Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2021, **19**(10): 732-740
doi: 10.1016/S1875-5364(21)60090-0
Chinese Journal of Natural Medicines

•Reaearch article•

Physalin B reduces Aβ secretion through down-regulation of BACE1 expression by activating FoxO1 and inhibiting STAT3 phosphorylation

ZHANG Wei[1], BAI Shan-Shan[1, 10], ZHANG Qi[2], SHI Ru-Ling[3], WANG He-Cheng[4], LIU You-Cai[5], NI Tian-Jun[1], WU Ying[6], YAO Zhao-Yang[1], SUN Yi[7*], WANG Ming-Yong[8, 9*]

[1] *School of Basic Medical Sciences, Xinxiang Medical University, Xinxiang 453003, China;*
[2] *Grade 2019, School of Basic Medical Sciences, Xinxiang Medical University, Xinxiang 453003, China;*
[3] *School of Public Health, Xinxiang Medical University, Xinxiang 453003, China;*
[4] *School of Life and Pharmaceutical Sciences, Dalian University of Technology, Panjin 124221, China;*
[5] *School of Basic Medical Sciences, Sanquan College of Xinxiang Medical University, Xinxiang 453003, China;*
[6] *School of Nursing Care, Xinxiang Medical University, Xinxiang 453003, China;*
[7] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China;*
[8] *School of Laboratory Medicine, Xinxiang Medical University, Xinxiang 453003, China;*
[9] *Xinxiang Key Laboratory of Immunoregulation and Molecular Diagnostics, Xinxiang 453003, China;*
[10] *Central Laboratory, the First Affiliated Hospital of Henan University of Science and Technology, Luoyang 471003, China*

Available online 20 Oct., 2021

[ABSTRACT] Physalin B (PB), one of the major active steroidal constituents of *Solanaceae Physalis* plants, has a wide variety of biological activities. We found that PB significantly down-regulated β-amyloid (Aβ) secretion in N2a/APPsw cells. However, the underlying mechanisms are not well understood. In the current study, we investigated the changes in key enzymes involved in β-amyloid precursor protein (APP) metabolism and other APP metabolites by treating N2a/APPsw cells with PB at different concentrations. The results indicated that PB reduced Aβ secretion, which was caused by down-regulation of β-secretase (BACE1) expression, as indicated at both the protein and mRNA levels. Further research revealed that PB regulated BACE1 expression by inducing the activation of forkhead box O1 (FoxO1) and inhibiting the phosphorylation of signal transducer and activator of transcription 3 (STAT3). In addition, the effect of PB on BACE1 expression and Aβ secretion was reversed by treatment with FoxO1 siRNA and STAT3 antagonist S3I-201. In conclusion, these data demonstrated that PB can effectively down-regulate the expression of BACE1 to reduce Aβ secretion by activating the expression of FoxO1 and inhibiting the phosphorylation of STAT3.

[KEY WORDS] Physalin B; β-amyloid; BACE1; FoxO1; Alzheimer's disease
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2021)10-0732-09

Introduction

Alzheimer's disease (AD), known as senile dementia, is an age-related neurodegenerative disease characterized by cognitive impairment, such as progressive memory loss, intellectual decline and behavioral changes [1]. With the growth of aging population, the number of AD patients is gradually increasing. Once dementia occurs, its progression is irreversible. However, there are still no effective methods to stop, cure, or prevent the disease [2]. Although the etiology of AD is not fully known yet, extracellular Aβ deposition has been recognized as the key factor leading to AD symptoms [3]. It is believed that the deposition of Aβ interferes with the transmission of intersynaptic neuron-to-neuron signals, which causes cell death [4]. Aβ peptides are generated by sequential cleavage of APP [5]. Full-length APP is a type I transmembrane protein, which can be metabolized *via* two routes: the

[Received on] 12-Apr.-2021
[Research funding] This research was supported by the National Natural Science Foundation (No. U1604108), Key Science and Technology Program of Henan Province (Nos. 202102310020 and 182102311148), the Program for Innovative Research Team (in Science and Technology) in University of Henan Province (No. 20IRTSTHN030), and the Graduate Scientific Research Innovation Support Project of Xinxiang Medical University (No. YJSCX201866Y).
[*]**Corresponding author]** ysun@icmm.ac.cn (SUN Yi); wmy118@126.com (WANG Ming-Yong)
These authors have no conflict of interest to declare.

non-amyloidogenic pathway and the amyloidogenic pathway. In the non-amyloidogenic pathway, APP is constitutively transferred to the plasma membrane which is rich in *α*-secretase, and cleaved to release a soluble N-terminal fragment, sAPP*α*, and a membrane-bound C-terminal fragment, CTF*α*. In contrast, in the amyloidogenic pathway, APP is cleaved by BACE1, which is mainly localized in endosomes, to yield sAPP*β* and CTF*β*, which is the *β*-secretase cleaved C-terminal fragment of APP. CTF*α* and CTF*β* are subsequently cleaved by *γ*-secretase to release P3 or A*β* [6-7]. Clearly, inhibition of *γ*-secretase/BACE1, or stimulation of *α*-secretase are three strategies to reduce A*β* production [8]. BACE1 is the initiating and rate-limiting enzyme in A*β* secretion [9]. Thus, BACE1 inhibition is an effective strategy to reduce A*β* levels so as to prevent and/or cure AD [10].

Forkhead box protein (Fox) is a kind of transcription factor in a family that includes FoxO1, FoxO3a, FoxO4, and FoxO6. Fox proteins are essential in several human intracellular pathways [11]. They are critical in transcriptional nucleus regulation of many metabolic processes [12]. FoxO1 is an important transcription factor of the FoxO family [13], which is widely expressed in almost all tissues and plays an essential role in regulating physiological processes such as cell proliferation, apoptosis and antioxidative stress [14-15]. Studies [16-17] indicated the association of FoxO1 with neurological diseases in humans. Our previous study found that FoxO1 was related to AD, suggesting the role of FoxO1 as a novel therapeutic target for AD treatment that reduced A*β* expression [18]. Another transcription factor, STAT3, is well known to regulate various genes and significantly attenuate A*β* generation by controlling BACE1 expression [19-20].

Currently, AD treatment does not achieve satisfactory clinical results. Researchers then turn their attention to natural herbals and their phytochemicals, some of which exhibit excellent effects against the pathological progression of AD[21]. Physalin is a sterane compound with a high oxidation level and a basic 13, 14-split ring-16, 24-cycloergosterane structure [22]. It is widely found in the family Solanaceae, such as *Physalis angulata*, *Franchet groundcherry calyx* and *fruit*, and other similar plants. It is named physalin due to slightly bitter taste[23]. Moreover, with respect to its unique chemical structure and wide distribution in many natural resources, physalin has attracted increasing attention within the pharmaceutical community. In particular, physalin B (PB) is gaining attention because it possesses a wide variety of biological activities, such as anti-tumor [24], anti-inflammatory [25], and immunomodulation activity [26]. However, the effects of PB on AD are rarely reported. Extracellular A*β* deposition is a major pathological hallmark of the progression of AD. Therefore, the present study is to investigate whether PB can inhibit the secretion of A*β* and explore the underlying mechanism involved.

Materials and methods

Materials

PB (96% HPLC purity) was isolated as previously described [27]. Rabbit anti-APP and CTF antibodies (A8717; 1 : 5000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against BACE1 (ab2077; 1 : 1000), STAT3 (ab109085; 1 : 1000), and p-STAT3 (ab76315; 1 : 5000) were obtained from Abcam (Cambridge, United Kingdom). The primary antibody against FoxO1 (C29H4; 1 : 1000) and BACE1 (5606s; 1 : 1000) was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). In addition, anti-*β*-actin antibody (1 : 4000) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and anti-GAPDH (bs-2188R; 1 : 2000) was purchased from Bioss Biotechnology Co., Ltd. (Beijing, China). Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) (code No.: 111-035-003; 1 : 5000) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The reagents used in the current study are listed as follows: Dulbecco's Modified Eagle's Medium (DMEM; high glucose) was obtained from HyClone (Logan, Utah, USA); 0.25% trypsin and 0.02% EDTA solution were obtained from Gino Biomedical Technology Co., Ltd. (Hangzhou, China); penicillin-streptomycin solution (100 ×) (C0222) was obtained from Beyotime (Shanghai, China); RIPA lysis buffer (C1053+) was obtained from Beijing Applygen Technologies Inc. (Beijing, China); *β*-secretase activity fluorometric assay kit was from BioVision (K360-100) (Milpitas, CA, USA). FoxO1 siRNA I (Mouse Specific) (56458) was purchased from Cell Signaling Technology Inc. (Boston, MA, USA); Immobilon Western chemiluminescent HRP substrate was obtained from Millipore Corporation (Billerica, MA, USA); and S3I-201 (HY-15146/CS-0512), AS1842856 (HY-100596) and Cell Counting Kit-8 (CCK-8) were obtained from MedChemExpress (Monmouth Junction, USA). All other chemicals used in this study were of analytical grade.

Cell culture

Mouse N2a neuroblastoma cells stably expressing wild-type Swedish mutant APP (APPsw) (abbreviated as N2a/APPsw cells for convenience) were kindly donated by Professor Dehua Chui (Peking University) [28] and cultured in the Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution, in an incubator at 37 °C with a humidified atmosphere of 95% air and 5% CO_2.

Cell viability test

To explore the influence of different concentrations of PB on cell viability, CCK-8 assay was performed to assess cell proliferation and toxicity in accordance with the manufacturer's instructions. N2a/APPsw cells were preincubated in 96-well plates at 37 °C for 24 h and then treated with various doses of PB (0, 0.3, 1.0, 3.0, 4.0, and 5.0 μmol·L^{-1}) for 24 h. Then, 10 μL of CCK-8 reagent was added to each well of the 96-well plates for 3 h, and the absorbance was measured on a microplate reader at 450 nm.

Protein extraction and Western blot

After treatment with different reagents and drugs, the N2a/APPsw cells were collected at the indicated time points,

and immediately placed on ice before removal of the medium. The cells were washed three times with PBS (pH 7.2) and then lysed with RIPA lysis buffer with a protein phosphatase inhibitor mixture (All-in-One, 100 ×, P1260) and protease inhibitor PMSF (#A1100, Applygen, Beijing, China). The lysates were collected and centrifuged at 12 000 rpm at 4 °C for 20 min, and the resultant supernatant was transferred to new tubes on ice. A Pierce™ BCA protein assay kit (#23227, Rockford, IL, USA) was used to quantify the total protein in the supernatant according to the manufacturer's instructions. The total protein content of each sample was maintained at 30.0 μg for Western blot. The proteins were first separated by SDS-PAGE and then transferred to 0.45 μm polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked in TBST (Tris-buffered saline (pH 7.5) supplemented with 0.1% Tween-20) with 5% nonfat milk at room temperature for 1.5 h and then incubated with the appropriate antibodies at 4 °C overnight. The membrane was washed three times (10 mins × 3) with TBST to which 5% nonfat milk was added, and then incubated with peroxidase-conjugated AffiniPure goat anti-rabbit or anti-mouse IgG antibodies at room temperature for 1.5 h. The washing procedure was repeated three more times. All the protein bands were observed by the ECL system, and normalized to *β*-actin or GAPDH.

Measurement of Aβ secretion by ELISA

Following treatment of N2a/APPsw cells with PB within a range of 0-3 μmol·L^{-1} for 24 h, the medium was harvested and then analyzed. The levels of A*β*40 and A*β*42 in the cell culture medium were measured with ELISA kits (#294-62501, #294-62601, Wako, Japan) according to the manufacturer's instructions. Data were normalized to that of the control. All ELISA assays were performed in triplicate.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

N2a/APPsw cells were treated with different concentrations (0, 0.3, 1.0, and 3.0 μmol·L^{-1}) of PB for 24 h, and then total RNA was carefully isolated by RNAiso plus (#9108, TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Upon extraction, the RNA concentration of each sample was immediately measured and reverse-transcribed into cDNA with an equal amount of total RNA (1 μg) using a PrimeScript™ RT reagent kit with gDNA Eraser (#RR047A, TaKaRa) in accordance with the manufacturer's instructions. The synthesized cDNA and the specific primers shown in Table 1 were used to amplify the target genes. The amplification was performed using TaqTM (#R001A, TaKaRa) in the following steps: 94 °C for 5 min and 94 °C for 30 s; 35 cycles of 94 °C for 30 s, 56.5 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min; and finally maintained at 4 °C. The PCR products were run on 1% agarose gels supplemented with ethidium bromide in electrophoretic fluid (1 ×). The gel analysis system was used to visualize the intensities of the amplified bands, and ImageJ software (NIH Image public domain, USA) was used to quantify the mRNA expression. All mRNA expression levels was normalized by *β*-actin mRNA.

Table 1 Primer pairs for RT-PCR

Gene	Sequence
Bace1	F: 5′-GCGGGAGTGGTATTATGAAGTG-3′
	R: 5′-ATGCGGAAGGACTGATTGG-3′
***β*-actin**	F: 5′-TCATCACTATTGGCAACGAGC-3′
	R: 5′-GAGGTCTTTACGGATGTCAACG-3′

β-Secretase activity assay

After the N2a/APPsw cells were treated with different concentrations (0, 0.3, 1.0, and 3.0 μmol·L^{-1}) of PB for 24 h, their *β*-secretase activity was measured by a *β*-secretase activity kit (#K360-100, BioVision, CA, USA). Ice-cold extraction buffer was used to extract the protein from the treated cells in accordance with the manufacturer's instructions. After incubation on ice for 10 min, the cell lysates were centrifuged at 10 000 × g at 4 °C for 5 mins. The resultant supernatant was transferred to a new tube, and the total protein was quantified using a Pierce™ BCA protein assay kit. Fifty microliters of cell lysate (30 μg of total protein) was prepared to add to each well in a 96-well plate, and then, 50 μL of 2 × reaction buffer was added, gently mixed and incubated at 37 °C for 20 min. Then, 2 μL of the *β*-secretase substrate was added and incubated in the darkness at 37 °C for 1 h. Fluorescence was read on a fluorescent plate reader with excitation/emission wavelengths of 345/500 nm.

siRNA transient transfection

Foxp1 siRNA was used to transfect N2a/APPsw cells for 24 h using LipofectamineTM 3000 reagent according to the manufacturer's instructions. Then, the transfected cells were treated with 3.0 μmol·L^{-1} PB for 24 h and harvested for later experiments.

Transfection and luciferase assay

A total of 10 × 10^4 N2a/APPsw cells were seeded in each well of 24-well plates for 24 h and then cotransfected with *Bace1* promoter constructs (0.5 μg) (kindly provided by Professor Hong Qing, Beijing Institute of Technology, Beijing, China) and the pRL-TK vector plasmid (0.02 μg), to normalize transfection efficiency, using Lipofectamine™ 3000 reagent for 24 h. Then, the cells were pretreated with 100 μmol·L^{-1} STAT3 antagonist S3I-201 and 50 μmol·L^{-1} FoxO1 antagonist AS1842856 for 30 mins, respectively. Then, the cells were treated with 3.0 μmol·L^{-1} PB for 24 h. Next, the *Bace1* promoter activity was evaluated in the following steps: the cells were lysed with 1 × passive lysis buffer for 15 mins at room temperature, and then, the cell lysate was collected in new tubes and centrifuged at 12 000 rpm for 10 mins. The supernatant was transferred into new tubes for the luciferase assay in accordance with the Dual-Luciferase Reporter Assay System protocol (#E1910, Promega, Madison, WI, USA) using a Luminometer (Promega, Madison, WI, USA). Renilla luciferase activity from the pRL-TK vector plasmid was used to normalize the luciferase activity.

Statistical analysis

All the experiments were independently repeated at least

three times. Data were analyzed in GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego CA, USA) and are presented as the mean ± SEM. One-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test was used to analyze whether the results were significantly different. $P < 0.05$ was considered statistically significant.

Results

Effect of PB on cell viability

The structure of PB is shown in Fig. 1A. CCK-8 assay was performed to explore the effect of PB on N2a/APPsw cell proliferation. The results illustrated that PB did not induce cytotoxicity at the concentrations of 0, 0.3, 1.0, or 3.0 μmol·L^{-1} (Fig. 1B). Therefore, these concentrations were used for the following experiments.

Effect of PB on Aβ secretion and APP processing

To evaluate the effect of PB on Aβ secretion, N2a/APPsw cells were treated with PB (0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h. According to ELISA results, compared with the control group, the levels of Aβ40 and Aβ42 decreased with the increase of PB concentrations (Fig. 2A). The secretion of Aβ40 and Aβ42 decreased at 0.3 μmol·L^{-1} PB and significantly decreased at 3.0 μmol·L^{-1} ($P < 0.01$). To explore how PB affected the APP process in N2a/APPsw cells, we evaluated the effect of PB on the expression of APP metabolism-related proteins by Western blot. The results showed that PB significantly down-regulated the expression of full-length APP (flAPP) and CTFβ compared with the control group (Fig. 2B). In contrast, the CTFα level remained unchanged (Fig. 2B). These data showed that PB can suppress the amyloidogenic pathway of APP processing, without ef-

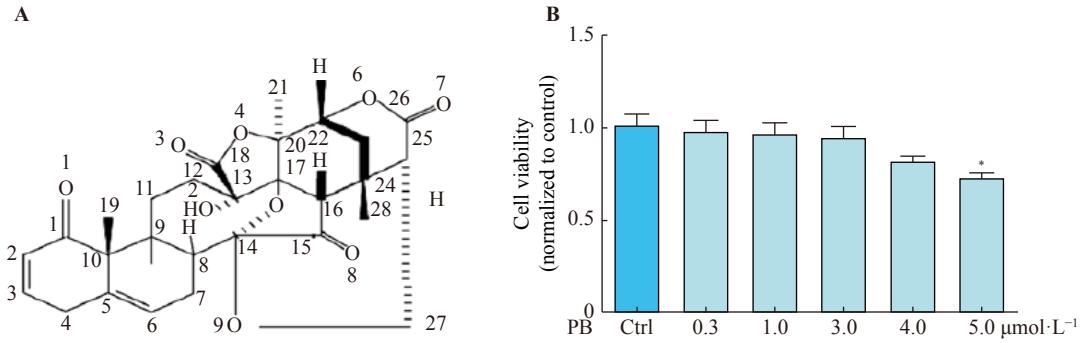

Fig. 1 Effect of PB on cell viability. N2a/APPsw cells were treated with PB (0, 0.3, 1.0, 3.0, 4.0, and 5.0 μmol·L^{-1}) for 24 h. (A) Chemical structure of PB. (B) The effect of PB on cell viability was assessed by CCK-8 assay. ($n = 3$), * $P < 0.05$ *vs* control group

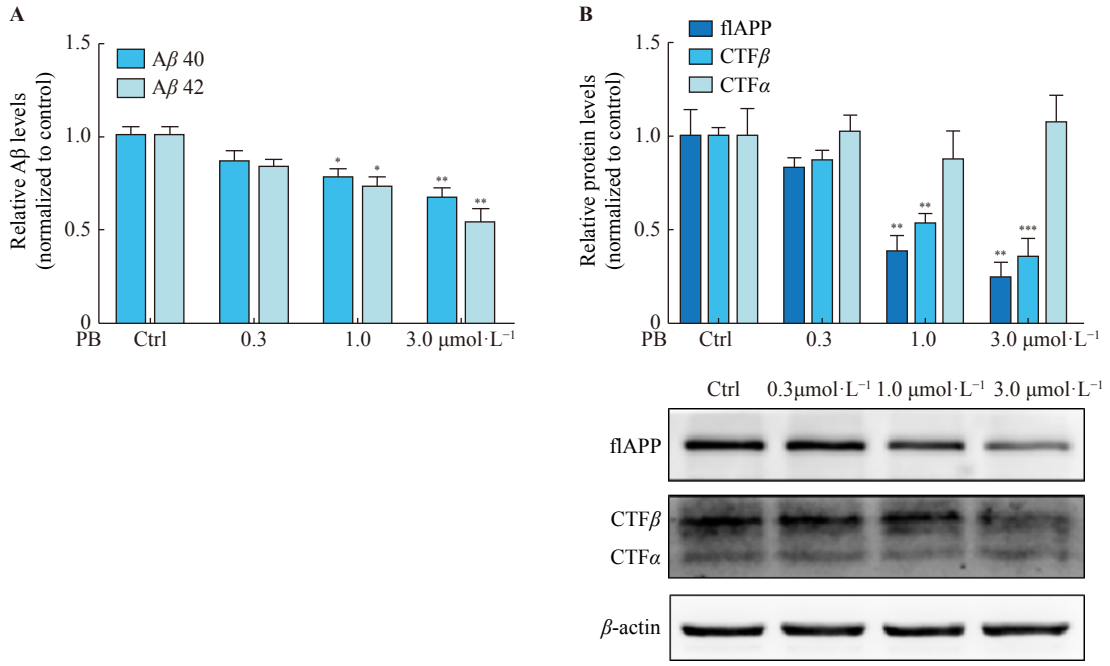

Fig. 2 Effect of PB on Aβ secretion and APP processing. N2a/APPsw cells were treated with PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h. (A) The effect of PB on Aβ40 and Aβ42 secretion was assessed by ELISA. (B) The effect of PB on the expression of flAPP, CTFα, and CTFβ was evaluated by Western blot. ($n = 3$), * $P < 0.05$, ** $P < 0.01$ *vs* control group

fects on the non-amyloidogenic pathway.

Effect of PB on BACE1 expression and activity

Furthermore, we tried to explore whether PB influenced the expression of BACE1. Western blot results showed that PB obviously inhibited BACE1 expression (Fig. 3A). BACE1 controls the first and rate-limiting step of Aβ generation, and inhibiting BACE1 activity is also very important. Therefore, we then detected the effect of PB on BACE1 activity by β-secretase activity fluorometric assay kit. The results indicated that BACE1 activity in the PB (0.3−3.0 μmol·L^{-1}) treated groups was significantly reduced conpared with the control group (Fig. 3B). These results demonstrated that PB decreased Aβ secretion by inhibiting BACE1 expression and activity.

Effect of PB on Bace1 mRNA expression

To further explore the underlying mechanisms of PB on the expression of *Bace1*, RT-PCR was performed to measure the expression of *Bace1* mRNA. The results showed that PB at a range of 0.3−3.0 μmol·L^{-1} effectively reduced the mRNA expression of *Bace1* (Fig. 4). These findings indicated that PB reduced the mRNA expression of *Bace1* through inhibiting its transcription.

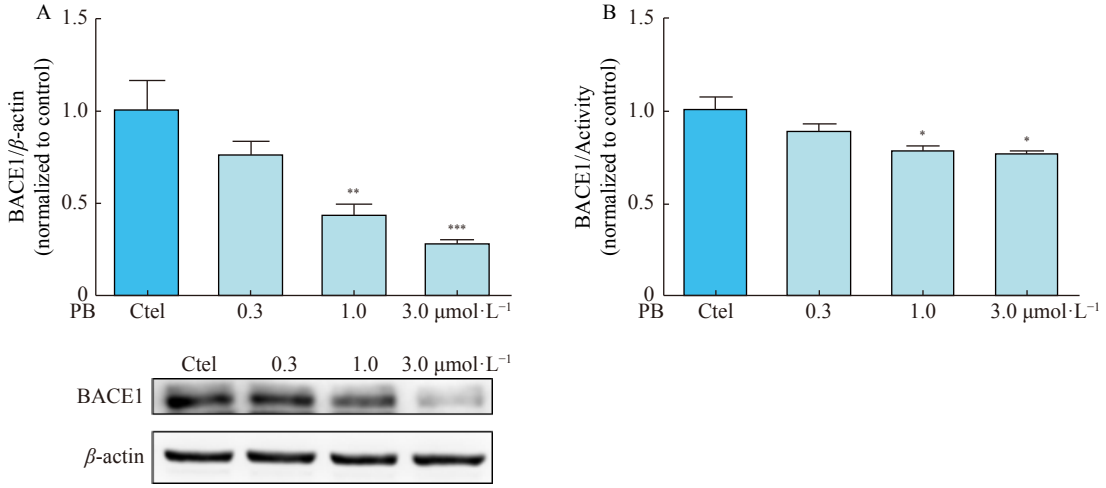

Fig. 3 Effect of PB on BACE1 expression and activity. N2a/APPsw cells were treated with PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h. (A) The effect of PB on the expression of BACE1 was assessed by Western blot. The enzyme β-secretase was isolated from the cells. (B) The effect of PB on BACE1 activity was evaluated by the β-secretase activity assay. (n = 3), * P < 0.05, ** P < 0.01, and *** P < 0.001 *vs* control group

Effect of PB on Bace1 transcription process

The expression of *Bace1* mRNA is strictly regulated at the transcriptional level, and there are many putative transcription factor binding sites in the upstream sequence of *Bace1* gene [10], *Foxo1*, which is considered the most vital transcription factor in the *Foxo* family, plays an essential role in several human intracellular signaling pathways [19-20]. Accumulated evidence [16-18] suggests that *Foxo1* is involved in the regulation of APP metabolic progression. Accordingly, we investigated the effect of PB on *Foxo1* expression. As shown in Fig. 5A, PB significantly increased *Foxo1* expression. To verify whether PB regulated *Bace1* expression mainly by regulating the expression of *Foxo1*, we transfected *Foxo1* siRNA into N2a/APPsw cells, and results showed that *Bace1* levels increased compared with that of the control group (Fig. 5B). However, compared with the N2a/APPsw cells treated with *Foxo1* siRNA alone, the increase of *Bace1* expression was attenuated when N2a/APPsw cells were co-treated with *Foxo1* siRNA and 3.0 μmol·L^{-1} PB (Fig. 5B). Moreover, we explored *Bace1* promoter activity using the Dual-Luciferase Reporter Assay System, and the results showed that compared with the FOXO1 antagonist AS1842856 treatment alone, the increase of *Bace1* promoter activity was also attenuated when N2a/APPsw cells were co-treated with AS1842856 and 3.0 μmol·L^{-1} PB (Fig. 5C).

STAT3 can effectively induce *Bace1* transcription [29-30].

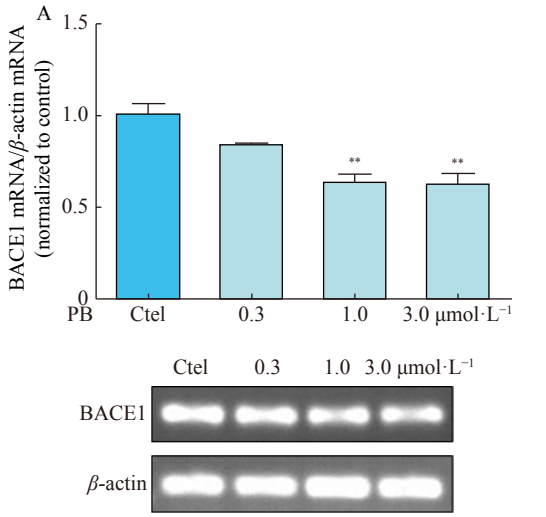

Fig. 4 Effect of PB on *Bace1* mRNA expression. N2a/APPsw cells were treated with PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h. The effect of PB on the expression of *Bace1* mRNA was assessed by RT-PCR. (n = 3), ** P < 0.01, and *** P < 0.001 *vs* control group

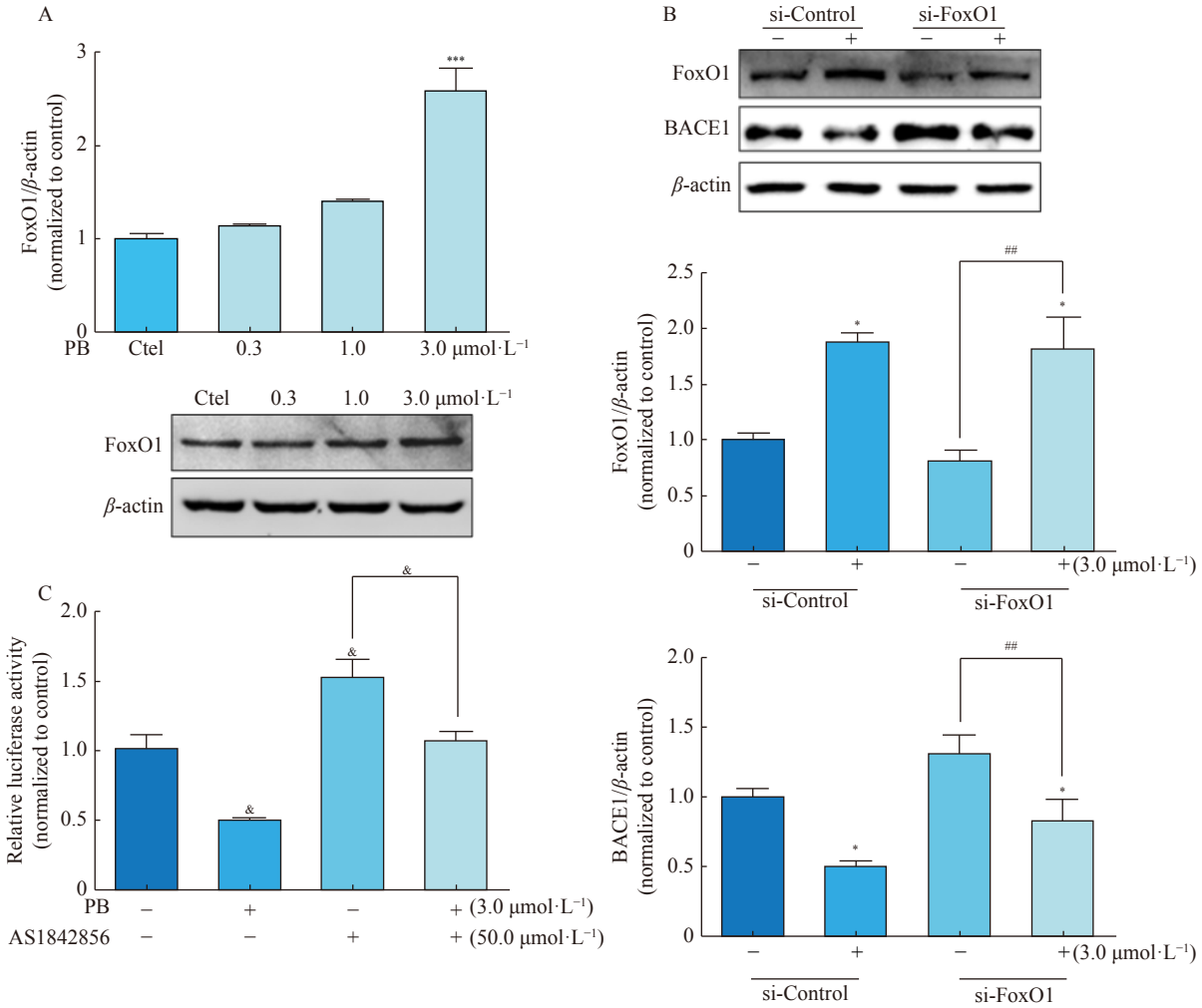

Fig. 5 The effect of PB on BACE1 expression is FoxO1-dependent. N2a/APPsw cells were treated with PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h, and the proteins were then extracted. (A) The effect of PB on FOXO1 expression was measrued by Western blot. The cells were treated with *Foxo1* siRNA for 12 h and then exposed to PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h. (B) The levels of FOXO1 and BACE1 were detected by Western blot, $^{\#\#}$ $P < 0.001$ (si-FoxO1 alone group *vs* PB and si-FoxO1 group). (C) *Bace1* promoter activity was evaluated by a dual-luciferase assay. (n=3), * $P < 0.05$, *** $P < 0.001$ *vs* control group; $^{\&}$ $P < 0.05$, $^{\&\&}$ $P < 0.001$ (AS1842856 alone group vs PB and AS1842856 group)

Phosphorylated STAT3 (p-STAT3) dimerizes and is translocated to the nucleus, where it positively modulates *Bace1* gene transcription [30]. To determine whether PB suppressed *Bace1* expression through inhibiting STAT3 phosphorylation, Western blot was performed to investigate the effect of PB on P-STAT3 in nuclear extracts. As illustrated in Fig. 6A, PB significantly decreased the expression of p-STAT3 compared with the control group. Then, N2a/APPsw cells were exposed to S3I-201, a STAT3 inhibitor, to prevent STAT3 phosphorylation [31]. As shown in Fig 6B, S3I-201 (100 μmol·L^{-1}) significantly inhibited STAT3 phosphorylation in a manner similar to that of PB, as determined by Western blot. Moreover, co-treatment with S3I-201 and PB inhibited STAT3 phosphorylation to a greater extent than treatment with S3I-201 alone (Fig 6B). This finding suggested that PB inhibited STAT3 phosphorylation through a different mechanism with S3I-201. In addition, *Bace1* promoter activity was evaluated. We found that both PB and S3I-201 weakened the activity of *Bace1* promoter, while cotreatment with S3I-201 and PB reduced *Bace1* promoter activity to a greater extent than treatment with S3I-201 alone (Fig. 6C). These results showed that PB reduced BACE1 expression mainly through activating FoxO1 and inhibiting STAT3 phosphorylation.

Discussion

Many factors contribute to the occurrence of AD; however, its pathological mechanism is not yet clear. Currently, targeting Aβ for the treatment of AD appears to be an accepted and feasible approach. Recent studies [32-33] have suggested that agents that can effectively decrease Aβ production and/or increase Aβ clearance are effective in retarding AD progression. In the present study, we found that PB significantly attenuated the secration of both Aβ40 and Aβ42. Therefore, we investigated the mechanism underlying the PB-

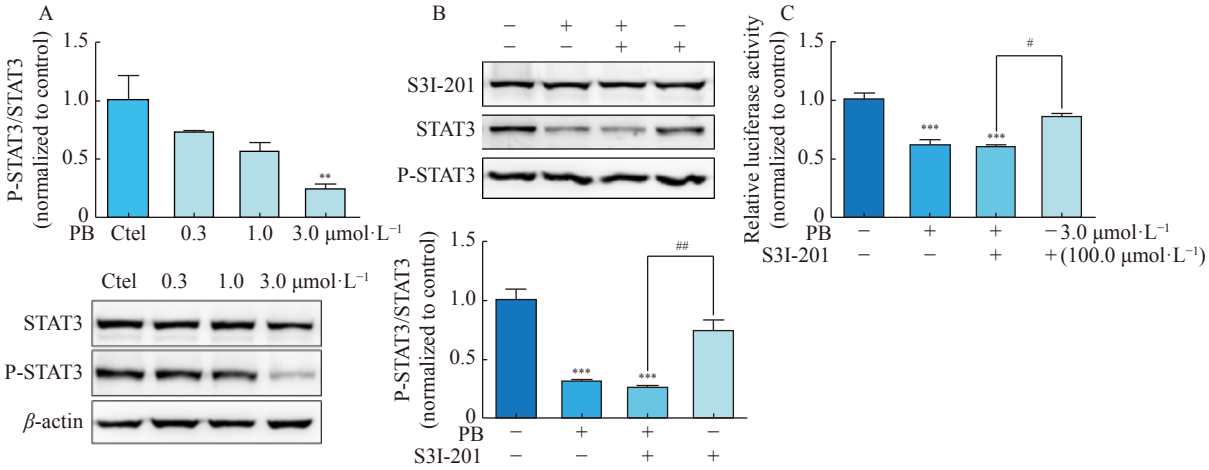

Fig. 6 Effect of PB on STAT3 phosphorylation. N2a/APPsw cells were treated with PB (0, 0.3, 1.0 and 3.0 μmol·L^{-1}) for 24 h, and the nuclear proteins were then extracted. (A) The effect of PB on p-STAT3 expression was assessed by Western blot. The cells were pretreated with 100 μmol·L^{-1} S3I-201 for 30 mins followed by treamtent with PB before incubation for 24 h. (B) The effect of PB on p-STAT3 and STAT3 expression was then evaluated by Western blot. (C) The effect of PB on *Bace1* promoter activity was evaluated by the dual-luciferase assay. (n = 3), ** P < 0.01 and *** P < 0.001 *vs* control group. $^{\#}$ P < 0.05 (PB and S3I-201 group *vs* S3I-201 alone group)

induced decrease in Aβ levels in N2a/APPsw cells in the experiments described.

First, we explored the effect of PB on other APP metabolite levels in N2a/APPsw cells. According to Western blot results, PB notably decreased the levels of flAPP and CTFβ, without any change in CTFα expression. These data indicated that PB decreased Aβ secretion mainly through inhibiting the amyloidogenic pathway of APP rather than the non-amyloidogenic pathway.

Then, we explored the changes in APP metabolism-related enzyme expression. We found that PB significantly reduced the level of BACE1. These results further proved that PB decreased Aβ secretion, which was mainly attributed to BACE1-mediated processes of the amyloidogenic APP pathway.We also found that PB decreased the expression of *Bace1* mRNA. These results indicated that PB down-regulated the expression of BACE1 mainly through inhibiting the transcription of *Bace1*. It is widely accepted that Aβ overproduction is the product of "overactivated" BACE1, as indicated by intensive research [34-35]. We further investigated the mechanism underlying the involvement of PB in attenuating BACE1 expression. We observed that PB seemed to specifically reduce the expression of BACE1, rather than all APP pathway proteins. A transcription factor must bind to a specific DNA sequence to exert its effect [10]. *Bace1* transcription is under the influence of many factors, such as specificity protein (SP1), hypoxia inducible factor 1α (HIF-1α), PPARγ and STAT3 [36-38]. Therefore, we investigated whether PB influenced STAT3 phosphorylation. Results showed that PB significantly attenuated the expresion of P-STAT3 and furthermore, we learned that PB had different active groups with S3I-201 to regulate STAT3 phosphorylation. However, STAT3 is just one of numerous transcription factors that regulate *Bace1* gene expression. Therefore, it cannot be ruled out that PB may regulate the activity of transcription factors in addition to STAT3 to affect *Bace1* promoter activity.

Forkhead box O1 (FoxO1) is considered the most important protein of the FoxO family, and a vital transcriptional regulatory factor widely expressed in almost all tissues, where it plays an essential role in regulating physiological processes such as cell proliferation, apoptosis and antioxidative stress [14-15]. Recent studies [16-18] have shown that FoxO1 is closely related to neurological diseases in humans. In the present study, we investigated whether down-regulation of BACE1 expression is mediated by FoxO1. We verified that PB significantly increased FoxO1 expression. Actually, our data indicated that the effect of PB on FoxO1 expression was abolished, while the level of BACE1 protein increased when *Foxo1* was knocked down *via* siRNA. These results demonstrated that the effect of PB on BACE1 expression, as well as on Aβ secretion, was FoxO1-dependent. Therefore, FoxO1 is expected to become a novel therapeutic target for AD treatment. Our data indicated that PB not only regulates STAT3 but also FoxO1. It is likely that PB exerts its effectiveness through acting on some upsteam proteins of FoxO1 and/or STAT3, but the underlying mechanism is not completely understood. Moreover, multiple synergistic pathways are involved in the development of AD. It is possible that numerous kinases regulate FoxO1 and/or STAT3, and further study will be performed to explore the mechanisms by which FoxO1 and STAT3 activities are regulated.

APP is cleaved by BACE1, the rate-limiting enzyme mediating the amyloidogenic pathway, to produce Aβ that is the main component of AD senile plaques [39]. This pathway primarily occurs in cells, where BACE1 plays an essential role [9]. BACE1 is an aspartyl protease, which is a stress-induced protease [10]. Many factors, such as inflammation, oxidative stress, and hypoxia, can activate BACE1 enzymatic

activity [40], and AD pathogenesis is obviously affected by subtle changes in BACE1 activity [41]. Our previous data indicated that AD seriously harms the physical and mental health of elderly people. With the growth of aging population, the incidence of AD is continuously increasing, which places a heavy burden on individuals and society. However, until now, all the drugs commercially available are unable to stop, cure or prevent AD. With respect to the serious situation, it is very important to research and develop new effective drugs to treat AD and/or adopt non-pharmacological interventions to improve the quality of life of AD patients [42].

In summary, the current study first confirmed that PB attenuated Aβ secretion by inhibiting the expression of BACE1 in N2a/APPsw cells and then verified the effect of PB on BACE1 by up-regulation of FoxO1 and reduction of STAT3 phosphorylation (Fig.7). Our findings suggest that PB provides beneficial neuroprotection against AD, which may be a promising potential drug against AD.

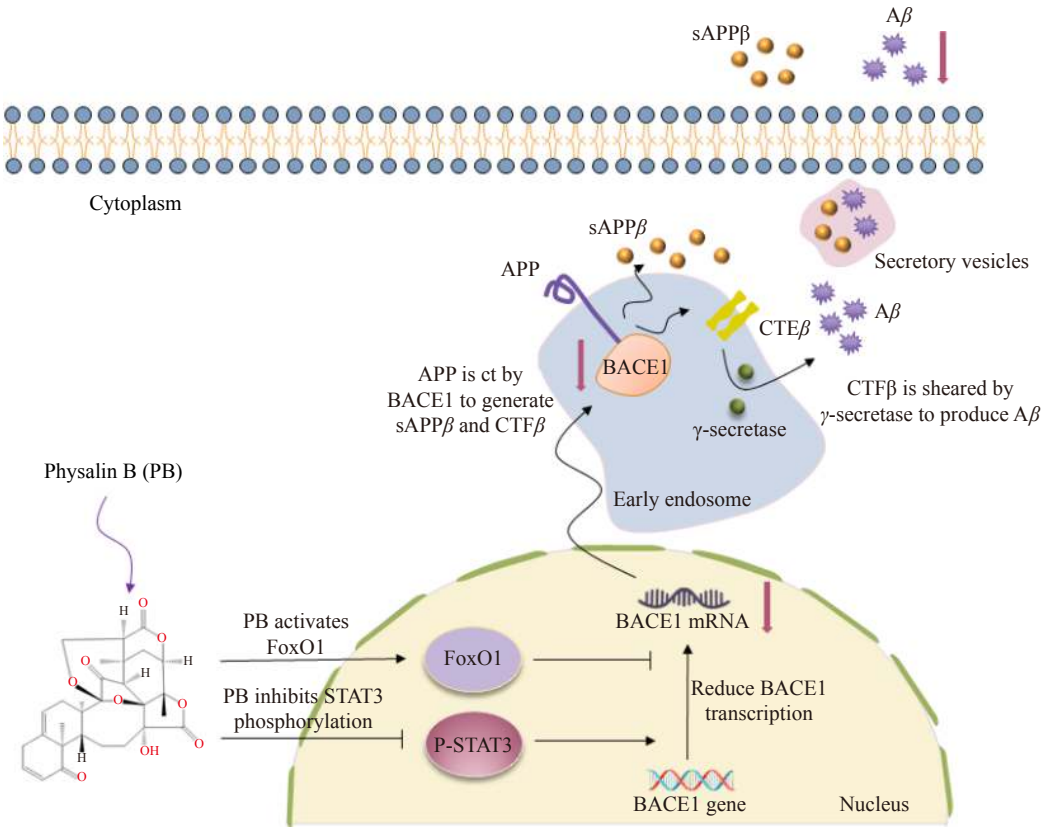

Fig. 7 Scheme summarizing PB regulates BACE1 transcription factors by inducing the activation of FoxO1 and inhibiting the phosphorylation of STAT3

References

[1] Ishii1 R, Canuet L, Aoki Y, *et al*. Healthy and pathological brain aging: from the perspective of oscillations, functional connectivity, and signal complexity [J]. *Neuropsychobiology*, 2017, **75**(4): 151-161.

[2] Wu YT, Ali GC, Guerchet M, *et al*. Prevalence of dementia in mainland China, Hong Kong and Taiwan: an updated systematic review and meta-analysis [J]. *Int J Epidemiol*, 2018, **47**(3): 709-719.

[3] Tiwari S, Atluri1 V, Kaushik A, *et al*. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics [J]. *Int J Nanomedicine*, 2019, **14**: 5541-5554.

[4] Parihar MS, Brewer GJ. Amyloid-β as a modulator of synaptic plasticity [J]. *J. Alzheimers Dis*, 2010, **22**(3): 741-763.

[5] Haass C, Kaether C, Thinakaran G, *et al*. Trafficking and proteolytic processing of APP [J]. *Cold Spring Harb Perspect Med*, 2012, **2**(5): a006270.

[6] Laulagnier K, Javalet C, Hemming FJ, *et al*. Amyloid precursor protein products concentrate in a subset of exosomes specifically endocytosed by neurons [J]. *Cell Mol Life Sci*, 2018, **75**(4): 757-773.

[7] Zhang C, Browne A, Child D, *et al*. Curcumin decreases amyloid-beta peptide levels by attenuating the maturation of amyloid-beta precursor protein [J]. *J Biol Chem*, 2010, **285**(37): 28472-28480.

[8] Citron M. Alzheimer's disease: strategies for disease modification [J]. *Nat Rev Drug Discov*, 2010, **9**(5): 387-398.

[9] Ranjan PS, Faundez V, Fang G, *et al*. Mint3 / X11gamma is an ADP-ribosylation factor-dependent adaptor that regulates the traffic of the Alzheimer's Precursor protein from the trans-Golgi network [J]. *Mol Biol Cell*, 2008, **19**(1): 51-64.

[10] Gu MY, Chun YS, Zhao D, *et al*. Glycyrrhiza uralensis and semilicoisoflavone B reduce Aβ secretion by increasing PPARγ expression and inhibiting STAT3 phosphorylation to inhibit BACE1 expression [J]. *Mol Nutr Food Res*, 2018, **62**(6): e1700633.

[11] Paroni G, Seripa D, Fontana A. *et al*. FOXO1 locus and acetylcholinesterase inhibitors in elderly patients with Alzheimer's disease [J]. *Clin Interv Aging*, 2014, **9**: 1783-1791.

[12] Gross DN, Wan M, Birnbaum MJ. The role of FOXO in the

regulation of metabolism [J]. *Curr Diab Rep*, 2009, **9**(3): 208-214.
[13] Manolopoulos KN, Klotz LO, Korsten P, *et al*. Linking Alzheimer's disease to insulin resistance: the FoxO response to oxidative stress [J]. *Mol Psychiatry*, 2010, **15**(11): 1046-1052.
[14] Zaballos MA, Santisteban P. FOXO1 controls thyroid cell proliferation in response to TSH and IGF-I and is involved in thyroid tumorigenesis [J]. *Mol Endocrinol*, 2013, **27**(1): 50-62.
[15] Wilhelm K, Happel K, Eelen G, *et al*. FOXO1 couples metabolic activity and growth state in the vascular endothelium [J]. *Nature*, 2016, **529**(7585): 216-220.
[16] Maiese K. FoxO proteins in the nervous system [J]. *Anal Cell Pathol (Amst)*, 2015, **2015**: 569392.
[17] Santo EE, Paik J. FOXO in neural cells and diseases of the nervous system [J]. *Curr Top Dev Biol*, 2018, **127**: 105-118.
[18] Zhang W, Bai SS, Yang JH. *et al*. FoxO1 overexpression reduces Aβ production and tau phosphorylation *in vitro* [J]. *Neurosci Lett*, 2020, **738**: 135322.
[19] Jin P, Kim JA, Choi DY, *et al*. Anti-inflammatory and anti-amyloidogenic effects of a small molecule, 2, 4-bis (p-hydroxyphenyl) -2-butenal in Tg2576 Alzheimer's disease mice model [J]. *J Neuroinflammation*, 2013, **10**: 2.
[20] Rebillat ASC, Pace C, Gourmaud S, *et al*. Neuroinflammation and Aβ accumulation linked to systemic inflammation are decreased by genetic PKR down-regulation [J]. *Sci Rep*, 2015, **5**: 8489.
[21] Prasansuklab A, Tencomnao T. Amyloidosis in Alzheimer's disease: The toxicity of Amyloid Beta (Aβ), mechanisms of its accumulation and implications of medicinal plants for therapy [J]. *Evid Based Complement Alternat Med*, 2013, **2013**: 413808.
[22] Choudhary MI, Yousaf S, Ahmed S, *et al*. Antileishmanial physalins from *Physalis minima* [J]. *Chem Biodivers*, 2005, **2**(9): 1164-1173.
[23] Li AL, Chen BJ, Li GH, *et al*. *Physalis alkekengi L. var. franchetii* (Mast.) Makino: An ethnomedical, phytochemical and pharmacological review [J]. *J Ethnopharmacol*, 2018, **210**: 260-274.
[24] Wang A, Wang SP, Zhou FY, *et al*. Physalin B induces cell cycle arrest and triggers apoptosis in breast cancer cells through modulating p53-dependent apoptotic pathway [J]. *Biomed Pharmacother*, 2018, **101**: 334-341.
[25] Mangwala Kimpende P, Lusakibanza M, Mesia K, *et al*. Isolation, pharmacological activity and structure determination of physalin B and 5β, 6β-epoxyphysalin B isolated from *Congolese Physalis angulata L.* [J]. *Acta Crystallogr C*, 2013, **69**(12): 1557-1562.
[26] Soares MBP, Bellintani MC, Ribeiro IM, *et al*. Inhibition of macrophage activation and lipopolysaccaride-induced death by seco-steroids purified from *Physalis angulata L* [J]. *Eur J Pharmacol*, 2003, **459**(1): 107-112.
[27] Fan J J, Zheng X L, Xia H, *et al*. Chemical constituents from whole herb of *Physalis angulata* and their cytotoxic activity [J]. *Chin Tradit Herb Drugs*, 2017, **48**(6): 1080-1086.
[28] Tong YW, Sun Y, Tian XS, *et al*. Phospholipid transfer protein (PLTP) deficiency accelerates memory dysfunction through altering amyloid precursor protein (APP) processing in a mouse model of Alzheimer's disease [J]. *Hum Mol Genet*, 2015, **24**(19): 5388-5403.
[29] Xiang Y, Meng SS, Wang JF, *et al*. Two novel DNA motifs are essential for BACE1 gene transcription [J]. *Sci Rep*, 2014, **4**: 6864.
[30] Liu L, Martin R, Kohler G, *et al*. Palmitate induces transcriptional regulation of BACE1 and presenilin by STAT3 in neurons mediated by astrocytes [J]. *Exp Neurol*, 2013, **248**: 482-490.
[31] Siddiquee K, Zhang S, Guida WC, *et al*. Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity [J]. *Proc Natl Acad Sci U S A*, 2007, **104**(18): 7391-7396.
[32] Yamin G, Ono K, Inayathullah M, *et al*. Amyloid beta-protein assembly as a therapeutic target of Alzheimer's disease [J]. *Curr Pharm Des*, 2008, **14**(30): 3231-3246.
[33] Panza F, Lozupone M, Logroscino G, *et al*. A critical appraisal of amyloid-β-targeting therapies for Alzheimer disease [J]. *Nature Reviews Neurology*, 2019, **15**(2): 73-88.
[34] Verduyckt M, Vignaud H, Bynens T, *et al*. Yeast as a model for Alzheimer's Disease: latest studies and advanced strategies [J]. *Methods Mol Biol*, 2016, **1303**: 197-215.
[35] Nelson PT, Alafuzoff I, Bigio EH, *et al*. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature [J]. *J Neuropathol Exp Neurol*, 2012, **71**(5): 362-381.
[36] Norstrom E. Metabolic processing of the amyloid precursor protein -- new pieces of the Alzheimer's puzzle [J]. *Discov Med*, 2017, **23**(127): 269-276.
[37] Chiba T, Yamada M, Sasabe J, *et al*. Amyloid-beta causes memory impairment by disturbing the JAK2/STAT3 axis in hippocampal neurons [J]. *Mol Psychiatry*, 2009, **14**(2): 206-222.
[38] Chen XF, Zhang YW, Xu H, *et al*. Transcriptional regulation and its misregulation in Alzheimer's disease [J]. *Mol Brain*, 2013, **6**: 44.
[39] Zhou ZD, Chan CH, Ma QH, *et al*. The roles of amyloid precursor protein (APP) in neurogenesis: Implications to pathogenesis and therapy of Alzheimer disease [J]. *Cell Adh Migr*, 2011, **5**(4): 280-292.
[40] Chami L, Checler F. BACE1 is at the crossroad of a toxic vicious cycle involving cellular stress and β-amyloid production in Alzheimer's disease [J]. *Mol Neurodegener*, 2012, **7**: 52.
[41] Takasugi N, Sasaki T, Suzuki K, *et al*. BACE1 activity is modulated by cell-associated sphingosine-1-phosphate [J]. *J Neurosci*, 2011, **31**(18): 6850-6857.
[42] Pivi GAK, Vieira NMdA, da Ponte JB, *et al*. Nutritional management for Alzheimer's disease in all stages: mild, moderate, and severe [J]. *Nutrire*, 2017, **42**: 1.

Cite this article as: ZHANG Wei, BAI Shan-Shan, ZHANG Qi, SHI Ru-Ling, WANG He-Cheng, LIU You-Cai, NI Tian-Jun, WU Ying, YAO Zhao-Yang, SUN Yi, WANG Ming-Yong. Physalin B reduces A*β* secretion through down-regulation of BACE1 expression by activating FoxO1 and inhibiting STAT3 phosphorylation [J]. *Chin J Nat Med*, 2021, **19**(10): 732-740.