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Original article

Linarin could protect myocardial tissue from the injury of Ischemia-reperfusion through activating Nrf-2

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ABSTRACT

Objectives: As we all know, oxidative stress was one of the most important causes of ischemia-reperfusion injury. And it was reported that Nrf-2 as an important regulator for oxidative stress could be activated by Linarin. Thus it would be interesting to find whether Linarin could inhibit ischemia-reperfusion injury through activating Nrf-2.
Methods: In this study, cell activity was detected by MTT assay and caspase-3 activity detection kit. And the expressions or activities of some signal proteins were evaluated by western-blot or activity detection kits. At last, the effect and mechanism of Linarin on heart tissues were verified in the ischemia-reperfusion model of isolated hearts.
Results: The proliferation activity of cell was inhibited while the apoptosis rate was increased after hypoxia-reoxygenation. However, Linarin could inhibit these two variations. It was found that these effects of Linarin were related with the activation of Nrf-2 through PI3 K/Akt signaling pathway. Meanwhile, the anti-oxidative enzymes, regulated by Nrf-2, were enhanced to against the oxidative stress caused by hypoxia-reoxygenation. And with the inhibition of oxidative stress, some proliferation and apoptosis related proteins such as NF-kB and Cytochrome C were adjusted to support the viability of cells. At last, these results were verified in the ischemia reperfusion experiment of isolated hearts.
Conclusions: From this study, we assured that LIN could protect myocardial tissue from ischemia-reperfusion through activating Nrf-2.

1. Introduction

In recent years, many organs and tissues could have gotten reperfusion after ischemia with the establishment and promotion of treatment methods, such as coronary artery bypass grafting, thrombolytic therapy, cardiac surgery extracorporeal circulation, cardiopulmonary cerebral resuscitation, organ transplantation and so on [1–3]. In most cases, ischemia-reperfusion(I/R) could help organs and tissues to restore. However, sometimes I/R also caused damage to them and became a serious threat to recovery. This phenomenon was called ischemia-reperfusion injury [4].

I/R injury was thought to be a complicated pathophysiologic process. According to prior reports, the pathogenic mechanisms had the following aspects: oxidative stress, calcium overload, inflammatory cytokines injury, energy metabolism disorder, endothelin and angiotension II effect and so on [5–11]. Oxidative stress, as one of the most important mechanisms, had been proved to be caused by reactive oxygen species(ROS) produced in the I/R process [12–14]. The strong chemical activity of ROS resulted in lipid peroxidation, protein function inhibition and structure damage of chromosome [15,16]. Meanwhile, oxidative stress also could aggravate cell apoptosis, calcium overload and inflammatory cytokines secretion which could promote the progression of I/R injury. At present, drug treatment for reducing oxidative stress was deemed to be an ideal method to avoid or reduce the injury of I/R [17–19].

Nuclear factor erythroid2-related factor 2(Nrf-2) is an important regulator for anti-oxidation. Its main function is to improve the expression of anti-oxidative gene, such as HO-1. Its activation has been proved to be related with many cardiovascular diseases. It is thought to be an important potential target for curing some cardiovascular diseases.

Linarin(LIN) as an natural compound has showed its anti-oxidative effect in vitro and vivo [20]. And its promotion for Nrf-2 activity was also found in previous papers [20]. But it was still not clear that whether it could inhibit myocardial ischemia-

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reperfusion injury through activating Nrf-2. In this study, we found that LIN could improve the proliferation activity and reduce the apoptosis of myocardial cell in the hypoxia-reoxygenation(H/R) model. The mechanism research indicated that the protective effect of LIN had a direct relationship with the activation of Nrf-2 mediated by PI3 K/Akt pathway. And these results were further verified in the experiments of isolated hearts after ischemia-reperfusion. It demonstrated that LIN could protect myocardial tissue from the injury of I/R through activating Nrf-2.

2. Materials and methods

2.1. Myocardial cell culture

The myocardial cell H9C2(purchased from ATCC) was maintained in DMEM(purchased from Invitrogen) containing 10% fetal bovine serum(FBS) in a water-saturated incubater(37 °C, 5%CO2). The H/R model was established as follows: H9C2 cells were seeded in a 96-well plate for 24 h to reach 80% confluence. Following the cells in serum-free medium were incubated in hypoxic environment(100% Nitrogen) for 4 h, then restored under normoxic incubater for 1 h. In this process, the cells of Vehicle-control group did not experience H/R modeling while the other groups did. And model group cells were treated with DMSO while the treated groups were treated with relevant drugs dissolved in DMSO in the process of H/R.

2.2. Cell viability detection

The cell viability was detected by MTT. After hypoxia-reoxygenation(H/R), the cells in the 96-well plate were incubated with 200 μl fresh DMEM containing 0.5 mg/ml MTT for another 4 h at 37 °C. Following the culture medium was moved and 100 μl DMSO was used to dissolve insoluble formazan crystals in the cells. The absorbance of DMSO solution was detected with a multimode reader at a wavelength of 490 nm.

2.3. Caspase-3 activity detection

Caspase-3 is a key enzyme in the initial period of cell apoptosis. The detection of caspase-3 activity has been an important method to evaluate cell apoptosis. In this study, the cells were collected to detect the caspase-3 activity with a commercial kit(Product ID: C1115, purchased from Beyotime). The operations were carried out according to the instruction of the kit strictly.

2.4. Anti-oxidative enzyme activity detection

Cell or tissue samples were homogenized to extract enzymes for detection. SOD and GSH-Px activity were both detected by kits (Product ID: S0101 and S0055 repectively) which were purchased from Beyotime according to the manufactures.

2.5. MDA and ROS detection

The MDA extracted from the homogenate of cell or tissue was detected by the kit(Product ID: S0131, purchased from Beyotime). ROS was labeled by fluorescence probe supplied by a kit from Beyotime(S0033) while the cell was labeled by DAPI(Product ID: C1002, purchased from Beyotime). The result of MDA was detected by a multimode reader at a wavelength of 532 nm. ROS was observed by a fluorescence microscope and the intensity of fluorescence was analyzed by Image-pro Plus 6.0.

2.6. Western-blot

According to different applications, the total protein, cytoplasmic protein or nuclear protein of cell or tissue was extracted with different protein extraction kits(Nuclear and cytoplasmic protein extraction Kit, KeyGEN; Total protein extraction kit, Bestbio), repectively. The extracted protein was quantified by BCA protein concentration detection kit(purchased from Beyotime). The method of western-blot was similar as described in Piao et al. [21] with some modifications: Protein was separated by different concentration SDS-PAGE according to the molecular weight of goal protein and transferred onto PVDF membrane (purchased from Invitrogen). It was blocked by 5%(w/v) bovine serum albumin in Tris-buffered saline containing 0.005%(v/v) Tween-20(TBST). Then, it was incubated overnight (4 °C) with first antibody. The membrane was washed in TBST and incubated with HRP-conjugated secondary antibody (1:50000) for 2 h. It was washed with TBST and visualized using ECL Prime Detection System(purchased from GE Healthcare). The anti-Nrf-2 and anti-pNrf-2 antibody were purchased from RabMAb, and the others were all purchased from Santa Cruz. The second antibodies were bought from Huamei Biological Company.

2.7. Isolated heart experiments

Langendorff heart model was used to evaluate the LIN effect on myocardial I/R injury. In this study, all animals experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals Act. The method was as described in KE [22] et al. with some modifications: 56 SD rats were divided into seven groups randomly. The rats were bleed to death with the help of sodium heparin(500 U/kg, intraperitoneal (i.p.)) below narcotism by sodium pentobarbital(60 mg/kg, Sigma). The hearts were harvested rapidly and fixed to the Langendorff apparatus, following was perfused retrogradely with Krebs-Henseleit buffer(Sigma) via the aorta under constant (70 mm Hg) at 37 °C. A balloon filled with Kreb's solution (the pressure in this balloon was 8 mmHg) was inserted into the left ventricle. The pressure signal was collected and analyzed by PowerLab Data Recording & Analysis System(Powerlab, Australia). The collected heart function indexs were as follows: heart rate (HR), left ventricular systolic pressure (LVSP) and the maximal rate of pressure rise (+dp/dtmax). The artery effluent was collected to evaluate the coronary flow. The heart was continuous perfusion with KHB for 30 min after it had reached a stable. In this process, the KHB used in the treated group contained different concentration drug. Then, the heart went through global ischemia for 30 min and reperfusion for 60 min orderly.

After I/R, the hearts were collected for infarct size assessment. The method was described in KE [22] et al. with some modifications: The harvested hearts were cut into 2 mm transverse slices. Following the slices were incubated in 1% triphenyl tetrazolium solution with shake at 37 °C for 10 min 0.1 M PBS buffer(pH 7.4) was used to wash the redundant dye for twice. At last DMSO(10 ml per 1 g tissue) was used to dissolve the red product formazan in the dyed heart slice after scrunch and the collected supernatant was examined at 490 nm with a multimode reader. The other parts of hearts were scrunched to homogenate for some examinations.

2.8. Statistical analysis

Mean ± error was used for all values. SPSS 17.0 for Windows were used in all analysis. $p < 0.05$ showed statistically significant.