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Original article

iRhom2 is involved in lipopolysaccharide-induced cardiac injury in vivo and in vitro through regulating inflammation response

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ABSTRACT

Heart is a complex assembly of many cell types constituting of myocardium, endocardium and epicardium that intensively communicate to each other in order to maintain the proper cardiac function. Previous research has demonstrated that lipopolysaccharide (LPS) can induce myocardial dysfunction. iRhom2 is encoded by the gene Rhbdf2, regulating inflammation via tumor necrosis factor-α (TNF-α). In this study, we attempted to investigate the role of iRhom2 in LPS-induced cardiac injury and clarify the potential mechanism. We found that in vivo cardiac histopathological changes were induced after LPS challenge, accompanied with increase of TNF-α, interleukin-1β (IL-1β), interleukin-18 (IL-18) and interleukin-6 (IL-6) in serum and in heart tissue samples, which was dependent on TLR-4/NF-κB activation. Of note, we found that iRhom2 was a positive regulator for LPS-induced inflammation. LPS treatment markedly up-regulated iRhom2 and its down-streaming signal of IGS56. iRhom2 silence significantly suppress pro-inflammatory cytokines releases, and inactivated Toll-like receptor-4/Nuclear Factor kappa-B (TLR-4/NF-κB) signaling pathway in cells after LPS administration, suggesting its possible relationship with heart injury via TLR-4/NF-κB. It is concluded that iRhom2 may be a promising therapeutic target for LPS-induced cardiac injury by regulating inflammatory res`ponse.

1. Introduction

Cardiovascular disease is one of the leading causes of death worldwide. Clinical study shows that its main cause of death is myocardial infarction [1,2]. Furthermore, heart injury is associated with high rates of morbidity and mortality in sepsis [3]. Pathological studies have shown that inflammation response is one of the most common underlying molecular and cellular pathophysiologies of heart injury [4]. Lipopolysaccharide (LPS) is a major constituent of the bacterial outer membrane, and serves a crucial function in the initiation of the pathophysiological cascades [5,6]. A reduction in LPS has been associated with improved outcomes in patients with heart disease [7]. Previous study has reported that cultured H9c2 cardiomyocytes have been shown to exhibit a marked inflammatory response to LPS stimulation [8]. In addition, LPS has been shown to induce acute injury in different organs, such as brain, kidney and liver, through accelerating inflammatory response [9–11]. Thus, the inhibition of inflammatory processes after LPS stimulation may exert a beneficial effect on cardiac dysfunction.

Toll like receptors (TLRs) are *trans*-membrane signaling molecules that participate in innate immune recognition [12]. Toll-like receptor-4 (TLR-4) is able to identify pathogenic micro-organisms in a natural immune system, bind the specific ligand and produce corresponding inflammation following identification [13,14]. And TLR-4 exogenous ligands include LPS [15]. Previous evidence has suggested that TLR-4 is expressed by numerous kinds of cells, and that the injury of these cells is associated with the release of pro-inflammatory cytokines after LPS challenge [16]. Thus, the inhibition of TLR-4 may reduce inflammatory responses and ameliorate the cell damage or tissue injury after LPS administration.

Rhomboid protease was initially discovered in *Drosophila* [17]. *Drosophila* rhomboid protease cuts epidermal growth factor receptor (EGFR) ligand Spitz and a homologue for mammalian tumor growth factor (TGF)-α [18]. The rhomboid protease family members have been shown to have a common structure [19]. iRhom1 and iRhom2 are inactive homologs of rhomboid intra-membrane serine proteases, which lack essential catalytic residues [20]. These are necessary for maturation and trafficking of tumor necrosis factor-alpha (TNF-α) converting enzyme (TACE) [21].

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TNF-α is considered as a 'fire alarm' of the body, as it helps body fight against infections. But TNF-α can also cause diseases such as inflammatory arthritis [22]. TACE and its regulator, iRhom2, can be rapidly activated by small amounts of cytokines, growth factors, and pro-inflammatory mediators, substantiating rapid alarm through TNF-α [23]. Additionally, iRhom2 was reported to modulate TNF-α induced by LPS and Listeria by controlling TACE [24,25]. Thus, although the mechanism remains unknown, iRhom2 has been associated with inflammation, which might be also involved in heart disease. Here, it was the first time that we identified the possible role of iRhom2, as a positive regulator, enhanced inflammation response in LPS-induced cardiac injury via promoting TLR-4 activity in vivo and in vitro, suggesting that iRhom2 is essential for LPS-triggered inflammation in heart and facilitates the development of novel therapeutic strategy for treatment of heart failure.

2. Materials and methods

2.1. Animals treatment

All animal procedures were done according to the guidelines for care and use of laboratory animals approved by Department of Cardiology Huaihe Hospital, Henan University. 50 male C57BL/6 mice weighed 18–22 g were purchased from Experimental Animal Center of Nanjing Medical University (Nanjing, China). All mice were housed in a temperature of 22 ± 2 °C and relative humidity of 60 ± 10% environment under 12 h light/dark cycles. All mice were mainly divided into 5 groups: (1) Control (Con) and (2) 10, 15, 20 and 30 mg/kg LPS [26]. Lipopolysaccharide (LPS) was obtained from Sigma–Aldrich (Shanghai, China) and prepared in phosphate buffer saline (PBS). After 10 days adaptation, the model group of mice were treated by intraperitoneal injection (i.p.) with different concentrations of LPS for six hours. The control group was also administrated with Hanks' buffer. Then, all mice were sacrificed. Eyeball blood was collected and centrifuged at 15,000 × *g* for 15 min prepared for the following investigation, and the whole heart tissues were carefully harvested on 4 °C glacial table.

2.2. Preparation of cardiac muscle cells

The isolation, purification and culture of cardiomyocytes from the mice (10 weeks of age) were performed as described before [27]. Briefly, the cardiacmyocytes were isolated from mouse hearts via coronary perfusion with collagenase type 2 (Invitrogen, USA). Then cells were seeded in laminin-coated tissue culture wells in Minimum Essential Medium (MEM) (Invitrogen, USA) with Hanks' Balanced Salt Solution (HBSS) (Invitrogen, USA), supplemented with 2 mmol/L ATP, 100 U/mL penicillin, 10% serum, and 10 mmol/L 2,3-butanedione monoxime (BDM) (Sigma, USA). Two hours later, the medium was changed to culture medium of MEM with HBSS, containing 0.1 % bovine serum albumin (Sigma, USA), 10 mmol/L BDM, and 100 U/mL penicillin-G. The cardiac myocytes were maintained in primary culture for 72 h with 2% CO_2.

2.3. Cells transfection and reporter analysis

The cells were transfected by lipofectamine 2000 (Thermo Fisher Scientific, USA). To normalize for transfection efficiency, 0.01 μg of pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega, USA).

2.4. iRhom2 RNA interference

Double-stranded oligonucleotides responding to the target sequences were cloned into the pSuper.Retro-RNAi plasmid (Oligoengine). The following sequences were targeted for iRhom2 mRNA: #1, 5′-GAT GCC CAA GAT TGT GGA T-3′; #2, 5′-TGA CCA TCC TGA GGG ACC T-3′. Chemically synthesized 21-nucleotide siRNA duplexes were obtained from Gene-Pharma and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA oligonucleotides are as follows: NC (negative control): 5′-UUC UCC GAA CGU GUC ACG UTT-3′.

2.5. Pro-inflammatory cytokines analysis

The levels of major inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-18 levels in serum or heart tissue samples from mice were determined by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN, USA). Serum was obtained from blood following centrifugation at 2000 rpm for 15 min and then used for ELISA. Six hours after injection of LPS, heart tissues were harvested and frozen in liquid nitrogen immediately until homogenization. Proteins were extracted from the lungs using T-PER Tissue Protein Extraction Reagent Kit (Thermo) according to the manufacturer's instructions. Protein concentrations were determined by BCA protein assay kit and then used for ELISA.

2.6. Wesertn blot analysis

Six hours after injection of LPS, heart tissues were harvested and frozen in liquid nitrogen immediately until homogenization. Proteins were extracted from the lungs using T-PER Tissue Protein Extraction Reagent Kit (Thermo) according to the manufacturer's instructions. Protein concentrations were determined by BCA protein assay kit, and equal amounts of protein were loaded per well on a 10% sodium dodecyl sulphatepolyacrylamide gel. Subsequently, proteins were transferred onto polyvinylidene difluoride membrane. The resulting membrane was blocked with Tris-buffered saline containing 0.05% Tween-20 (TBS-T), supplemented with 5% skim milk (Sigma) at room temperature for 2 h on a rotary shaker, and followed by TBS-T washing. The specific primary antibody, diluted in TBST, was incubated with the membrane at 4 °C overnight. Subsequently, the membrane was washed with TBS-T followed by incubation with the peroxidase-conjugated secondary antibody at room temperature for 1 h. The immunoactive proteins were detected by using an enhanced chemiluminescence western blotting detection kit. Western blot bands were observed using GE Healthcare ECL Western Blotting Analysis System and exposed to x-ray film of Kodak. The primary antibodies used in our study are shown as followings: rabbit anti-GAPDH (ab2118, 1:1000, Cell Signaling Technology), rabbit anti-IL-1β (ab31202, 1:1000, Cell Signaling Technology), rabbit anti-IL-18 (ab71495, 1:1000, Abcam), rabbit anti-IL-6 (ab, 1:1000, Abcam, USA), rabbit anti-TNF-α (ab6671, 1:1000, Abcam), rabbit anti-IKKα (ab32041, 1:1000, Abcam), mouse anti-TLR-4 (ab22048, 1:1000, Abcam), rabbit anti-MyD88 (ab2068, 1:1000, Abcam), rabbit anti-p-IκBα (ab2859, 1:1000, Cell Signaling Technology), rabbit anti-p-NF-κB (ab13346, 1:1000, Cell Signaling Technology), rabbit anti-NF-κB (ab4746, 1:1000, Cell Signaling Technology), mouse anti-iRhom2 (ab116139, 1:1000, Abcam) and rabbit anti-ISG56 (ab118062, 1:1000, Abcam).