β-Asarone protects PC12 cells against OGD/R-induced injury via attenuating Beclin-1-dependent autophagy

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Aim: To explore the effects of β-asarone from Acorus Tatarinowii Schott on autophagy in an ischemic stroke model of PC12 cells.

Methods: The ischemic stroke model of PC12 cells was made by OGD/R (2 h oxygen-glucose deprivation followed by 24 h reperfusion). Drug administration was started 1 h before OGD and last for 3 h. Then the cells were incubated in the drug-free and full culture medium under normoxic conditions for 24 h. After the treatments, Beclin-1, intracellular free calcium concentration ([Ca2+]i) and mitochondrial membrane potential (MMP) were analyzed using flow cytometry. Cell viability was measured using MTT assay. Cell morphology was studied under inverted phase contrast microscope, and autophagosomes were observed under transmission electron microscope.

Results: Pretreatment with β-asarone (20, 30, or 45 μg/mL) or the calcium channel antagonist nimodipine (10 μmol/L) significantly increased the cell viability and MMP, and decreased Beclin-1 expression and [Ca2+]i in OGD/R-treated PC12 cells. Under inverted phase contrast microscope, pretreatment with β-asarone or nimodipine dramatically increase the number of cells and improved the cellular morphology. Autophagosomes were found in OGD/R-treated PC12 cells as well as in drug plus OGD/R-treated PC12 cells.

Conclusion: β-Asarone protects PC12 cells against OGD/R-induced injury partly due to attenuating Beclin-1-dependent autophagy caused by decreasing [Ca2+]i and increasing MMP.

Keywords: β-asarone; PC12 cells; oxygen-glucose deprivation/reperfusion; autophagy; Beclin-1; [Ca2+]; MMP; nimodipine

Introduction

Oxygen-glucose deprivation and reperfusion (OGD/R) leads to neuronal insult. Autophagy, a process of self-eating, is usually induced by starvation, ischemia and hypoxia, growth factor deficiency, etc. It helps to maintain cell homeostasis[1-3], but excessive autophagy may also leads to autophagic neuron death and apoptosis[4-6]. β-asarone, one of chief constituents from Acorus Tatarinowii Schott, can easily pass through the blood brain barrier[7], and shows various neuroprotective effects such as protecting neuron against apoptosis[8-11]. However, the effect of β-asarone on autophagy in a model of ischemic stroke in vitro is rarely reported. PC12 cell line is derived from a pheochromocytoma of the rat adrenal medulla. It can acquire neuron-like properties when exposed to nerve growth factor (NGF)[12]. Therefore, it is widely used as an in vitro model of cerebral ischemia/reperfusion[13, 14]. Intracellular free calcium concentration ([Ca2+]i) and mitochondrial membrane potential (MMP) are important indicators to reflect cell injury. It is reported that autophagy can be induced by increased [Ca2+], and decreased MMP[15, 16]. Beclin-1, an autophagy related gene, is an essential indicator of autophagy. Flow cytometry is adopted as an important quantitative analysis, but Beclin-1 analysis by flow cytometry was merely reported until we established a method of quantitative analysis of Beclin-1[17]. In this research, we studied the protective mechanism of β-asarone in OGD/R treated PC12 cells by evaluation of Beclin-1, [Ca2+]i, MMP and cell viability and observation of autophagosomes and cellular morphology. Control group treated with nimodipine (a calcium antagonist) was established to explore whether β-asarone has effects on [Ca2+]i, and MMP so as to reduce autophagy.

Materials and methods

β-asarone preparation

β-asarone (cis forms of 2,4,5-trimethoxy-1-propenylbenzene) is a fat-soluble substance with a small molecular weight. It was extracted from Acorus Tatarinowii Schott according to the procedure that we have reported[18]. β-asarone, the purity of
which was up to 99.55%, was confirmed by gas chromatography-mass (GC-MS), infrared spectrum (IR), and nuclear magnetic resonance (NMR) detection.

Cell cultures
PC12 cells (Culture Collection of Chinese Academy of Science, Shanghai, China) were seeded in 25-cm² polystyrene flasks (Corning Costar Corp, USA) with 4.5 g/mL glucose in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA), containing 5% heat-inactivated foetal bovine serum (Gibco, USA) and 5% horse serum (Gibco, USA). The cells were incubated under an atmosphere of 95% air and 5% CO₂. Culture medium was replaced every 48 h.

Glucose deprivation and hypoxia/reperfusion
PC12 cells were washed with phosphate buffer solution (PBS) for one time and incubated in Earle’s balanced salt solution (116 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L MgSO₄, 1 mmol/L NaH₂PO₄, 0.9 mmol/L CaCl₂, and 10 mg/L phenol red). And then the cells were incubated in a hypoxia chamber (Themo scientific, USA) with a compact gas oxygen controller to maintain oxygen concentration at 1% by injecting a gas mixture of 94% N₂ and 5% CO₂ for 2 h. After hypoxia, the cells were transferred back to full culture medium with oxygen for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions.

Drugs
PC12 cells were incubated with full culture medium containing β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) under normoxic conditions for 1 h before hypoxia. The full culture medium containing drug was discarded. The cells were rinsed once with PBS, and incubated with Earle’s balanced salt solution containing β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) for 2 h of hypoxia. The Earle’s balanced salt solution was discarded, and then PC12 cells were incubated with full culture medium free of drug under normoxic conditions.

Flow cytometric analysis of Beclin-1 expression
PC12 cells were seeded into 24-well plastic plates with 1 mL at the density of 1×10⁵ cells/mL. It contained 10 samples in normal control group, OGD/R treated group and OGD/R+drug treated groups, respectively. PC12 cells were harvested by trypsinization [0.25% trypsin/2.6 mmol/L ethylenediaminetetraacetic acid (EDTA), Gibco, USA] and incubated in 2 mL 2% (v/v) bovine serum albumin (BSA)/PBS for 30 min. Permeabilization of the cells was done using fixation and permeabilization (Invitrogen, Beijing, China), according to the manufacturer instructions. Then the cells were incubated with mouse anti-human Beclin-1 monoclonal antibody (100 μL, 2: 100 dilution, Santa Cruz Biotechnology, catalog No sc-48381, USA) at room temperature for 30 min. After washing with 2% BSA/PBS once, cells were incubated with PE-conjugated goat anti-mouse IgG1 antibody (0.005 μg/μL, Mutiscience, catalog No gam0041, China) in the darkness at room temperature for 30 min. The control cells were incubated with the secondary antibody alone. After washing once with 2% BSA/PBS, the fluorescence of 10,000 cells was analyzed by flow cytometer (Beckman Coulter, Florida, American, 488 nm) using the EXPO32 data analysis system (Beckman Coulter, Florida, USA).

Flow cytometric analysis of MMP and [Ca²⁺]
PC12 cells were seeded in 24-well plastic plates with 1 mL at the density of 1×10⁵ cells/mL. Then after growth for 48 h, the cells were treated with OGD/R as described above. Normal control group, OGD/R treated group and OGD/R+drug groups contained 10 samples, respectively. After 24 h reperfusion, cells were harvested, washed with 2 mL PBS once, centrifuged for 5 min at 1000 r/min (TDL-5-A Low Speed Centrifuge, Shanghai, China) and pelleted. The cells were resuspended in 0.5 mL PBS. For MMP detection, cell suspensions were incubated with Rhodamine123 (Rho123) (final concentration 10 μg/mL, ALEXIS, USA, catalog No alx-610-018-m005) in the darkness at 37°C for 30 min. For [Ca²⁺] detection, cell suspensions were incubated with Flura 3-acetoxymethyl ester (Fluo-3am) (final concentration 5 μmol/L, BIOTIUM, USA, catalog No Fluo-3am) in the darkness at 37°C for 20 min. Then the cells were trypsinized and pelleted. The cells were resuspended in 0.5 mL PBS. After washing once in 2 mL PBS, the cells were resuspended in 0.5 mL PBS. The fluorescence of 10,000 cells was analyzed by flow cytometry (488 nm). The mean fluorescence intensity of Fluo-3 was used as the indication of [Ca²⁺] quantity.

Inverted phase contrast microscope for observation of cellular morphology
After normoxic, hypoxic or hypoxic+drug treatment (OGD 2 h+R 24 h), cellular morphology were observed under inverted phase contrast microscope (×200, Olympus, Japan).

Transmission electron microscope for observation of autophagy
After normoxic, hypoxic or hypoxic+drug treatment (OGD 2 h+R 24 h), the cells were trypsinized and pelleted. The cells were suspended and fixed with 2% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) at 4°C for 2 h and postfixed with 1%
Beclin-1 expression and \([\text{Ca}^{2+}]_i\) in PC12 cells

β-asarone (45 μg/mL) 10 0.56±0.08
β-asarone (20 μg/mL) 10 0.64±0.07

Mean±SD for 10 samples. Significantly increased cell viability and MMP in OGD/R treated cells.

β-asarone (20, 30, or 45 μg/mL) and nimodipine (10 μmol/L) for 3 h (1 h before OGD and 2 h throughout OGD). They were incubated with full and drug-free culture medium respectively, and \([\text{Ca}^{2+}]_i\) was dramatically increased. Cell viability was restored to near normal levels (0.79±0.04 in OD value) at the dose of 30 μg/mL of β-asarone or 10 μmol/L of nimodipine (0.75±0.09, 0.74±0.04 in OD value, respectively, P>0.05) (Table 1, Figure 1).

Effect of β-asarone on Beclin-1 and \([\text{Ca}^{2+}]_i\) in OGD/R treated PC12 cells

Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were 6.3%±0.8%, 124.7±14.9 in normal cultured cells respectively. They were dramatically increased after OGD/R treatment (28.5%±2.3% in Beclin-1, 294.9±42.7 in \([\text{Ca}^{2+}]_i\), respectively, P<0.001). After incubation with β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) respectively, Beclin-1 expression and \([\text{Ca}^{2+}]_i\) were both significantly reduced (P<0.001). As for β-asarone (30 μg/mL) group and nimodipine (10 μmol/L) group, Beclin-1 expression and \([\text{Ca}^{2+}]_i\) were both significantly reduced (P<0.001). After incubation with β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L) respectively, Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were both significantly reduced (P<0.001). As for β-asarone (30 μg/mL) group and nimodipine (10 μmol/L) group, Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were both significantly reduced (P<0.001). As for β-asarone (30 μg/mL) group and nimodipine (10 μmol/L) group, Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were both significantly reduced (P<0.001). As for β-asarone (30 μg/mL) group and nimodipine (10 μmol/L) group, Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were both significantly reduced (P<0.001). As for β-asarone (30 μg/mL) group and nimodipine (10 μmol/L) group, Beclin-1 expression and \([\text{Ca}^{2+}]_i\), were both significantly reduced (P<0.001).

Table 1. Effect of β-asarone on cell viability and MMP in OGD/R treated PC12 cells.

| Groups          | n | Cell viability (OD value) |
|-----------------|---|--------------------------|
| Normal control  | 10| 0.79±0.04                |
| Model control   | 10| 0.46±0.02                |
| Nimodipine (10 μmol/L) | 10| 0.74±0.04                |
| β-asarone (20 μg/mL) | 10| 0.64±0.07                |
| β-asarone (30 μg/mL) | 10| 0.75±0.09                |
| β-asarone (45 μg/mL) | 10| 0.56±0.08                |

Normal control cells were grown in full culture medium under normoxic conditions. Model control cells were treated with OGD/R (2 h OGD+24 h R). Therapeutic cells were incubated with nimodipine (10 μmol/L) or β-asarone (20, 30, or 45 μg/mL) respectively for 3 h (1 h before OGD and 2 h OGD). They were incubated with full and drug-free culture medium for 24 h. β-asarone (20, 30, or 45 μg/mL) and nimodipine (10 μmol/L) both significantly increased cell viability and MMP in OGD/R treated cells. Mean±SD for 10 samples. *P<0.01 vs normal control. **P<0.01 vs OGD/R treated group.

Figure 1. Effect of β-asarone on MMP in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 μmol/L) or β-asarone (20, 30, or 45 μg/mL) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, MMP was analyzed using flow cytometry. The mean fluorescence intensity of Rhodamin represented the state of depolarization of MMP. Mean±SD for 10 samples. *P<0.01 vs normal control group. **P<0.01 vs model control group.

Observation of cellular morphology

Cellular morphology was observed under inverted phase contrast microscope. The number of neurons showed a significant reduction. The PC12 cells exhibited round, slender and degenerated morphology after OGD/R treatment. However, the number of cells showed a dramatic increase. The cells exhibited improved cellular morphology after treatment with β-asarone (20, 30, or 45 μg/mL) or nimodipine (10 μmol/L), respectively. These observations were in accordance with the results of MTT assay (Figure 4).

Observation of ultrastructural morphology of autophagosome

Representative ultrastructural morphology of autophagy was observed under transmission electron microscopy. Normal morphology was observed in cells cultured under normal conditions (Figure 5A), autophagic morphology could be observed in cells treated with OGD/R, OGD/R+nimodipine.
β-asarone can reduce neuronal apoptosis and protect neurons. However, its effect on autophagy in an ischemic stroke model of PC12 cells is rarely reported. Since PC12 cells possess the neuron-like properties, the protective effect of β-asarone on OGD/R induced injury in PC12 cells can demonstrate the possible protective mechanism of β-asarone on neurons following cerebral ischemia/reperfusion in a certain extent. Therefore, PC12 cells were established to an in vitro model of ischemia/reperfusion in this study. Autophagy is cellular process of degrading long-lived protein and organelles through the lysosomal pathway. It plays an important role in providing energy and turning over useless, superfluous and damaged organelles to maintain intracellular homeostasis. Activity of autophagy increases when cell injury, nutritional deficiencies, growth factors deficit or high energy demand occurs. However, excessive autophagy also leads to autophagic neuron death and apoptosis[4–6]. Beclin-1, a key regulatory gene of autophagy, is first discovered in mamma-

Discussion

β-asarone (cis forms of 2,4,5-trimethoxy-1-propenylbenzene) is a fat-soluble substance with a small molecular weight. It is extracted from Acorus Tatarinowii Schott. The absorption and elimination of β-asarone are rapid in vivo. β-asarone is easy to pass through blood brain barrier. Brain is an important organ of distribution of β-asarone. In the rat serum, elimination half-life and peak time are 54 and 12 min, respectively[7]. The half lives of β-asarone in blood, hippocampus, cortex, brain stem, thalamus, and cerebellum of rabbits are 1.3801, 1.300, 1.937, 7.142, 2.832, and 8.149 h, respectively[19]. β-asarone is excreted in urine, feces and bile, and the excretion efficiency is about 62% in urine, 22% in feces, and 16% in bile of rabbits. About 22% β-asarone is converted into α-asarone. Most β-asarone is excreted in 12 h[20].

β-asarone has significant pharmacological effects on central nervous system. It has effects on multi-target genes in mouse brain[9]. It can obviously increase the expression of c-fos in epilepsy rat brain[9], and attenuate neuronal apoptosis induced by β-amyloid in rat hippocampus and in PC12 cells[10, 11]. But it may also have side effects. It is reported that β-asarone could cause acute respiratory disturbance by inhibition of neurotransmission in the medullary respiratory neuronal network[21]. In this study, we observed that β-asarone could aggravate OGD/R induced injury of the cells when they had been incubated with β-asarone for too long, such as 24 h.

β-asarone can reduce neuronal apoptosis and protect neurons. However, its effect on autophagy in an ischemic stroke model of PC12 cells is rarely reported. Since PC12 cells possess the neuron-like properties, the protective effect of β-asarone on OGD/R induced injury in PC12 cells can demonstrate the possible protective mechanism of β-asarone on neurons following cerebral ischemia/reperfusion in a certain extent. Therefore, PC12 cells were established to an in vitro model of ischemia/reperfusion in this study. Autophagy is cellular process of degrading long-lived protein and organelles through the lysosomal pathway. It plays an important role in providing energy and turning over useless, superfluous and damaged organelles to maintain intracellular homeostasis. Activity of autophagy increases when cell injury, nutritional deficiencies, growth factors deficit or high energy demand occurs. However, excessive autophagy also leads to autophagic neuron death and apoptosis[4–6]. Beclin-1, a key regulatory gene of autophagy, is first discovered in mammalian. It regulates localization of other autophagy-related proteins to autophagosome[22, 23].

It is widely accepted that mitochondria are factories for energy production. And they are also the targets for autophagy. MMP is a sensitive indicator for evaluation of mitochondrial function. When mitochondrial membrane is damaged due to hypoxia, MMP decreases, which leads to ATP deficit and engulfment of damaged mitochondrial by the autophagosomes[16, 24]. ATP deficit during ischemia and reperfusion leads to damage of energy-dependent ion pump on cellular membrane, concentration disorder of intracellular and extracellular ion, depolarization, and overload of intracellular Ca2+ which in turn leads to a decrease of MMP[25]. Ca2+ partici-

Figure 2. Effect of β-asarone on Beclin-1 expression in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 μmol/L) or β-asarone (20, 30, or 45 μg/mL) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, Beclin-1 expression was analyzed using flow cytometry. Beclin-1 was represented as a percentage of positive expression. Mean±SD for 10 samples. *P<0.01 vs normal control group. **P<0.01 vs model control group.

Figure 3. Effect of β-asarone on [Ca2+]i in OGD/R treated PC12 cells. Model control cells were treated with 2 h OGD followed by 24 h reperfusion. The treated cells were incubated with nimodipine (10 μmol/L) or β-asarone (20, 30, or 45 μg/mL) 1 h before OGD and 2 h throughout OGD, followed by their transfer to full and drug-free culture medium under normoxic conditions for 24 h. Normal control cells were incubated in a regular cell culture incubator under normoxic conditions. After these treatments, [Ca2+]i was analyzed using flow cytometry. The mean fluorescent intensity of Fluo-3 was used as the indication of [Ca2+]i quantity. Mean±SD for 10 samples. *P<0.01 vs normal control group. **P<0.01 vs model control group.
pates in formation of autophagosome as an important regula-
tory factor. It modulates localization of atg18 to autophago-
somal membranes\[15\]. Removal of intracellular calcium can inhibit autophagy. For example, thapsigargin inhibits release of sarcoplasmic reticulum calcium, which also reduces autophagic flux by 50%\[26\]. Ca\(^{2+}\) leads to endoplasmic reticu-
lum misfolded proteins and mitochondria depolarization, which induces autophagy of general and specific organelles so as to reestablish cellular homeostasis\[27\]. Taken together, MMP and Ca\(^{2+}\) are both closely related with autophagy.

In this study, we showed that β-asarone and nimodipine both significantly increased cell viability and MMP, and
improved cellular morphology in OGD/R treated PC12 cells but decreased Beclin-1 and [Ca2+]i. These results suggested that increased [Ca2+]i and decreased MMP may induced Beclin-1 dependent autophagy, which is in accordance with above listed studies. β-asarone can protect PC12 cells against OGD/R induced injury. It may attenuate Beclin-1 expression above listed studies. β-asarone can protect PC12 cells against Beclin-1 dependent autophagy, which is accordance with dipine does.

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Author contribution
Zhen-tao MO and Yong-qi FANG designed the research; Zhen-tao MO, Yu-ping HE, and Sheng ZHANG performed the research; Zhen-tao MO analyzed the data and wrote the paper.

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