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Beta-asarone attenuates amyloid beta-induced autophagy via Akt/mTOR pathway in PC12 cells

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ABSTRACT

Alzheimer's disease (AD) is an age related and progressive neurodegenerative disease. Autophagy is a self-degradative process and plays a critical role in removing long-lived proteins and damaged organelles. Recent evidence suggests that autophagy might be involved in the pathogenesis of AD. β-asarone have various neuroprotective effects. However, the effect of β-asarone on autophagy in amyloid β-peptide (Aβ) induced cell injury is unclear, and little is known about the signaling pathway of β-asarone in autophagy regulation. The aim of the present study was to determine whether β-asarone protects cells from $A\beta_{1-42}$ induced cytotoxicity via regulation of Beclin-1 dependent autophagy and its regulating signaling pathway. We examined effects of β-asarone on cell morphology, cell viability, neuron specific enolase (NSE) levels, autophagosomes and regulating Beclin-1, p-Akt and p-mTOR expressions in $A\beta_{1-42}$ treated PC12 cells. We found that β-asarone could maintain the original morphology of cells and increase cell viability and decrease NSE levels significantly. Meanwhile, β-asarone decreased Beclin-1 expression significantly. In addition, β-asarone can increase levels of p-Akt and p-mTOR. These results showed that β-asarone protected cells from $A\beta_{1-42}$ induced cytotoxicity and attenuated autophagy via activation of Akt-mTOR signaling pathway, which could be involved in neuroprotection of β-asarone against Aβ toxicity. Our findings suggest that β-asarone might be a potential preventive drug for AD.

1. Introduction

Alzheimer's disease (AD) is an age related and progressive neurodegenerative disease. Amyloid β (Aβ) is central to the pathology of AD, because of its stronger aggregative ability and neurotoxicity (Haass and Selkoe, 2007). Neuron specific enolase (NSE) specifically localized in neurons (Kaiser et al., 1989). NSE levels increase in cerebrospinal fluid or blood in several brain pathologies, which may reflect neuronal damage and be a marker for morphological status in AD (Van Eldik and Wainwright, 2003; Steiner et al., 2006; Chaves et al., 2010).

Autophagy is a self-degradative process and plays a critical role in removing long-lived proteins and damaged organelles (Rami, 2009). Autophagy is always activated by nutrient deprivation, cell injury and other forms of cell stress (Mortimore and Schworer, 1977). Beclin-1, the autophagy-related protein, is essential for the initiation of autophagy (Liang et al., 1999). Recent evidence suggests that autophagy might be involved in the pathogenesis of AD. On one hand, APP processing and Aβ generation are associated with the autophagic pathway (Nixon et al., 2005), but autophagic dysfunction in AD pathology and the levels of Beclin-1 are reduced in early AD (Li et al., 2010a, 2010b). On the other hand, autophagy also plays an important role in clearing Aβ aggregates and preserving neuronal function in AD (Pickford et al., 2008). Usually, the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) signaling pathway is important for autophagy. AKT, located downstream of class I PI3K, is a serine-threonine kinase that activates the kinase mTOR and suppresses autophagy. mTOR, which functions downstream of AKT (Schmelzle and Hall, 2000; Gingras et al., 2001), is inhibited and then induces autophagy.

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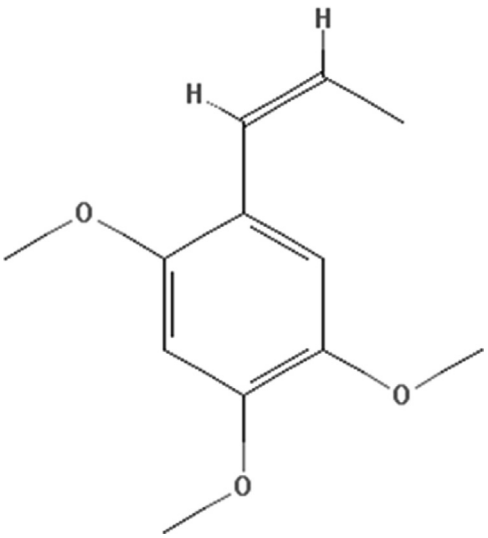

Fig. 1. Chemical structure of β-asarone.

β-asarone (for its structure, see Fig. 1), the major component of *Acorus tatarinowii* Schott, can easily pass through the blood–brain barrier (BBB) (Wu and Fang, 2004), and shows various neuroprotective effects such as protecting neuron against apoptosis (Fang et al., 2003, 2008). However, the effect of β-asarone on autophagy in Aβ-induced cell injury is unclear, and little is known about the signaling pathway of β-asarone in autophagy regulation. In this study, we examined effects of β-asarone on cell viability, NSE levels, cell morphology and autophagosomes in $A\beta_{1-42}$ treated PC12 cells. Furthermore, we investigated the regulating effects of β-asarone on Beclin-1, p-Akt and p-mTOR expressions. In addition, we also used autophagy inhibitor 3-methyladenine (3-MA), autophagy activator rapamycin and PI3K-Akt-mTOR signaling pathway inhibitor NVP-BEZ235 as control for making sure that the results of β-asarone on autophagy were accurate.

2. Materials and methods

2.1. β-asarone preparation

β-asarone (cis forms of 2, 4, 5-trimethoxy-1-propenylbenzene) is a small molecular weight (208) substance with a strong fat-soluble, which is extracted from *Acorus tatarinowii* Schott according to the procedure that we have reported (Liu and Fang, 2011). The purity of β-asarone was up to 99.55% that was confirmed by gas chromatography-mass spectrometry, infrared spectrum and nuclear magnetic resonance detection.

2.2. $A\beta_{1-42}$ preparation

$A\beta_{1-42}$ (GL Biochem, China) was dissolved in sterile double deionized water at a concentration of 100 μM, and incubated at 37 °C for 7 d to obtain the aggregated form. Then the aggregated $A\beta_{1-42}$ was diluted to the desired concentration in serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA).

2.3. Cell culture

PC12 cells (Culture Collection of Chinese Academy of Science, China) were cultured in DMEM containing 5% heat-inactivated fetal bovine serum (Gibco, USA) and 5% horse serum (Gibco, USA). The cells were seeded in 25-cm^2 polystyrene flasks (Corning Costar Corp, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Determination of cell viability

The cells were cultured at a density of 1×10^4 cells/well in 96-well culture plates at 37 °C in a 5% CO_2 incubator. After incubation for 48 h, cells were preincubated with or without β-asarone for 1 h following incubation with aggregated $A\beta_{1-42}$ for 24 h. Ten μl/well of MTT solution (Final concentration, 1 mg/ml) (Sigma, USA) was added and cells were incubated at 37 °C for 4 h. After discarding the culture medium, 100 μl DMSO (Sigma, USA) was added to dissolve the formazan crystals for 10 min. The number of viable cells in each well was determined at 570 nm on a microplate reader (Multiskan Mk3, Thermo Scientific, USA).

2.5. NSE analyses

The cells were treated as described above. The medium was collected for NSE measurement using an ELISA method according to the manufacturer's protocol (R&D Systems, USA).

2.6. Flow cytometric evaluation of Beclin-1, p-mTOR and p-Akt expressions

2.6.1. Sample preparations

The cells were seeded in 24-well or 12-well plates at a cell density of 1×10^5 or 2×10^5 cells/well. Then the cells were treated with or without β-asarone at concentrations of 7.5, 15, and 30 μg/ml, respectively. Following 1 h incubation, aggregated $A\beta_{1-42}$ (1.25 μM) was added. The cells were collected 24 h later. The cells were washed once in phosphate buffered saline (PBS) and then blocked with protein block solution (PBS with 2% BSA) for 20 min, fixed with 1% paraformaldehyde for 20 min followed by permeabilization for 15 min in 0.5% Triton-X (Invitrogen, USA) at room temperature. The cells were undivided or divided into three, and were used to evaluate the Beclin-1 or p-Akt, p-mTOR and Beclin-1 expressions, respectively, as the method that we have established (He et al., 2013).

2.6.2. Flow cytometric evaluation of Beclin-1 and p-mTOR expressions

The cells were incubated with rabbit anti-rat Beclin-1 primary antibody (2:100, Santa Cruz Biotechnology, USA) or rabbit anti-rat p-$mTOR^{S2448}$ primary antibody (2:100, Cell Signaling, USA) in darkness at room temperature for 30 min. After incubation, cells were washed once with protein block solution, and then incubated with PE-conjugated goat anti-rabbit IgG secondary antibody (1:100, Santa Cruz Biotechnology, USA) in darkness at room temperature for 30 min. After incubation, cells were washed once with PBS. Set the isotype controls. More than 10,000 events were captured for every analysis. FACS data were collected using an EPICS ALTRA flow cytometer (Beckman Coulter, USA) with EXPOTM32 software.

2.6.3. Flow cytometric evaluation of p-Akt expression

The cells were incubated with PE-conjugated rabbit anti-rat p-Akt^{S473} antibody (2:100, Cell Signaling, USA) in darkness at room temperature for 30 min. After incubation, cells were washed once with PBS. Set the isotype controls. More than 10,000 events were captured for every analysis. FACS data were collected using an EPICS ALTRA flow cytometer (Beckman Coulter, USA) with EXPOTM32 software.