes from A β_{1-42} -induced apoptosis.

In the progression of AD, the examination of brain tissues of patients with AD and animal models has demonstrated that

ERS has some association with the toxicity of A β (17,20), thereby disturbing the functions of ER and leading to the overactivation of ERS in neural cells (19) and deposition of A β . In addition, A β and hyperphosphorylated tau protein interact with each other and move into blood circulation, aggravating the condition of patients with AD (39,40). Moderate ERS can protect and recover homeostasis but long-standing ERS induces apoptosis. That is to say excessive ERS results in apoptosis through CHOP (41). In the present study experiments

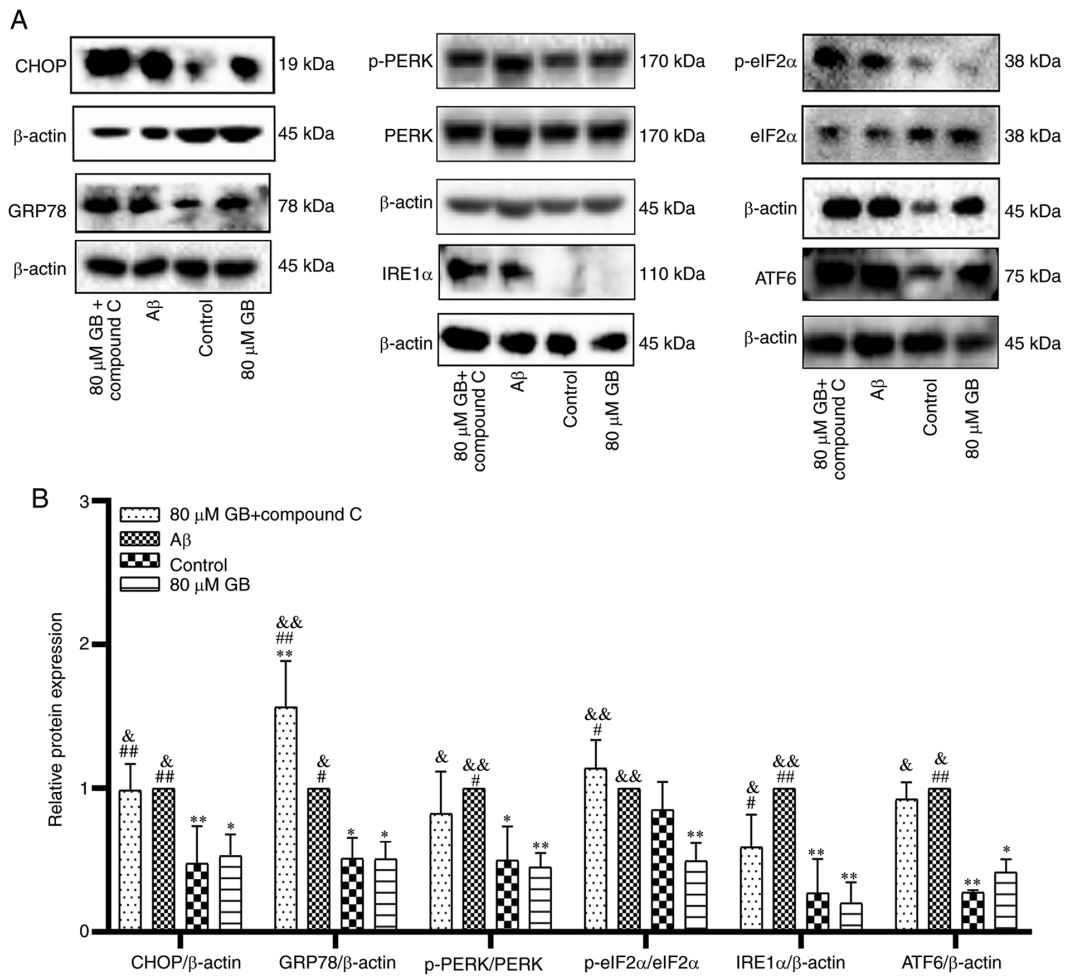


Figure 4. Compound C prevents GB-induced protein expressions of ERS in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of ERS following treatment with 10 μ M A β ₁₋₄₂ with 80 μ M GB or treated with 10 μ M A β ₁₋₄₂ or 10 μ M A β ₁₋₄₂ + 80 μ M GB + 10 μ M compound C for 24 h. [#]P<0.05 and ^{##}P<0.01 vs. control group, ^{*}P<0.05 and ^{**}P<0.01 vs. A β group, [&]P<0.05 and ^{&&}P<0.01 vs. 80 μ M GB group. GB, ginkgolide B; ERS, endoplasmic reticulum stress; A β , β -amyloid; p-, phosphorylated; GRP78, binding immunoglobulin protein; PERK, protein kinase R like endoplasmic reticulum kinase; eIF2 α , eukaryotic translation initiation factor 2 subunit 1; IRE1 α , inositol-requiring enzyme 1 α ; ATF6, activating transcription factor 6.

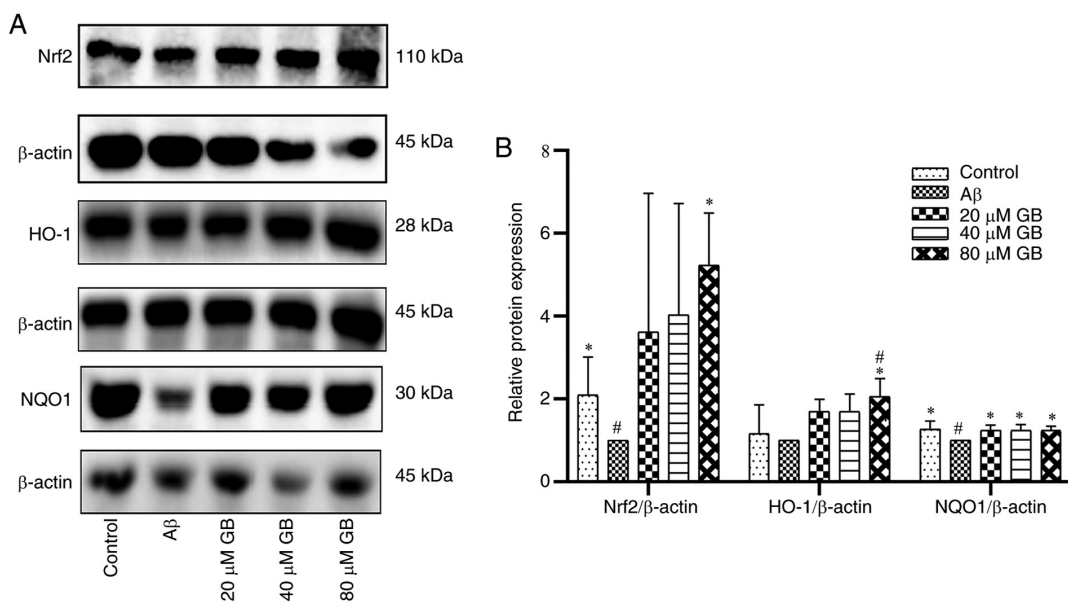


Figure 5. Effect of GB on protein expressions in Nrf2-HO-1-NQO1 induced by A β ₁₋₄₂ in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of Nrf2, HO-1 and NQO1 following treatment with 10 μ M A β ₁₋₄₂ with different GB concentration (20, 40 and 80 μ M GB) or treated with 10 μ M A β ₁₋₄₂ for 24 h. [#]P<0.05 vs. control group, ^{*}P<0.05 vs. A β group. GB, ginkgolide B; A β , β -amyloid; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase (quinone 1).

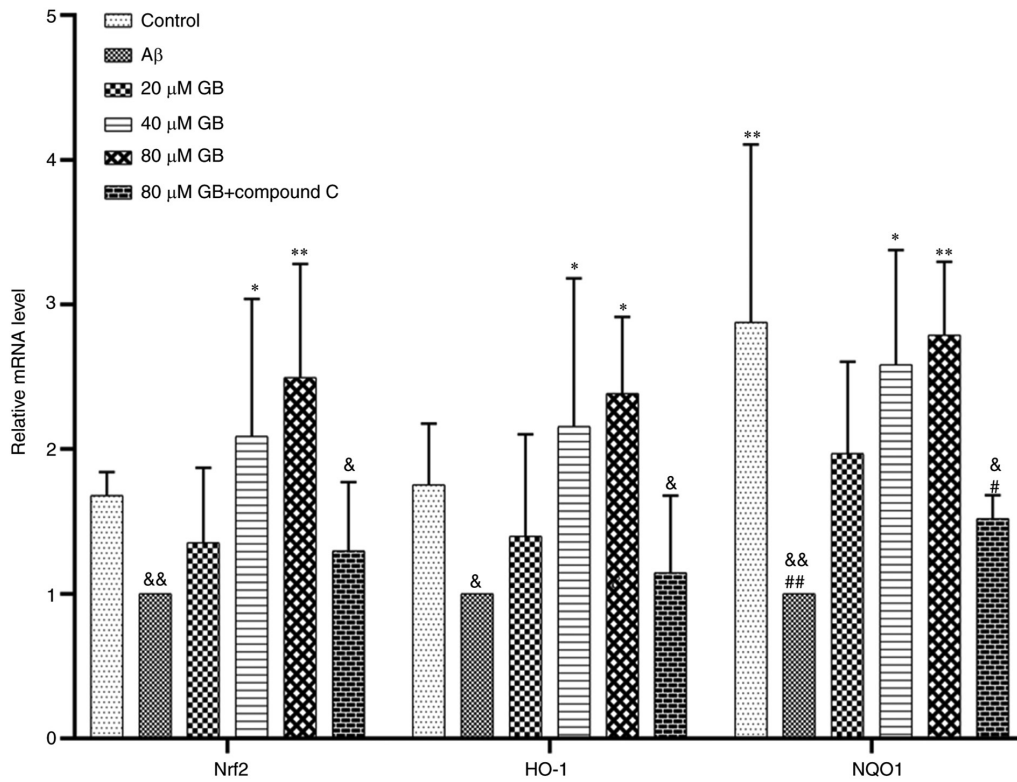


Figure 6. Effect of GB on gene expressions of Nrf2-HO-1-NQO1 induced by Aβ₁₋₄₂ in astrocytes. Reverse transcription-quantitative PCR data of relative mRNA expression levels of Nrf2, HO-1 and NQO1 following treatment with 10 μM Aβ₁₋₄₂ with different GB concentration (20, 40, 80 μM GB) or treated with 10 μM Aβ₁₋₄₂ or treated with 10 μM Aβ₁₋₄₂ + 80 μM GB + 10 μM compound C for 24 h. #P<0.05 and ##P<0.01 vs. control group, *P<0.05 and **P<0.01 vs. Aβ group, &P<0.05 and &&P<0.01 vs. 80 μM GB group. GB, ginkgolide B; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase (quinone 1); Aβ, β-amyloid.

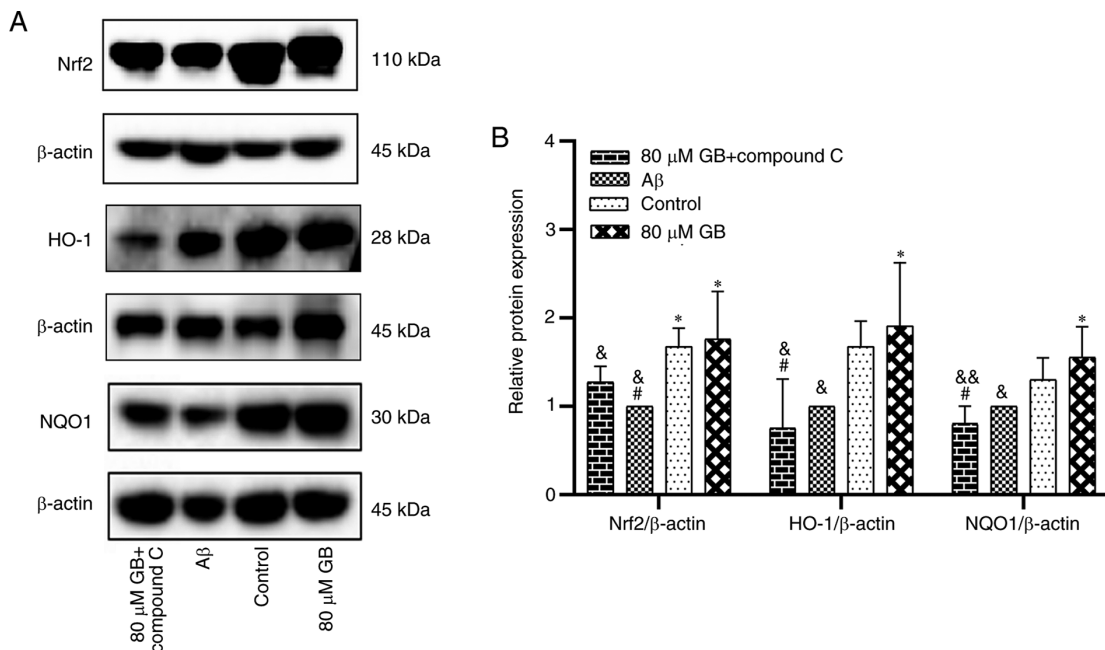


Figure 7. Compound C inhibits GB-activated protein expressions of Nrf2-HO-1-NQO1 in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of Nrf2, HO-1 and NQO1 following treatment with 10 μM Aβ₁₋₄₂ with 80 μM GB or treated with 10 μM Aβ₁₋₄₂ or 10 μM Aβ₁₋₄₂ + 80 μM GB + 10 μM compound C for 24 h. #P<0.05 vs. control group, *P<0.05 vs. Aβ group, &P<0.05 and &&P<0.01 vs. 80 μM GB group. GB, ginkgolide B; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase (quinone 1); Aβ, β-amyloid.

were performed to confirm that Aβ₁₋₄₂ increased the expression of astrocyte ERS markers, leading to increased expression

of apoptotic protein CHOP. However, GB could protect astrocytes from apoptosis by halting Aβ₁₋₄₂-induced ERS. This

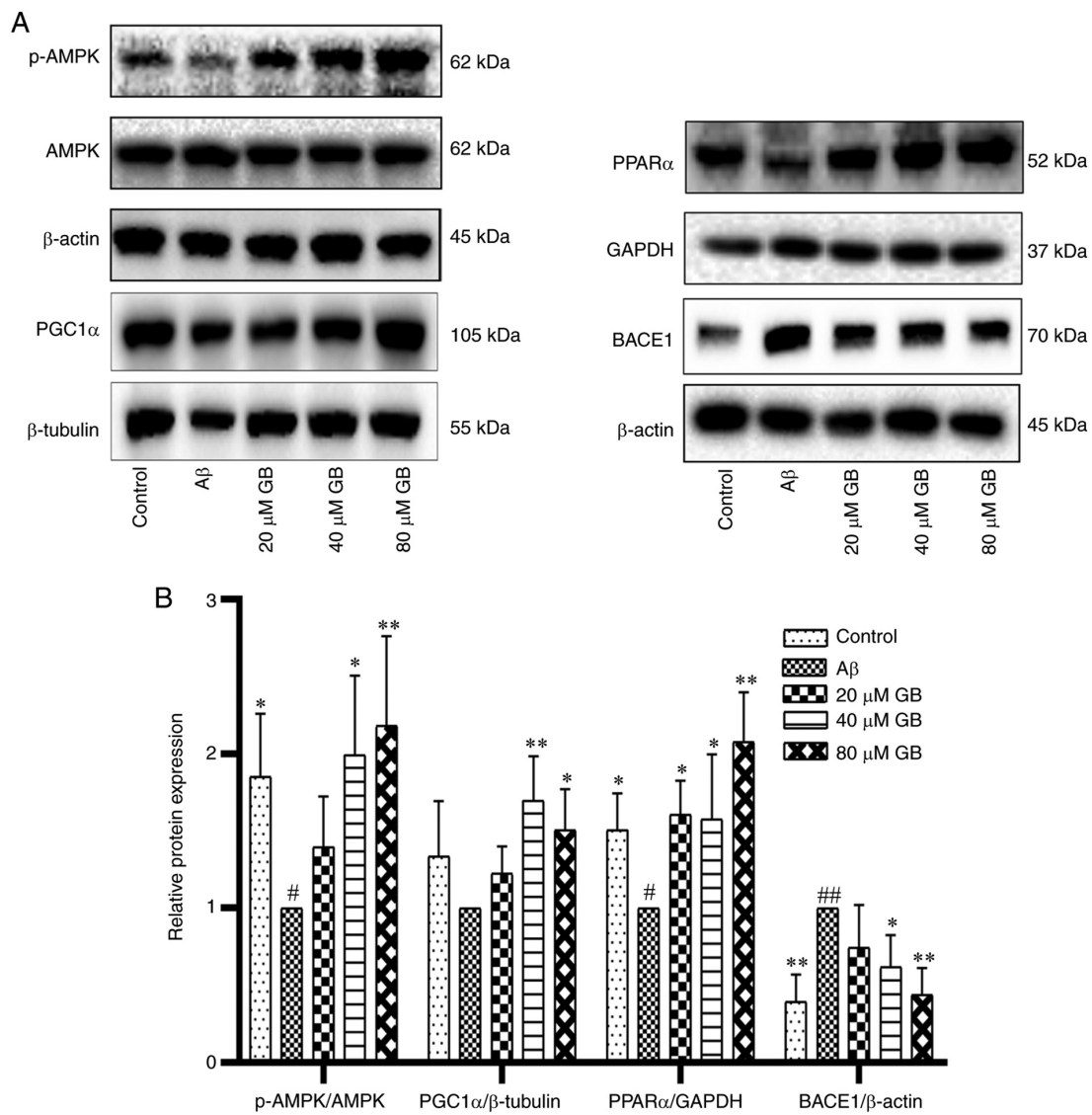


Figure 8. Effect of GB on the expression of related proteins by AMPK pathway induced by A β_{1-42} in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of p-AMPK, PGC1 α , PPAR α and BACE1 following treatment with 10 μ M A β_{1-42} with different GB concentration (20, 40 and 80 μ M GB) or treated with 10 μ M A β_{1-42} for 24 h. * P <0.05 and # P <0.01 vs. control group, * P <0.05 and ** P <0.01 vs. A β group. GB, ginkgolide B; AMPK, 5' adenosine monophosphate-activated protein kinase; A β , β -amyloid; p-, phosphorylated; PGC1 α , proliferator-activated receptor γ coactivator 1 α ; PPAR α , peroxisome proliferator-activated receptor α ; BACE1, amylose β secretase 1.

finding showed that A β_{1-42} protected astrocytes by decreasing the expression of ERS-related proteins in AD.

Evidence demonstrates that OS is an incipient factor that leads to cognitive dysfunction (42). In addition, the levels of OS products is clearly increased in the brain tissues of patients with AD (43,44) so that the brains are in a state of high oxidative stress and oxidative injury occurs earlier than senile plaques and neurofibrillary tangles (45,46). In addition, mitochondrial OS, abnormal mitochondrial functions, their interaction (47,48) and ROS take part in the pathological progression of AD (49). *In vivo* and *in vitro* studies confirm that A β stimulates OS in the brain tissues via varied pathways (50,51). The products of OS also induced A β deposition to cause abnormal energy metabolism, mitochondrial dysfunction, even resulting in apoptosis which induces memory loss and cognitive dysfunction (52). Astrocytes participate in the physiological processes of all nervous system diseases and are critical in protecting neurons (53). The experimental results from the present study

demonstrated that GB improved the activity of A β_{1-42} -induced antioxidant enzymes SOD and GSH-Px and decreased the content of MDA and ROS, thus inhibiting the increase in the level of A β_{1-42} -induced mitochondrial superoxides to decrease the generation of ROS. The present study also explored the involvement of the Nrf2 pathway in the mechanism of GB for improving A β_{1-42} -induced OS. Nrf2 has been verified as a protective factor in the pathological changes in AD (54). Hence, cognitive dysfunction in Nrf2-deficient mice was more serious and A β deposition and astrocyte activation clearly increased. In addition, the expression of the Nrf2 pathway in the brain tissues of animals with AD distinctly decreased. Activating the Nrf2 signaling pathway ameliorates OS, actively protects nerves and improves the spatial learning ability of mice with AD (39,40). The Nrf2-HO-1-NQO1 pathway is critical in preventing OS and also serves as an antioxidant transcription factor. When an organism is stimulated by external factors, Nrf2 dissociates from Kelch-like ECH-associated protein 1

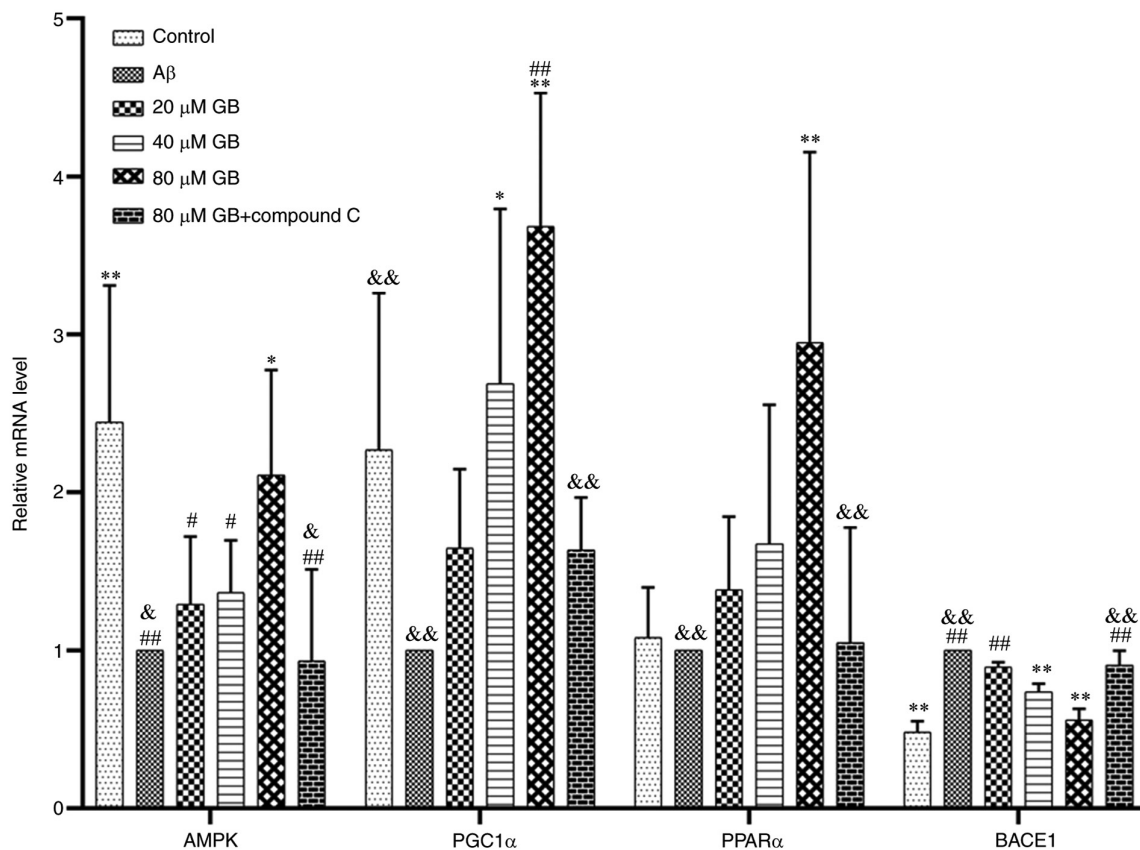


Figure 9. Effect of GB on gene expressions of AMPK pathway induced by $A\beta_{1-42}$ in astrocytes. Reverse transcription-quantitative PCR data of relative mRNA expression levels of AMPK, PGC1 α , PPAR α and BACE1 were treated with 10 μ M $A\beta_{1-42}$ with different GB concentration (20, 40, 80 μ M GB) or treated with 10 μ M $A\beta_{1-42}$ or treated with 10 μ M $A\beta_{1-42}$ + 80 μ M GB + 10 μ M compound C for 24 h. * P <0.05 and ** P <0.01 vs. control group, # P <0.05 and ## P <0.01 vs. A β group, & P <0.05 and && P <0.01 vs. 80 μ M GB group. GB, ginkgolide B; AMPK, 5' adenosine monophosphate-activated protein kinase; A β , β -amyloid; PGC1 α , proliferator-activated receptor γ coactivator 1 α ; PPAR α , peroxisome proliferator-activated receptor α ; BACE1, amylose β secretase 1.

in the cytoplasm and moves to the nucleus after activation to recognize antioxidant response elements and initiate the transcription of downstream antioxidant genes, including HO-1 and NQO1 (55). The results of the present study showed that GB increases the gene and protein expression levels of Nrf2-HO-1-NQO1 in astrocytes, thus exerting antioxidant activity to prevent OS and apoptosis and protect astrocytes from $A\beta_{1-42}$ -induced injury.

Energy metabolism disorder is a key pathological event in the early progression of AD because ATP can inhibit polymer of A β , weaken its neutral toxicity and decrease the formation of senile plaques, thus antagonizing AD (56,57). Insufficient levels of ATP accelerate the pathogenesis of AD (58,59). β -amyloid precursor protein (APP) and A β accumulate on the mitochondrial membranes and cause functional and structural injury to mitochondria and a metabolic decline in mitochondrial energy, thus decreasing the generation of ATP (60) and disrupting the normal functions of neural cells. The experimental results from the present study identified that GB improved the levels of $A\beta_{1-42}$ -induced ATP, thereby improving mitochondrial damage and hence stabilizing energy metabolism. However, AMPK inhibitor compound C inhibited the GB-induced improvement, which was probably associated with AMPK. AMPK is vital in metabolic homeostasis (61). A previous study found that the levels of A β increase when the levels of hippocampal p-AMPK decrease in 6-month-old

APP/PS1 mice. That is, decreasing AMPK activity exacerbates the pathology of AD (62). BACE1 mediates the first cleavage of APP and is the key and speed-limiting enzyme in the process of A β generation (63). AMPK inhibits the expression and activity of BACE1 and then regulates APP cleavage to reduce A β production (64). AMPK activates PGC-1 α (59), which is the transcriptional co-activator regulating the expression of energy metabolism-related genes. PGC-1 α improves the synthesis and metabolism in mitochondria, increases the number of mitochondria and increases the content of ATP (65). PGC-1 family members possess multifunctional transcriptional co-activation effects. They combine with PPARs and act as a 'molecular switch' in a number of energy metabolic signaling pathways, which regulate glycolipid metabolism and energy metabolism (66,67). A previous study demonstrates that the expression of PGC1 α in the brain tissues of patients with AD declines, leading to abnormal functioning of mitochondria (68). PGC1 α inhibits the generation of A β and a decrease in the expression of PGC1 α may be involved in the pathological progression of AD (69). PPAR α does not influence the generation of A β , but its excessive expression decreases the expression level of A β (70,71). In the present study, $A\beta_{1-42}$ inhibited the expression of the AMPK-PGC1 α -PPAR α pathway and increased the expression of BACE1 in astrocytes, which was in accordance with the aforementioned findings. That is, $A\beta_{1-42}$ induced the dysfunction of mitochondrial energy metabolism and also

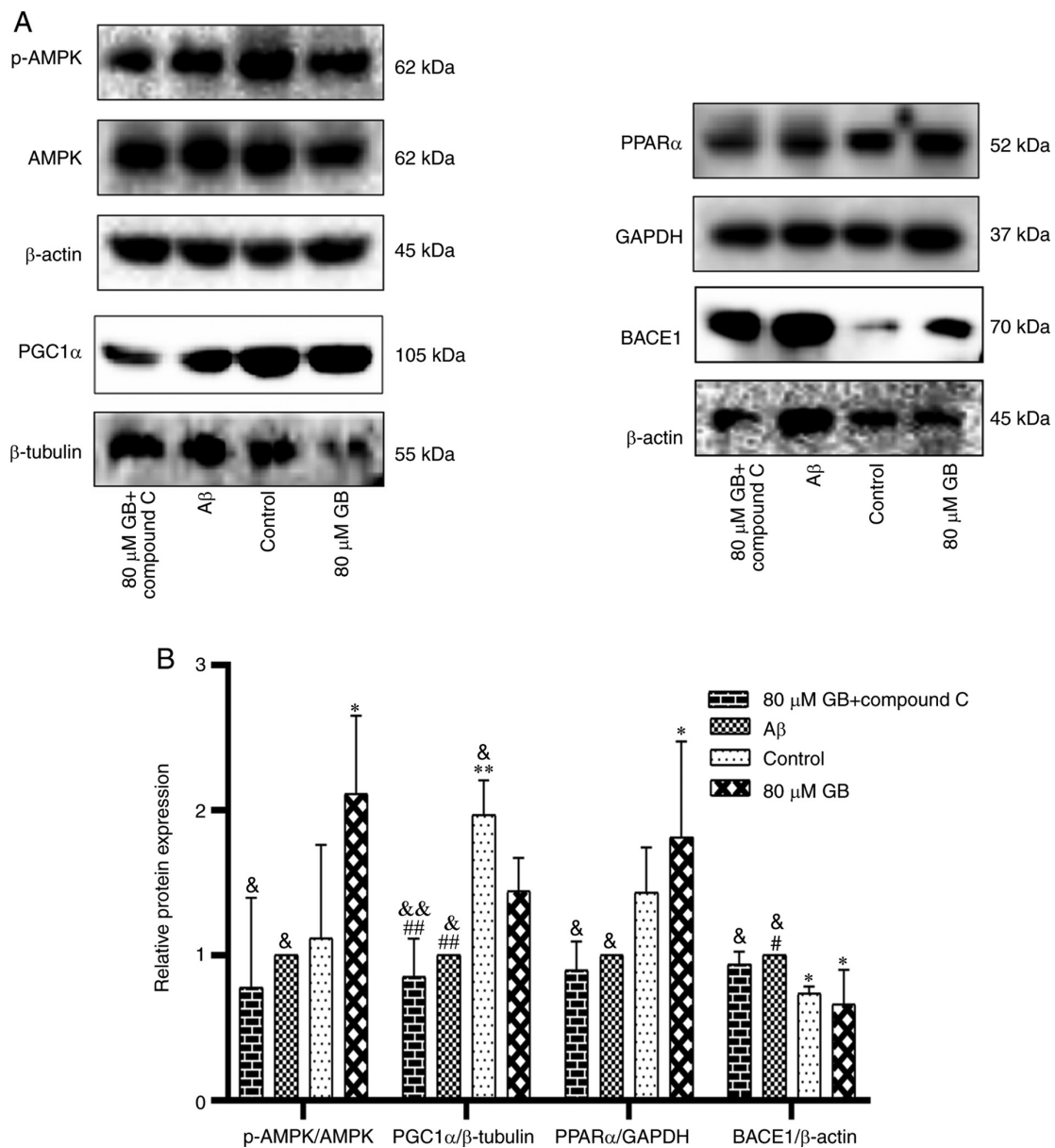


Figure 10. Compound C inhibits GB-activated protein expressions of AMPK pathway in astrocytes. (A) Western blotting image and (B) western blotting data of protein expression levels of p-AMPK, PGC1 α , PPAR α and BACE1 following treatment with 10 μ M A β ₁₋₄₂ with 80 μ M GB or treated with 10 μ M A β ₁₋₄₂ or A β ₁₋₄₂ + 80 μ M GB + 10 μ M compound C for 24 h. *P<0.05 and **P<0.01 vs. control group, *P<0.05 and **P<0.01 vs. A β group, &P<0.05 and &&P<0.01 vs. 80 μ M GB group. GB, ginkgolide B; AMPK, 5' adenosine monophosphate-activated protein kinase; p-, phosphorylated; PGC1 α , proliferator-activated receptor γ coactivator 1 α ; PPAR α , peroxisome proliferator-activated receptor α ; BACE1, amylose β secretase 1; A β , β -amyloid.

A β generation. However, GB increased the expression of the AMPK-PGC1 α -PPAR α signaling pathway, indicating that GB protected astrocytes via suppressing the inhibition of energy metabolism of A β ₁₋₄₂ to reduce the generation of A β . However, AMPK inhibitor compound C inhibited the functions of GB, implying that GB regulated energy metabolism and A β generation via likely activating the AMPK-PGC1 α -PPAR α pathway.

Energy metabolism causes environmental disorders of the endoplasmic reticulum to result in ERS (72). However, the persistence of metabolic injury causes neural damage resulting from prolonged activation of ERS (73,74). AMPK is the key sensor of the state of energy in eukaryotic cells and has strong effects on ERS, insulin resistance and lipid metabolism (75,76). Activating AMPK can help prevent hypoxic

injury, atherosclerosis and heart injury caused by ERS (77,78). In the present study, A β ₁₋₄₂ increased the expression of astrocyte ERS markers and resulted in higher expression levels of the apoptotic protein CHOP. GB inhibited ERS and apoptosis, while AMPK inhibitor compound C inhibited GB-induced ERS and apoptosis. These findings indicated that GB impeded the activation of ERS caused by energy metabolism disorders and suppressed ERS-related signaling pathways through the AMPK signaling pathway to inhibit A β ₁₋₄₂-induced astrocyte injury and protect astrocytes. ERS can increase the generation of ROS. Conversely, the generation of ROS can induce ERS. Hence, a strong correlation exists between ROS and ERS (79,80). A previous study noted that activated AMPK is associated with the regulation of the Nrf2 signaling pathway (81). The present study demonstrated that

the antioxidant activity of GB prevented OS and apoptosis via increasing the expression of Nrf2-HO-1-NQO1 in astrocytes to protect astrocytes from A β ₁₋₄₂-induced injury. AMPK inhibitor compound C inhibited the improvement in GB-induced OS and apoptosis. Hence, the GB probably acted by promoting the activation of AMPK and Nrf2 to increase the gene and protein expression levels of HO-1 and NQO1. In other words, GB ameliorated A β ₁₋₄₂-induced OS responses, which was probably related to the activation of AMPK-Nrf2-HO-1-NQO1 signaling pathways.

In conclusion, GB protects against A β ₁₋₄₂-induced cell apoptosis by inhibiting ERS, OS and energy metabolism disorders via activating AMPK signaling pathways. These findings might provide an innovative insight into the treatment of A β -related AD using GB.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW and LZ confirm the authenticity of all the raw data. JW designed the experiments, performed the experimental procedures, analyzed the data, and wrote the manuscript. YD and LZ performed experimental procedures, and analyzed the data. ZW designed experiments and reviewed the manuscript. WX and JZ conceived the project, designed experiments, and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures related to the use and care of animals in the present study were approved by the Ethics Committee for the Use of Experimental Animals of Jiangsu Kanion Pharmaceutical Co. Ltd. State Key Laboratory of New Pharmaceutical Process for Traditional Chinese Medicine (approval no. 2019012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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