Galangin decreases p-tau, $A\beta_{42}$ and β-secretase levels, and suppresses autophagy in okadaic acid-induced PC12 cells via an Akt/GSK3β/mTOR signaling-dependent mechanism

LIPING HUANG[1,2], MINGQIN LIN[2], XIAOQIN ZHONG[3-5], HONGYAN YANG[2] and MINZHEN DENG[3-5]

[1]School of Chemistry and Chemical Engineering, Lingnan Normal University, Zhanjiang, Guangdong 524048; [2]College of Pharmacy, Hainan Medical University, Haikou, Hainan 571199; [3]Department of Neurology, Guangdong Provincial Hospital of Chinese Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine; [4]Guangdong Provincial Academy of Chinese Medical Sciences; [5]The Second Clinical College of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510120, P.R. China

Received January 21, 2018; Accepted October 19, 2018

DOI: 10.3892/mmr.2019.9824

Abstract. Okadaic acid (OA)-induced neurotoxicity may be considered a novel tool used to study Alzheimer's disease (AD) pathology, and may be helpful in the development of a novel therapeutic approach. It has been reported that galangin inhibits β-site amyloid precursor protein-cleaving enzyme 1 expression, which is a key enzyme for amyloid β (Aβ) generation and is a potential drug candidate for AD therapy. However, further studies are required to confirm its neuroprotective effects in other AD models. The present study aimed to explore the neuroprotective effects of galangin on OA-induced neurotoxicity in PC12 cells. The cells were divided into the following groups: Control group, model group (175 nM OA for 48 h) and galangin groups (0.25, 0.5 and 1 μg/ml). Beclin-1, phosphorylated (p)-protein kinase B (Akt), p-glycogen synthase kinase (GSK)3β and p-mechanistic target of rapamycin (mTOR) expression was also measured in the following PC12 cell groups: Control group, model group, 3-methyladenine group (5 nM), rapamycin group (100 nM) and galangin group (1 μg/ml). The levels of β-secretase, $A\beta_{42}$ and p-tau were detected by ELISA, Beclin-1 expression was examined by immunohistochemistry and the protein expression levels of p-Akt, p-mTOR p-GSK3β, and Beclin-1 were detected by western blotting. Galangin treatment enhanced cell viability in cells treated with OA, and decreased β-secretase, $A\beta_{42}$ and p-tau levels. In addition, it suppressed Beclin-1 and p-GSK3β expression, but promoted p-Akt and p-mTOR expression by regulating the Akt/GSK3β/mTOR pathway. These results indicated that galangin protected PC12 cells from OA-induced cytotoxicity and inhibited autophagy via the Akt/GSK3β/mTOR pathway, thus suggesting that it may be considered a potential therapeutic agent for AD.

Correspondence to: Ms. Mingqin Lin, College of Pharmacy, Hainan Medical University, 3 Xueyuan Road, Haikou, Hainan 571199, P.R. China
E-mail: lin-mingqin@163.com

Dr Minzhen Deng, Department of Neurology, Guangdong Provincial Hospital of Chinese Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, 111 Dade Road, Guangzhou, Guangdong 510120, P.R. China
E-mail: dengmz1@126.com

Key words: galangin, Alzheimer's disease, okadaic acid, autophagy, Akt/GSK3β/mTOR pathway, β-secretase

Introduction

Alzheimer's disease (AD) is a common progressive neurodegenerative disease associated with increasing age, which is characterized by the abnormal deposition of senile plaques and intracellular neurofibrillary tangles owing to the formation of extracellular amyloid β (Aβ) protein and hyperphosphorylation of tau protein in the brain (1). Enhanced brain Aβ and amyloid precursor protein (APP) aggregation is closely associated with the development of AD (2). Extracellular amyloid plaques identified in AD, including $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, are derived from the larger APP by β-secretase and γ-secretase (3). An inhibitor of γ-secretase, semagacestat, has been reported to cause neurotoxicity, thus its clinical application is limited; therefore, a safe and effective suppressor of β-secretase may be considered a promising treatment approach (4,5).

Galangin is a natural flavonol, which is extracted from *Alpinia officinarum* (6). It has been reported that galangin exerts anti-inflammatory effects on mast cell-derived allergies and collagen-induced arthritis via the suppression of receptor activator of nuclear factor (NF)-κB ligand-induced activation of Janus kinase, p38 and NF-κB pathways (7). In addition to these effects, galangin has been considered as a potential drug candidate for AD therapy. It may decrease levels of β-secretase and acetylated H3 in the β-secretase promoter regions via upregulation of endogenous histone deacetylase 1 (HDAC1)-mediated deacetylation in SH-SY5Y cells (8).

It has been reported that there is a close association between abnormal degradation of misfolded proteins and

AD pathogenesis (9); overaccumulation of Aβ can lead to it autonomously aggregating into oligomers, which block proteasome function and clog neuronal processes (10). Hyperphosphorylation of tau means it cannot bind to microtubules, thus resulting in self-aggregation of tau into neurofibrillary tangles, which injure axonal transport and normal neuronal function (11). The effects of autophagy in AD are contradictory, or the level of autophagy initiation varies with disease progression. On the one hand, the autophagy-initiation Beclin-1 protein is reduced in early AD (12). However, transcriptional data indicate the factors of autophagy activation were upregulation in AD brains (13). Further studies are required to clarify the level of autophagic activity during the different stages of AD. Okadaic acid (OA) has also been used to study AD in numerous species. OA is able to inhibit protein phosphatase 2A (PP2A) activity, thereby causing tau hyperphosphorylation in the AD brain (14). The present study aimed to determine the potential mechanisms underlying the effects of galangin on autophagy in OA-induced PC12 cells.

Materials and methods

Materials and reagents. Galangin (lot no. 111699-200501) was purchased from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). OA (cat. no. 78111-17-8) was purchased from Shanghai Adamas Reagent Co. Ltd. (Shanghai, China). 3-methyladenine (3MA; cat. no. M9281) and rapamycin (cat. no. V900930) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), PBS and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The ELISA kits for phosphorylated (p)-tau (cat. no. P261FC), β-secretase enzyme (cat. no. B096FC) and $A\beta_{42}$ (cat. no. A227FC) were purchased from Hermes Criterion Biotechnology (Elixir Canada Medicine Company Ltd., Vancouver, Canada).

OA and galangin preparation. OA was diluted to 10 μg/ml with PBS containing 0.001% dimethyl sulfoxide (DMSO), and was stored in sterile microcentrifuge tubes and maintained at 20˚C. For use, it was diluted to various concentrations with high glucose DMEM containing 10% FBS. Galangin was diluted to 1 μg/ml with PBS containing 0.001% DMSO, and was stored in sterile microcentrifuge tubes and maintained at -20˚C. For use, it was diluted to various concentrations with high glucose DMEM medium containing 10% FBS.

Experimental design. Highly differentiated PC12 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were grown in DMEM supplemented with 10% FBS. The cells were incubated at 37˚C, in an atmosphere containing 5% CO_2 for 48 h; the media were replaced daily. Cells were divided into a control group, AD model group, galangin groups (0.25, 0.50 or 1.00 μg/ml galangin), 3MA group (5 nM 3MA) and rapamycin group (100 nM rapamycin). With the exception of the control group cells, PC12 cells in all other groups were treated with OA (175 nM) for 48 h, following pretreatment for 0.5 h with galangin (0.25, 0.50 and 1.00 μg/ml), 3MA (5 nM) or rapamycin (100 nM).

Cell Counting Kit (CCK)-8 assay. Cell viability was determined using a CCK-8 kit (Dojindo Molecular Technologies, Inc., Japan), according to the manufacturer's protocol. The PC12 cell groups with various concentrations of OA (0-250 nM) were seeded at a density of $1x10^5$ cells/well in 96-well plates and were cultured at 37˚C in an atmosphere containing 5% CO_2 for 12, 24 and 48 h. The PC12 cell groups treated with 175 nM OA + galangin (0.25, 0.50, 0.75 and 1.00 μg/ml) were seeded at a density of $1x10^5$ cells/well in 96-well plates and were cultured at 37˚C in an atmosphere containing 5% CO_2 for 48 h. Subsequently, 10 μl CCK-8 (0.5 g/l) was added and the cells were incubated for 2 h at 37˚C in an atmosphere containing 5% CO_2. The optical density (OD) of each group was measured at a wavelength of 450 nm; OD values were used to calculate cell viability.

Analysis of p-tau, $A\beta_{42}$ and β-secretase. The cells were treated as aforementioned prior to ELISA. Briefly, the media from treated PC12 cells were collected and centrifuged at 10,000 x g for 10 min at 4˚C. Subsequently, the supernatant was collected, and p-tau, $A\beta_{42}$ and β-secretase expression was measured using ELISA kits, according to the manufacturer's protocol (Hermes Criterion Biotechnology; Elixir Canada Medicine Company Ltd.).

Western blot analysis of p-Akt, p-GSK3β, p-mTOR and Beclin-1 expression. PC12 cells were harvested and lysed using phenylmethanesulfonyl fluoride lysis buffer (Sigma-Aldrich; Merck KGaA). The lysates were incubated for 30 min at 4˚C, centrifuged at 13,000 x g for 15 min at 4˚C, and total protein was extracted and quantified using a bicinchoninic acid kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, 40 μg protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% bovine serum albumin (BSA; cat. no. 810652, Merck KGaA) for 1 h at 4˚C and were incubated with primary antibodies against GAPDH (cat. no. ab8245; Abcam), p-Akt (cat. no. 9275S; Cell Signaling Technology, Inc., Danvers, MA, USA), Akt (cat no. 4691S; Cell Signaling Technology, Inc.), p-GSK3β (cat. no. ab75745; Abcam), GSK3β (cat. no. ab131356; Abcam), p-mTOR (cat. no. ab109268; Abcam), mTOR (cat. no. ab134903; Abcam) and Beclin-1 (cat. no. ab62557; Abcam) overnight at 4˚C (all 1:1,000). Subsequently, the blots were washed and incubated with their respective horseradish peroxidase (HRP)-conjugated immunoglobulin G (anti-mouse and anti-rabbit) secondary antibodies (1:2,000; cat nos. 7076S and 7074S, respectively; Cell Signaling Technology, Inc.) at room temperature for 1 h. GAPDH was used as an internal control. Bound secondary antibodies were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using Image Lab 4.1 software (Bio-Rad Laboratories, Inc.). Western blotting was repeated at least three times for each condition. After development, the band intensities were semi-quantified with Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Immunocytochemistry. The cells ($1x10^6$ cells/well) were treated with cell culture medium (control group), 175 nM OA (model group), 3MA (5 nM), rapamycin (100 nM) or

galangin (1 μg/ml) + 175 nM OA for 48 h. Following fixation in 4% paraformaldehyde at 4˚C for 60 min, the cells were washed with PBS and incubated with 300 μl BSA for 20 min at room temperature. Subsequently, cells were incubated with anti-Beclin-1 antibody (cat. no. ab62557; 1:50 dilution; Abcam) for 1 h at 37˚C. After washing with PBS, cells were incubated with HRP-conjugated anti-rabbit immunoglobulin (Ig)G (1:200; cat no. 7074S; Cell Signaling Technology, Inc.) for 20 min at 37˚C. The cells were washed again with PBS and amplified with avidin biotin-peroxidase complex (ABC) labeling by adding 300 μl streptavidin (S)ABC (cat no. SA1021; Wuhan Boster Biological Technology, Ltd.) for 20 min overnight. Thereafter, the cells were washed and were stained with 300 μl 3,3'-diaminobenzidine (cat no. AR1022; Wuhan Boster Biological Technology, Ltd.) for 5 min at room temperature. Subsequently, the nuclei were stained with hematoxylin at room temperature for 5 min. Finally, images were obtained using a light microscope (U-SPT; Olympus Corporation, Tokyo, Japan). Brown staining indicated positive expression, and the darker the color, the more expression of Beclin-1. Data were analyzed using ImageJ software v1.48 (National Institutes of Health, Bethesda, MD, USA); the integrated optical density (IOD) of the positive neurons and the area of image were used to calculate the average optical density (AOD), according the following formula:

$$\mathrm{AOD} = \frac{IOD}{Area} \times 100$$

Immunofluorescence. The cells (1x10^6 cells/well) were treated with cell culture medium (control), 175 nM OA (model), 3MA (5 nM), rapamycin (100 nM) or galangin (1 μg/ml) + 175 nM OA for 48 h. After fixation in 4% paraformaldehyde for 60 min at 4˚C, cells were washed with PBS and 300 μl BSA was added for 20 min at room temperature. Subsequently, the cells were incubated with rabbit anti-Beclin-1 antibody (1:50 dilution; Abcam) for 1 h at 37˚C. After washing with PBS, cells were incubated with Alexa 488-conjugated anti-rabbit IgG (1:200; cat no. 4412S; Cell Signaling Technology, Inc.) for 30 min at 37˚C. The cells were washed again with PBS and 300 μl SABC-fluorescein isothiocyanate was added for 30 min at 37˚C in the dark. Thereafter, the cells were washed and 300 μl DAPI was added for 5 min at room temperature to stain the nuclei (Wuhan Boster Biological Engineering, Wuhan, China). Finally, images of the cells were captured using a light microscope (U-SPT; Olympus, Tokyo, Japan). Data were analyzed using ImageJ software v1.48 (National Institutes of Health); the AOD was calculated as aforementioned.

Statistical analysis. Data are expressed as the means ± standard deviation, and significant differences among different groups were determined by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. The experiments were repeated at least three times. Correlations among p-tau, Aβ_{42}, β-secretase, p-Akt, p-GSK3β, p-mTOR and Beclin-1 expression were determined using Pearson's correlation analysis. All statistical analyses were performed using SPSS statistical software version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of OA on the viability rates of PC12 cells. The effects of various concentrations of OA on the viability rates of PC12 cells are shown in Fig. 1. Cell viability rates were similar at 12 and 24 h, but were reduced at 48 h following exposure to 50 and 175-225 nM OA. In addition, the cell viability rates were highest at 24 h, followed by at 12 and 48 h following treatment with 75-150 nM OA, and were highest at 12 h, followed by at 24 and 48 h following treatment with 225-250 nM OA. Therefore, treatment with 175 nM OA for 48 h was selected in this experiment, as it reduced viability by 50%.

Effects of various concentrations of galangin on OA-induced cytotoxicity in PC12 cells. PC12 cells were incubated with various concentrations of galangin (0.25, 0.50, 0.75 and 1.00 μg/ml) in the presence of 175 nM OA for 48 h, and CCK-8 assays were used to detect the effect of galangin. The results demonstrated that, compared with in the control group, OA decreased cell viability, whereas galangin significantly increased the viability of PC12 cells in a concentration-dependent manner (P<0.01; Fig. 2). These results indicated that galangin may alleviate cellular damage caused by OA, thus suggesting that galangin has a neuroprotective effect.

Galangin decreases β-secretase, p-tau and Aβ42 levels. The cells were treated as aforementioned and media were collected for p-tau, Aβ_{42} and β-secretase measurement. p-tau, Aβ_{42} and β-secretase levels were increased in the model group compared with in the control group (P<0.01). Conversely, p-tau, Aβ_{42} and β-secretase levels were significantly decreased in the galangin-treated groups compared with in the model group (P<0.05). The findings indicated that p-tau, Aβ_{42} and β-secretase levels were reduced with increases in the dose of galangin (Fig. 3).

Effects of galangin on the expression levels of p-Akt, p-GSK3β, p-mTOR and Beclin-1 in PC12 cells treated with OA. The cells were treated as aforementioned, with untreated cells used as a control. The expression levels of p-Akt and p-mTOR were decreased (P<0.01), whereas the expression levels of p-GSK3β and Beclin-1 were increased (P<0.05), in the model group compared with in the control group. In addition, galangin evidently increased p-Akt and p-mTOR expression (P<0.01), and significantly attenuated p-GSK3β and Beclin-1 expression (P<0.01), compared with in the model group. In addition, pretreatment with the autophagy inhibitor 3MA significantly increased p-Akt and p-mTOR expression, but decreased p-GSK3β and Beclin-1 expression, compared with in the model group (P<0.01). Conversely, pretreatment with the autophagy activator rapamycin significantly reduced p-Akt and p-mTOR expression, but increased p-GSK3β and Beclin-1 expression compared with in the model group (P<0.05; Fig. 4).

Galangin inhibits OA-induced autophagy in PC12 cells. The detailed mechanism underlying the suppressive effects of galangin was investigated. OA significantly increased Beclin-1 expression compared with in the control group (P<0.05). Conversely, pretreatment with galangin prior to exposure to OA significantly suppressed Beclin-1 expression (P<0.01). In

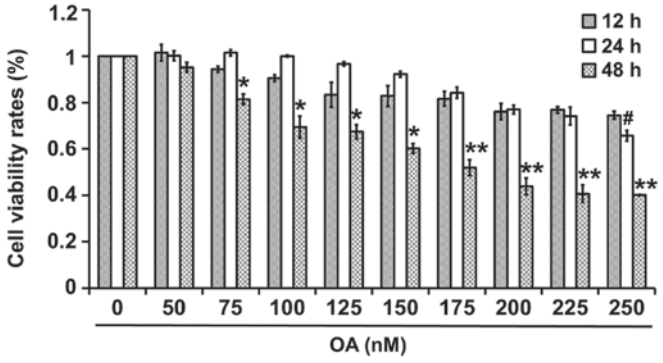

Figure 1. Effects of various concentrations of OA on the viability rates of PC12 cells. Cell viability rates were decreased in a dose-dependent manner; 175 nM OA was selected for subsequent experiments, as it reduced viability by 50%. *P<0.05, **P<0.01 vs. the 0 nM group in 48 h. $^{\#}$P<0.05 vs. the 0 nM group in 24 h. OA, okadaic acid.

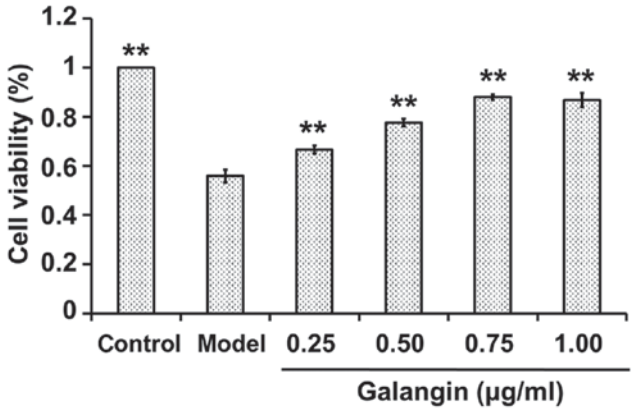

Figure 2. Viability of OA-stimulated PC12 cells treated with various concentrations of galangin (0.25, 0.50, 0.75 and 1.00 μg/ml) for 48 h. **P<0.01 vs. the model group. OA, okadaic acid.

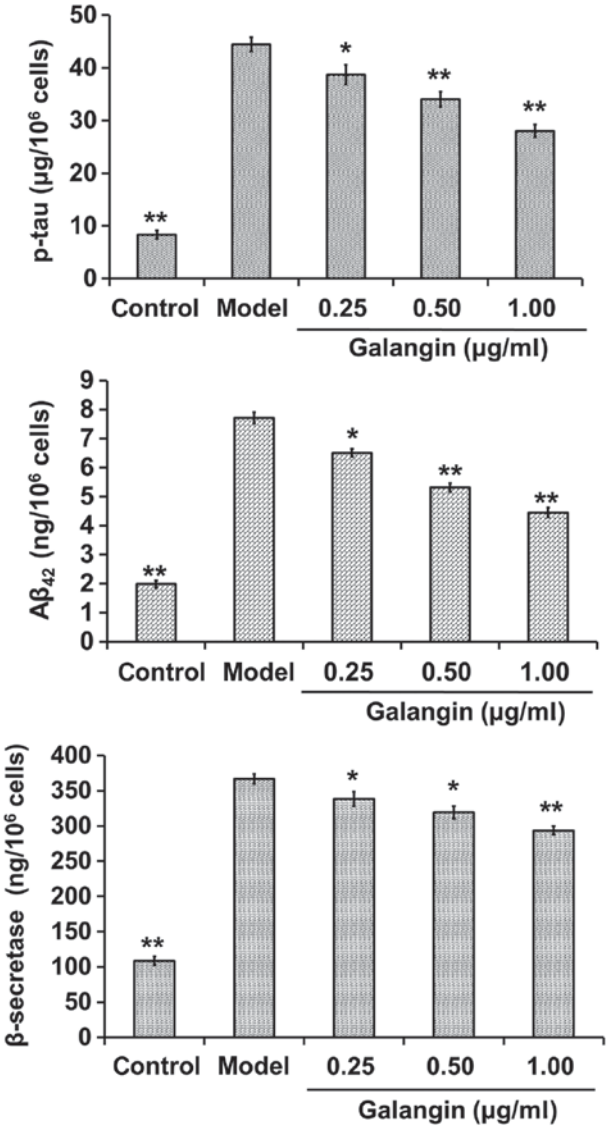

Figure 3. Galangin decreases β-secretase, p-tau and $A\beta_{42}$ levels. PC12 cells were pretreated with or without galangin (0.25, 0.50 and 1.00 μg/ml) for 0.5 h prior to treatment with OA (175 nM) for 48 h. The media were collected for β-secretase, p-tau and $A\beta_{42}$ measurement using ELISA. Data are expressed as the means ± standard deviation. *P<0.05, **P<0.01 vs. the model group. Aβ, amyloid β; OA, okadaic acid; p, phosphorylated.

addition, pretreatment with the autophagy activator rapamycin promoted activation of Beclin-1 expression (P<0.05), whereas pretreatment with the autophagy inhibitor 3MA suppressed Beclin-1 expression (P<0.01; Fig. 5).

Correlation analysis. Finally, the associations between p-tau, $A\beta_{42}$, β-secretase, p-Akt, p-GSK3β, p-mTOR and Beclin-1 expression levels were investigated using Pearson correlation analysis (Table I). Pearson analysis demonstrated that there were significant positive correlations between p-tau and $A\beta_{42}$/β-secretase/p-GSK3β (0.839/0.733/0.789, respectively; P<0.01), $A\beta_{42}$ and β-secretase/p-GSK3β/Beclin-1 (0.978/0.507/0.618, respectively; P<0.01 or P<0.05), β-secretase and Beclin-1 (0.701; P<0.05), and p-Akt and p-mTOR (0.826; P<0.01). In addition, there were significant negative correlations between β-secretase and p-Akt/p-mTOR (-0.692/-0.715; P<0.05), p-Akt and Beclin-1 (-0.990; P<0.01), and p-mTOR and p-GSK3β/Beclin-1 (-0.761/-0.918; P<0.01).

Discussion

Autophagy is an essential degradation pathway involved in the clearance of abnormal protein aggregates and protein homeostasis in neuronal and non-neuronal cells (15). A previous study suggested that autophagy initiation by Beclin-1 may be involved in the pathogenesis of AD (16). Targeting Aβ, tau and β-secretase has long been believed to be a viable strategy for drug discovery against AD (13). Aβ has been reported to be associated with autophagy, and is able to induce reactive oxygen species (ROS) accumulation and apoptotic cell death (17). Excessive ROS production has been suggested to serve a role in the initiation of autophagic cell death (18). In addition, $A\beta_{1\text{-}42}$ can induce autophagic cell death in human glioma and human neuroblastoma cell lines (19). The Aβ-loading of autophagosomes could lead not only of the extracellular secretion of Aβ, but of its lysosomal degradation, in which APP, β-secretase (BACE1) and γ-secretase reside. Studies have confirmed that autophagy is essential for Aβ secretion effectively, since loss of the Atg7 gene in APP transgenic mice results in reduced extracellular

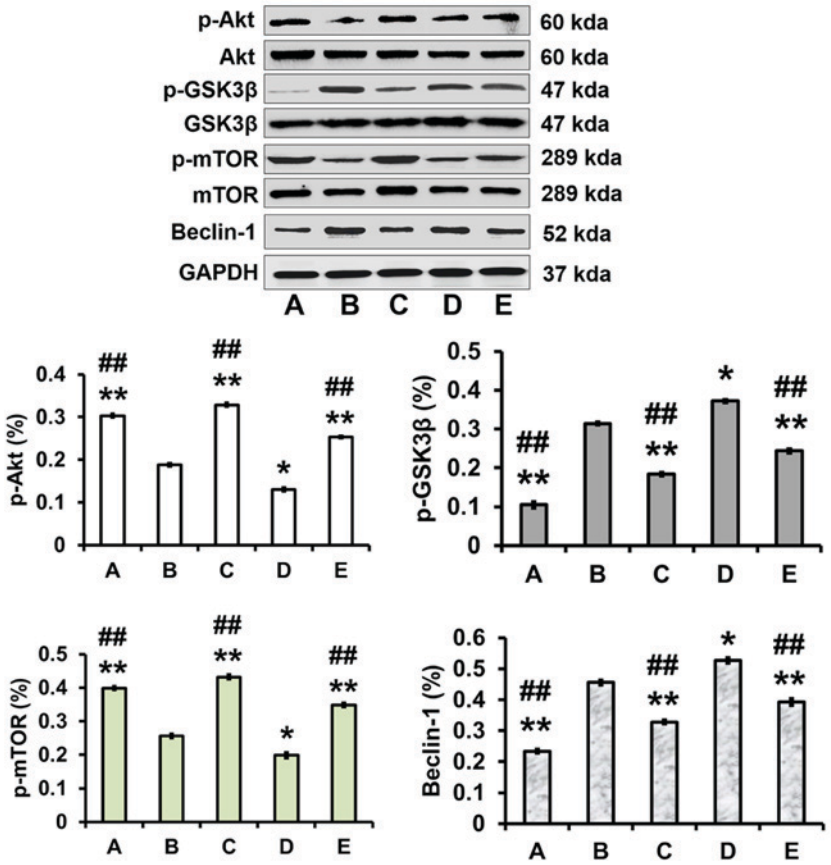

Figure 4. Effects of galangin on the protein expression levels of p-Akt, p-GSK3β, p-mTOR and Beclin-1. PC12 cells were pretreated with 3MA (5 nM), rapamycin (100 nM) and galangin (1.00 μg/ml) for 0.5 h prior to exposure to OA (175 nM) for 48 h. Protein expression levels of p-Akt and p-mTOR were significantly increased; however, p-GSK3β and Beclin-1 protein expression was clearly decreased in response to galangin. Representative western blot images are shown. A, control group; B, model group; C, 3-methyladenine group; D, rapamycin group; E, galangin group. Data are expressed as the means ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the model group; $^{\#\#}$P<0.01 vs. the rapamycin group. Akt, protein kinase B; GSK, glycogen synthase kinase; mTOR, mechanistic target of rapamycin; OA, okadaic acid; p, phosphorylated.

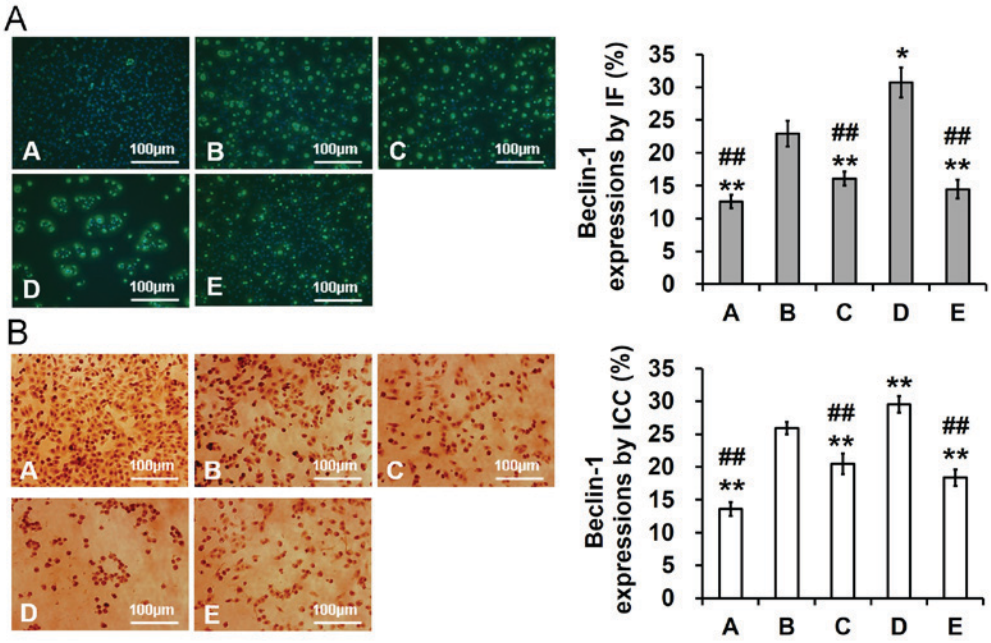

Figure 5. Effects of galangin on Beclin-1 expression in OA-stimulated PC12 cells (magnification, x200). Cells were pretreated (0.5 h) with 5 nM 3MA, 100 nM rapamycin and 1.00 μg/ml galangin and were then exposed to OA (175 nM) for 48 h. (A) Beclin-1 expression, as determined by IF and AOD analysis. (B) Beclin-1 expression, as determined by immunocytochemistry and AOD analysis. Data are expressed as the means ± standard deviation. *P<0.05, **P<0.01 vs. the model group; $^{\#\#}$P<0.01 vs. the rapamycin group. A, control group; AOD, average optical density; B, model group; C, 3-methyladenine group; D, rapamycin group; E, galangin group; IF, immunofluorescence; OA, okadaic acid.

Table I. Correlations between p-tau, $A\beta_{42}$, β-secretase, p-Akt, p-GSK3β, p-mTOR and Beclin-1 in the OA model group.

	Gene						
Gene	p-tau	$A\beta_{42}$	β-secretase	p-Akt	p-GSK3β	p-mTOR	Beclin-1
p-tau	1.000	0.839[b]	0.733[b]	-0.231	0.789[b]	-0.282	0.240
$A_{\beta 42}$		1.000	0.978[b]	-0.622[a]	0.507[a]	-0.688[a]	0.618[a]
β-secretase			1.000	-0.692[a]	0.434	-0.715[a]	0.701[a]
p-Akt				1.000	-0.171	0.826[b]	-0.990[b]
p-$GSK3_{\beta}$					1.000	-0.761[b]	0.242
p-mTOR						1.000	-0.918[b]
Beclin-1							1.000

[a]P<0.05 (two-tailed) and [b]P<0.01 (two-tailed). Aβ, amyloid β; Akt, protein kinase B; GSK, glycogen synthase kinase; mTOR, mechanistic target of rapamycin; OA, okadaic acid; p, phosphorylated.

Aβ plaques and promoted Aβ accumulation (20,21). Research revealed that autophagy plays dual roles in the release of Aβ to the extracellular space. Exposing the neurons to rapamycin, autophagy enhanced and Aβ secretion decreased. Conversely, inhibition of autophagy by spautin-1, which decreased Aβ secretion (22). According to one study, age-induced reduction of autophagy-related gene expression is associated with onset of AD and tau seems to mediate the neuronal toxicity of Aβ (23). Pimozide reduces toxic forms of tau in tauC3 mice via AMP-activated protein kinase-mediated autophagy (24). AD is the most common neurodegenerative disorder in the elderly, and it has previously been confirmed that BACE1 at the mRNA and protein level is decreased in SH-SY5Y cells treated with galangin (8). However, to the best of our knowledge, the effects and mechanism of galangin on autophagy in the OA-induced PC12 cell model has not been reported.

In vivo and *in vitro*, OA leads to Aβ deposition and subsequent neuronal degeneration, synaptic loss, memory impairment and tau hyperphosphorylation, all of which resemble AD pathology (25,26). OA also induces neurotoxicity of the SH-SY5Y cell line and cultured neuronal cells, resulting in the development of pathological conditions similar to AD (27). Neurotoxicity induced by OA, which is associated with abnormally phosphorylated tau protein, promotes AD-like pathology (28). The results of the present study indicated that there was evident neurotoxicity in PC12 cells treated with OA (75-250 nM) for 48 h. In addition, galangin exerted dose-dependent neuroprotective effects on PC12 cells treated with 175 nM OA.

Aβ is generated through serial cleavage of APP. β-secretase is a key enzyme for Aβ production, and is a promising target for AD therapy (29). OA-induced PP2A gene regulation and kinase remodeling serve a crucial role in the generation of abnormal p-tau, which results in neurotoxicity and an AD-like pathology (30). To investigate the neuroprotective effects of galangin on OA-induced PC12 cell injury, β-secretase, p-tau and $A\beta_{42}$ levels were detected following treatment with galangin (0.5 h) and OA (48 h). A recent study indicated that galangin reduces β-secretase at the mRNA and protein level, and Aβ levels in SH-SY5Y cells through the upregulation of endogenous HDAC1-mediated deacetylation (8). The results of the present study also demonstrated that galangin increased cell viability and significantly decreased β-secretase, p-tau and $A\beta_{42}$ levels. These results indicated that pretreatment with galangin protected PC12 cells from OA-induced cytotoxicity.

The mechanism underlying the effects of galangin on OA-induced PC12 cell autophagy was also examined. Autophagy serves an important role in clearing Aβ aggregates and preserving neuronal function in AD (31). Beclin-1 is an autophagy-associated protein, which is involved in the initiation of autophagy (32). It has previously been reported that Beclin-1 deficiency increases the expression of APP and APP-like proteins, accelerates Aβ accumulation and promotes neurodegeneration in mice, indicating that Beclin-1 may be involved in the pathogenesis of AD (33); the present study results in OA-induced PC12 cells are consistent with this finding. The present results demonstrated that OA could activate autophagy and increase the expression of Beclin-1. Conversely, galangin significantly decreased Beclin-1 expression, thus suggesting that galangin may suppress OA-induced autophagy. In other words, galangin has the pharmacological effect of alleviating AD: the autophagy of the model group is up-regulated, and the autophagy is down-regulated after treatment with galangin. It is concluded that galangin has a certain effect of autophagy inhibition to OA induced PC12 cells. Furthermore, Beclin-1 expression was significantly reduced in response to pretreatment with the autophagy inhibitor 3MA; however, Beclin-1 expression was significantly enhanced following pretreatment with the autophagy activator rapamycin.

To elucidate the precise molecular mechanism underlying the effects of galangin on autophagy in OA-treated PC12 cells, the present study focused on the Akt/mTOR and Akt/GSK-3β pathways. Akt has a key role in regulating cell signals that are important for cell death and survival (34). mTOR, which functions downstream of Akt, is mainly mediated by phosphoinositide 3-kinase/Akt signaling transduction and suppresses autophagy (35); therefore, the present study used the classic mTOR inhibitor rapamycin to investigate the effects of mTOR on autophagy. GSK3, as an integrator of Akt and Wnt signals, also serves a central role in the regulation of mTOR during synaptic plasticity (36). GSK3β has also

been identified as serving a prominent role in the formation of abnormal protein accumulation, which is important in AD (37). The present study revealed that p-Akt and p-mTOR levels were decreased, whereas p-GSK3β was increased in the OA treatment group compared with in the normal control group. However, galangin increased levels of p-Akt and p-mTOR, but reduced levels of p-GSK3β, compared in with the OA treatment group. These results indicated that galangin attenuated autophagy through modulation of the Akt/GSK3β/mTOR pathway.

Finally, significant positive correlations were detected in the present study, including between Beclin-1 and β-secretase, and $A\beta_{42}$ and p-GSK3β/p-tau, whereas there were significant negative correlations between Beclin-1 and p-Akt/p-mTOR, β-secretase and p-Akt/p-mTOR, and $A\beta_{42}$ and p-Akt/p-mTOR. These findings indicated that galangin decreased β-secretase, $A\beta_{42}$, p-tau and Beclin-1 levels, which may interact with the Akt/GSK3β/mTOR pathway in PC12 cells following OA treatment.

In conclusion, galangin attenuated OA-induced autophagy via the Akt/GSK3β/mTOR pathway. Galangin promoted Akt and mTOR phosphorylation, but suppressed GSK3β phosphorylation, which served to inhibit autophagy. The findings of the present study indicated that galangin may be a potential preventive drug for AD.

Acknowledgements

Not applicable.

Funding

The present study was supported by The Lingnan Normal University-Level Talent Project (grant no. ZL1801), The Natural Science Foundation of Guangdong Province of China (grant nos. 2018A030307037, 2016A030310362 and 2017A030310604), The Hainan Natural Science Foundation of China (grant nos. 20168266 and 817140), The Program of Hainan Association for Science and Technology Plans to Youth R&D Innovation (grant no. HAST201635), The Scientific Research Cultivating Fund of Hainan Medical University (grant nos. HY2015-01 and HY2016-02), The Scientific Research Project of Guangdong Provincial Administration of Traditional Chinese Medicine (grant nos. 20181114) and The National Natural Science Foundation of China (grant no. 201705071).

Availability of data and materials

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

Authors' contributions

LH and MD conceived the study, designed the experiments, analyzed the data and prepared the manuscript. LH, ML, XZ, HY and MD selected the subjects and obtained samples for the present study. XZ, MD and LH performed the experiments. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

References

1. Swomley AM, Förster S, Keeney JT, Triplett J, Zhang Z, Sultana R and Butterfield DA: Abeta, oxidative stress in Alzheimer disease: Evidence based on proteomics studies. Biochim Biophys Acta 1842: 1248-1257, 2014.
2. Guerreiro RJ, Gustafson DR and Hardy J: The genetic architecture of Alzheimer's disease: Beyond APP, PSENs and APOE. Neurobiol Aging 33: 437-456, 2012.
3. Yen CH, Wang CH, Wu WT and Chen HL: Fructo-oligosaccharide improved brain β-amyloid, β-secretase, cognitive function, and plasma antioxidant levels in D-galactose-treated Balb/cJ mice. Nutr Neurosci 20: 228-237, 2017.
4. Wolfe MS: Processive proteolysis by γ-secretase and the mechanism of Alzheimer's disease. Biol Chem 393: 899-905, 2012.
5. Imbimbo BP and Giardina GA: γ-secretase inhibitors and modulators for the treatment of Alzheimer's disease: Disappointments and hopes. Curr Top Med Chem 11: 1555-1570, 2011.
6. Wang X, Gong G, Yang W, Li Y, Jiang M and Li L: Antifibrotic activity of galangin, a novel function evaluated in animal liver fibrosis model. Environ Toxicol Pharmacol 36: 288-295, 2013.
7. Huh JE, Jung IT, Choi J, Baek YH, Lee JD, Park DS and Choi DY: The natural flavonoid galangin inhibits osteoclastic bone destruction and osteoclastogenesis by suppressing NF-κB in collagen-induced arthritis and bone marrow-derived macrophages. Eur J Pharmacol 698: 57-66, 2013.
8. Zeng H, Huang P, Wang X, Wu J, Wu M and Huang J: Galangin-induced down-regulation of BACE1 by epigenetic mechanisms in SH-SY5Y cells. Neuroscience 294: 172-181, 2015.
9. Penke B, Bogár F and Fülöp L: Protein folding and misfolding, endoplasmic reticulum stress in neurodegenerative diseases: In trace of novel drug targets. Curr Protein Pept Sci 17: 169-182, 2016.
10. Viola KL and Klein WL: Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis. Acta Neuropathol 129: 183-206, 2015.
11. Guo JL and Lee VM: Seeding of normal Tau by pathological Tau conformers drives pathogenesis of Alzheimer-like tangles. J Biol Chem 286: 15317-15331, 2011.
12. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B and Wyss-Coray T: The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest 118: 2190-2199, 2008.
13. Lipinski MM, Zheng B, Lu T, Yan Z, Py BF, Ng A, Xavier RJ, Li C, Yankner BA, Scherzer CR and Yuan J: Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc Natl Acad Sci USA 107: 14164-14169, 2010.
14. Kamat PK and Nath C: Okadaic acid: A tool to study regulatory mechanisms for neurodegeneration and regeneration in Alzheimer's disease. Neural Regen Res 10: 365-367, 2015.
15. Li Q, Liu Y and Sun M: Autophagy and Alzheimer's disease. Cell Mol Neurobiol 37: 377-388, 2017.
16. Salminen A, Kaarniranta K, Kauppinen A, Ojala J, Haapasalo A, Soininen H and Hiltunen M: Impaired autophagy and APP processing in Alzheimer's disease: The potential role of Beclin 1 interactome. Prog Neurobiol 106-107: 33-54, 2013.
17. Li RC, Pouranfar F, Lee SK, Morris MW, Wang Y and Gozal D: Neuroglobin protects PC12 cells against beta-amyloid-induced cell injury. Neurobiol Aging 29: 1815-1822, 2008.

18. Chen Y, McMillan-Ward E, Kong J, Israels SJ and Gibson SB: Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 15: 171-182, 2008.
19. Wang H, Ma J, Tan Y, Wang Z, Sheng C, Chen S and Ding J: Amyloid-beta1-42 induces reactive oxygen species-mediated autophagic cell death in U87 and SH-SY5Y cells. J Alzheimers Dis 21: 597-610, 2010.
20. Cataldo AM, Petanceska S, Terio NB, Peterhoff CM, Durham R, Mercken M, Mehta PD, Buxbaum J, Haroutunian V and Nixon RA: Abeta localization in abnormal endosomes: Association with earliest Abeta elevations in AD and Down syndrome. Neurobiol Aging 25: 1263-1272, 2004.
21. LaFerla FM, Green KN and Oddo S: Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8: 499-509, 2007.
22. Choy RW, Cheng Z and Schekman R: Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. Proc Natl Acad Sci USA 109: E2077-E2082, 2012.
23. Omata Y, Lim YM, Akao Y and Tsuda L: Age-induced reduction of autophagy-related gene expression is associated with onset of Alzheimer's disease. Am J Neurodegener Dis 3: 134-142, 2014.
24. Kim YD, Jeong EI, Nah J, Yoo SM, Lee WJ, Kim Y, Moon S, Hong SH and Jung YK: Pimozide reduces toxic forms of tau in TauC3 mice via 5' adenosine monophosphate-activated protein kinase-mediated autophagy. J Neurochem 142: 734-746, 2017.
25. Cakir M, Duzova H, Tekin S, Taslıdere E, Kaya GB, Cigremis Y, Ozgocer T, Yologlu S: ACA, an inhibitor phospholipases A2 and transient receptor potential melastatin-2 channels, attenuates okadaic acid induced neurodegeneration in rats. Life Sci 176: 10-20, 2017.
26. Wu X, Kosaraju J and Tam KY: SLM, a novel carbazole-based fluorophore attenuates okadaic acid-induced tau hyperphosphorylation via down-regulating GSK-3β activity in SH-SY5Y cells. Eur J Pharm Sci 110: 101-108, 2017.
27. Zhang J, Cheng Y and Zhang JT: Protective effect of (-) clausenamide against neurotoxicity induced by okadaic acid and beta-amyloid peptide25-3. Yao Xue Xue Bao 42: 935-942, 2007 (In Chinese).
28. Kamat PK, Rai S and Nath C: Okadaic acid induced neurotoxicity: An emerging tool to study Alzheimer's disease pathology. Neurotoxicology 37: 163-172, 2013.
29. Zhao XJ, Gong DM, Jiang YR, Guo D, Zhu Y and Deng YC: Multipotent AChE and BACE-1 inhibitors for the treatment of Alzheimer's disease: Design, synthesis and bio-analysis of 7-amino-1,4-dihydro-2H-isoquilin-3-one derivates. Eur J Med Chem 138: 738-747, 2017.
30. Yang CC, Kuai XX, Gao WB, Yu JC, Wang Q, Li L and Zhang L: Morroniside-induced PP2A activation antagonizes Tau hyperphosphorylation in a cellular model of neurodegeneration. J Alzheimers Dis 51: 33-44, 2016.
31. Aminyavari S, Zahmatkesh M, Farahmandfar M, Khodagholi F, Dargahi L and Zarrindast MR: Protective role of Apelin-13 on amyloid β25-35-induced memory deficit; Involvement of autophagy and apoptosis process. Prog Neuropsychopharmacol Biol Psychiatry 89: 322-334, 2019.
32. Yu J, Lan L, Lewin SJ, Rogers SA, Roy A, Wu X, Gao P, Karanicolas J, Aubé J, Sun B and Xu L: Identification of novel small molecule Beclin 1 mimetics activating autophagy. Oncotarget 8: 51355-51369, 2017.
33. Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E and Wyss-Coray T: Regulation of amyloid precursor protein processing by the Beclin 1 complex. PLoS One 5: e11102, 2010.
34. Hotfilder M, Sondermann P, Senss A, van Valen F, Jürgens H and Vormoor J: PI3K/AKT is involved in mediating survival signals that rescue Ewing tumour cells from fibroblast growth factor 2-induced cell death. Br J Cancer 92: 705-710, 2005.
35. Du C, Zhang T, Xiao X, Shi Y, Duan H and Ren Y: Protease-Activated Receptor-2 promotes kidney tubular epithelial inflammation by inhibiting autophagy via the PI3K/Akt/mTOR signalling pathway. Biochem J 474: 2733-2747, 2017.
36. Ma T, Tzavaras N, Tsokas P, Landau EM and Blitzer RD: Synaptic stimulation of mTOR is mediated by Wnt signaling and regulation of glycogen synthetase kinase-3. J Neurosci 31: 17537-17546, 2011.
37. Yang S, Chen Z, Cao M, Li R, Wang Z and Zhang M: Pioglitazone ameliorates Aβ42 deposition in rats with diet-induced insulin resistance associated with AKT/GSK3β activation. Mol Med Rep 15: 2588-2594, 2017.