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Ripk3 regulates cardiac microvascular reperfusion injury: The role of IP3R-dependent calcium overload, XO-mediated oxidative stress and F-action/filopodia-based cellular migration

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ABSTRACT

Ripk3-mediated cellular apoptosis is a major contributor to the pathogenesis of myocardial ischemia reperfusion (IR) injury. However, the mechanisms by which Ripk3 influences microvascular homeostasis and endothelial apoptosis are not completely understood. In this study, loss of Ripk3 inhibited endothelial apoptosis, alleviated luminal swelling, maintained microvasculature patency, reduced the expression of adhesion molecules and limited the myocardial inflammatory response. In vitro, Ripk3 deficiency protected endothelial cells from apoptosis and migratory arrest induced by HR injury. Mechanistically, Ripk3 had the ability to migrate onto the endoplasmic reticulum (ER), leading to ER damage, as evidenced by increased IP3R and XO expression. The higher IP3R content was associated with cellular calcium overload, and increased XO expression was involved in cellular oxidative injury. Furthermore, IP3R-mediated calcium overload and XO-dependent oxidative damage were able to initiate cellular apoptosis. More importantly, IP3R and XO also caused F-actin degradation into G-actin via post-transcriptional modification of cofilin, impairing the formation of the filopodia and limiting the migratory response of endothelial cells. Altogether, our data confirmed that Ripk3 was involved in microvascular IR injury via regulation of IP3R-mediated calcium overload, XO-dependent oxidative damage and filopodia-related cellular migration, ultimately leading to endothelial apoptosis and migratory inhibition. These findings provide a potential target for treating cardiac microcirculatory IR injury.

1. Introduction

Cardiac ischemia is defined as a blockade of the coronary artery. The strategies for a timely opening of the epicardial artery are the standard treatments for patients with myocardial infarction including percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) [1]. By dredging blocked vessels, cardiac circulation is stabilized and restored, which significantly reduces cardiovascular mortality resulting from myocardial infarction. Notably, reperfusion after ischemia causes ischemia/reperfusion injury (IR injury), also known as the “no-reflow” phenomenon in clinical practice [2]. Our team has conducted several clinical and basic experiments to dissect the underlying mechanism of cardiac IR injury [3]. We found that the ‘no-reflow’ phenomenon or cardiac IR injury could be primarily attributed to cardiac microvascular damage [4]. Mechanistically, in response to reperfusion after ischemia, the regaining of blood induces microvascular death via multiple mechanisms [5,6]. Subsequently, the collapse of the cardiac microcirculation results into an undersupply of energy, oxygen and nutrients to the cardiomyocytes despite the patency of the epicardium artery, which further aggravates damage to the heart [7]. Therefore, understanding the cellular and molecular mechanisms of microvascular IR injury may pave the road to new treatment modalities, which is needed for the treatment of cardiac IR injury in clinical practice [8].

Our team has explored several targets that are associated with microvascular damage including mitochondrial fission factor (Mff)-mediated mitochondrial fission, [9,10], Parkin-related mitophagy [11], FUN14 domain containing 1 (FUNDC1)-required platelet activation [12], xanthine oxidase (XO)-dependent endothelial oxidative stress [13] and IP3R-activated cytoplasmic and mitochondrial calcium overload [14]. Recently, some investigators have reported that receptor-interacting serine/threonine-protein kinase 3 (Ripk3) was closely involved in the reperfusion injury [15,16]. Ripk3 was largely upregulated in the infarcted heart and positively associated with cardiac death. Mechanistically, Ripk3 is a serine/threonine-protein kinase that prefers to interact with phospholipid structures. After Ripk3 binding to the

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cellular membrane, Ripk3 forms several pores in the membrane, leading to the activation of necroptosis, which is the classical function of Ripk3 [16]. Moreover, our previous results have demonstrated that Ripk3 has the ability to localize to the mitochondria by interacting with the mitochondrial receptor FUNDC1, leading to defective mitophagy [15]. Poorly structured mitophagy fails to remove damaged mitochondria, boosting mitochondria-related apoptosis in cardiomyocytes [10,17], which is the non-classical function of Ripk3. Notably, little evidence is available to establish the role of Ripk3 in endothelial damage.

Apart from the cellular and mitochondrial membrane, the endoplasmic reticulum (ER) also contains phospholipid structures [18]. Whether Ripk3 is able to interact with the ER remains unclear. If so, what is the consequence of the Ripk3-ER interaction? Previous studies have shown that ER damage could induce endothelial apoptosis via calcium overload and XO-mediated cellular oxidative stress [13,19]. Accordingly, we hypothesize that the above mechanisms may be responsible for the Ripk3-induced microvascular damage. Notably, in addition to cellular apoptosis, endothelial migration is an important element of infarcted tissue repair that occurs via promotion of vessel formation [20]. Our recent work has hinted that F-actin-based cytoskeleton rearrangement is the determinant of endothelial migration [15,21]. Cell movement in a certain direction is dependent on the F-actin-based filopodia, which determines the movement direction and provides the adhesive force [20]. However, whether Ripk3 is also associated with F-actin-based endothelial migration remains unknown. Therefore, in the present study, we conduct Ripk3 loss-of-function assays in vivo and in vitro to demonstrate whether Ripk3 promotes cardiac microvascular damage by regulating ER damage, calcium overload, oxidative stress and endothelial migration. Our results indicated that Ripk3 increased the expression of inositol trisphosphate receptor (IP3R) and XO under IR injury, leading to calcium overload and cellular oxidative stress, respectively, which finally contributed to the endothelial apoptosis. More importantly, Ripk3-mediated calcium overload and cellular oxidative injury inhibited cellular migration by impairing the F-actin balance and blunting filopodia formation. These findings provide new information regarding Ripk3 in cardiac reperfusion injury, which highlight Ripk3 as a potential target for reducing cardiac microvascular damage.

2. Materials and methods

2.1. Cardiac IR injury model in vivo

All protocols were approved by the Department of Cardiology, PLA general hospital Animal Care and Use Committee. The Ripk3$^{-/-}$ mice (C57BL/6 background) were generated as our previously described [15]. Then, the wild-type (WT) mice and Ripk3$^{-/-}$ mice (12 weeks old, male) were underwent the cardiac ischemia reperfusion injury model. The model was conducted in vivo via a 8.0 surgical suture ligation of left anterior descending coronary artery for about 2 h to induce the ischemia damage. Then, the slipknot was loosened for about 4 h to cause the reperfusion injury. After the cardiac ischemia reperfusion injury, the blood was collected and analyzed via ELSIA about the CK-MB, troponin T and LDH according to our previous study [11].

2.2. The gelatin-ink staining

The gelatin-ink staining was used to observe the patency of microvasculature according to our previous study [11]. Firstly, once the IR injury was completed, the gelatin-ink (3% gelatin and ink) was injected into the heart via jugular vein at the room temperature of 30 °C. Then, the hearts were cut and maintained at 4 °C for about one hours. Finally, after 4% paraformaldehyde fixation in, cryosectioning was carried out and observed under microscope.

2.3. Hypoxia reoxygenation injury (HR injury) model in vitro

The human umbilical vein endothelial cells (HUVEC) were used to induce the hypoxia reoxygenation injury model in vitro. The hypoxia preconditioning was performed as cells cultured in a tri-gas incubator for with N_2 concentration in 95% and CO_2 concentration at 5% for about 2 h. Then, cells were under normal culture condition for about 4 h to induce the reperfusion injury.

2.4. Western blots

Tissues and cultured HUVEC were lysed in ice-cold RIPA buffer supplemented with a cocktail of protease. Then, the samples were centrifuged at 14,000 rpm for 20 min at 4 °C. Equal amounts of protein were separated via SDS-PAGE and then transferred to PVDF membrane (Millipore, Billerica, MA, USA) [22]. The 5% bovine serum albumin was used to block the samples for 1 h at room temperature. Then, the membranes were incubated overnight at 4 °C with primary antibodies: Ripk3 (1:1000, Cell Signaling Technology, #15828), p-eNOS (1:1000, Cell Signaling Technology, #9572), G-actin (1:1000, Abcam, #ab200046), IP3R (1:1000, Cell Signaling Technology, #8548), pro-caspase3 (1:1000, Cell Signaling Technology, #9662), cleaved caspase3 (1:1000, Cell Signaling Technology, #9664), c-IAP1 (1:2000, Cell Signaling Technology, #7065), Bax (1:2000, Abcam, #ab90435), caspase9 (1:1000, Abcam, #ab32539), XO (1:1000, Abcam, #ab109235). Then the membranes were incubated with secondary antibodies at room temperature for 1 h. Band intensity was quantified using the Image-Pro Plus 6.0 software [23].

2.5. MTT, caspase-3 activity and TUNEL assay

The MTT assay was used to detect the cellular viability. In brief, after treatment, MTT solution (5 mg/ml for about 20 μl) was added into the medium for about 4 h. Then, the supernatant was discarded and 100 μl DMSO was applied into the culture for about 30 min. Then, the optical density value of 490 nm (OD) was measured.

The caspase-3 activity and TUNEL assay were detected to reflect the cellular apoptosis according to our previous study. The level of caspase-3 activity was expressed as a percentage of the control group. The TUNEL positive cells were imaged and counted.

2.6. Immunofluorescence

Tissues and cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 for 10 min. Then, the 5% normal goat serum was used to block the sample for 1 h at room temperature. Then, the samples were incubated with primary antibodies overnight at 4 °C [24]. After extensively washed, samples were observed under an Axio Observer Z1 microscope [25]. The primary antibodies used in the present study were as follows: Calreticulin, the ER marker (Abcam, #ab39897), Ripk3 (Cell Signaling Technology, #15828), F-actin (Abcam, #ab205), tubulin (Cell Signaling Technology, #5335), ICAM1 (Abcam, #ab53013), F4/80 (Abcam, #ab6640) and Troponin T (Abcam, #ab8295).

2.7. Immunohistochemistry

The infarcted tissues were fixed in 4% paraformaldehyde followed by dehydration in graded ethanol [26]. The 4-mm sections of the heart were obtained and stained with primary antibodies: VCAM1 (Abcam, #ab106777).

2.8. The siRNA transfection

Transient knockdown assays were performed using DharrmaFECT 1 (Dharmacon, Lafayette, CO), according to the manufacturer's manual