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Esculetin inhibits oxidative stress and apoptosis in H9c2 cardiomyocytes following hypoxia/reoxygenation injury

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ABSTRACT

Esculetin (6,7-dihydroxycoumarin), a natural coumarin compound extracted from natural plants, was reported to be involved in ischemia/reperfusion (I/R) injury. However, the role of esculetin in myocardial I/R injury remains unclear. This study was designed to investigate the protective effects of esculetin on cardiomyocytes induced by hypoxia/reoxygenation (H/R), and explore the underlying mechanisms. Our results showed that esculetin improved the cell viability and decreased lactate dehydrogenase (LDH) release in H/R-stimulated H9c2 cells. In addition, esculetin significantly suppressed oxidative stress and apoptosis in H9c2 cells exposed to H/R treatment. Exploration of the underlying mechanisms of its action indicated that esculetin enhanced the activation of JAK2/STAT3 pathway in H/R-stimulated H9c2 cells. Taken together, these findings indicated that esculetin inhibits oxidative stress and apoptosis in H9c2 cardiomyocytes following H/R injury through the activation of JAK2/STAT3 pathway.

1. Introduction

Myocardial infarction is a leading cause of heart failure and associated with a high rate of mortality in humans [1]. Although the treatment of myocardial infarction has been improved in the past years, it was still a major cause of disability in the world [2,3]. In China alone, over 700,000 people reportedly die from this disease every year [4]. Currently, rapid restoration of blood flow is the most effective therapeutic strategy for improving clinical outcomes. However, reperfusion itself may aggravate myocardial damage which was called myocardial ischemia/reperfusion (I/R) injury [5]. Although the molecular mechanisms underlying myocardial I/R injury is poorly understood, oxidative stress and apoptosis play important roles in the pathogenesis of myocardial I/R injury [6,7]. Thus, suppressing oxidative stress and apoptosis may be a good strategy against myocardial infarction.

Esculetin (6,7-dihydroxycoumarin), a natural coumarin compound extracted from natural plants, possesses a variety of pharmacological activities, including anti-inflammatory, anti-nociceptive, antioxidant and anti-tumor activities [8–10]. Additionally, esculetin was found to be involved in ischemia/reperfusion (I/R) injury. For example, Wang et al. reported that esculetin attenuated cerebral I/R injury in a middle cerebral artery occlusion model in mice [11]. However, the role of esculetin in myocardial I/R injury remains unclear. This study was designed to investigate the protective effects of esculetin on cardiomyocytes induced by hypoxia/reoxygenation (H/R), and explore the underlying mechanisms. Our data indicated that esculetin inhibits the apoptosis in H9c2 cardiomyocytes following H/R injury through the activation of JAK2/STAT3 signaling pathway.

2. Materials and methods

2.1. Cell culture

The rat cardiomyocyte-derived H9c2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C.

2.2. Hypoxia/reoxygenation (H/R) model

To induce hypoxia, H9c2 cells were exposed to a hypoxic

Abbreviations: I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; MDA, malondialdehyde; PVDF, polyvinylidene difluoride.

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environment of 95% N_2 and 5% CO_2 for 3 h at 37 °C. Afterwards, the medium was replaced with fresh oxygenated culture medium, and the culture vessels were transferred to a normoxic incubator (5% CO_2) at 37 °C for 6 h of reoxygenation. Cells under normoxic conditions served as a control.

2.3. Cell viability assay

Cell viability was measured using the WST-1 assay. Briefly, H9c2 cells at a density of 1×10^4 cells per well were treated with different concentrations of esculetin (0–40 μM) for 24 h or pretreated with different concentrations of esculetin (0–20 μM) for 2 h, followed by H/R stimulation for 3 h/6 h. Then, the WST-1 solution (10 μl/well) was added, and cells were further incubated at 37 °C for 2 h. Absorbance was measured at 450 nm by using a microplate spectrophotometer (Bio-Rad, CA, USA).

2.4. Cell cytotoxicity assay

After treatment, cell cytotoxicity was detected via detecting the activity of lactate dehydrogenase (LDH) enzyme released into medium with the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions.

2.5. Measurement of cellular ROS production, SOD activity and malondialdehyde (MDA) level

Intracellular ROS production was assessed by the conversion of non-fluorescent 2,7-dichlorofluorescein diacetate (DCF-DA). In brief, after treatment, H9c2 cells were stained with 10 μM DCFH-DA for 30 min at 37 °C in dark and then were washed with PBS for 3 times. Fluorescence of DCFH-DA was captured with a fluorescence microscope (excitation wavelength at 488 nm and emission wavelength at 585 nm) and quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD) and normalized to cell area.

After treatment, the cell culture medium was centrifuged, and the supernatant was collected. The activity of SOD in cell supernatant was measured using a commercially available kit according to the manufacturer's instructions (Sigma). The level of MDA was evaluated by a MDA assay kit (Nanjing Jiancheng Bioengineering Institute; Nanjing, China).

2.6. Western blot analysis

H9c2 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) and the protein concentration was quantified by the Bradford assay. A total of 30 μg of protein from each sample was separated on 12% SDS PAGE electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 °C. The primary antibodies were anti-Bax, anti-Bcl-2, anti-p-JAK2, anti-JAK2, anti-p-STAT3, anti-STAT3 and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then washed three times with TBST, and incubated with secondary anti-primary IgG conjugated with horseradish peroxidase (Cell Signaling Technology, Boston, USA) for 2 h at room temperature. Finally, the blots were determined using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Bio-Science AB, Uppsala, Sweden).

2.7. Caspase 3 activity assay

The activity of caspase-3 in cell supernatant was detected using a commercially available kit according to the manufacturer's instructions (Sigma).

2.8. Statistical analysis

Results are expressed as mean ± SD. Comparisons of parameters were performed with the unpaired Student's *t*-test or ANOVA followed by post hoc Tukey's HSD test for multiple comparisons. A p value less than 0.05 was considered significant.

3. Results

3.1. Esculetin improved the cell viability and decreased LDH release in H/R-stimulated H9c2 cells

First, we examined the effect of esculetin on cell viability using the WST-1 assay. As shown in Fig. 1A, treatment with 40 μM esculetin had significant effect on H9c2 cell viability; however, there was no difference from control group after incubation of H9c2 cells with esculetin at the concentrations between 5 and 20 μM. Thus, 5–20 μM of esculetin was used in the following experiments. In addition, H/R treatment significantly decreased the viability of H9c2 cells. However, esculetin pretreatment reversed the viability of H9c2 cells stimulated H/R (Fig. 1B). The results of LDH assay indicated that H/R treatment increased LDH release to 367.2%. After incubation with esculetin prior to H/R treatment, LDH releases were decreased to 316.3%, 246.7% and 178.4%, respectively, in H9c2 cells (Fig. 1C).

3.2. Esculetin suppressed oxidative stress in H9c2 cells exposed to H/R treatment

Oxidative stress was involved in the development of myocardial I/R injury. Thus, we examined the effect of esculetin on oxidative stress in H/R-stimulated H9c2 cells. As shown in Fig. 2A, the activity of SOD significantly decreased in H/R-treated H9c2 cells compared with that in the control group. When H/R-injured H9c2 cells were incubated with esculetin, SOD activity was significantly increased compared with the H/R group. In contrast, esculetin pretreatment efficiently suppressed H/R-induced ROS level and MDA production in H9c2 cells (Fig. 2B and C).

3.3. Esculetin inhibited apoptosis in H9c2 cells exposed to H/R treatment

We then determined the effect of esculetin on H9c2 cell apoptosis. As shown in Fig. 3A, as compared with the control group, Bcl-2 protein expression was significantly decreased in H/R-stimulated H9c2 cells, whereas an enhanced expression of Bax was observed. However, pretreatment with esculetin efficiently up-regulated Bcl-2 expression and down-regulated Bax expression in H9c2 cells in response to H/R. Additionally, esculetin pretreatment suppressed the H/R-induced caspase-3 activity in H9c2 cells (Fig. 3C).

3.4. Esculetin promoted JAK2/STAT3 pathway activation in H/R-stimulated H9c2 cells

In order to explore the protective effects of esculetin on cardiomyocytes, we investigated the effects of esculetin on JAK2/STAT3 activation in H9c2 cells exposed to H/R. As shown in Fig. 4A, the levels of p-JAK2 and p-STAT3 were significantly lower in H9c2