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Original article

Hydroxysafflor yellow A cardioprotection in ischemia–reperfusion (I/R) injury mainly via Akt/hexokinase II independent of ERK/GSK-3β pathway

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ABSTRACT

Hydroxysafflor yellow A (HSYA) is the main active component of Carthamus tinctorius L which has been used for hundreds of years in Chinese folk medicine in the treatment cardiovascular disease. This study was designed to investigate whether HSYA exerts cardioprotection in ischemia–reperfusion (I/R) injury heart and the mechanisms involved. The protective effect and mechanisms in myocardial ischemia reperfusion injury of HSYA was evaluated by hypoxia–recover (H/R) injury cell model which induced by hypoxia and recovered with oxygen in H9c2 cells. PI3K/Akt and ERK as the reperfusion injury salvage kinase (RISK) pathway and Hexokinase II (HKII) were both examined. In H/R cell model, HSYA significantly reduced dehydrogenase (LDH), Caspase 3 level, alleviated oxidative stress injury and apoptosis, meanwhile restored mitochondrial energy metabolism. Pretreatment with PI3K inhibitor (LY294002) or hexokinase II inhibitor (3-BrPA), the protective effect of HSYA was significantly attenuated. On the contrary, pretreatment with ERK inhibitor (PD98059), the protective effect of HSYA on myocardial cells was decreased slightly, not as significant as PI3K inhibitor or hexokinase II inhibitor. ERK play a protective role in myocardial protection by phosphorylation of GSK3-β, but the effect of HSYA on phosphorylation of GSK3-β is weakly, however the effect of HSYA on Akt and hexokinase II were significantly up-regulated. Meanwhile, the phosphorylation of GSK3-β by HSYA was significantly reduced after gave the ERK inhibitor and had no significant difference between the model group. The cardioprotection effect of HSYA appears to be mainly mediated via the PI3K/Akt/hexokinase II.

1. Introduction

Ischemia and reperfusion (IR) injury (IRI) is a primary cause of cardiac failure, morbidity, and mortality after cardiac operations or heart infarctions [1,2]. Recently, evidence has suggested that the reperfusion injury salvage kinase (RISK) pathway is a key regulator of mechanisms underlying cardioprotection in ischemia–reperfusion (I/R) injury [3,4]. Activation of PI3K/Akt and extracellular signal-regulated kinase ERK(1/2) are two key signals of the RISK pathway during reperfusion [5,6]. PI3K/Akt and ERK(1/2) are involved in regulating mitochondrial function and cell apoptosis. Research suggests that mitochondrial dysfunction is involved in stress induced cardiac cell damage and death. Recently, Hexokinase II which regulated by Akt, also has been found play a key role in the regulated mitochondrial function of myocardial cells [7].

Carthamus tinctorius L. is a medicinal plant, officially listed in the Chinese Pharmacopoeia (named in Honghua), and has been used for hundreds of years in Chinese folk medicine for the treatment of a series of pathological conditions, including cardiovascular disease [8]. Hydroxysafflor yellow A (HSYA) is an important active component of Honghua and has been widely used for the treatment of coronary heart disease [9–11]. But the mechanism is not clear.

In the present experiment, cultured H9c2 cells were used to investigate the influence of HSYA on hypoxia cardiomyocytes, and to explore the mechanism of HSYA on mitochondrial protection, thus providing the experimental reliance for its clinical application of preventing and treating myocardial ischemia reperfusion injury.

2. Materials and methods

2.1. Reagents

The rat heart-derived H9c2 cell line was provided by the Cell Culture Center, Institute of Basic Medical Science, and Chinese

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Academy of Medical Science. Hydroxysafflor yellow A (Sigma Aldrich, St. Louis, MO, USA). The kits for determining the superoxidedismutase (SOD) activity, malondialdehyde (MDA) and the lactate dehydrogenase (LDH) level were obtained from Jian cheng Bioengineering Institute (Nanjing, China). MTT(3-(4,5-dimethylthia2-yl)-2,5-di-phenyltetrazolium), LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor), 3-bromopyruvate (3-BrPA, hexokinase II inhibitor), and the Caspase 3 Activity Assay Kit were from Sigma Chemical Co. phosphorylated Akt (Ser473), Akt, hexokinase II, p-GSK-3β (Ser9), GSK-3β, β-actin, GAPDH (Abcom, UK)

2.2. To establish hypoxia–recover (H/R) injury cell model induced by hypoxia and recovered with oxygen in H9c2 cells

H9c2 myocardial cells were inoculated at the concentration of 2×10^6/ml. Twenty four hours before treatment, To simulate hypoxia, the culture medium was replaced by hypoxia buffer (139 mM NaCl, 4.7 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5 mM Hepes, 20 mM sodiumlactate), and then incubated for 12 h in a Low oxygen gas (95% N_2, 5% CO_2). Then, the condition was recovered with oxygen, the cells were incubated in serum-free DMEM under normal conditions (20% O_2, 5% CO_2) at 37 °C for 4 h and different drugs were applied simultaneously.

2.3. Experimental groups

The cardiomyocytes were randomly divided into different groups as follows:

(1) The control group, in which the cardiomyocytes were incubated in normal condition throughout the experimental period;
(2) Hypoxia and recovered with oxygen in H9c2 cells: the H/R model group; The rest of the group was given drug intervention in H/R model.
(3) HSYA group, in which the cardiomyocytes were treated as in the H/R group with the addition of HSYA at a final concentration of 1.25, 5 or 20 μM to the culture during reoxygenation for 4 h. (4) HL: HSYA (5 μM) and LY294002 (20 nM); (5) HP: HSYA (5 μM) and PD98059 (30 nM); (6) HB: HSYA (5 μM) and 3-BrPA (30 nM); (7) LY: LY294002 (20 nM); (8) PD: PD98059 (30 nM); (9) Br: 3-BrPA (30 nM)

2.4. Methyl thiazolyl tetrazolium (MTT) assay

Culture media was refreshed with media containing MTT reagent (5 mg/ml) and cells were incubated under standard conditions for an additional 4 h. The culture media was carefully aspirated and 100 μl dimethylsulfoxide was added per well to solubilize the formazan crystals. Following agitation, absorbance was measured spectrophotometrically at a wavelength of 490 nm using a Tecan infinite M200pro Microplate Spectrophotometer (Tecan Laboratories, Inc., Switzerland). Viabilities of the challenged cells were expressed relative to control cells.

2.5. MDA, SOD and LDH assay

SOD and MDA content were determined when experiment completed, cells from different groups were collected and washed with cold PBS for three times, and cold cell lysis buffer was added for cell lysis. Supernatant was collected for detection after centrifuging at 1800 × g for 5 min at 4 °C. The detection process was done according to manufacturer's instruction of the SOD and MDA assay kits. Cell culture media in different groups were taken for determination of LDH using the LDH assay kits as described by the manufacturer. Protein concentrations of cell lysates were determined using a BCA protein assay kit (Sigma Aldrich, St. Louis, MO, USA).

2.6. ATP assay

The level of ATP in cardiomyocytes cell lines was determined using the ATP assay Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Briefly, harvested cultured cells were lysed with a lysis buffer, followed by centrifugation at 10,000 × g for 2 min at 4 °C. The level of ATP was determined by mixing 50 μl of the supernatant with 50 μl of luciferase reagent, which catalyzed the light production from ATP and luciferin. The emitted light was linearly related to the ATP concentration and measured using a microplate illuminometer.

2.7. Caspase 3 activity assay

The H9c2 cells were cultured in 6-wellplates at 7×10^4 cells/ml. The cells and protein lysates were then collected following the

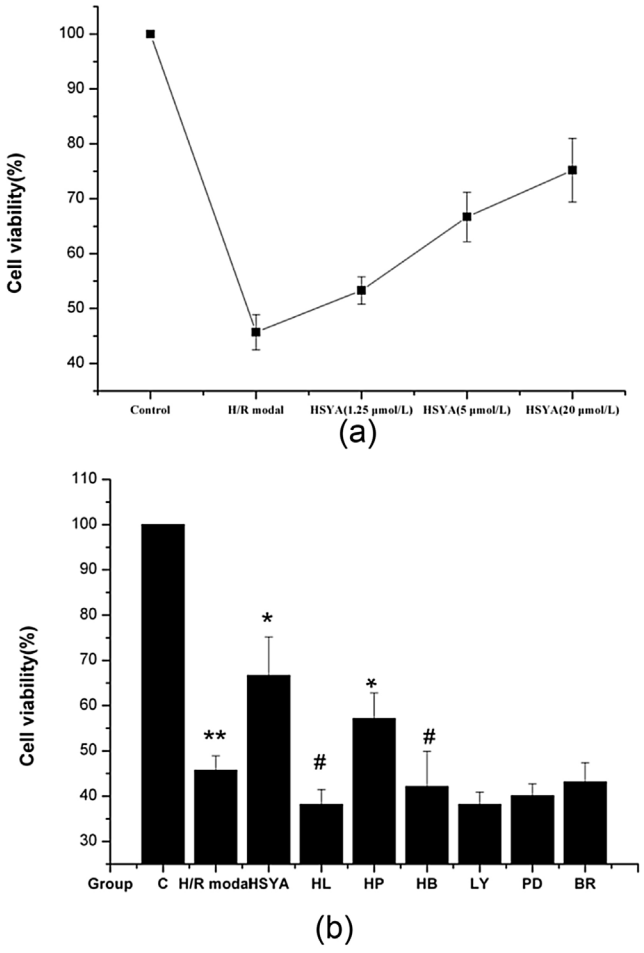

Fig. 1. (a) Effect of HSYA on the viability of H9c2 cells, HSYA at a final concentration of 1.25, 5 or 20 μM to the H/R model; (b) Relative cell viability determined by MTT assay on treatment with HSYA (5 μM)and HL(HSYA 5 μM + LY294002); HP(HSYA 5 μM+ PD98059); HB (HSYA 5 μM + 3-BrPA); LY: LY294002(PI3 K inhibitor); PD: PD98059(ERK inhibitor); Br: 3-BrPA(hexokinase II inhibitor); C: control. Results shown are mean ± SD of three independent experiments performed in duplicate. $^{**}p < 0.01$ relative to control, $^{*}p < 0.05$ relative to H/R group, $^{\#}p < 0.05$ relative to HSYA group.