Protocatechuic acid attenuates β-secretase activity and okadaic acid-induced autophagy via the Akt/GSK-3β/MEF2D pathway in PC12 cells

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Abstract. Okadaic acid (OA) can be used to induce an Alzheimer's disease (AD) model characterized by tau hyperphosphorylation, the formation of neurofibrillary tangles formation and β-amyloid (Aβ) deposition. Previous studies have shown that the upregulation of Beclin-1-dependent autophagy may contribute to the elimination of aggregated Aβ. However, the effects of protocatechuic acid (PA) on the levels of $Aβ_{42}$, phosphorylated (p)-tau and β-secretase in OA-induced cell injury are unclear, and little is known concerning the role of the PA signaling pathway in the regulation of autophagy. The present study aimed to determine whether PA protects cells from OA-induced cytotoxicity via the regulation of Beclin-1-dependent autophagy and its regulatory signaling pathway. PC12 cells were treated with OA with or without PA for 24 h. Enzymatic assays were performed to measure p-tau, Aβ42 and β-secretase activity. Western blotting was performed to detect p-Akt, p-glycogen synthase kinase-3β (p-GSK-3β), Akt, GSK-3β, myocyte enhancer factor 2D (MEF2D) and Beclin-1 protein expression levels. Immunofluorescence and immunocytochemistry were used to measure Beclin-1 expression levels. The results from this study showed that PA could increase cell viability and significantly decrease the levels of $Aβ_{42}$, p-tau, β-secretase and Beclin-1. PA can also promote the expression of p-Akt and MEF2D while suppressing the expression of p-GSK-3β. These results indicated that PA protects PC12 cells from OA-induced cytotoxicity, and attenuates autophagy via regulation of the Akt/GSK-3β/MEF2D pathway, therefore potentially contributing to the neuroprotective effects of PA against OA toxicity. These findings suggested that PA may have potential as a drug candidate in preventative AD therapy.

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Introduction

Alzheimer's disease (AD) is one of the most debilitating neurodegenerative diseases (1); it is characterized by the excessive accumulation of β-amyloid (Aβ) and severe neuronal loss in the brains of patients with AD (1). The microtubule-associated protein tau is an axonal phosphoprotein. It has been shown that total tau, phosphorylated (p)-tau and $Aβ_{42}$ are key biomarkers for AD pathophysiology, and the deposition of peptides into plaques in patients with AD is closely associated with neuronal degeneration, p-tau and Aβ aggregation (2). Additionally, β-secretase is a key protease that controls the formation of the Aβ peptide, which is hypothesized to be a key mediator of the amyloid-driven pathology of AD (3).

In vitro and *in vivo* studies have suggested that protocatechuic acid (PA), a phenolic compound from *Radix Salviae miltiorrhizae*, may be an effective neuroprotective agent for AD therapy. PA has a protective effect in cultured rat cortical neurons against $Aβ_{25-35}$-induced cytotoxicity, and can inhibit the mRNA expression of amyloid precursor protein (APP) in double-transfected [human *APP* gene and presenilin-1 (*PS1*) gene] Chinese hamster ovary cells (M146L) (4,5). Moreover, PA has also been shown to improve cognitive deficits and increase the level of brain-derived neurotrophic factor, as well as attenuating amyloid deposits and the inflammatory response in aged APP/PS1 double transgenic mice (6). Previous studies have shown that PA is an efficient and safe substance in the prevention of AD progression (4-6).

Autophagy is a cellular process that degrades proteins and recycles cellular components (7). There are three main types of autophagy in mammals: Macroautophagy; microautophagy; and chaperone-mediated autophagy (CMA) (7). Beclin-1, an autophagy-related protein, is involved in the initiation of

autophagy (8). Previous report has shown that autophagy may be related to the pathogenesis of AD (9). Autophagy contributes to the clearance of Aβ aggregates and helps to preserve neuronal function in AD (9). The PI3K-Akt-mTOR signaling pathway is generally considered to be important for autophagy. Akt, which is located downstream of class I PI3K, is a serine threonine kinase that suppresses autophagy (10). A previous study has indicated that CMA plays a role in the direct degradation of the neuronal transcription factor myocyte enhancer factor 2D (MEF2D), a protein commonly known to promote neuronal survival (11). Recent studies have suggested that the administration of methylene blue also promotes the phosphorylation of Akt and glycogen synthase kinase (GSK)-3β, which leads to an increased concentration of MEF2D in the nucleus (12-14). In the present study, an okadaic acid (OA)-induced PC12 cell injury model was used to further understand the molecular mechanisms underlying the neuroprotective effects of PA on the biomarkers of AD, and to study its effects on the signaling pathways in autophagy. The effects of PA on cell viability, p-tau, $A\beta_{42}$ and β-secretase levels, as well as its effect on the expression levels of p-Akt, p-GSK-3β, MEF2D and Beclin-1 channel proteins in OA-treated PC12 cells were examined.

Materials and methods

Cell culture. The PC12 cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in high glucose DMEM containing 10% FBS (both purchased from Gibco; Thermo Fisher Scientific, Inc.). The cells were seeded in 25-cm^2 polystyrene flasks (Costar; Corning Inc.) at 37˚C in a humidified atmosphere of 5% CO_2.

OA and PA preparation. OA (cat. no. P112508; Adamas Reagent, Ltd.) was dissolved in PBS containing 0.001% DMSO (15) at a concentration of 10 μg/ml, and stored in sterile Eppendorf Tubes® at -20˚C for 7 days to obtain the aggregated form. The aggregated OA was then diluted to the desired concentration (0-250 nM) in high-glucose DMEM containing 10% FBS.

PA (cat. no. A0617-1, LOT: Cat. no. 110809-201605; National Institutes for Food and Drug Control) was diluted to 1 μg/ml in PBS containing 0.001% DMSO (15), and stored in sterile Eppendorf Tubes® that were incubated at -20˚C. Upon use, it was diluted to different concentrations (25, 50 or 100 μg/ml) in high-glucose DMEM medium containing 10% FBS. In addition, PC12 cells were incubated with the autophagy inhibitor 3-methyladenine (3-MA; 100 mM; cat no. M9281; Sigma-Aldrich; Merck KGaA) and the autophagy activator rapamycin (0.2 mg/ml; cat no. R0395; Sigma-Aldrich; Merck KGaA) at 37˚C for 24 h, which were used as controls to ensure that the neuroprotective effects of PA on autophagy were regulated by the Akt/GSK-3β/MEF2D pathway.

Determination of cell viability. PC12 cells were cultured at a density of $1x10^4$ cells/well in 96-well culture plates at 37˚C in an incubator with a humidified atmosphere of 5% CO_2. After 48 h incubation, the PC12 cells were pre-incubated with 50 μl PA at different concentrations (25, 50 or 100 μg/ml) or without PA (16) for 0.5 h at 37˚C, followed by incubation with OA (175 nM) for 24 h at 37˚C. Cell Counting Kit-8 (CCK-8; cat. no. CK04; Dojindo Molecular Technologies, Inc.) solution (10 μl/well; concentration of 0.5 g/l) was added and the cells were incubated at 37˚C for 2 h. The number of viable cells in each well was determined at 450 nm on a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.). The cell viability rate of the PC12 cells was calculated as follows: Cell viability rate (%)=$(OD_{\text{test group}}-OD_{\text{blank of test group}})/(OD_{\text{control group}}-OD_{\text{blank of control group}})$ x100%.

Enzymatic assay analyzing p-tau, $A\beta_{42}$ and β-secretase activity. PC12 cells were seeded into a 6-well culture plate at a density of $1x10^5$ cells/well. The seeded cells were then subjected to various treatments as described above for the CCK-8 cell viability assay. Briefly, the media from the treated PC12 cells was collected and centrifuged at 10,000 x g at 4˚C for 10 min. Then, the supernatant was collected and measured for p-tau (cat. no. P261FC), $A\beta_{42}$ (cat. no. A227FC) and β-secretase (cat. no. B096FC) using ELISA according to the manufacturer's protocol (Elixir Medical Corporation).

Western blot analysis. Western blotting was performed to detect p-Akt, p-GSK-3β, Akt, GSK-3β, MEF2D and Beclin-1. These proteins of similar molecular weight were detected by the membrane regeneration method; the corresponding internal reference was used for quantitative analysis (17). PC12 cells were harvested and lysed using a 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 the total proteins were extracted and quantified using a bicinchoninic acid kit (Wuhan Boster Biological Technology, Ltd.). Electrophoresis was performed with 40 μg of the total proteins using 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked in 5% BSA (cat. no. 810652; EMD Millipore) for 1 h at 4˚C and incubated with primary antibodies against GAPDH (cat. no. ab8245; Abcam), p-Akt (cat. no. 9275S; Cell Signaling Technology, Inc.), p-GSK-3β (cat. no. ab75745; Abcam), Akt (cat. no. 9272S; Cell Signaling Technology, Inc.), GSK-3β (cat. no. ab131356; Abcam), MEF2D (cat. no. ab32845; Abcam) and Beclin-1 (cat. no. ab62557; Abcam) proteins overnight at 4˚C (all 1:1,000 dilution). The blots were subsequently washed and incubated with their respective horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG; anti-mouse and anti-rabbit) secondary antibodies (1:2,000; cat. nos.7076S and 7074S; Cell Signaling Technology, Inc.) at 37˚C for 1 h. GAPDH was used as an internal control. The bound secondary antibodies were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Inc.) with the ChemiDoc XRS™ with Quantity One® 1-D analysis software (Bio-Rad Laboratories, Inc.). Blots were repeated at least three times for each condition. After development, the band intensities were quantified with Image-Pro Plus 6.0 analysis software (Media Cybernetics, Inc.).

Immunofluorescence. The cells were treated with cell culture medium containing OA (175 nM) and PA (25, 50 or 100 μg/ml) for 24 h. Following a 60-min fixation in 4% paraformaldehyde at 4˚C, the cells were washed with PBS and treated with

300 μl of BSA (1:100) for 20 min at 37˚C. The cells were then incubated with Rabbit anti-Beclin-1 primary antibodies (1:50; Abcam) for 1 h at 37˚C. After washing with PBS, the cells were then incubated with Alexa 488-conjugated anti-rabbit IgG (1:200; cat no. 4412S; Cell Signaling Technology, Inc.) for 30 min at 37˚C. Thereafter, the cells were washed and treated with 300 μl of DAPI (Wuhan Boster Biological Technology, Ltd.) for 5 min at 37˚C to stain the cell nuclei. Finally, the cells were imaged using a light microscope (U-SPT; Olympus Corporation) at a magnification x200, in five random fields of view. Data analyses were performed using the ImageJ software v1.48 (National Institutes of Health).

Immunocytochemistry. The methods used to fix the cells for the immunocytochemistry experiments were the same as those performed for the immunofluorescence experiments. Following these steps, the cells were incubated with mouse anti-Beclin-1 primary antibodies (1:50 dilution; Abcam) for 1 h at 37˚C. After washing with PBS, the cells were incubated with HRP-conjugated anti-Rabbit IgG for 20 min at 37˚C (1:200; cat no. 7074S; Cell Signaling Technology, Inc.). The cells were subsequently washed with PBS and amplified via avidin biotin-peroxidase complex labeling by adding 300 μl of streptavidin-biotin complex (cat no. SA1021; Wuhan Boster Biological Technology, Ltd.) for 20 min overnight. The cells were then washed and treated with 300 μl of 3,3'-diaminobenzidine (cat no. AR1022; Wuhan Boster Biological Technology, Ltd.) for 5 min at 37˚C to stain the cell nuclei. Finally, cell imaging was performed using a light microscope (U-SPT; Olympus Corporation) at a magnification x200, in five random fields of view. Data were analyzed using the ImageJ software v1.48 (National Institutes of Health).

Statistical analysis. Data were expressed as the mean ± SD; significant differences between different groups were determined by one-way or two-way ANOVA followed by post hoc testing with Bonferroni correction for multiple comparisons, with a significance threshold of $P<0.05$. The experiments were repeated ≥3 times. Correlations between p-tau, $A\beta_{42}$, β-secretase, p-Akt, p-GSK-3β, MEF2D and Beclin-1 expression were identified by Pearson correlation analysis. $P<0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS 13.0 (SPSS, Inc.) statistical software package.

Results

CCK-8 assay. The CCK-8 assay was used to profile cell viability and determine the optimal concentration and length of time for OA treatment in PC12 cells. OA can lead to a reduction in cell viability, as indicated by the negative association between the cell viability rates, and the concentration and duration of OA treatment. The cell viability rate of OA-induced PC12 cells was significantly reduced at 48 h compared to 12 and 24 h. The 50% inhibitory concentration of OA was determined to be 175 nM at 48 h (Fig. 1).

OA treatment alone decreased the cell viability rate of PC12 cells in the model group (OA treated group without PA pretreatment) when compared to the control group (PC12 cells without OA and PA treatment). The PC12 cells were

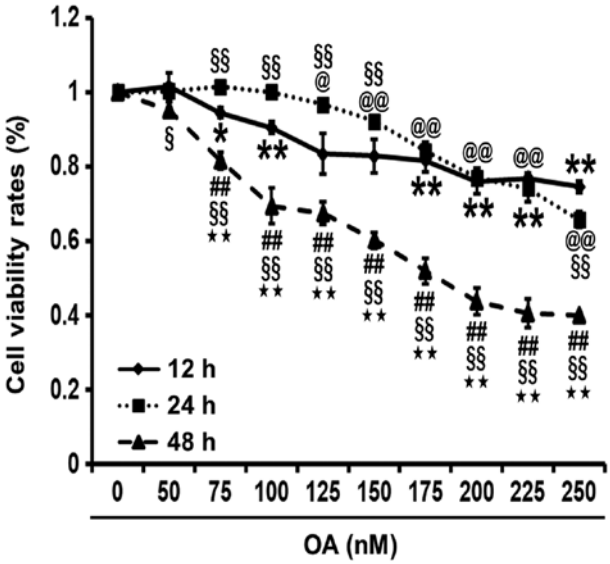

Figure 1. Effect of OA-induced cytotoxicity on cell viability rates in PC12 cells. Increasing concentrations of OA led to decreased cell viability rates for PC12 cells. The largest change in cell viability rates for OA-induced PC12 cells, during the three sampling time points, was at 48 h; the 50% inhibition concentration of OA was 175 nM. Data are presented as the mean ± SD (n=6). $^{*}P<0.05$, $^{**}P<0.01$ vs. 0 nM at 12 h; $^{@}P<0.05$, $^{@@}P<0.01$ vs. 0 nM at 24 h; $^{\#\#}P<0.01$ vs. 0 nM at 48 h; $^{\S}P<0.05$, $^{\S\S}P<0.05$ vs. 12 h at the indicated concentration; $^{\star\star}P<0.01$ vs. 24 h at the indicated concentration. OA, okadaic acid.

then pre-treated with PA at different concentrations for 0.5 h, followed by the addition of 175 nM OA in a combined cultured for 48 h. It was found that PA could increase the viability rate of the OA-treated PC12 cells in a dose-dependent manner when compared to the model group ($P<0.01$; Fig. 2).

p-tau, $A\beta_{42}$ and β-secretase levels by ELISA assay. Treatment with OA yielded a significant increase in p-tau, $A\beta_{42}$ and β-secretase levels in the model group when compared to the control group ($P<0.01$). However, cells exposed to pretreatment with PA followed by the addition of OA showed a decrease in p-tau (Fig. 3A), $A\beta_{42}$ (Fig. 3B) and β-secretase (Fig. 3C) levels at all doses for the PA-treated groups when compared to the model group ($P<0.05$).

p-Akt, p-GSK-3β, MEF2D and Beclin-1 expression in PA-treated PC12 cells. As shown in Fig. 4A, there was a significant decrease in the levels of p-Akt (Fig. 4B) and MEF2D (Fig. 4C) expression, and a significant increase in p-GSK-3β (Fig. 4D) and Beclin-1 (Fig. 4E) expression in the model group (group B) compared with the control group (group A; $P<0.01$). In addition, the expression of p-Akt (Fig. 4B) and MEF2D (Fig. 4C) increased, while the expression of p-GSK-3β (Fig. 4D) and Beclin-1 (Fig. 4E) decreased in the autophagy-inhibiting 3-MA-treated (group C) and 100 μg/ml PA-treated (group E) PC12 groups when compared to the model group (group B) ($P<0.01$ or $P<0.05$). It was found that 100 μg/ml PA decreased he expression levels of p-tau, $A\beta_{42}$ and β-secretase to a greater extend compared with 25 and 50 μg/ml PA; therefore, 100 μg/ml PA was used for the mechanism experiments. In contrast, p-Akt (Fig. 4B) and MEF2D (Fig. 4C) expression decreased, and GSK-3β (Fig. 4D) and Beclin-1 (Fig. 4E) expression increased, in the

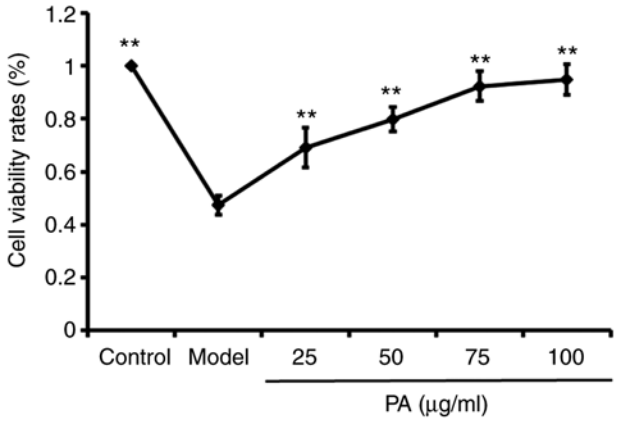

Figure 2. Effects of various concentrations of PA on okadaic acid-induced cytotoxicity in PC12 cells. PA had a significant effect on attenuating the cell viability rate and displayed a dose-effect relationship from 25-100 μg/ml PA. Data are presented as the mean ± SD (n=6). **P<0.01 vs. model group. PA, protocatechuic acid.

autophagy-activating rapamycin-treated group (group D) when compared to the model group (group B; P<0.01 or P<0.05). Furthermore, the expression of p-Akt (Fig. 4B) and MEF2D (Fig. 4C) was significantly upregulated, whereas the expression of p-GSK-3β (Fig. 4D) and Beclin-1 (Fig. 4E) was significantly downregulated, in the rapamycin-treated group (group D) when compared to the 3-MA treated group (group C; P<0.01 or P<0.05, respectively).

Beclin-1-positive staining in PA-treated PC12 cells. To investigate the effects of PA on OA-induced autophagy, Beclin-1 expression was detected in the PC12 cells using immunocytochemistry and immunofluorescence staining methods (Fig. 5A). Beclin-1-positive expression was increased in the model group (group b) when compared to the control group (group a; P<0.05; Fig. 5B and C). In contrast, pretreatment with 3-MA (group c) or 100 μg/ml PA (group e) led to a clear reduction in Beclin-1-positive expression when compared to the model group (group B) or the rapamycin-treated group (group d; P<0.01).

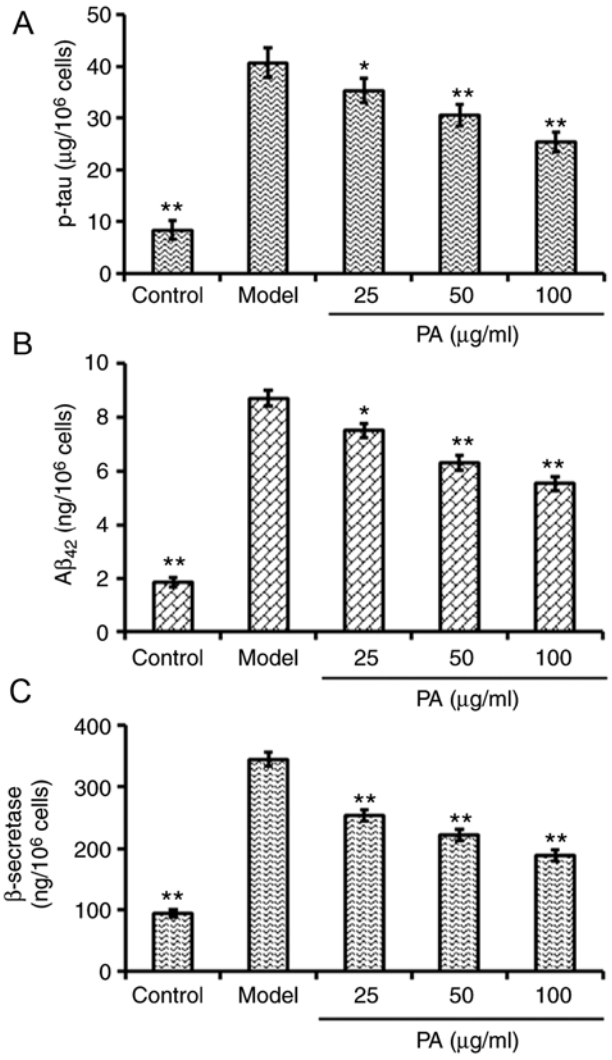

Figure 3. Effects of PA on p-tau, $A\beta_{42}$ and β-secretase levels in okadaic acid-induced PC12 cells. Levels of (A) p-tau, (B) $A\beta_{42}$ and (C) β-secretase levels decreased in the PA-treated cells when compared to the model group. Data are presented as mean ± SD (n=6). *P<0.05, **P<0.01 vs. model group. PA, protocatechuic acid; p, phosphorylated; Aβ, β-amyloid.

Correlation analysis. Pearson correlation analysis was performed to investigate the relationships between the reduction of p-tau, $A\beta_{42}$ and β-secretase, and the expression of pathway proteins (including p-Akt, p-GSK-3β, MEF2D and Beclin-1 proteins) in the PC12 cells that were pre-treated with PA followed by the addition of OA. The data are shown in Table I. The Pearson analysis showed that in the PA-treated cells there were significant positive correlations between: p-tau and p-GSK-3β, $A\beta_{42}$ and β-secretase; $A\beta_{42}$ and β-secretase, p-GSK-3β and Beclin-1; and β-secretase and Beclin-1 (P<0.01 or P<0.05). Conversely, there were significant negative correlations between: $A\beta_{42}$ and p-Akt; β-secretase and p-Akt; p-Akt and Beclin-1; p-GSK-3β and MEF2D; and MEF2D and Beclin-1 (P<0.01 or P<0.05).

Discussion

OA is a potent polyether marine toxin obtained from the black sponge *Halichondria okadai* that effectively and selectively inhibits serine/threonine residues of protein phosphatase 1 and protein phosphatase 2A (PP2A) (18). A previous study has shown that in the brains of patients with AD there is decreased activity of PP2A (an important tau dephosphorylating enzyme) and increased phosphorylation of tau (19). Intracerebral injections of OA can lead to tau hyperphosphorylation, the formation of neurofibrillary tangles and Aβ deposition, as well as memory loss and neurodegeneration (20), which could be used to produce a useful model of AD. In the present study, the aim was to develop OA-induced PC12 cells as a research model. Cell viability testing by CCK-8 showed that different concentrations of OA were negatively associated with the cell viability rate of PC12 cells from 12 to 48 h. However, pretreatment with 25-100 μg/ml of PA in the OA-induced PC12 cells was sufficient to increase the cell viability rate in a dose-dependent manner. This suggested that PA could attenuate the OA-induced damage to PC12 cells.

A growing body of evidence indicates that PA has beneficial effects on improving the cognitive deficits of patients with AD. PA has neuroprotective effects against oxidative stress, nitrosative stress and excitotoxicity, as well

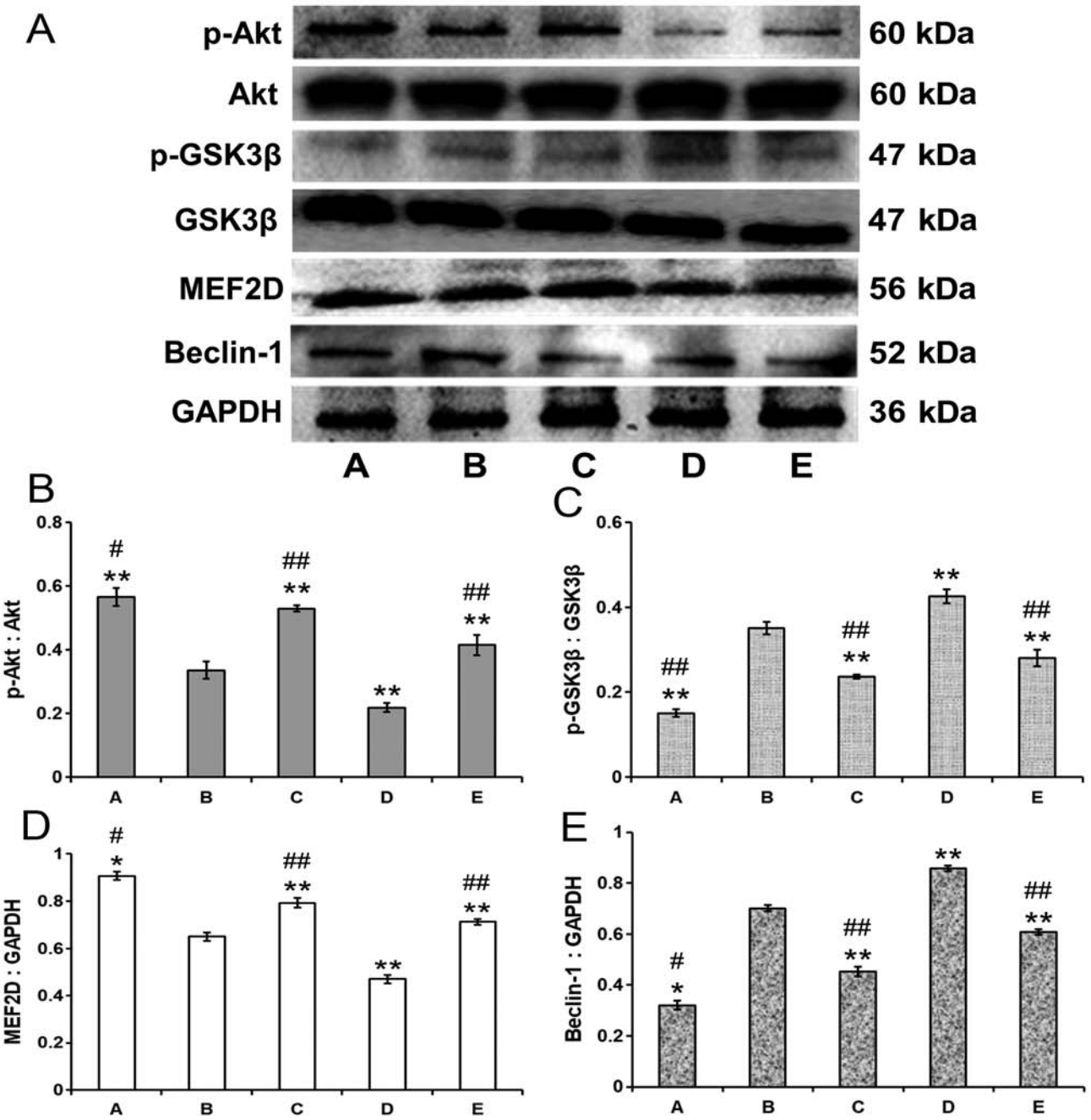

Figure 4. Effects of PA on the expression of p-Akt, p-GSK-3β, MEF2D and Beclin-1 proteins in the OA-induced PC12 cells. (A) Western blotting results. PA treatment in OA-induced PC12 cells enhanced the expression of (B) p-Akt and (C) MEF2D, and reduced the expression of (D) p-GSK-3β and (E) Beclin-1 when compared to the model group. Values are expressed as the mean ± SD (n=3). *P<0.05, **P<0.01 vs. model group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. rapamycin group. A, control group; B, model group; C, 3-methyladenine group; D, rapamycin group; E, PA group. Each blot was repeated at least three times for every condition. PA, protocatechuic acid; p, phosphorylated; OA, okadaic acid; MEF2D, myocyte enhancer factor 2D; GSK, glycogen synthase kinase.

as anti-neuroinflammatory and anti-apoptotic effects in cerebellar granule neurons (21-23). PA has also been shown to alleviate oxidative stress, and can be used as an effective agent to treat renal ischemia and reperfusion injuries (24). Additionally, it has been shown that PA can enhance learning and memory performance, and alleviate apoptosis and glial proliferation following exposure to chronic intermittent hypoxia in rats (25). PA also improves cognitive deficits and decreases Aβ deposition, as well as decreasing the expression levels of APP in aged APP/PS1 double transgenic mice (6). In the present study, it was found that the expression levels of p-tau, $A\beta_{42}$ and β-secretase decreased in the OA-induced PC12 cells that were incubated with different concentrations of PA. These results suggested a negative association between cell viability and p-tau, $A\beta_{42}$ and β-secretase.

Moreover, the possible mechanisms via which PA affected cell autophagy induced by OA were examined. Autophagy regulates the pathological markers of AD in a bidirectional manner, and disorders of autophagy may lead to abnormal protein deposition in the nervous system (26). Therefore, autophagy is considered to be closely related to AD; it is not only involved in the generation and clearance of Aβ, but also

Table I. Correlation analysis.

Protein	p-tau	$A\beta_{42}$	β-secretase	p-Akt	p-GSK3β	MEF2D	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]	-0.188	0.618[a]
β-secretase			1.000	-0.692[a]	0.434	-0.215	0.701[a]
p-Akt				1.000	-0.171	0.426	-0.990[b]
p-GSK3β					1.000	-0.761[b]	0.242
MEF2D						1.000	-0.518[a]
Beclin-1							1.000

p, phosphorylated; Aβ, β-amyloid; GSK, glycogen synthase kinase; MEF2D, myocyte enhancer factor 2D; n=6; [a]P<0.05, [b]P<0.01.

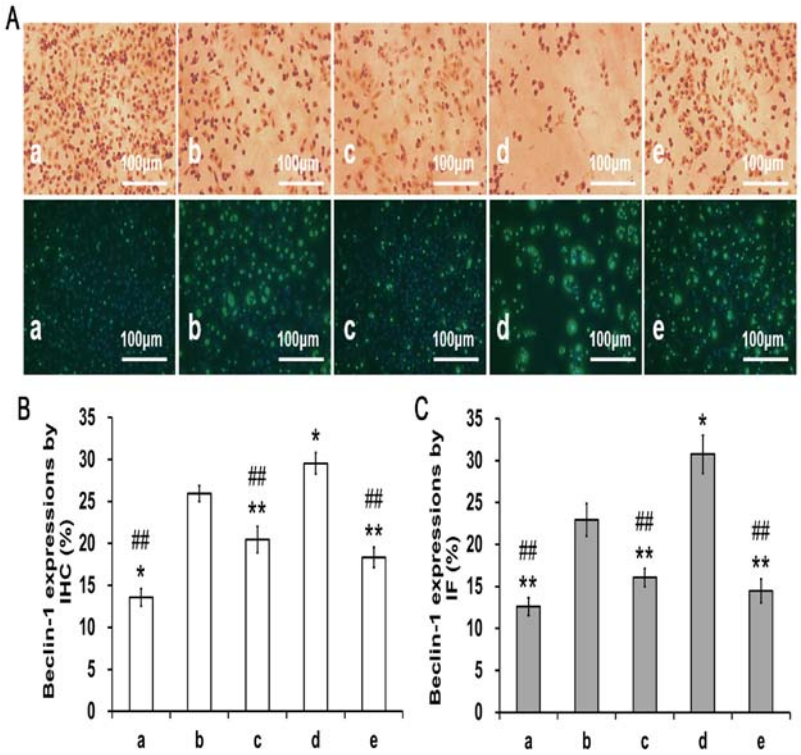

Figure 5. Effects of PA on Beclin-1 expression in the OA induced PC12 cells (magnification, x200). Cells were pre-treated with PA and then exposed to OA (175 nM) for 48 h, during which the same treatments were applied. (A) Immunohistochemistry and immunofluorescent results. (B) Quantification of Beclin-1 expression from immunohistochemistry. (C) Quantification of Beclin-1 expression from immunofluorescence experiment. PA treatment in OA-induced PC12 cells reduced the expression of Beclin-1 compared with the model group. Data are presented as the mean ± SD. n=3. *P<0.05, **P<0.01 vs. model group; $^{\#\#}$P<0.01 vs. rapamycin group. a, control group; b, model group; c, 3-methyladenine group; d, rapamycin group; e, PA group; PA, protocatechuic acid; OA, okadaic acid.

plays a key role in the metabolism of tau proteins (27). A key protein involved in the initiation of autophagy is Beclin-1, which is a 60-kDa coiled-coil protein that is expressed in neurons and glia (28). A recent study found reduced levels of Beclin-1, disruptions to autophagy and accumulations of Aβ in a transgenic AD mouse model (28). Additionally, it was found that β-asarone has neuroprotective effects against 6-hydroxydopamine-induced Parkinson's disease via the JNK/Bcl-2/Beclin-1 pathway, and it can increase Bcl-2 expression and inhibit Beclin-1 expression (29). MEF2D is also strongly associated with autophagy (30). APP processing and Aβ generation are associated with the autophagic pathway (31), which suggests that the upregulation of Beclin-1-dependent autophagy may contribute to the elimination of aggregated Aβ. In the present study, it was shown that OA could induce autophagy and increase the expression of Beclin-1. In contrast, PA significantly decreased the expression of Beclin-1, which suggested that PA can attenuate OA-induced autophagy. In comparison, pretreatment with the autophagy inhibitor 3-MA or the autophagy activator rapamycin significantly inhibited or enhanced the expression of Beclin-1, respectively. Beclin-1 was used to assess autophagy; however, autophagy morphological detection may be more effective in determining the occurrence of autophagy. Due to the limitation of experimental conditions, autophagy morphological detection was not carried out in this study.

Activated Akt plays important roles in cellular growth, cell cycle progression and cell survival (32). Akt/GSK-3β is the classical Akt pathway; GSK-3β is a downstream signaling molecule of Akt, and Akt can be directly suppressed by GSK-3β (33). It has been shown that osthol can decrease p-tau levels via the PI3K/Akt/GSK-3β signaling pathway in AD, which suggests that the phosphorylation of tau is closely related to this signaling pathway (34). Moreover, the phosphorylation of Akt and GSK-3β can lead to an increased concentration of MEF2D in the nucleus (12). In the present study, proteins from the autophagic pathway were detected, which were similarly controlled by 3-MA and rapamycin. A limitation of this study is that the levels of MEF2D were detected in total cell lysates instead of analyzing the MEF2D content in the nucleus. It was found that levels of p-Akt and MEF2D were downregulated significantly, while the levels of p-GSK-3β were upregulated significantly in the OA treatment group when compared to the normal control group. However, PA could significantly upregulate the levels of p-Akt and MEF2D, and reduce the levels of p-GSK-3β, when compared to the OA treatment group. According to previous research, suppressing the activity of GSK-3β can increase the expression of MEF2D (30,35). The results of the present study are consistent with previous studies; overall, it was shown that PA could attenuate the autophagy responses induced by OA, and its neuroprotective effects may be exerted via the activation of the Akt/GSK-3β/MEF2D signaling pathway.

Finally, significant positive correlations, including p-tau/p-GSK-3β, $A\beta_{42}$ and p-GSK-3β/Beclin-1 and β-secretase/Beclin-1, and significant negative correlations, including $A\beta_{42}$/p-Akt, β-secretase/p-Akt, MEF2D and p-GSK-3β/Beclin-1, and p-Akt/Beclin-1, were found. These findings indicated that the downregulation of β-secretase, $A\beta_{42}$ and p-tau levels, in OA-induced PC12 cells after treatment with PA, may be caused by the effect of PA on autophagy via the regulation of the Akt/GSK-3β/MEF2D pathway. Further experiments are required to identify the mechanisms by which PA regulates autophagy.

In conclusion, these results indicated that PA attenuates OA-induced autophagy via the Akt/GSK-3β/MEF2D pathway. PA enhanced Akt phosphorylation and increased levels of MEF2D, but suppressed GSK-3β phosphorylation, which is known to inhibit autophagy. These findings suggested that PA could be a potential drug candidate for the prevention of AD.

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Availability of data and materials

The datasets used and/or analyzed during the present 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, XZ, SQ and MD contributed to conception 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 interests.

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