Biomedicine & Pharmacotherapy 104 (2018) 712–717

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Shikonin protects H9C2 cardiomyocytes against hypoxia/reoxygenation injury through activation of PI3K/Akt signaling pathway

Shuang Wang[a,1], Yanfang Zhu[a,1], Ruixia Qiu[b,*]

[a] Department of Emergency, No.1 People's Hospital of Jining City, Jining, Shandong, China
[b] Department of Medical Affairs, No.1 People's Hospital of Jining City, No.6 Jiankang Road, Jining, 272011, Shandong, China

ARTICLE INFO

Keywords:
Shikonin
H9C2 cells
Hypoxia/reoxygenation injury
Viability
Mitochondria-dependent apoptosis
PI3K/Akt signaling

ABSTRACT

Myocardial ischemic/reperfusion (I/R) injury often leads to irreversible myocardial cell death and even heart failure, with limited therapeutic possibilities. In the present study, we evaluated the protective effects of shikonin (SHK) against hypoxia/reoxygenation (H/R)-induced cardiomyocyte damage and explored the underlying mechanisms. H9C2 cardiomyocytes were pretreated with different doses of SHK prior to H/R exposure. We observed that SHK pretreatment significantly increased cell viability, attenuated LDH release, and suppressed cardiomyocyte apoptosis induced by H/R exposure. SHK pretreatment also restored the loss of mitochondrial membrane potential (MMP) and cytochrome c release. In addition, SHK significantly enhanced the phosphorylation of Akt and GSK-3β in H/R-treated H9C2 cells. These protective effects of SHK were partially reversed by LY294002, a specific PI3K/Akt inhibitor. Therefore, our findings suggested that SHK might be a promising agent for myocardial I/R injury, and PI3K/Akt signaling plays a crucial role during this process.

1. Introduction

Myocardial infarction (MI) remains the main cause of morbidity and mortality around the world [1]. MI can cause injury or death of myocardium due to prolonged ischemia and hypoxia. Reperfusion strategy is the current standard therapy to protect the myocardium against MI, but it also has side effect which was called ischemia/reperfusion (I/R) injury [2]. Cardiomyocyte apoptosis, one of the important pathological mechanisms of myocardial I/R injury, leads to widespread myocardial cell damage, cardiac dysfunction and ultimately heart failure. Therefore, identification of effective anti-apoptotic agents is a promising therapeutic approach for this disease [3].

Many experimental studies demonstrated that a wide variety of natural products have potential pharmacological benefits for cardiovascular diseases [4]. Shikonin (SHK; Fig. 1A), a natural naphthoquinone pigment purified from the root of *lithospermum erythrorhizon* (purple gromwell), has been identified as a multifunctional bioactive natural product [5]. Recently, it has been frequently demonstrated that SHK exerts beneficial effects in alleviating I/R injury in brain and liver [6,7]. Besides, SHK treatment could attenuate isoproterenol-induced heart injury in mice [8]. However, there are few documents about the pharmacological role of SHK in myocardial I/R injury.

Accordingly, the purpose of the present study was to determine the role of SHK in cardiomyocyte cell line H9C2 cells against hypoxia/reoxygenation (H/R)-induced apoptosis and mitochondrial dysfunction. The potential underlying molecular mechanisms through which SHK exerts its functions was also investigated.

2. Materials and methods

2.1. Cell culture and treatment

The rat cardiomyocyte-derived cell line H9C2, purchased from American Type Culture Collection (ATCC; Rockville, MD, USA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. H9C2 cells from passage 5–10 were used in this study.

For normoxic condition, cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. To induce H/R injury, cells were incubated in a hypoxic incubator in serum-free and low-glucose DMEM at 37 °C for 12 h in a humidified atmosphere of 94% N_2, 5% CO_2 and 1% O_2, and then incubated with fresh medium and restored to normoxic condition for reoxygenation for another 24 h.

2 h prior to H/R treatment, cells were randomly pretreated with 10, 20 and 40 μM SHK (Sigma–Aldrich, St. Louis, MO, USA) with DMSO

* Corresponding author.
E-mail address: qrxjzk@163.com (R. Qiu).
[1] These authors contributed equally to this work and should be considered as co-first authors.

https://doi.org/10.1016/j.biopha.2018.04.144
Received 12 March 2018; Received in revised form 17 April 2018; Accepted 19 April 2018

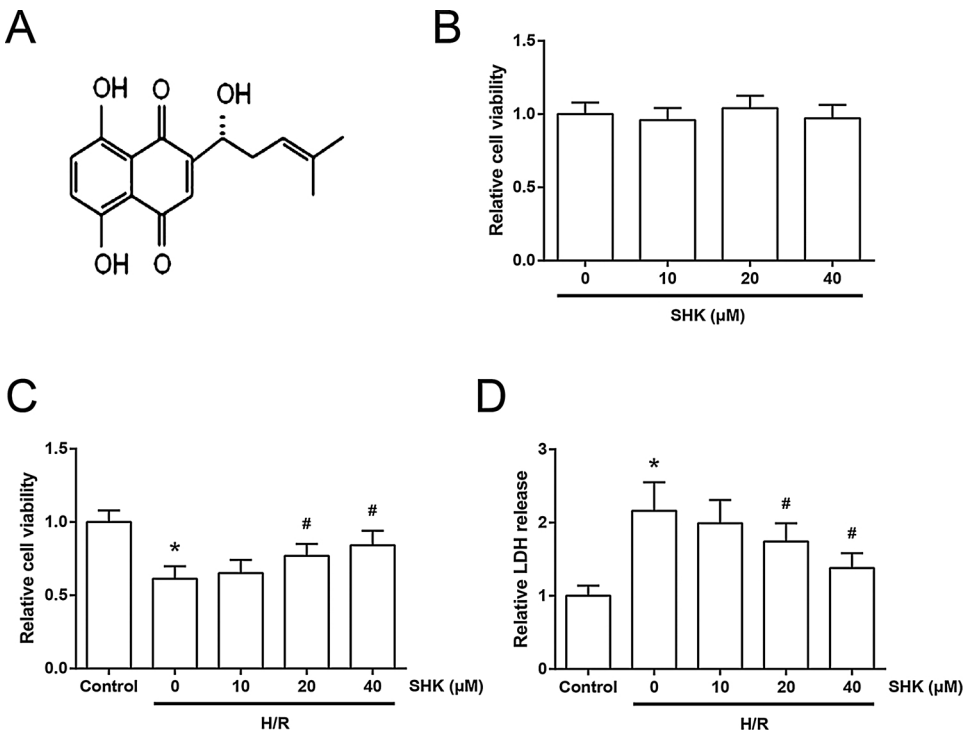

Fig. 1. SHK protects against H/R injury in H9C2 cells. (A) Chemical structure of SHK. (B) Cytotoxicity of SHK in H9C2 cells under normoxic condition was determined by CCK-8 assay following exposure to different doses of SHK for 48 h. (C) H9C2 cells were pretreatment with different doses of SHK for 2 h prior to H/R exposure, and the cell viability was detected by CCK-8 assay. (D) The cell injury was assessed by measuring the amount of LDH release in H9C2 cells. All values are expressed as mean ± SD. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. H/R.

(Sigma–Aldrich) as a vehicle, respectively. LY294002 (20 μM; Beyotime, Shanghai, China) was applied in the medium for 1 h before the administration of SHK when necessary.

2.2. Cell viability assay

Cell viability was assessed using the cell counting kit-8 (CCK-8; Dojindo, Tokyo, Japan). H9C2 cells were seeded at a density of 3×10^3 cells/well in 96-well plates. After the aforementioned treatments, cells were incubated with 10 μl CCK-8 solution for additional 1 h, and the absorbance value was detected at a wavelength of 450 nm using a microplate reader (Dynex, Chantilly, VA, USA).

2.3. LDH release assay

Cell injury was verified by measuring the amount of LDH released into the extracellular fluid from damaged cells. 0.2 ml culture medium was collected after the treatments, and the amount of LDH was detected by spectrophotometry via an LDH assay kit (Jiancheng, Nanjing, China). Cellular LDH release was expressed as the percentage of total cell LDH activity.

2.4. Annexin V-FITC/PI apoptosis assay

Cellular apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (Beyotime). Following different treatments, cells were harvested, washed twice with PBS, resuspended in 100 μl binding buffer, and then mixed with 10 μl Annexin V-FITC and 5 μl PI. The above reaction was performed at room temperature in the dark. Then the cells were subjected to FACScan flow cytometer (BD Biosciences, Franklin lakes, NJ, USA).

2.5. Measurement of intracellular ROS level

The intracellular level of ROS was monitored using the membrane-permeable fluorescent probe 2′-7′-dichlorofluorescein diacetate (DCFH-DA; Sigma–Aldrich) [9]. Briefly, cells were washed twice with PBS after experimental treatments, and incubated with 10 μM DCFH-DA at 37 °C for 20 min. ROS level was then detected by FACScan flow cytometer.

2.6. Analysis of mitochondrial membrane potential (MMP)

MMP detection was performed by a mitochondrial membrane potential assay kit (Beyotime) with cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). In normal cells, JC-1 aggregates in mitochondria, fluorescencing red. In apoptotic cells, JC-1 accrues in the cytosol, as a green fluorescencing monomer [10]. After experimental treatments, H9C2 cells were rinsed with PBS twice and stained with 0.5 ml JC-1-fluorescent dye for 30 min at 37 °C. Cells were then washed with PBS for three times and analyzed immediately with a microplate reader. The results were expressed as the ratio of red to green fluorescence.

2.7. Western blot analysis

The cells were washed with PBS and lysed using a protein extraction kit (Beyotime). The lysates of cytoplasm and mitochondria were prepared using the Mitochondria/Cytosol Fractionation kit (Abcam, Cambridge, UK). The protein concentration was quantified using BCA Protein Assay (Pierce, Rockford, IL, USA) [11]. Equal amounts of protein were loaded into each lane, separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then incubated overnight at 4 °C with the appropriate primary antibodies, including anti-Bax (1:1000; cat. no. 2772; Cell Signaling Technology, Danvers, MA, USA), anti-Bcl-2 (1:1000; cat. no. ab196495; Abcam), anti-cleaved caspase-3 (1:1000; cat. no. 9664; Cell Signaling Technology), anti-cytochrome c (1:1500; cat. no. ab13575; Abcam), anti-p-Akt (Ser 473) (1:2000; cat no. 4060; Cell Signaling Technology), anti-Akt (1:5000; cat no. 4685; Cell Signaling Technology), anti-p-GSK-3β (Ser 9) (1:2000; cat no. 5558; Cell Signaling Technology), anti-GSK-3β (1:2000, cat no. 9315; Cell Signaling Technology), anti-GAPDH (1:1000; cat. no. 2118, Cell Signaling Technology) and anti-COX IV (1:2000; cat. no. ab16056; Abcam). Thereafter, the membranes were incubated with the corresponding goat anti-rabbit or goat anti-mouse secondary antibodies (1:1000; Beyotime) at room temperature for 1 h. The signals were visualized using an