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Astragalus polysaccharide protects hypoxia-induced injury by up-regulation of miR-138 in rat neural stem cells

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A R T I C L E I N F O

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A B S T R A C T

Astragalus polysaccharide (APS) is the main active ingredient of *astragalus* and exhibits various pharmacological effects. This study aimed to investigate the effect of APS on hypoxia-induced injury in neural stem cells (NSCs). The NSCs derived from the hippocampus of rat were subjected to hypoxia incubator to establish a hypoxia model. NSCs were pretreated with APS before hypoxia injury to investigate the effect of APS. The expression of miR-138 was inhibited by transfection with miR-138 inhibitor and the miR-138 level was measured by qRT-PCR. Cell viability and apoptotic cell rates were respectively assessed by CCK-8 assay and flow cytometry assay. Western blot was performed to determine the expression of apoptosis-, JNK pathway- and p38MAPK pathway-related factors. Hypoxia exposure caused the reduction of cell viability and induction of cell apoptosis of NSCs. However, APS pretreatment attenuated the cell injury induced by hypoxia, as evidenced by increased cell viability, reduced apoptotic cells, inhibited expression of pro-apoptotic factors and enhanced expression of anti-apoptotic factor. Interestingly, higher miR-138 expression was observed in the hypoxia-injured NSCs compared with normoxia, and miR-138 expression was further up-regulated by APS pretreatment. Furthermore, miR-138 inhibitor blocked the protective effect of APS on hypoxia-injured NSCs. In addition, we found that APS inhibited the JNK and p38MAPK pathways through miR-138. In conclusion, this study demonstrated a protective effect of APS on hypoxia-induced NSC injury. The regulatory mechanism might be mediated by up-regulation of miR-138 and inhibition of the JNK and p38MAPK pathways.

1. Introduction

Perinatal hypoxic-ischemic encephalopathy (HIE) is a critical cause of brain injury in the newborn and can bring about permanent and devastating consequences, thus making it become a major burden for the patient, the family and even society [1,2]. It is of great significance to identify and develop promising therapeutic strategies to attenuate brain injury in newborns with HIE. Brain is particularly sensitive to hypoxia injury due to their high demand but low reserve of oxygen, and brain consumes almost 20% of the total body oxygen [3]. Neural stem cells (NSCs), mainly derived from hippocampus of the brain, have been indicated to possess the capacity of self-renewal and multi-lineage differentiation [4]. These cells exhibit the utilizing potential to develop the transplantation strategies and screen the candidate agents for neurogenesis in neurodegenerative diseases [5].

MicroRNAs (miRNAs/miRs) are a group of small and endogenous non-coding RNAs that regulate gene expression through directly cleaving target messenger RNA (mRNA) or translational repression [6]. MiR-138 has been identified as a tumor suppressor in various kinds of cancers [7–9]. Interestingly, it has been proved that miR-138, up-regulated in hypoxia-induced pulmonary artery smooth muscle cells (PASMCs), can enhance proliferation and inhibit apoptosis of PASMCs [10]. Moreover, miR-138 has been reported to exert a protective effect on hypoxia-induced apoptosis through the MLK3/JNK/c-Jun pathway in cardiomyocytes [11]. It still remains unclear whether miR-138 could be involved in the hypoxia-induced NSCs.

Astragalus polysaccharide (APS) is one of the main active ingredients of *astragalus* and has been identified to possess various effects, such as anti-inflammatory, anti-oxidative, anti-aging, anti-viral and anti-tumor functions [12]. A previous study has revealed that APS

Abbreviation: APS, *Astragalus* polysaccharide; NSCs, neural stem cells; HIE, hypoxic-ischemic encephalopathy; miRNA/miR, microRNA; mRNA, messenger RNA; PASMCs, pulmonary artery smooth muscle cells; SD, Sprague-Dawley; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; CCK-8, Cell Counting Kit-8; PI, propidium iodide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-poly-acrylamide gel electrophoresis; PVDF, polyvinylidene fluoride membrane; BSA, bovine serum albumin; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; ANOVA, one-way analysis of variance; CNS, central nervous system; HCMECs, human cardiac microvascular endothelial cells; GSCs, glioma stem cells

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protects myocardial microvascular endothelial cells against the hypoxia/reoxygenation-induced cell damages via mediation of the PI3K/Akt/eNOS signaling pathway [13]. In addition, *astragalus* extract has been reported to exhibit the protective effect on intermittent hypoxia-induced hippocampal neurons impairment in rats [14]. However, the effect of APS on NSCs has not been fully explored yet. On account of the observations talked above, we hypothesized that APS might also exert a protective role in hypoxia-induced injury of NSCs. The present study aimed to explore the effect of APS on hypoxia-induced injury in NSCs. We found that APS significantly increased the cell viability and reduced cell apoptosis. Furthermore, we found that miR-138 was involved in the regulatory effect of APS on hypoxia-injured NSCs and miR-138 inhibitor reversed the effect of APS on cell viability and apoptosis of NSCs. In addition, we also found that miR-138 mediated the inhibitory effects of APS on the JNK and p38MAPK pathways in hypoxia-injured NSCs. Our study might provide new insights of APS pharmacological effects on NSCs and enrich the function of APS in clinical application.

2. Materials and methods

2.1. Experimental animals

Timed-pregnant Sprague-Dawley (SD) rats weighing 220–250 g were purchased from Yangzhou University Medical Centre (Yangzhou, China). All procedures and assessments were approved in accordance with the Animal Care and Use Committee guideline of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in a 12 h light-dark cycle and allowed free access to food and water.

2.2. Cell culture and treatment

Primary NSCs were obtained from the hippocampus of embryonic day 14 SD rats following the previously described method [15]. Briefly, SD rats were anesthetized with intraperitoneal chloral hydrate (2 ml/kg of body weight) (Sigma, St. Louis, MO, USA). The hippocampus was mechanically isolated from the brain tissues and was trypsinized in 0.125% trypsin (Gibco, Grand Island, New York, USA). Then, the cell suspension was centrifuged at 1000 rpm for 5 min and the cell pellet was dissociated to a single-cell suspension. NSCs were seeded at a density of 5×10^4 cells/ml on the culture flask (Corning, Acton, MA, USA) containing 1:1 Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco), 2% B27 (Invitrogen, Carlsbad, CA, USA), 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA), 10 ng/ml epidermal growth factor (EGF; Peprotech), 1% penicillin/ streptomycin and 1% L-glutamine (Gibco). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and were passaged via weekly digestion with Accutase (Millipore, Bed-ford, MA, USA) in the medium described above. All NSCs used in this study were between passages 2 and 4.

For hypoxia treatment, cell culture was performed in a hypoxia chamber (STEMCELL Technologies, Vancouver, British Columbia, Canada) filled with an anaerobic gas mixture of 94% N_2, 5% CO_2, and 1% O_2. APS were purchased from Pharmagenesis, Inc. (Boster Biology Co., Wuhan, China). APS was dissolved in DMEM/F12 to generate a 10 mg/ml working solution, and were further diluted with DMEM/F12 at different concentrations (2.5, 5 and 7.5 mg/ml). For the measurements of APS toxicity, NSCs were treated with APS (0–10 mg/ml) for 24 h. For the analysis of the protective effect of APS, NSCs were pretreated with APS (0–5 mg/ml) for 2 h before the establishment of hypoxia injury.

2.3. Cell transfection

The inhibitor control and miR-138 inhibitor were synthesized by GenePharma Co. (Shanghai, China). MiR-138 inhibitor or the inhibitor control was transfected into NSCs using Lipofectamine 3000 reagent (Invitrogen) on the basis of the manufacturer's instructions. After transfection for 48 h, cells were collected for the following experiments.

2.4. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNAs were reverse transcribed to cDNA through using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The Taqman Universal Master Mix II (Applied Biosystems) was used for the real-time PCR analysis to measure the expression levels of miR-138. Data were analyzed by relative quantification using the $2^{-\Delta\Delta Ct}$ method [16], normalizing to U6. Each sample was analyzed for three times, and all reactions were run in triplicate.

2.5. Cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA). Briefly, cells were seeded on the 96-well plates at a density of 5×10^3 cells/well. After treatment, 20 μl of CCK-8 solution was added to the culture medium, and the mixtures were incubated for another 1 h at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. Then, the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA). Each sample was analyzed for three times.

2.6. Apoptosis assay

Cell apoptosis was assessed by flow cytometry using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). In brief, cells were collected and washed with cold phosphate-buffered saline (PBS) for three times. Then, cells were resuspended in binding buffer and were mixed with 10 μl of Annexin V-FITC and 10 μl of PI according to the manufacturer's instructions. After incubation for 30 min at room temperature in the dark, apoptotic cells were determined by a FACS can (Beckman Coulter, Fullerton, CA, USA) and data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). Each sample was analyzed for three times.

2.7. Western blot analysis

The protein samples were extracted from cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing the protease inhibitors (Roche, Basel, Switzerland) according to the manufacturer's protocol. A BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used to quantify the concentration of protein samples. Equal amounts of protein (30 μg for each sample) were electrophoresed on sodium dodecyl sulfate-poly-acrylamide gel electrophoresis ((SDS-PAGE)) and then were transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore). These membranes were blocked in 5% bovine serum albumin (BSA; sigma) for 2 h and then were incubated at 4 °C overnight with the responding primary antibodies (at a dilution of 1:1000 in 5% BSA): Bax (#5023), Cleaved caspase 3 (#9662), Cleaved caspase-9 (#20,750), Bcl-2 (#4223), β-actin (#4970), p-p38MAPK (#9211), p38MAPK (#9212), JNK (#9252), p-JNK (#4668), c-Jun (#9165) and p-c-Jun (#3270; Cell Signaling Technology, Beverly, MA, USA). Then, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution; Santa Cruz, Santa Cruz, CA, USA) for 2 h at room temperature. The blots were visualized by enhanced chemiluminescence (ECL) kit (Pierce) and the optical density of bands was analyzed by Image J software (National Institute of Health, Bethesda, MA, USA).

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