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Astragaloside IV reduces the hypoxia-induced injury in PC-12 cells by inhibiting expression of miR-124

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ABSTRACT

Background: *Astragalus membranaceus* has been clinically used in cerebral ischemia treatment in China and its main component, Astragaloside IV (Ast IV) shows anti-hypoxia activity, but the underlying mechanism has not been clearly clarified. This study was aimed to investigate the effects of Ast IV on hypoxia-induced injury in PC-12 cells as well as the underlying mechanism.

Methods: Relative miR-124 expression was detected by qRT-PCR. Hic-5 expression was analyzed by qRT-PCR and Western blot. To alter miR-124 and Hic-5 expressions, cells were respectively transfected with miR-124 mimic and pEX-Hic-5. Cell proliferation and apoptosis were measured by BrdU assay and Annexin V-fluorescein isothiocynate (FITC)/propidium iodide (PI) double staining method, respectively. Besides, apoptotic proteins and cell proliferation-associated factors were analyzed by Western blot.

Results: Ast IV alleviated hypoxia-induced injury in PC-12 cells by decreasing apoptosis ($P < 0.01$). Ast IV inhibited up-regulation of miR-124 induced by hypoxia ($P < 0.01$). miR-124 mimic impaired the anti-apoptotic effect of Ast IV on PC-12 cells ($P < 0.01$). Hic-5 expression was significantly down-regulated in miR-124 overexpressed cells ($P < 0.001$) and Hic-5 overexpression activated Sp1/Survivin signaling pathway ($P < 0.001$).

Conclusion: Ast IV could ameliorate hypoxia-induced injury in PC-12 cells by decreasing miR-124 expression and then up-regulating Hic-5 expression.

1. Introduction

Cerebral ischemia is one type of central nervous system (CNS) diseases, which might be triggered by ischemia and hypoxia [1]. Cerebral ischemia can be clinically divided into acute and chronic cerebral ischemia [2]. Acute cerebral ischemia is a common disease in the elderly and thereby it causes people's attention and prevention [3]. The chronic cerebral ischemia can induce impaired memory, dizziness, sleep disturbance and other complications [4]. Cerebral ischemia is accompanied with hypoxia-induced injury of nerve cells [5]. Even though, understanding the etiology of brain ischemia is advanced and deeper, effective treatment to ameliorate ischemic injury still remains limited. Nowadays, more attention was turned to traditional medicine. *Astragalus membranaceus*, a traditional medicine, has been used in China for inhibiting stroke and other cardiovascular and cerebrovascular diseases [6,7]. Astragaloside IV (Ast IV) is a depurated small molecular saponin belonging to the principal active ingredients of *Astragalus membranaceus* [8,9]. Increasing evidence showed that Ast IV possesses a variety of protective roles, including anti-oxidative injury [10], anti-inflammation [11], anti-hepatitis [12], anti-cancer [13], anti-diabetes [14], and anti-chronic heart failure [15]. In addition, the preclinical evidence revealed that Ast IV could ameliorate neurological deficit and decrease infarct volume [16]. It could also suppress neuroinflammation response and further exert the neuroprotective effect against cerebral ischemia in mice [17]. Importantly, Ast IV was found to have function in protecting against ischemic brain injury in a murine model of transient focal ischemia [8]. The moderating effect of Ast IV on hypoxia-ischemia induced blood-brain barrier disruption was not novel, but the underlying mechanisms have not been clarified yet.

microRNAs (miRNAs) play crucial roles in the modulation of genes' expression at the post-transcriptional level, via degradation or translational inhibition of their target mRNAs [18]. The growing studies

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demonstrated the functions of miRNAs in ischemic diseases [19–21]. Among these identified miRNAs, miR-124 is a specific miRNA in nervous system and its aberrant expression contributes to the pathological condition related with CNS [22]. The role of miR-124 in cerebral ischemia was investigated by a series of studies [22–24], but its participation in traditional Chinese medicines to regulate their functions in treatment of cerebral ischemia was rarely reported.

This study was undertaken to explore whether there existed a potential protective effect of Ast IV on hypoxia-induced injury in PC-12 cells via regulating miR-124.

2. Materials and methods

2.1. Cell culture and treatment

PC-12 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) (density of 1×10^4 cells/ml) containing fetal bovine serum (FBS, 10% (v/v), Life science, UT, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in the incubator with suitable humidity. The O_2 concentration of 3% and 21% were used as hypoxia and normoxia culture condition, respectively. Cells were treated with Ast IV (ref: 74777, > 98.0%, Sigma-Aldrich, St. Louis, USA) at the concentration of 10 μg/ml.

2.2. Quantitative real time polymerase chain reaction (qRT-PCR) analysis

As described previously [25], qRT-PCR for miR-124 was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). After the total RNA was extracted using Trizol reagent and treated with DNAse I (Life Technologies Corporation, Carlsbad, CA, USA), RNA was reverse transcribed with reverse transcriptase (ReverTra Ace, Toyobo). The level of miR-124 analyzed by qRT-PCR was normalized to values of U6. qRT-PCR for Hydrogen peroxide-inducible clone-5 (Hic-5) was performed as Wang et al. described [26]. The data were standardized to level of GAPDH. Their expressions were calculated by relative quantification $2^{-\Delta\Delta Ct}$ method.

2.3. Cell transfection

For miR-124 overexpression, miR-124 mimic synthesized by Life Technologies Corporation was used in this study. For Hic-5 overexpression, the whole length of Hic-5 was cloned to pEX-2, which was transfected into PC-12 cells using lipofectamine 3000 reagent. Transfected cells were harvested after post-transfection of 48 h.

2.4. Cell proliferation assay

PC-12 cells spread to the bottom of the dish with diameter of 3.5 cm were incubated for 72 h. Bromodeoxyuridine (BrdU, Sigma-Aldrich) was added to the cultured cells at a concentration of 1 mg/ml. After the labeling process for 3 h, cells were incubated with the mouse anti-BrdU antibody (ab8152) and goat anti mouse IgG (ab6785, Abcam, Cambridge, MA, USA). BrdU-positive cells were observed and counted under the fluorescence microscope from 10 selected visual fields.

2.5. Apoptosis assay

Cells were collected by centrifugation at 2000 *g* for 10 min and then rinsed with phosphate buffer saline (PBS). Afterwards, cells were suspended in 300 μl binding buffer and then 5 μl Annexin V- fluorescein isothiocynate (FITC) solution was added in the cell suspension. After incubation at room temperature away from light for 15 min, 5 μl propidium iodide (PI) solution was added in cell suspension. Before flow cytometry analysis, 200 μl binding buffer was supplemented. FACScan (Beckman Coulter, Fullerton, CA, USA) was used and the data were analyzed by using FlowJo software (Tree Star Software, San Carlos, California, USA).

2.6. Western blot

After protein was extracted and quantified, 50 μg protein was separated using 12% sodium dodecyl sulfate-polyacrykamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane. PVDF membranes were incubated with a series of primary antibodies prepared in 5% blocking buffer at a dilution of 1:1000 at 4 °C overnight. The primary antibodies used here were listed as follows: Bcl-2 (ab196495), Bax (ab32503), pro-Caspase-3 (ab44976), cleaved-Caspase-3 (ab49822), pro-Caspase-9 (ab2013), cleaved-Caspase-9 (ab52298), proliferating cell nuclear antigen (PCNA) (ab18197), Cyclin A (ab137769), Cyclin E1 (ab71535), CDK2 (ab32147), Cyclin D1 (ab134175), CDK4 (ab199728), Hic-5 (ab42476), Sp1 (ab227383), and Survivin (ab469, Abcam, Cambridge, UK). Then PVDF membranes were incubated in the horseradish peroxidase-conjugated second antibody for 1 h at room temperature. Signals were captured by enhanced chemiluminescence method.

2.7. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. We evaluated the data with Student's *t*-test and a one-way analysis of variance for multiple comparisons. The data with *P* value less than 0.05 was considered significant.

3. Results

3.1. Hypoxia induced growth-inhibition of PC-12 cells

Oxygen-deprivation induced injury in PC-12 cells along with significantly increase of apoptotic cell rate ($P < 0.001$, Fig. 1A), up-regulation of pro-apoptotic proteins (Bax, cleaved-Caspase-3, and cleaved Caspase-9), and down-regulation of anti-apoptotic protein (Bcl-2) (Fig. 1B). In addition, cell proliferation was significantly decreased ($P < 0.001$, Fig. 1C) and tested pro-proliferation factors were down-regulated (PCNA, Cyclin A, Cyclin E1, CDK2, Cyclin D1, and CDK4) (Fig. 1D) after oxygen-deprivation. Thus, the growth of PC-12 cells was inhibited in response to hypoxia.

3.2. Ast IV suppressed hypoxia-induced apoptosis of PC-12 cells

The effects of Ast IV on growth of hypoxia-stimulated PC-12 cells were analyzed. The apoptotic cell rate was significantly decreased after Ast IV treatment ($P < 0.01$, Fig. 2A), indicating that Ast IV attenuated the pro-apoptotic effect of hypoxia. Simultaneously, Bcl-2 expression was increased and expressions of Bax as well as cleaved-Caspase-3/9 were decreased (Fig. 2B). However, Ast IV had no significant proliferation-promoting effect on PC-12 cells ($P > 0.05$, Fig. 2C) and also no significant effects on expressions of proliferation-associated genes (Fig. 2D). To sum up, Ast IV exerted anti-apoptotic role in PC-12 cells exposed to hypoxia injury and thereby the anti-apoptosis molecular mechanism of Ast IV was investigated.

3.3. Ast IV reduced apoptosis of PC-12 cells by down-regulating miR-124

miR-124 expression was found to be up-regulated in hypoxia-treated cells but further down-regulated by Ast IV treatment (both $P < 0.01$, Fig. 3A). The role of miR-124 in the function of Ast IV was investigated. miR-124 overexpression was achieved by transfection with miR-124 mimic ($P < 0.01$, Fig. 3B). It was found that miR-124 mimic impaired the anti-apoptotic effect of Ast IV ($P < 0.01$, Fig. 3C); and compared with Ast IV treatment group, Bcl-2 expression was down-