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Research Paper

Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury

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ABSTRACT

Ripk3-required necroptosis and mitochondria-mediated apoptosis are the predominant types of cell death that largely account for the development of cardiac ischemia reperfusion injury (IRI). Here, we explored the effect of Ripk3 on mitochondrial apoptosis. Compared with wild-type mice, the infarcted area in Ripk3-deficient ($Ripk3^{-/-}$) mice had a relatively low abundance of apoptotic cells. Moreover, the loss of Ripk3 protected the mitochondria against IRI and inhibited caspase9 apoptotic pathways. These protective effects of Ripk3 deficiency were relied on mitophagy activation. However, inhibition of mitophagy under Ripk3 deficiency enhanced cardiomyocyte and endothelia apoptosis, augmented infarcted area and induced microvascular dysfunction. Furthermore, ischemia activated mitophagy by modifying FUNDC1 dephosphorylation, which substantively engulfed mitochondria debris and cytochrome-c, thus blocking apoptosis signal. However, reperfusion injury elevated the expression of Ripk3 which disrupted FUNDC1 activation and abated mitophagy, increasing the likelihood of apoptosis. In summary, this study confirms the promotive effect of Ripk3 on mitochondria-mediated apoptosis via inhibition of FUNDC1-dependent mitophagy in cardiac IRI. These findings provide new insight into the roles of Ripk3-related necroptosis, mitochondria-mediated apoptosis and FUNDC1-required mitophagy in cardiac IRI.

1. Introduction

Reperfusion itself results in additional damage, known as cardiac ischemia-reperfusion injury (IRI) [1]. During reperfusion, the death of cardiomyocytes [2] and cardiac microvascular endothelia cells [3] (CMECs) are the key factors that determine the disease course and prognosis. Necrosis and apoptosis are the two major cell death mechanisms in IRI [4]. Over the past years, necroptosis is determined to be the prominent mode of programmed cell death (PCD) in response to IRI via the Ripk1/Ripk3/MLKL pathways [4–6]. Interestingly, many studies have suggested that necroptosis has multiple interactions in apoptosis [7,8]. Ripk1 is the upstream signal of caspase8-mediated apoptosis [9], but activated caspase 8 degrades Ripk3 and inhibits subsequent necroptosis [10]. Whether the disruption of Ripk3 could aggregate or alleviate apoptosis remains unclear.

Mitophagy is also a protective mechanism enabling the efficient and selective elimination of damaged mitochondria to attenuate mitochondria-mediated apoptosis via the engulfment of ruptured mitochondria and neutralization of pro-apoptotic factors [11]. However, no information is available about the fundamental relationship between Ripk3 and mitophagy in cardiac IRI. Considering the regulatory role of mitophagy in apoptosis [12–14], we propose that shared signaling pathway elements between apoptosis and necroptosis may be involved in mitophagy. Furthermore, mitophagy could be activated via PINK/Parkin pathways and FUNDC1 pathways according to previous studies [15,16]. As for the PINK/Parkin, little solid evidence is available for the role of Parkin in cardiac IRI when compared with FUNDC1. Furthermore, the activator of Parkin is PINK which locates in the mitochondria but not in the cytoplasm [17]. However, Ripk3 is a cytoplasmic protein which has the kinases property (for example, Ripk3 could induce the MLKL phosphorylation modification) [18]. Based on such two reasons, we think that Parkin may not be indirectly regulated by Ripk3. On the other hand, FUNDC1 is the receptor of LC3II and locates on the surface of mitochondria outer membrane [19]. Moreover, several studies have

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indicated the protective role of FUNDC1 in cardiac IRI [15]. Meanwhile, FUNDC1 could be inactivated or activated via phosphorylation modification [20]. These information hint that Ripk3 has the ability to regulate mitophagy via FUNDC1. Thus, in the present study, using loss- and gain-of-function experiments related to Ripk3 in vitro and in vivo, we observed that Ripk3 deficiency suppressed cellular apoptosis. Specifically, upregulated Ripk3 during reperfusion disrupted ischemia-induced mitophagy via the post-transcriptional modification of FUNDC1, committing cells to mitochondria-mediated apoptosis and amplifying cardiac myocytes and microvascular reperfusion injury.

2. Methods

2.1. Animal and ex vivo models of cardiac reperfusion injury

All protocols were approved by the PLA General Hospital Institutional Animal Care and Use Committee. Ripk3$^{-/-}$ mice with a C57BL/6 background were generated as previously described [21]. Ripk3$^{-/-}$ and wild-type (WT) male mice (12-wk-old) underwent a procedure to induce cardiac IRI according to our previous studies. A 7-0 silk suture was placed around the left anterior descending coronary artery, and a reversible slipknot was tied for 30 min and subsequently loosened for 2 h for reperfusion. Then, blood samples were collected for analysis of LDH, Troponin T and CK-MB levels. The hearts (n = 6/group) were used for infarct size measurement using 2% Evans blue and 1% TTC.

2.2. Cardiomyocyte and CMEC isolation, experimental groups and reperfusion injury induction in vitro

Cardiomyocytes and CMECs were isolated from the hearts of Ripk3$^{-/-}$ and WT mice using the enzyme dissociation method as described by our previous studies [22,23]. For experimental groups in vitro, cardiomyocytes and CMECs were obtained from WT and Ripk3$^{-/-}$ mice, named the WT and Ripk3$^{-/-}$ groups, respectively. In addition, Ripk3 gain-of-function experiments (Ad + Ripk3$^{-/-}$) were performed by Ripk3 adenovirus vector overexpression in cells isolated from Ripk3$^{-/-}$ mice. Reperfusion injury was mimicked in vitro according to our previous study [24]. Hypoxia was conducted in a 37 °C airtight chamber saturated with 95% N_2/5% CO_2 for 30 min with H-DMEM/serum starvation, and reoxygenation was performed in a 37 °C/5% CO_2 incubator under H-DMEM/10% FBS for 2 h. To trigger mitophagy, FCCP (5 μm) was added for 120 min to induce mitophagy according to previous study [25]. To inhibit mitophagy, 3-MA (10 mM) was pretreated for 2 h.

2.3. Echocardiogram and microvascular imaging using gelatin-ink perfusion

Echocardiography was performed by echocardiogram (14.0 MHz, Sequoia C512; Acuson, Germany) to observe the change of cardiac function (n = 6/group) according to our previous study [26].

The 37 °C ink plus 3% gelatin (gelatin-ink staining via the jugular vein) was applied for gelatin-ink perfusion after a 2-h reperfusion. Rapid ligature of the great vessel of the cardiac base and the superior and inferior vena cava was performed once the limbs appeared black. Then, the heart was maintained at 4 °C for at least 1 h to promote gelatin coagulation. Thereafter, the heart was fixed in 4% paraformaldehyde and processed for cryosectioning.

2.4. Immunohistochemistry and immunofluorescence staining

The following primary antibodies were used for immunohistochemistry and immunofluorescence staining: Ripk3 (1:200), TOMM20 (1:1000), LC3II (1:500), F-actin (1:1000), Tubulin (1:1000), all from Abcam, USA. DAPI (Sigma-Aldrich, USA), lysosome stain (Abcam, USA) and a mitochondrion-selective MitoFluor™ stain (Molecular Probes, USA) were used to detect the nuclei, lysosomes and mitochondria, respectively.

2.5. Mitochondrial membrane potential (ΔΨm) measurement, mitochondrial permeability transition pore (mPTP) opening evaluation and mitochondrial ROS (mROS) detection

The ΔΨm was visualized using the JC-1 Kit (Beyotime, China) and the mPTP opening was assessed as the rapid dissipation of tetramethylrhodamine ethyl ester (TMRE) fluorescence according to our previous study [27]. The arbitrary mPTP opening time was determined as the time when TMRE fluorescence intensity decreased to halfway between the initial and residual fluorescence intensity. The mROS was detected using a MitoSOX™ Red Mitochondrial Superoxide Indicator (Molecular Probes, USA), observed using a microscope or analyzed by flow cytometry.

2.6. Caspase3/9 activity, LDH release and TUNEL assays

Caspase3/9 activity (Beyotime, China), LDH release (Beyotime, China) and TUNEL late-apoptotic (Roche Applied Bio Sciences, USA) analyses were performed using commercial kits as previously described [28].

2.7. Trypan blue staining and MTT assay for cellular death evaluation

Trypan blue is a vital stain used to selectively color dead cells blue. After treatment, the cells were treated with 0.4% trypan blue. Then, trypan blue-positive cell images were acquired and analyzed using a fluorescence microscope. MTT was conducted in a 96-well plate in triplicate. After reperfusion injury, 20 μl of MTT (5 mg/ml PBS, pH 7.4) was added, and the plate was incubated for another 4 h. The supernatant was subsequently discarded, and 100 μl of DMSO was added to each well for 10 min. Finally, the optical density (OD) value was measured at A490 nm, reflecting cell viability according to our previous study [23].

2.8. Migration assay

The migration assay of CMECs was evaluated using 24-well transwell chamber (Coring, U.S.A.) according to our previous study [22]. Firstly, 10^5 CMECs were seeded in the upper chamber in serum-free medium. The chemotactic agent SDF-1 (Sigma-Aldrich, U.S.A., 100 ng/ml) was added to the lower chamber to induce CMEC migration. After 12 h incubation at 37 °C, non-migrating cells in the upper chamber were carefully removed with cotton swab, cells that had traversed the membrane were fixed in methanol, stained with 0.05% crystal violet.

2.9. Calcium imaging

After treatment, cardiomyocytes were placed on a glass coverslip and were incubated with 1 μM Fura-2/AM for 30 min at room temperature. After washing three times with PBS to remove extracellular dye, the coverslip was fixed in in the Warner model RC-26 chamber (Warner Instruments, Hamden, CT, USA) and PH-1 heated platform (Warner Instruments, Hamden, CT, USA). Then cells were observed on an inverted microscope (Olympus America, Melville, NY, USA) and Fura-2 fluorescence was alternately excited at the wavelengths of 340 nm and 380 nm with a monochrometer (TILL Photonics, Polychrome V, Munich, Bavaria, Germany). Intracellular free calcium concentration was calculated according to the previous study [29].

2.10. Western blot analysis and co-immunoprecipitation

The following primary antibodies were used for western blot analyses: caspase3 (1:1000), caspase9, (1:1000), caspase12 (1:1500),