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Protective effect of berberine against cardiac ischemia/reperfusion injury by inhibiting apoptosis through the activation of Smad7

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ABSTRACT

Berberine (BBR) is an isoquinnoline derivative alkaloid extracted from *Rhizoma Coptidis* that has the potential to protect myocardial tissues from ischemia/reperfusion (I/R) injuries. We attempted to evaluate the effect of BBR on the proliferation and apoptosis of a hypoxia/reoxygenation (H/R) cell model and to reveal the mechanism driving the improving function of BBR myocardial tissues. The H/R cell model was established using H9c2 rat cardiac myoblasts. The cell viability, apoptotic rates, and cell cycle distribution were measured with CCK-8 assay and flow cytometry. The expression of Smad7 and caspase-3 were determined both at mRNA and protein levels. In addition, expression of Smad7 was knocked down with specific siRNA and the effect of the interference was assessed. The proliferation ability of H/R cells was enhanced after the administration of BBR, and the apoptosis and cell cycle arrest due to H/R injury were also alleviated by BBR treatment. Moreover, the treatment of BBR on H/R injury functioned through the Smad7-activation-induced attenuating of apoptosis by activating Smad7 pathway which resulted suppression of caspase 3 expression and activity. The knockdown of Smad7 confirmed our conclusion about the key role of Smad7 in the function of BBR administration. However, our results as well as some previous studies also demonstrated that the effect of BBR was tissue and protocol specific, and the underlying mechanism related to the BBR treatment was so complicated that practical application should be carefully investigated based on certain diseases and patients.

1. Introduction

Coronary heart disease (CHD) is one of the critical causable factors of deaths and disabilities worldwide. Based on the World Health Organization's investigation in 2012, CHD is the major factor contributing tonon-communication diseases death worldwide (17.5 million) (http://www.who.int/gho/ncd/mortality_morbidity/cvd/en/). The detriments of CHD are usually attributable to the effect of cardiac ischemiawhich is characterized by the deficient supply of blood flow and energy generating nutrients to the myocardium. In recent years, various medical and coronary interventions have been made to control the mortality rates related to CHD. Treatments based on re-establish blood flow to minimize damage to the heart, such as primary percutaneous coronary intervention (PPCI) and thrombolytic therapies, are the currently most effective treating methods of CHD. However, these therapies can themselves lead to further cardiomyocyte death due to ischemia/reperfusion (I/R) injuries during the reperfusion process [1]. Although techniques and agents against CHD are being rapidly advanced, few therapies for preventing cardiac I/R injury are available. Thus, development of novel gentle methods to improve the outcome and survival of cardiac I/R injury is imperative.

Generally, I/R injury is initiated by release of reactive oxygen species (ROS) including excessive apoptosis [2,3], which triggers extensive degradation of cytosolic essential proteins and leads to dys-regulation of cellular functions [4]. Apoptosis-related genes or pathways regarding cardiac I/R injury have been substantially studied. Previous study of Okado et al. [5] inferred that activation of caspase-3 by Smad7 contributed to the transforming growth factor (TGF)-induced apoptosis in mesangial cells during I/R injury. Therefore, it reminds that specific inhibition of Smad7 might serve as a potential therapeutic strategy to inhibit apoptosis associated with I/R-induced damages, i.e., myocardial I/R injury. Based on the information, numerous natural products with antioxidant and antiapoptotic potential have been extensively investigated for their effect in I/R managements. Berberine (BBR) is an isoquinnoline derivative alkaloid extracted from *Rhizoma Coptidis,* also known as “Huanglian” [6] which has been long time used as a folk medicine in China and other Asian countries against gastrointestinal infections [7]. Multiple beneficial effects of BBR against various diseases have been reported [8–12]. The study of Chang et al. revealed that animal models administrated with 10 mg/kg/day BBR for 14 days

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reversed the damages due to I/R injury [8]. Regarding I/R injuries attacking heart, it has been reported that BBR contributed to the amelioration of the injuries via multiple pathways, including AMPK, PI3K/Akt, Notch1/Hes-PTEN/Akt, and etc. [7,13,14], which all confirmed the cardioprotective function of BBR. To further provide information on the mechanism through which BBR alleviates myocardial injuries, in the present study, we investigated the cardioprotective function of BBR against cardiac I/R injury with hypoxia-reoxygenation (H/R) H9c2 cells as the *in vitro* model. The protecting effect of BBR on the cell viability and apoptosis was detected using CCK-8 assay and flow cytometry method. Moreover, we also assessed the regulating effect of BBR on caspase-3 and Smad7 pathways using real-time quantitative PCR (qPCR) and western blotting assay, respectively. Then the expression of Smad7 was knocked down in model cells to verify the key role of Smad7 in the cardioprotective effect of BBR.

2. Methods

2.1. Chemicals and cell cultures

BBR was purchased from Sigma-Aldrich (St. Lousi, MO USA). H9c2 rat cardiac myoblasts were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and cultured in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics mixture in an atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. H/R cell model establishment

H9c2 cells at concentration of 5×10^4/mL were incubated on slides in one well of 24-well plates for 24 h. For hypoxia treatment, log-growth cells were subjected to an atmosphere consisting of 1% O_2, 94% N_2, and 5% CO_2 for 0, 6, 8, and 12 h. Reoxygenation was conducted by incubating the cells in a condition of 95% air, 5% CO_2, and 37 °C for 24 h, respectively.

2.3. Flow cytometry detection of apoptosis

To determine the best hypoxia time course, the apoptotic rates of H9c2 cells under different treatments were determined using flow cytometry technique: briefly, after completion of reoxygenation of H9c2 cells, 5 μL FITC was added to different wells. After incubation with FITC for 10 min at room temperature, the cells were resuspended with 1 × Binding buffer and added with 5 μL Propidium Iodide (PI). Then the apoptotic rates of the cell lines were detected with flow cytometer. Apoptotic cell rate (UR + LR-all apoptosis cell percentage) was equal to the sum of the cell death rate (UR, upper right quadrant-advanced stage apoptosis cell percentage) and the early apoptosis rate (LR, lower right quadrant-prophase apoptosis cell percentage).

2.4. Determination of the concentration of BBR for administration of H/R H9c2 cells

H/R H9c2 cells were incubated with BBR at different concentrations according to previous studies [15–17] to assess the cytotoxicity of BBR: A) control group, H/R H9c2 cells incubated with dissolvent (DMSO) of BBR agent. B) 25 μM BBR group, H/R H9c2 cells incubated with 25 μM BBR. C) 50 μM BBR group, H/R H9c2 cells incubated with 50 μM BBR. D) 75 μM BBR group, H/R H9c2 cells incubated with 75 μM BBR.

2.5. CCK-8 assay

For different groups, cell samples were collected at 0, 24, 48, and 72 h of incubation, respectively. Then the cytotoxicity of BBR on cells were assessed using CCK-8 assay: briefly, CCK-8 solution was added to cells from different time points and cultured at 37 °C for 90 min. OD value at 450 nm was determined using a Microplate Reader.

2.6. Grouping of H/R H9c2 cells and BBR administration

According to the results of cell viability assessment assay, the most suitable concentration of BBR for further experiments was determined as 50 μM and grouping of H/R H9c2 cells were as followings: A) control group, H/R H9c2 cells incubated for 72 h. B) mock group, H/R H9c2 cells incubated with dissolvent of BBR vehicle (DMSO) for 72 h. C) BBR group, H/R H9c2 cells incubated with 50 μM BBR for 72 h. For different group, samplings of cells were conducted at 0, 24, 48, and 72 h of incubation, respectively. Then the cell viabilities of different groups at different time points were measured using CCK-8 method as described above. Then apoptotic rates and cell cycles in different groups were also determined using flow cytometry as mentioned above.

2.7. Real-time quantitative PCR

The whole RNA in different treatments at 48 h was extracted using Trizol method according to the manufacture's instruction. GAPDH was selected as the reference gene. cDNA templates were achieved by reversely transcribing the RNA using RT-PCR kit (Fermentas), and the final qPCR reaction mixture of volume 20 μL contained 10 μL of SYS BR Primix Ex Taq 2, 0.5 μL of each primers (Smad7, forward: 5′-CCAACT GCAGACTGTCCAGA-3′, reverse: 5′-TTCTCCTCCCAGTATGCCAC-3′. caspase-3, forward: 5′-CATGGCCCTGAAATACGAAGTC-3′, reverse: 5′-GCAGGCCTGAATGATGAAGAGTTT-3′. GAPDH forward: 5′-TATGAT GATATCAAGAGGGTAGT-5′, reverse, 5′-TGTATCCAAACTCATTGTCA TAC-3′), 1 μL of the cDNA template, and 8 μL of Rnase free H_2O. Thermal cycling parameters for the amplification were as follows: a denaturation step at 94 °C for 30 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 15 s. Relative gene expression was evaluated with Data Assist Software version 3.0 (Applied Biosystems/Life Technologies). The relative expression levels of Smad7 and caspase-3 were determined according to the expression of $2^{-\triangle\triangle ct}$.

2.8. Western blotting assay

The protein products of Smad7 and caspase-3 were extracted from different cell samples at 48 h. GAPDH was used as reference protein. All the extracts were boiled in loading buffer for 5 min and then subject to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. Then targeted proteins were transferred onto polyvinylidene difluoride sheets. The membranes were washed with TBST three times, 20 min each time. Then primary antibodies (1:2000) against Smad7 (Abcam, #190987), caspase-3 (cell signal technology, #9662), cleaved caspase-3 (cell signal technology, #9654), and GAPDH (Kangchen, #KC-5G5) were incubated with the membranes overnight at room temperature. After additional three washes, secondary HRP goat anti-rabbit IgG antibodies (1:2000) (Boster, #BA1054) were added and incubated with the membranes for 5 h. After another three washes, the blots were developed using Beyo ECL Plus reagent and the results were detected in the Gel Imaging System. The relative expression levels of different proteins were calculated with Bio-Rad Quantity One.

2.9. siRNA interference of Smad7 gene

To further explore the function of Smad7, we specifically interfered the expression of Smad7 in H/R H9c2 cells. Lentivirus-mediated *Smad7*-specific siRNA (5'-AGGCAUUCCUCGGAAGUCATT-3') and the negative control siRNA (NC) were obtained from Genechem Biotech (Shanghai, China). For transfection, H/R H9c2 cells was adjusted to 1×10^4/mL and incubated on slides in one well of 24-well plates with different lentivirus particles for 24 h, respectively. Then the cells were grouped into different treatments: A) control group, H/R H9c2 cells incubated with 50 μM BBR for 48 h. B) NC group, H9c2 cells transfected with NC siRNA incubated with 50 μM BBR for 48 h. C) siRNA group, H9c2 cells transfected with *Smad7*-specific siRNA incubated with 50 μM BBR for