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Angelica sinensis polysaccharide protects rat cardiomyocytes H9c2 from hypoxia-induced injury by down-regulation of microRNA-22

Hui Pan, Linlin Zhu*

Department of Cardiology, Affiliated Hospital of Jining Medical University, Jining, Shandong 272000, China

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ABSTRACT

Background: The cardioprotective role of *Angelica sinensis* has been proven in previous studies. However, the effects of *Angelica sinensis* polysaccharide (ASP, major bioactive component of *Angelica sinensis*) on myocardial infarction (MI) remain unclear. This study aimed to investigate the effects of ASP on hypoxia-induced H9c2 cell injury as well as the underlying mechanisms.

Methods: We constructed *in vitro* hypoxic model to mimic MI. Cell viability, proliferation and apoptosis were respectively measured by using CCK-8 assay, Western blot analysis, and flow cytometry assay/Western blot analysis, to evaluate cell injury after treatments. The effects of ASP pretreatment on hypoxia-induced injury were explored. Expression of miR-22 after treatments was determined by stem-loop RT-PCR, and whether ASP affected H9c2 cells via miR-22 was studied. Involvements of the PI3K/AKT and JAK1/STAT3 pathways were finally explored.

Results: Hypoxia-induced decreases of cell viability and proliferation as well as increase of apoptosis were attenuated by ASP pretreatments. Hypoxia treatment up-regulated miR-22 expression, and the up-regulation was mitigated by ASP pretreatment. Effects of ASP pretreatment on hypoxia-treated H9c2 cells were mitigated by miR-22 overexpression while were augmented by miR-22 inhibition. Phosphorylation levels of PI3K, AKT, JAK1 and STAT3 were increased by ASP through down-regulating miR-22 in hypoxia-treated H9c2 cells.

Conclusion: ASP pretreatment attenuated hypoxia-induced H9c2 cell injury, possibly through down-regulating miR-22 expression. The PI3K/AKT and JAK1/STAT3 pathways were activated by ASP pretreatment via miR-22 in hypoxia-treated cells.

1. Introduction

Myocardial infarction (MI) resulting from temporary or permanent occlusion of the main coronary arteries is a leading cause of death and disability globally [1]. MI may reduce blood supply to the beating heart muscle of the left ventricle, leading to formation of fibrous cicatricial tissues and remodeling of ventricular tissues. Ultimately, the contraction ability of the myocardium was decreased, resulting in heart failure [2,3]. Currently, the reperfusion therapy with thrombolytic drugs can effectively restore blood supply and reduce injury of MI to some extent [4]. However, the reperfusion itself may induce secondary injury to the myocardium, limiting the therapeutic effects [5]. There is an unmet need to develop innovative drugs for improving outcome of MI.

Angelica sinensis polysaccharide (ASP) is a β-D-pyranoid polysaccharide, extracted from the roots of a Chinese herbal medicine, *Angelica sinensis* [6]. For thousands of years, *Angelica sinensis* has been widely applied for treatments of numerous diseases including cardiovascular disease [7]. The aqueous extract of *Angelica sinensis* showed a cardioprotective role in doxorubicin-induced chronic cardiotoxicity [8]. Therefore, we hypothesized that ASP as the major bioactive component of *angelica sinensis* might play an important role in MI-induced myocardial dysfunction. Recently, multiple biological activities of ASP have been identified, such as hematopoiesis, immunomodulation, anti-oxidation, anti-tumor, radioprotection, and *etc*. [6,9]. However, the specific role of ASP in MI which needs more attention remains unclear.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs with a length of 19–24 nucleotides, which participate in diverse biological processes through regulating gene expression [10,11]. For MI, a previous study has shown that miR-125b-5p can protect the heart against MI through suppressing pro-apoptotic bak1 and klf13 in cardiomyocytes [12]. Likewise, miR-375 inhibition was reported to attenuate post-MI pathological response through the PDK-1-AKT signaling axis [13]. Down-regulation of miR-675 was observed in SH-SY5Y cells under stimulation with ASP, suggesting that miRNAs might be dysregulated after ASP treatments and thereby affected the downstream responses [14].

* Corresponding author at: Department of Cardiology, Affiliated Hospital of Jining Medical University, No.89, Guhuai Road, Jining, Shandong 272000, China.
E-mail address: zhulinlin64@yeah.net (L. Zhu).

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The irreversible injury of myocardium occurs within 20 min of ischemia. The first phase in the progression from healthy to infarcted myocardium is cardiomyocyte loss [3]. Therefore, proliferation and apoptosis of cardiomyocytes after MI is of great importance. Herein, *in vitro* cell model to mimic MI was induced by hypoxia in rat cardiomyocytes H9c2. The *in vitro* effects of ASP on proliferation and apoptosis of H9c2 cells under hypoxia were studied. We also explored the possible downstream miRNAs as well as signaling pathways.

2. Materials and methods

2.1. Cell culture and treatment

H9c2 cells (ATCC® CRL-1446™) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were derived from rat embryonic ventricular cardiomyocytes. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with fetal bovine serum (FBS; 10%, Hyclone) at 37 °C. Normally, H9c2 cells were maintained in a humidified incubator filled with 5% CO_2 and 95% air. Cells under hypoxic condition were incubated in a hypoxic incubator containing 94% N_2, 5% CO_2 and 1% O_2. ASP was purchased from Ci Yuan Biotechnology Co., Ltd., Shaanxi (Xian, China). For stimulation with ASP, cells were incubated in DMEM containing 50, 100, 150 and 200 μg/mL ASP for 48 h prior to hypoxia treatment.

2.2. miRNA transfection

miR-22 mimic, miR-22 inhibitor, and negative control (NC) were all synthesized by GenePharma Co. (Shanghai, China). miRNAs were respectively transfected into H9c2 cells using the Lipofectamine™ 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.3. Cell viability assay

The Cell Counting Kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was utilized to testify cell viability. H9c2 cells were plated in 96-well plates at a density of 5×10^3 cells/well and maintained at 37 °C for overnight. After treatments, culture medium was replaced by DMEM containing 10% CCK-8 solution. Then, cells were incubated at 37 °C for 1 h, followed by measurements of absorbance at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.4. Apoptosis assay

The FITC Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI, for Flow Cytometry (Invitrogen) was used to identify early apoptotic cells. H9c2 cells grown in 6-well plates were collected after treatments, and then washed in cold phosphate buffered saline (PBS). Cells were suspended in binding buffer and incubated with 5 μL of FITC annexin V and 100 ng of propidium iodide (PI) according to the manufacturer's instructions. Early apoptotic cells (Annexin-V positive and PI-negative) were detected by using a LSRII/Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany). Percentage of early apoptotic cells was analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA).

2.5. Stem-loop RT-PCR

After treatments, total RNA of H9c2 cells was extracted using a miRNeasy Mini Kit (Qiagen GmBH, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). The mixture consisting of 2 μL 5× PrimeScript RT Master Mix, 1 μL RNA and 7 μL RNase free dH_2O was subjected to reactions for 15 min at 37 °C and 5 s at 85 °C. Real-time PCR was performed with TB Green™ Premix Ex Taq™ II (TaKaRa) at the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters: 95 °C 10 s, and 40 cycles of 95 °C 5 s and 60 °C 31 s. Expression levels of miR-22 were normalized to the endogenous snRNA U6 control. Relative expression ratio of miR-22 was calculated by the $2^{-\Delta\Delta Ct}$ method [15]. PCR reactions were repeated three times. Independent experiments were done in triplicate.

2.6. Western blot analysis

After treatments, H9c2 cells were harvested and lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with 1 mM PMSF (Beyotime). The whole cell lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and the protein content in the supernatant was determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk, PVDF membranes were immunoblotted with primary antibody and HRP-conjugated IgG (goat anti-rabbit ab205718 or goat anti-mouse ab6789, Abcam, Cambridge, UK), successively. Primary antibodies included anti-p53 antibody (ab131442), anti-p16 antibody (ab51243), anti-cyclinD1 antibody (ab134175), anti-Bax antibody (ab32503), anti-pro caspase-3 antibody (ab90437), anti-cleaved caspase-3 antibody (ab49822), anti-PI3K antibody (ab133595), anti-phospho (p)-PI3K antibody (ab182651), anti-β-actin antibody (ab8227, all Abcam), anti-caspase-9 antibody (#9508), anti-AKT antibody (#9272), anti-p-AKT antibody (#9271), anti-JAK1 antibody (#3344), anti-p-JAK1 antibody (#74,129), anti-STAT3 antibody (#4904) and anti-p-STAT3 antibody (#9134, all Cell Signaling Technology, Beverly, MA, USA). Proteins in the PVDF membranes were visualized using the enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Optical density of the bands was determined by ImageJ software (National Institutes of Health, Bethesda, MA, USA).

2.7. Statistical analysis

Experiments were performed in triplicate with three repeats. Results were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). Data were evaluated using a one-way analysis of variance (ANOVA) or unpaired two-tailed *t*-test. The *P*-value lower than 0.05 was considered statistically significant.

3. Results

3.1. Hypoxia induced H9c2 cell injury

H9c2 cells were incubated under hypoxia for different times, and cell viability was analyzed. As shown in Fig. 1A, cell viability was decreased significantly when the hypoxic duration was 4 h, 8 h (both $P < 0.05$), 16 h ($P < 0.01$) and 24 h ($P < 0.001$), as compared with untreated cells. Cells were incubated under hypoxia for 16 h in subsequent experiments since the cell viability was reduced by half when the hypoxic duration was 16 h. When compared with the Control group, expression of p53 and p16 was up-regulated while expression of cyclinD1 was down-regulated by hypoxia significantly (all $P < 0.05$, Fig. 1B). The percentage of apoptotic cells in the Hypoxia group was dramatically higher than the Control group ($P < 0.001$, Fig. 1C), and expression of pro-apoptotic Bax, cleaved caspase-3 and cleaved caspase-9 was observably up-regulated by hypoxia (Fig. 1D). Those results collectively illustrated that hypoxia induced H9c2 cell injury.

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