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Amelioration of myocardial ischemia-reperfusion injury by SIRT4 involves mitochondrial protection and reduced apoptosis

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ABSTRACT

Apoptosis and mitochondria dysfunction are key contributors to myocardial ischemia-reperfusion (MI-R) injury. SIRT4, a mitochondrial-localized sirtuin, controls cellular energy metabolism and stress response, and is abundantly present in the heart, however, its role in MI-R injury is not clear. In the current study, we demonstrate that SIRT4 is downregulated in cardiomyocytes both in vitro and in vivo models after MI-R. Functionally, SIRT4 overexpression decreases myocardial infarct size and serum creatine phosphokinase (CPK) level, and vice versa, SIRT4 depletion by siRNA increases myocardial infarct size and serum CPK level. Furthermore, we show that these protective roles of SIRT4 against MI-R injury are associated with preserved mitochondrial function and reduced myocardial apoptosis. Taken together, our findings indicate that SIRT4 ameliorates MI-R injury through regulating mitochondrial function and apoptosis, and suggest that manipulating SIRT4 may be of clinical benefit in MI-R injury.

1. Introduction

Acute myocardial ischemia-reperfusion (MI-R) injury has detrimental effects on coronary heart disease (CHD), which is the leading cause of death worldwide [1]. MI-R injury typically occurs in patients with an acute ST-segment elevation myocardial infarction (STEMI), and the most effective therapeutic intervention for alleviating myocardial ischemic injury is myocardial reperfusion achieved through either thrombolytic therapy or primary percutaneous coronary intervention (PPCI) [2]. However, myocardial reperfusion arouses further injuries, including cardiomyocyte dysfunction, apoptosis and cell death [3]. On the other hand, mitochondria are critical targets of ischemia and subsequent reperfusion, and they have emerged as key participants and regulators of MI-R injury [4]. Following coronary occlusion, a severe reduction in blood flow generates deleterious cellular processes, especially damage to mitochondria, which result in eventual cardiomyocyte death through activating apoptosis either from mitochondrial permeability transition pore (MPTP) or via mitochondrial outer membrane permeabilization (MOMP) [5,6]. Therefore, therapeutic strategies of preventing cardiomyocyte apoptosis during reperfusion may reduce reperfusion-induced cell death and improve heart function [7].

Sirtuins belong to a highly conserved family of nicotinamide adenine dinucleotide-(NAD)-dependent enzymes [8]. SIRT4 is a mitochondrial-localized member of the sirtuin family and plays key roles in a wide variety of cellular processes, such as energy metabolism, stress response and longevity [9]. SIRT4 is highly expressed in the heart and is recently implicated in cardiovascular diseases [10]. However, its physiological function in MI-R injury has not yet been established. In the present study, we uncover the protective role of SIRT4 in MI/R injury, which may relate to mitochondrial protection and reduced apoptosis of cardiomyocytes.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies and reagents were purchased from the following sources: SIRT4 (Abcam, ab124521), GAPDH (6C5) (Santa Cruz, sc-32233), Cox IV (Cell Signaling, 4844), Cleaved caspase3 (Asp175) (Cell Signaling, 9661), Caspase3 (Cell Signaling, 9662), β-actin (C4) (Santa Cruz, sc-47778), Goat anti-rabbit IgG-HRP (Abcam, ab6721), Goat anti-mouse IgG-HRP (Abcam, ab6789), Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma.

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2.2. Animals

All procedures involved in animal experiments were conducted in accordance with protocols and guidelines approved by the Institutional Animal Care and Use Committees of Tangdu Hospital, the Fourth Millitary University for animal welfare. Adult wild-type C57BL/6 male mice were used as animal models in this study. Generally, mice were fed and maintained under specific pathogen-free conditions, and at 12-week-old, mice were randomly divided into different groups by weight prior to experimental treatment.

2.3. Mouse model of myocardial ischemia-reperfusion

The mouse model of myocardial ischemia-reperfusion (MI-R) was established as previously described [11]. Briefly, mice were anaesthetized by intraperitoneally injecting a mix solution of 50 mg/kg sodium pentobarbital and 60 mg/kg ketamine. In addition, before surgery, 200 units/kg sodium heparin was also administrated to prevent clot formation. Anaesthetized mice were placed in a supine position, and intubated with an endotracheal tube (PE-60) and effectively ventilated with 100% oxygen (0.5 L/min) using a ventilator (HugoSachs, Model 845), which was set with conditions of a rate of 110 strokes/minute, 230-μl tidal volume and constant 37 °C. The mice were then denuded and the exposed regions were sterilized with alcohol and Betadine solution. The ribcage was exposed by making a midline incision along the sternum. A thoracotomy was performed at the left of the midline using an electrocautery. The second and third ribs were cauterized to make a vertical opening, and the left coronary artery (LCA) was visualized under an Olympus SZ61 stereomicroscope. The LCA was then ligated with a 7-0 silk suture underneath the coronary artery. A small piece of PE-10 tubing was then placed along with the LCA and the 7-0 suture was tightly tied to compress the LCA and render the left ventricle ischemic for 30 min. 7-0 silk suture was then removed to reperfuse the LCA for 48 h. Eventually, the sternum and skin was closed.

2.4. Assessment of area at risk, infarct size and serum CPK release

The assessments of AAR and infarct area were performed as previously documented [12]. The LCA arteries of mice were reoccluded and injected 1 ml of 1.0% Evans blue through the jugular vein to delineate the nonischemic tissue. Hearts were then excised, washed with PBS and cut into four transverse slices, which were stained for 5 min at 23 °C with 1.0 ml of 1.5% TTC to determine infarct area. The final stained samples were photographed under a microscope. The left ventricular area, AAR and infarct area were measured by computerized planimetry using Image J software. The infarct area was expressed as a percentage of the AAR and left ventricular area. For analyzing the serum CPK release as an index of myocyte injury, the blood samples of mice were collected from tail vein at 6 h after operation. The serum level of CPK was determined using Creatine Kinase (CK) (CPK) kit (Catachem, V184-12) according to the manufactural instructions.

2.5. Isolation, culture and treatment of cardiomyocytes

The isolation of adult mouse cardiomyocytes was performed with primary cardiomyocyte isolation kit (Pierce, 88281) according to the manufactural instructions.

The final isolated cardiomyocytes were cultured in MEM. Hypoxia-reoxygenation (HR) of cultured cardiomyocytes was completed by 6-h of hypoxia (95% N, 5% CO_2) and followed by 12-h reoxygenation (5% CO_2) to mimic myocardial ischemia-reperfusion.

2.6. Measurement of mitochondrial respiratory rate

The oxygen consumption of purified cardiac mitochondrial was measured as adapted from Ref. [11]. Briefly, isolated mitochondria (1 mg/ml) were initially incubated in respiration buffer composed of 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 25 mM sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.4. Complex I respiration was measured by adding 5 mM pyruvate and 2.5 mM malate into the respiration buffer prior to the mitochondria, which was followed by 1 mM ADP addition. Alternatively, for measuring Complex II mediated oxygen consumption, 8 mM succinate, 4 mM glycerol-3-phosphate and 5 mM rotenone were added into the respiration buffer prior to the addition of mitochondria followed by 1 mM ADP addition.

2.7. Western blotting

Protocols were described as previously [13]. PVDF membranes were immersed in the enhanced chemiluminescence (GE Healthcare, RPN2209) for protein detection via GE ImageQuant LAS 4000 instrument. The band intensity was quantified using ImageJ software.

2.8. qRT-PCR

Protocols were described as previously [13]. The sequences of primers were listed as follows: Sirt4 forward 5′-CGAGGGGACAAGGAGGATTT-3′, reverse 5′-GTCGGCCTGAAAGTCAATCC-3′; Actb forward 5′-ACTGGGACGACATGGAGAAG-3′, reverse 5′-GTCTCCGGAGTCCATCACAA-3′.

2.9. Statistics

Statistics was analyzed with GraphPad Prism 6 software. All data are representative of at least 3 independent experiments and presented as mean ± s.d. The statistical difference was calculated by unpaired Student's *t*-test, unless indicated otherwise. $P < 0.001$, $P < 0.01$ and $P < 0.05$ indicate a statistical difference, and NS indicates no statistical difference.

3. Results

3.1. SIRT4 is downregulated in cardiomyocytes after MI-R

To explore whether SIRT4 could play a possible role in MI-R injury, we first examined its expression level in cultured adult mouse cardiomyocytes subjected to treatments of 6 h of hypoxia and subsequent 12 h of reoxygenation in vitro for mimicking MI-R model [11]. Surprisingly, compared with sham treatment, the protein level of SIRT4 was decreased nearly by half ($P < 0.01$) in cardiomyocytes after MI-R in vitro (Fig. 1A). Consistent with this result, the transcript level of SIRT4 was also parallelly decreased ($P < 0.01$), although with less extent of change (Fig. 1B). To test whether this phenomenon could be extended to in vivo scenario, we next examined the expression level of SIRT4 in MI-R mice model subjected to 30 min of left ventricle (LV) ligation followed by 48 h of reperfusion. The results showed that compared with non-ischemic hearts (sham), the protein level of SIRT4 in cardiomyocytes from non-ischemic zone (NIZ) was not obviously affected (Fig. 1C, lane 1 and lane 2), whereas, the protein levels of SIRT4 in cardiomyocytes from both the border zone and ischemic zone of ischemic myocardium were significantly decreased, and with the latter being the lowest one (Fig. 1C). In addition, consistently, the transcript level of SIRT4 showed similar tendency compared with its protein level (Fig. 1D). Collectively, these results indicate that SIRT4 is downregulated in both transcript and protein levels in

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