Original article

Parthenolide attenuates cerebral ischemia/reperfusion injury via Akt/GSK-3β pathway in PC12 cells

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A B S T R A C T

Parthenolide (PN), a sesquiterpene lactone isolated from the herbal medicine feverfew (Tanacetum parthenium), was reported to possess neuroprotective activity. However, the neuroprotective effect of PN against cerebral ischemia/reperfusion (I/R) injury remains unclear. Therefore, the aim of the present study was to explore the neuroprotective effects of PN against oxygen-glucose deprivation (OGD)-induced apoptosis in PC12 cells and the underlying mechanisms. Our results demonstrated that PN ameliorated OGD/R-evoked neuronal injury and oxidative stress in PC12 cells. In addition, PN notably decreased HIF-1α expression, as well as inhibited apoptosis in PC12 cells after OGD/R. Furthermore, PN pretreatment significantly enhanced the phosphorylation of Akt and GSK-3β in PC12 cells exposed to OGD/R. In conclusion, the present study demonstrated that PN exhibits a neuroprotective effect against OGD/R through activation of the Akt/GSK-3β signaling pathway. Our findings suggest that PN has the potential to serve as a novel therapeutic agent for cerebral I/R injury.

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1. Introduction

Cerebral ischemia/reperfusion (I/R) is a major cause of severe disability and death in the world [1]. Despite the improvement in diagnosis and treatment for cerebral I/R during the last decade [2–4], therapeutic options to minimize the detrimental effects of cerebral I/R injury are limited. A better understanding of the molecular mechanisms responsible for cerebral I/R is urgent. Cerebral I/R is a complex pathophysiological process. Increasing evidence has demonstrated that oxidative stress plays a pivotal role in the pathogenesis of cerebral I/R injury. Excessive reactive oxygen species (ROS) can affect mitochondrial enzymes, lipids, and DNA, causing deleterious effects leading to mitochondrial dysfunction [5–7]. Thus, inhibiting oxidative stress may be a good way for the treatment of cerebral I/R injury.

Parthenolide (PN) is a sesquiterpene lactone isolated from the herbal medicine feverfew (Tanacetum parthenium). Previous studies have demonstrated that PN possesses anti-microbial, anti-inflammatory, anti-tumor and neuroprotective activities [8–10]. Dong et al. reported that PN administration dramatically ameliorated neurological deficit, brain water content, and infarct volume in a permanent middle cerebral artery occlusion (MCAO) model [11]. However, the neuroprotective effect of PN against cerebral ischemia/reperfusion injury remains unclear. Therefore, the aim of the present study was to explore the neuroprotective effects of PN against oxygen-glucose deprivation (OGD)-induced apoptosis in PC12 cells and the underlying mechanisms. Our results demonstrated that PN attenuated the OGD-induced neuronal damage through activation of the Akt/GSK-3β signaling pathway.

2. Materials and methods

2.1. Cell culture

PC12 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10%
pre-treated with various concentrations of PN (1, 5 and 10 μM) and subsequently exposed to OGD/R. Then, we added 10 μl of reagent from CCK-8 (Dojindo, Kumamoto, Japan) to each well. After 1 h of incubation at 37 °C, the absorbance at 490 nm was measured using a spectrophotometer.

### 2.4. Cell cytotoxicity assay

Cell cytotoxicity was measured by lactate dehydrogenase (LDH) assay (Promega, Poland). Briefly, after treatment, LDH release into the surrounding medium was measured according to the manufacturer’s protocol. Cellular LDH release was expressed as a percentage of the total LDH released from cells into the culture medium.

### 2.5. Determination of ROS and malondialdehyde (MDA) levels

The intracellular ROS levels were determined using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay kit (Beyotime Institute of Biotechnology, Nan tong, China). Briefly, treated cells were incubated with DCFH-DA at a final concentration of 20 μM for 30 min at 37 °C in the dark and then were washed with PBS for 3 times. The fluorescence intensity was measured at an excitation and emission wavelength of 488 nm and 525 nm, using a fluorescent microplate reader (Molecular Devices, Gemini XS, USA).

MDA content was estimated using thiobarbituric acid (TBA) method [12]. Briefly, treated cells were harvested and sonicated with phosphate buffer (pH 6.8) containing 1.0 mM phenylmethylsulfonyl fluoride to obtain cell homogenates. After centrifuged at 4000 rpm/min for 10 min at 4 °C, supernatant was collected. Absorbance was measured at 532 nm by spectrometry.

### 2.6. Determination of antioxidant enzymatic activities

After treatment, the antioxidant enzymatic activities of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured using commercially available kits, respectively, according to the manufacturer’s instructions.

### 2.7. Caspase-3 activity assay

The caspase-3 activity was measured using a commercially available kit (Abcam, Cambridge, UK). Briefly, after treatment, PC12 cells were lysed and then incubated on ice for 20 min to ensure complete cell lysis. Cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant fraction was collected for the determination.

### 2.8. Western blotting

Cells were harvested and lysed in RIPA lysis buffer (Beyotime, Haimen, China). Protein concentration was assessed using a BCA protein assay kit (Beyotime, China). The equal amount of protein samples was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After being blocked by 5% non-fat milk at room temperature for 1, the membranes were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used: anti-HIF-1α, anti-Bcl-2, anti-Bax, anti-p-PI3K, anti-PI3K, anti-p-Akt, anti-Akt and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the membrane was incubated with the corresponding horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Band was visualized via an enhanced chemiluminescence by an enhanced...
chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

2.9. Statistical analysis

All statistical analyses were performed using the SPSS software (ver. 13.0; SPSS, Chicago, IL). The quantitative data derived from three independent experiments are expressed as means ± SD. Statistical comparisons were performed using one-way analysis of variance followed by the Student’s t-test. P < 0.05 was considered statistically significant.

3. Results

3.1. Pretreatment with PN increased cell viability and prevented LDH release in OGD-treated PC12 cells

First, we examined the effects of PN on cell viability using the CCK-8 assay. After being subject to OGD, cellular viability was significantly reduced to 39% ± 1.2% compared to control. PN pretreatment at 1 to 10 μM substantially decreased OGD-induced cell death, respectively increasing viability rate to 48% ± 2.3%, 62% ± 4.1%, and 73% ± 5.4%. As the concentration was increased to 20 μM, PN did not show further protection (Fig. 1A). These results were further confirmed by the LDH release assay. The results demonstrated that OGD/R induced an obvious increase of LDH release in PC12 cells, which was significantly attenuated by PN treatment (Fig. 1B).

3.2. Pretreatment with PN attenuated ROS level and increased the activities of enzymes in OGD –treated PC12 cells

Oxidative stress is frequently implicated in the pathology of secondary neuronal damage following cerebral I/R. Thus, we examined the effect of PN on intracellular ROS level in PC12 cells exposed to OGD/R. As shown in Fig. 2A, OGD/R treatment provoked an elevation of ROS level, with a 2.8-fold increase compared with the control group. However, pretreatment of PN prior to OGD/R significantly decreased the level of intracellular ROS in PC12 cells. Furthermore, we observed that OGD/R treatment significantly decreased the activities of catalase (Fig. 2B), SOD (Fig. 2C) and GPx (Fig. 2D) in PC12 cells, respectively. PN pretreatment obviously restored the activities of catalase, SOD and GPx in PC12 cells exposed to OGD/R, respectively.
3.3. Pretreatment with PN decreased HIF-1α expression in PC12 cells after OGD/R

Hypoxia increases the expression of HIF-1α which induces apoptosis. Thus, we examined the effect of PN on HIF-1α expression in PC12 cells exposed to OGD/R. As shown in Fig. 3, OGD/R resulted in the up-regulation of HIF-1α. However, pretreatment of PN markedly decreased HIF-1α expression in PC12 cells exposed to OGD/R (Fig. 3).

3.4. PN notably inhibited apoptosis in PC12 cells after OGD/R

Next, we investigated the effects of PN on apoptosis-related genes expression in PC12 cells exposed to OGD/R. As shown in Fig. 4A, OGD/R treatment decreased Bcl-2 (an anti-apoptotic protein) expression and increased Bax (a pro-apoptotic protein) expression in PC12 cells, as compared with the control group. However, these changes were significantly reversed by PN pretreatment. Furthermore, we investigated the effects of PN on caspase-3 activity in PC12 cells under OGD/R condition. As indicated in Fig. 4B, the OGD group exhibited significantly increased caspase-3 activity. PN treatment markedly reduced caspase-3 activity compared to the OGD group (1.9 ± 0.2 versus 3.2 ± 0.3).

3.5. PN promoted the phosphorylation of Akt and GSK-3β in OGD-treated PC12 cells

To further investigate the molecular mechanism underlying PN-mediated neuroprotection, we determined p-Akt/Akt and p-GSK-3β/GSK-3β in PC12 cells exposed to OGD/R. Western blot analysis showed that the protein expression levels of p-Akt and p-GSK-3β were significantly decreased in PC12 cells exposed to OGD/R, compared to the control group; while the protein levels of total Akt and GSK-3β showed no difference. In addition, PN pretreatment significantly enhanced the phosphorylation of Akt and GSK-3β in PC12 cells (Fig. 5).

4. Discussion

In the present study, our results demonstrated that PN ameliorated OGD/R-evoked neuronal injury and oxidative stress in PC12 cells. In addition, PN notably decreased HIF-1α expression, as well as inhibited apoptosis in PC12 cells after OGD/R. Furthermore, PN pretreatment significantly enhanced the phosphorylation of Akt and GSK-3β in PC12 cells exposed to OGD/R.

OGD/R has been widely used as an in vitro ischemic model. Previous studies have demonstrated that OGD results in cell damage [13,14]. In this study, to evaluate the neuroprotective effects of PN on cerebral I/R injury, an OGD culture system was employed in vitro. Our data showed that OGD/R significantly decreased neuronal viability and increased LDH release in PC12 cells, which is consistent with the results of previous studies [15–17]. Conversely, PN pretreatment effectively increased neuron survival and prevented LDH release. These results suggest that PN exerts a protective effect in PC12 cells against OGD-induced cell viability.

OGD/R-induced neuronal cell damage is accompanied with oxidative stress. Several studies have shown that the ROS generation is aberrant in the processes of cerebral I/R injury [6,18,19]. Pharmacutes inhibiting ROS formation or antagonizing ROS activity are neuroprotective against reperfusion injury. In the current study, we found that OGD/R-induced ROS production was significantly inhibited by PN treatment. In addition, PN pretreatment obviously restored the activities of catalase, SOD and GPx in PC12 cells exposed to OGD/R. These data suggest that PN inhibited OGD/R-induced oxidative stress partially through suppressing the accumulation of ROS and increasing the activities of enzymes.

Emerging evidence has suggested that apoptosis is a major event leading to cerebral I/R injury [20–22]. Bcl-2 family proteins consist of antiapoptotic proteins such as Bcl-2 and Bcl-xL and proapoptotic proteins such as Bax and Bak. Bcl-2/Bax ratio is a well-established determinant in the regulation of apoptosis [23]. Caspase-3 is also as a key mediator of apoptosis in animal models of ischemic stroke [24]. In this study, we found that PN pretreatment greatly down-regulated the expression of Bax and caspase-3 activity, and up-regulated the expression of Bcl-2 in PC12 cells exposed to OGD/R. These data strongly suggest that PN protected PC12 cells in vitro from OGD-induced death by reducing the OGD-mediated induction of caspase-3 activation and Bax and inducing Bcl-2.

Increasing evidences suggest that the Akt/GSK-3β signaling pathway plays an important role in the pathogenesis of cerebral I/R injury [25–27]. Akt is a downstream kinase of PI3K that is activated by phosphorylation and plays an important role in cell death and survival processes [28]. In addition, it was reported that the activation of Akt leads to phosphorylation of the downstream effector GSK-3β, which is considered as a key player in regulating mitochondrial signaling and cell apoptosis during cerebral ischemia [29]. Valerio et al. reported that the GSK-3 inhibitor SB216763 obviously reduced ischemic cerebral damage, restored impaired mitochondrial biogenesis and prevented ROS production in ischemic stroke [30]. In this study, we found that PN pretreatment significantly enhanced the phosphorylation of Akt and GSK-3β in PC12 cells exposed to OGD/R. These data suggest that PN protected PC12 cells against OGD/R-induced injury through activation of the Akt/GSK-3β signaling pathway.

In conclusion, the present study demonstrated that PN exhibits a neuroprotective effect against OGD/R through activation of the Akt/GSK-3β signaling pathway. Our findings suggest that PN has
PN notably inhibited apoptosis in PC12 cells after OGD/R. Cultures were exposed or not exposed to 2 h oxygen-glucose deprivation and 24 h reoxygenation. 10 µM PN was added to the cultures 1 h before oxygen-glucose deprivation. A, The protein expression levels of Bcl-2 and Bax were detected using western blot. B, The caspase-3 activity was measured using a commercially available kit. Data are expressed as means ± SD of three independent experiments. *P < 0.01, #P < 0.01 versus OGD.
Fig. 5. PN promoted the phosphorylation of Akt and GSK-3β in OGD-treated PC12 cells. Cultures were exposed or not exposed to 2 h oxygen-glucose deprivation and 24 h reoxygenation. 10 μM PN was added to the cultures 1 h before oxygen-glucose deprivation. (A) The protein expression levels of p-Akt, Akt, p-GSK-3β and GSK-3β were detected using western blot. (B) Quantitative analysis of p-Akt levels normalized to t-Akt levels; (C) Quantitative analysis of p-GSK-3β levels normalized to t-GSK-3β levels. Data are expressed as means ± SD of three independent experiments. *P < 0.01, **P < 0.01 versus OGD.

the potential to serve as a novel therapeutic agent for cerebral I/R injury.

Conflicts of interest

The authors declare that they have no potential or actual conflicts of interest.

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