RCAN1 deficiency aggravates sepsis-induced cardiac remodeling and dysfunction through accelerating mitochondrial pathological fission

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Abstract

Background Cardiac dysfunction and remodeling are serious complications of sepsis and are correlated with high mortality. RCAN1 is a feedback regulator of cardiac hypertrophy. Here we aim to investigate role of RCAN1 in septic cardiomyopathy.

Methods RCAN1−/− mice and WT mice were randomly divided into control and LPS-induced groups, some with Midiv-1 or KN93 treatment. The protein levels of RCAN1, p-ERK1/2, NFAT3, Drp1, p-Drp1, p-CaMKII in cardiac tissue or cultured cardiomyocytes were detected by Western blotting. Myocardial function was assessed by echocardiography. Cardiac hypertrophy and fibrosis were detected by H&E and Masson's trichrome staining. Mitochondrial morphology was examined by transmission electron microscope. Serum level of LDH was detected by ELISA.

Results Our data showed that RCAN1 was downregulated in septic mouse heart and LPS-induced cardiomyocytes, and RCAN1−/− mice had impaired cardiac function and severe myocardial hypertrophy and fibrosis. NFAT3 and p-ERK1/2 were significantly increased in heart tissue of RCAN1−/− mice. Further, RCAN1 deficiency aggravated sepsis-induced cardiac mitochondrial injury as indicated by increased ROS production, pathological fission and the loss of mitochondrial membrane potential. Inhibition of fission by Mdivi-1 reversed LPS-induced cardiac hypertrophy, fibrosis and dysfunction in RCAN1−/− mice. Moreover, RCAN1 depletion caused mitochondrial translocation of CaMKII, which promoted fission and septic hypertrophy. Inhibition of CaMKII with KN93 reduced excessive fission, improved LPS-induced cardiac remodeling and dysfunction in RCAN1−/− mice.

Conclusions Our finding demonstrated that RCAN1 deficiency aggravated mitochondrial injury and septic cardiomyopathy through activating CaMKII. RCAN1 serves as a novel therapeutic target for treatment of sepsis-related cardiac remodeling and dysfunction.

Introduction

Sepsis is a common disease in emergency medicine. It is defined as life-threatening organ dysfunction caused by the maladjusted response of the host to infection, which results in septic shock and even death[1, 2]. There are more than 20 million people worldwide suffered from sepsis or septic shock and about 8 million people died[3]. Sepsis induced myocardial dysfunction (SIMD) is one of the main complications of clinical sepsis, which usually leads to myocardial remodeling and dysfunction[4]. However, the mechanism for septic cardiomyopathy and dysfunction remains unclear and related treatment options are very limited.

Calcineurin, also known as protein phosphatase 2B, is a ubiquitous calcium-dependent protein phosphatase that critically involved in the regulation of pathological hypertrophy, calcium homeostasis and intracellular trafficking of cardiomyocytes[5, 6]. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria that caused sepsis. Calcineurin is activated both in vivo and in vitro of
LPS-induced in cardiomyocytes[7, 8]. Regulator of calcineurin 1 (RCAN1), as a feedback inhibitor of calcineurin, prevents cardiac injury and remodeling through suppressing excessive activation of calcium signaling in cardiomyocytes[9, 10]. However, the potential role of RCAN1 in the pathogenesis of sepsis-related cardiac remodeling and dysfunction, especially LPS-induced septic cardiomyopathy has not been fully elucidated.

In the present study, we hypothesized that RCAN1 might be a protective regulator of LPS-mediated SIMD by reversing cardiac remodeling and dysfunction. We found that LPS-induced cardiac hypertrophy and fibrosis were exaggerated in RCAN1 gene knockout (RCAN1<sup>−/−</sup>) mice. RCAN1 deficiency increased mitochondrial fission, ROS production and the loss of mitochondrial membrane potential. Loss of RCAN1 promoted CaMKII activation and CaMKII-dependent mitochondrial dysfunction and cardiac hypertrophy. Our data shed light on the role of mitochondrial injury in the process of LPS-mediated cardiac remodeling, and we identified the RCAN1-calcineurin–CaMKII–Drp1 pathway in cardiomyocytes as a potential therapeutic target for septic cardiomyopathy.

**Materials And Methods**

**Animals and Reagents**

All animal experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China). 8 ~ 10 weeks old male wild-type (WT) C57BL/6J mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. RCAN1 knockout mice were kindly provided by Dr. Jian Wu from Institute of Biomedical Sciences, Fudan University. Generation of RCAN1<sup>−/−</sup> mice has been described previously [11]. Exon 2 ~ 3 was removed from RCAN1 gene (ENSMUST00000060005) using CRISPR/Cas9 strategy. Mouse genotyping was performed by PCR and agarose gel electrophoresis. All the mice were raised alone in specific pathogen-free conditions. Mice were randomly divided into four groups: the wild-type (WT) control group, RCAN1<sup>−/−</sup> control group, WT mice with intraperitoneal LPS (10 mg/kg) injection (WT-LPS group) and RCAN1<sup>−/−</sup>-LPS group. In addition, mice from RCAN1<sup>−/−</sup>-LPS group were randomly selected for intraperitoneal injection of Mdivi-1 (10 mg/kg, twice per week, 4 weeks, S716201, Selleck.cn, USA); or for intraperitoneal injection of KN93 (5 mg/kg, twice per week, 4 weeks, S6787, Selleck.cn, USA).

**Measurement of echocardiography**

Cardiac function of mice was measured by a preclinical ultrasound system (Vevo 2100, FUJIFILM Visual Sonics, Canada) after LPS injection for 4 weeks. In brief, mice were anesthetized with inhaled 1.5% isoflurane and were placed on a heating pad to maintain normal body temperature and heart rate at about 500 beats/min. The intercept angle between the Doppler beam line and flow direction was controlled within 60°, and M-mode echocardiographic images were recorded. The left ventricular end systolic inner diameter (LVIDs) and left ventricular end diastolic inner diameter (LVIDd) were measured.
from the M-mode view; and left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated and analyzed. All the measurements were double-blind.

**Cell culture and isolation of cardiomyocytes**

H9c2 cells, a cardiomyoblast cell line originally from the left ventricle of rat heart, was purchased from Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China). The cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 37°C, 5% CO2 incubator.

Adult mouse (6 ~ 8 weeks) ventricular cardiomyocytes were isolated from RCAN1−/− and WT mice with C57BL/6 background using standard enzymatic method as previously described. The animal procedure was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China). In brief, mice were anesthetized and hearts were excised. Then the hearts were transferred to a 6-cm dish containing fresh EDTA buffer. Cell digestion was completed by a sequential injection with EDTA buffer, perfusion buffer and collagenase buffer into the left ventricle. After stopping digestion and tissue dissociation, the isolated cardiomyocytes were stabilized in 1.0 mM Ca^{2+} Tyrode solution (126 mM NaCl, 5.4 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 0.33 mM NaH2PO4, 10 mM HEPES, and 10 mM glucose) for 10 min, and then suspended in Dulbecco's modified Eagle medium Minimum Essential Medium (DMEM) with 10% FBS. Cells were dispersed in 3-cm dishes precoating with laminin (10 µg/ml) at the glass bottom, and cultured in 5% CO2 and 37°C incubator. After 1 hour and every 24 hours thereafter, the media was changed with fresh, prewarmed culture medium.

For laser confocal scanning, the isolated cardiomyocytes were cultured with FBS free medium for 2 hours and stimulated by LPS for 6 hours, then cells were fixed by 0.5% paraformaldehyde for 1 hour. After pretreating with 0.1 Triton X-100 for 10 min, cells were blocked with 1% BSA for 1 hour, and then were incubated with anti-p-Drp1^{Ser616} (1:200, #3455, CST, US) and anti-p-CaMKII^{Thr286} (1:200, ab171095, Abcam, US) at 4°C overnight. After incubating with the respective fluorescent labeled second antibodies, cells were visualized under laser confocal microscopy.

**Serum LDH determination**

Blood samples were collected by extracting the eyeball blood after mice were anesthetized with isoflurane. Then, all the samples were centrifugated at 3000 rpm for 5 min, serum supernatant was carefully collected and stored at −80°C for subsequent analysis. Serum level of lactate dehydrogenase (LDH), one of the major cardiac injury markers, was determined using commercial kits (A020-1, Nanjing Jiancheng Biotechnology Institute, Nanjing, China) according to the manufacturer's instructions.

**Transmission electron microscope (TEM) analysis**

Left ventricular tissues of mice were minced into 0.5 cm³ small pieces and were prefixed in 2.5% glutaraldehyde in PBS at 4°C overnight and post-fixed with 1% buffered osmium tetroxide for 2 h at 4°C. Then, the specimens went dehydrated with graded ethanol and two final 15min. rinses in 100% ethanol.
After being embedded, the specimens were taken for ultrathin sections and were doubly stained with uranyl acetate and lead citrate. Ultrathin sections were examined using a transmission electron microscope (JEM-1010, JEOL, Japan).

**Mitochondrial ROS and TMRE examination**

Mouse hearts were rapidly removed and placed into ice-cold Krebs-Henseleit buffer containing 2.5mM CaCl_2, 115mM NaCl, 25mM NaHCO_3, 5.9mM KCl, 1.18mM MgCl_2, 1.23 mM NaH_2PO_4, 1.2 mM Na_2SO_4 and 10 mM glucose. Hearts were then perfused with Krebs-Henseleit medium for 10 min at 37°C, and the fluorescent probe, 2’,7’-Dichlorofluorescein diacetate (DCFH-DA) or tetramethylrhodamine ethyl ester (TMRE) was infused into the perfusion medium immediately prior to the perfusion at a final concentration of 50 µM or 5 µM. Then, mitochondria from the perfused heart tissues were isolated by a rapid tissue mitochondrial isolation kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Mitochondrial particles were washed, resuspended and ultrasonic crushed in 1.5 ml ice-old PBS buffer. After centrifuging at maximum speed 14000 g for 30 sec, the supernatant was collected and placed on ice, and fluorescence intensity was detected on a Biotek Synergy HT fluorometric plate reader (Biotek). DCF fluorescence was examined at an excitation wavelength of 488 nm and an emission wavelength of 525 nm; and TMRE fluorescence was examined at an excitation wavelength of 535 nm and an emission wavelength of 587 nm.

**H&E and Masson’s trichrome staining**

A thin midsection of heart was fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5-µm-thick sections. The sections were then deparaffinized in xylene, dehydrated with graded concentrations of ethanol. The sections were stained with H&E for cardiomyocyte size determination and Masson's trichrome for collagen deposition. For each image, collagen volume fraction was calculated as the ratio of collagen surface area (aniline blue) with respect to myocardial surface area (red).

**Western blot analysis**

LV tissue or isolated cardiomyocytes were homogenized in protein lysis buffer with 1mM PMSF (Beyotime Biotechnology, Shanghai, China). Protein supernatants were extracted from the lysis buffer after centrifuging at maximum speed 14000 g for 15 min at 4°C and heated at 95°C for 3 min for denaturation. Proteins (20 µg) were separated on 8 ~ 12% SDS–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked in PBS containing 1% skim milk powder for 1 h at room temperature and incubated with the primary antibodies at 4°C overnight, including anti-RCAN1(1:1000, ab140131, Abcam, US), anti-p-ERK1/2(1:1000, #4370, CST, US), anti-NFAT3(1:2000, A302-769A, Bethyl Laboratories, US), anti-Drp1(1:1000, 8570, CST, US), anti-p-Drp1Ser616(1:1000, 3455, CST, US), anti-p-CaMKIIThr286(1:1000, ab171095, Abcam, US), anti-β-actin (1:1000, #3700, CST), anti-GAPDH(1:1000, #2118, CST). Then, the membranes were washed with TBST (Tris-buffered saline plus 0.1% Tween-20) for 3 times and were incubated with the secondary antibodies (peroxidase-affinipure goat anti-rabbit IgG) for 0.5 hour at room temperature. The membranes were again washed with TBST for 3 times and were treated with enhanced ECL chemiluminescence.
Reagents (Thermo Fisher Scientific). Blots from the membranes were visualized by using the ChemiDoc Imaging System (Bio-Rad).

**Statistical analyses**

Data were reported as means ± SEM. The comparisons were performed using unpaired Student’s T-test or one-way ANOVA followed by a Tukey multiple comparison posthoc test. A $P$ value less than 0.05 was considered significant difference.

**Results**

**RCAN1 deficiency aggravated sepsis-related myocardial injury and dysfunction**

Previous studies have shown that calcineurin inactivation protect heart against LPS-induced toxicity[7]. However, the role of RCAN1, an endogenous feedback regulator of calcineurin, in sepsis-related myocardial injury and dysfunction has not been investigated. To elucidate the role of RCAN1 in sepsis-induced cardiac injury, the endogenous RCAN1 protein level was determined in cultured cardiomyocytes. As shown by Western blotting, the protein level of RCAN1 was time-dependently decreased in cardiomyocytes in response to LPS stimulation (Fig. 1A-B). Consistently, in vivo expression of RCAN1 was also significantly reduced in heart of LPS-treated mice (Fig. 1C and I).

To investigate the impact of RCAN1 decline on septic heart, we next generated RCAN1$^{-/-}$ mice and evaluated the cardiac function of RCAN1$^{-/-}$ mice and WT controls by echocardiography at 4 weeks after LPS induction. Results showed that the left ventricular (LV) function was markedly aggravated in RCAN1$^{-/-}$ mice when compared with that in WT controls, as indicated by increased LVIDs (3.20 mm ± 0.16 mm vs. 2.60 mm ± 0.11 mm, $P < 0.0001$) and LVIDd (4.04 mm ± 0.21 mm vs. 3.69 mm ± 0.16 mm, $P = 0.0086$), but decreased LVEF(42.00% ± 11.27% vs. 57.03% ± 4.64%, $P = 0.0128$) and LVFS (20.57% ± 6.10% vs. 29.37% ± 3.08%, $P = 0.0102$) values (Fig. 1D-H). However, no significant difference in LV function was observed between PBS-treated WT mice and RCAN1$^{-/-}$ mice, suggesting that RCAN1 deficiency only aggravated cardiac dysfunction under septic condition. To confirm whether cardiac dysfunction aggravation led to myocardial injury in LPS-induced RCAN1$^{-/-}$ mice, Lactate dehydrogenase (LDH), one of the major biomarkers of acute myocardial injury, was determined. A higher level of serum LDH was observed in RCAN1$^{-/-}$ mice compared with that in WT mice after LDH stimulation (Fig. 1J). In addition, a remarkable weight loss was observed in RCAN1$^{-/-}$ mice at 4 weeks after LPS injection compared with WT mice (Fig. 1K); meanwhile, heart to body weight ratio induced by LPS was significantly higher in RCAN1$^{-/-}$ mice than that in WT mice (Fig. 1L). Collectively, these data indicated that deletion of RCAN1 aggravated myocardial injury and dysfunction in LPS-induced mouse heart.

**RCAN1 deficiency enhanced LPS-induced myocardial remodeling**
Given that RCAN1 is critically involved in LPS-induced cardiac injury and dysfunction, we next investigated the effect of RCAN1 deficiency on cardiac pathophysiological significance in LPS induced mouse heart. Cardiac hypertrophy and fibrosis as indicated by hematoxylin-eosin (HE) and Masson's trichrome staining were significantly enhanced in WT mice and in RCAN1\(^{-/-}\) mice after LPS injection for 4 weeks when compared with those in control groups. However, LPS-induced septic myocardial hypertrophy and fibrosis were significantly aggravated in RCAN1\(^{-/-}\) mice compared with those in WT mice (Fig. 2A). Of note, LPS-induced a higher proportion of perivascular fibrosis in RCAN1\(^{-/-}\) mice, but no significant difference in the formation of interstitial fibrosis was observed between RCAN1\(^{-/-}\) mice and WT mice (Fig. 2B-E), which indicated a possible link between LPS-mediated vascular inflammation and the consequence of cardiac perivascular fibrosis. In addition to cardiac fibrosis, cardiac hypertrophy measured by the cross sectional area (CSA) of cardiac myocytes was also augmented in RCAN1\(^{-/-}\) mice compared with that in WT mice. As shown by the wheat germ agglutinin (WGA) staining (Fig. 2A, the bottom panel), no significant difference in CSA between RCAN1\(^{-/-}\) mice and WT mice was observed under normal condition, however, but the surface size of single cardiomyocyte was significantly increased in LPS-induced RCAN1\(^{-/-}\) mice compared with that in LPS-induced WT mice (Fig. 2F-G). Moreover, cardiac hypertrophy was verified by the change of hypertrophic genes. LPS-induced expressions of p-ERK1/2 and NFAT3 were significantly upregulated in the LV tissue of RCAN1\(^{-/-}\) mice compared with those of WT mice (Fig. 2H-J), suggesting a protective role of RCAN1 in LPS-induced heart against sepsis-related cardiac hypertrophy.

**RCAN1 deletion exacerbated LPS-induced cardiac mitochondrial damage**

A large body of evidence has shown that mitochondrial dysfunction plays a crucial role in sepsis-related cardiac injury and remodeling\[12, 13\], however role of RCAN1 in LPS-induced mitochondrial dysfunction has not been well defined. Here, we found that mitochondrial oxidative stress indicated by ROS production was significantly higher in RCAN1\(^{-/-}\) mouse hearts than WT mouse hearts after LPS injection for 24 hours (Fig. 3B). By transmission electron microscope (TEM) examination, we observed that mitochondrial fission was increased in LPS-induced septic mouse heart, and LPS-induced fission response was further enhanced in RCAN1 deficient mouse heart (Fig. 3A), and RCAN1 depletion resulted in enhanced mitochondrial fission was confirmed by the mitochondrial count in heart tissue of septic mice (Fig. 3C).

To confirm whether increased fission leads to mitochondrial injury in septic cardiomyocytes, we next measured the impact of RCAN1 deficiency on LPS-induced mitochondrial membrane potential (\(\Delta \Psi_m\)), an important indicator of mitochondrial function that examined by TMRE fluorescent, in isolated adult cardiomyocytes. Results showed that \(\Delta \Psi_m\) was downregulated in septic mouse heart, and was further downregulated in LPS-induced RCAN1-deficient cardiomyocytes when compared with that in WT cardiomyocytes (Fig. 3D). Dynamin-related protein 1 (Drp1) is known as one of the major pro-fission
proteins whose activity is tightly related to its phosphorylation at serine 616, and Drp1\textsuperscript{Ser616} phosphorylation preceded its mitochondrial localization and subsequent mitochondrial fission and damage. Here, we found that the phosphorylation level of p-Drp1\textsuperscript{Ser616} induced by LPS was significantly higher in RCAN1\textsuperscript{−/−} cardiomyocytes than in WT cardiomyocytes (Fig. 3E-F). Therefore, these data suggested that the deletion of RCAN1 aggravated LPS-induced cardiac mitochondrial injury which might be due to oxidative stress-related excessive fission and a rapid loss of mitochondrial membrane potential. **Pharmacologic inhibition of Drp1 attenuated LPS-induced cardiac dysfunction and remodeling in RCAN1\textsuperscript{−/−} mice**

To determine whether enhanced Drp1 activity and increased mitochondrial fission due to RCAN1 deficiency contributed to LPS-mediated cardiac remodeling and dysfunction, RCAN1\textsuperscript{−/−} mice were treated with Mdivi-1, an inhibitor of Drp1, or vehicle during LPS infection. After LPS induction for 4 weeks, cardiac hypertrophy and fibrosis reflected by HE and Masson’s trichrome staining both were significantly attenuated in mdivi-1-treated mice compared with those in vehicle controls (Fig. 4A-B). Echocardiographic analysis showed that cardiac dysfunction 4 weeks post-LPS stimulation was largely improved by Mdivi-1 (Fig. 4C). Compared with those with vehicle treatment, Mdivi-1 treatment increased EF value by 27.3% and FS value by 26.7% in LPS-induced RCAN1\textsuperscript{−/−} mice. In addition, both LVIDs (2.75 mm ± 0.20 mm vs. 3.14 mm ± 0.21 mm, P = 0.0089) and LVIDd (3.82 mm ± 0.27 mm vs. 4.17 mm ± 0.26 mm, P < 0.05) were significantly decreased by Mdivi-1 treatment (Fig. 4D-G). These data suggested that inhibition of mitochondrial fission rescued cardiac function and reversed cardiac remodeling in LPS-induced RCAN1\textsuperscript{−/−} mice.

**RCAN1 suppressed Drp1 phosphorylation and preserved mitochondrial function through regulating CaMKII**

To elucidate the mechanism by which RCAN1 deficiency leads to Drp1 phosphorylation, we focused on Calcium/calmodulin-dependent protein kinase II (CaMKII). Previous studies identified CaMKII as a modulator of mitochondrial Ca\textsuperscript{2+} homeostasis and inhibition of CaMKII suppressed mitochondrial Ca\textsuperscript{2+} uniporter (MCU) and mPTP opening\cite{14, 15}, which partly due to the regulation of Drp1 activity\cite{16, 17}. Therefore, we determined the CaMKII activity in isolated adult mouse cardiomyocytes after stimulating by LPS for 24 hours. As shown in Fig. 5, LPS induced the elevation of CaMKII phosphorylation in cardiomyocytes, which was further elevated in RCAN1 deficient cardiomyocytes (Fig. 5A-B). Of note, deletion of RCAN1 also enhanced the accumulation of mitochondrial p-CaMKII in LPS-treated cardiomyocytes (Fig. 5C-D). To confirm whether mitochondrial translocation of the phosphorylated CaMKII contributed to septic mitochondrial injury and dysfunction, LPS-induced cardiomyocytes were triple stained by anti-Ryr2, anti-p-CaMKII and anti-p-Drp1. Colonization fluorescence density within individual cardiomyocyte showed that protein expression and interaction between p-CaMKII and p-Drp1 were strengthened and T-tube structure was severely impaired in RCAN1 deficient cardiomyocytes (Fig. 5E), suggesting that RCAN1 deficiency promoted LPS-induced interaction between p-Drp1 and
mitochondrial translocated p-CaMKII, and mitochondrial p-CaMKII might determine mitochondrial fitness and mitochondrial dysfunction-mediated cardiomyocyte remodeling and death.

**Inhibition of CaMKII improved LPS-induced cardiac injury and remodeling in RCAN1−/− mice**

To further confirm the role of CaMKII activation in RCAN1 deficiency-mediated mitochondrial dysfunction and cardiac remodeling, the RCAN1−/− mice were pretreated with KN93, the specific CaMKII inhibitor. After LPS injection for 4 weeks, mitochondrial morphology of cardiac tissue was observed by TEM. As shown in Fig. 6A, LPS-induced excessive mitochondrial fission and mitochondrial disarrangement in RCAN1−/− mice compared with PBS-induced RCAN1−/− mice, which were largely improved RCAN1−/− mice by KN93 treatment. Cardiac hypertrophy and fibrosis reflected by HE and Masson staining were both enhanced in LPS-induced RCAN1−/− mice, which were also reversed by inhibiting CaMKII (Fig. 6B-C). In addition, echocardiography showed that KN93 treatment significantly improved cardiac dysfunction of LPS-induced RCAN1−/− mice (Fig. 6D-H). The changes of cardiac hypertrophic genes were determined. As shown by Western blotting, the protein levels of NFAT3 and p-ERK1/2 in heart tissue of RCAN1−/− mice were significantly downregulated by KN93 (Fig. 6I-K). In addition, a relative lower level of serum LDH was observed in RCAN1−/− mice treated with KN93 (Fig. 6L). All these data indicated that inhibition of CaMKII effectively rescued septic cardiac injury, dysfunction and remodeling due to RCAN1 deficiency in cardiomyocytes.

**Discussion**

Septic induced myocardial dysfunction and remodeling is one of the main complications that increase mortality of clinical sepsis[18]. However the potential mechanism has not been well clarified. In the present study, we demonstrate that RCAN1 plays a crucial role in protecting heart against sepsis-induced myocardial remodeling and dysfunction. Cardiac hypertrophy and fibrosis are both enhanced in RCAN1 gene deficient mice after LPS injection, and the decline of LVEF and LVFS indicates serious myocardial dysfunction in RCAN1−/− mice. Our further investigation reveals that mitochondrial injury is critically involved in the development of SIMD. Excessive mitochondrial fission due to the phosphorylation of Drp1 not only promotes mitochondrial dysfunction but also promotes cardiac hypertrophic response via activating CaMKII signaling (Fig. 7).

Mitochondrion is the major place for aerobic respiration and reactive oxygen species (ROS) production. Mitochondrial injury and dysfunction is highly associated with SIMD[4, 19]. Increased ROS generation leads to oxidative stress, impairs mitochondrial biogenesis, the fission/fusion balance and mitophagy, and thereby promoting apoptosis and cell death which has been widely reported in sepsis-related heart, kidney and lung injury[12, 13, 20]. In this study, we found that RCAN1 deficiency resulted in increased mitochondrial ROS production and a rapid loss of mitochondrial membrane potential. Inhibition of excessive fission by blocking the interaction between Drp1 and mitochondrial adaptor fission 1 (Fis1)
rescued mitochondrial function, promoted survival of cardiomyocytes and reduced morbidity and mortality of septic cardiomyopathy[13, 21]. By Western blotting, we found that the phosphorylation level of Drp1, the major pro-fission marker located at the outer mitochondrial membrane, was significantly increased in LPS-induced RCAN1−/− mice. In fact, RCAN1 depletion resulted in mitochondrial fragmentation in a variety of cell types, including mouse embryonic fibroblasts and primary neonatal and adult rodent cardiomyocytes, which was mainly due to the pharmacological inhibition of calcineurin[5, 22], because calcineurin-mediated dephosphorylation of serine 637 in DRP1 triggered its mitochondrial translocation and fission process[23, 24]. Consistent with these studies, here we found that long-term treatment with Mdivi-1, a specific mitochondrial fission inhibitor, effectively reversed sepsis-mediated pathological hypertrophy, fibrosis and cardiac dysfunction in RCAN1−/− mice, further confirmed a protective role of RCAN1 in protecting sepsis-mediated pathologic fission and mitochondrial injury, which might prevent septic cardiomyopathy through regulating Drp1 activity and mitochondrial dynamics.

RCAN1 is an endogenous suppressor of the Ca2+-dependent protein phosphatase calcineurin[10, 22]. We have previously reported that a CnB1 variant that highly associating with calcineurin activity is involved in the development of human dilated cardiomyopathy (DCM)[25]. A novel mutation of g.482G > T in RCAN1 gene resulted in congenital heart defect in children[26]. RCAN1 overexpression inhibited calcineurin-mediated hypertrophy and apoptosis of cardiac myocytes[27], whereas deletion of RCAN1 in the mouse exacerbated myocardial ischemia/reperfusion injury and dysfunction[10, 22]. Previous studies showed that calcineurin-mediated dephosphorylation of Drp1 at serine 637 triggered its mitochondrial translocation[23, 24]. However, the specific role of RCAN1 in mitochondria function remained unclear. Here, we found that RCAN1 deficiency resulted in the activation of CaMKII in cardiomyocytes stimulated by LPS, increased expressions of both protein level and phosphorylation level of CaMKII were observed in cytoplasm and mitochondria fractions, suggesting that RCAN1 depletion promoted CaMKII mitochondria import in septic heart. Previous studies showed that CaMKII determines mitochondrial stress responses in heart[14]. Mitochondrial translocation of CaMKII leads to increased ROS production, enhanced mitochondrial fragmentation and mitochondrial ΔΨm deterioration, thereby promoting cell remodeling and death in septic heart[21, 28–30]. Unlike the effect of Drp1 dephosphorylation at Ser637 triggering its mitochondrial translocation, CaMKII induced Drp1 phosphorylation at Ser616 further activated Drp1-dependent mitochondrial fission, fragmentation and mPTP opening[16, 31]. In our study, inhibition of mitochondrial fission with Mdivi-1 effectively ameliorated cardiac hypertrophy and fibrosis in LPS-induced RCAN1−/− mice. Therefore, RCAN1 might preserve cardiac function and prevent cardiac remodeling through regulating CaMKII-dependent Drp1 phosphorylation and mitochondrial quality control.

Imbalance of fission and fusion disrupted mitochondrial homoeostasis, and excessive fission usually leads to ΔΨm deterioration and mitochondrial mPTP opening, which accelerated septic cardiac remodeling such as hypertrophy and apoptosis[19, 31, 32]. However, overexpression of RCAN1-1L in endothelial cells prevented fission and stabilized the mitochondrial permeability transition pores, which in turn contributed to cell survival under the condition of oxidative stress[27]. Consistent with these data, we
observed that RCAN1 increased mitochondrial fission and decreased ΔΨm in LPS-induced septic mouse heart. Mitochondrial expression of phosphorylated Drp1Ser616 was markedly increased in isolated cardiomyocytes from RCAN1−/− mice. Under high resolution of confocal microscope, we observed that RCAN1 deficiency also enhanced the interaction between CaMKII and Drp1 in LPS-induced cardiomyocytes and increased hypertrophic response and cell death. Excessive ethanol uptake promoted CaMKII-dependent Drp1 activation and Drp1-related mitochondrial fragmentation and dysfunction in neuronal cells[21]. Therefore, RCAN1 deficiency-mediated calcineurin/CaMKII pathway and impaired Ca2+ handling might be the cause of cardiac remodeling at least partly due to CaMKII-dependent Drp1 activation and pathological mitochondrial fission[16, 31]. Knockout CaMKII decreased sarcoplasmic reticulum (SR)mitochondria distance and improved mitochondrial metabolism in early diabetic hearts, which prevented myocardial remodeling and dysfunction[33, 34]. Here we also found that mitochondria fragmentation and disarrangement were widely appeared in LPS-induced RCAN1−/− hearts, and KN93 treatment could adjust mitochondrial morphology and reduced LPS-induced ROS production and pathological fission[35], further confirming a critical role CaMKII mitochondrial translocation in mitochondrial dysfunction and cardiac remodeling in RCAN1 deficient septic hearts.

In summary, our findings demonstrate that RCAN1 deficiency protects septic heart against mitochondrial injury and pathological remodeling through negative regulating CaMKII/Drp1 pathway. In view of the lack of effective therapeutic targets for septic cardiomyopathy, here we provide new insights into the molecular mechanism of RCAN1 in pathogenesis and therapy of sepsis-related cardiac dysfunction and remodeling.

Declarations

Author Contributions All the authors contributed to this manuscript. J.Z. and L.C. performed most of the experiments and wrote the original draft; S.W. measured mouse cardiac function; L.X., G.L. and J.L. analyzed the data; X.T. performed the cell experiments; S.W. and R.Y. designed and supervised the study. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials All the data had been included in the manuscript, and the original data could be obtained from the corresponding author upon reasonable request.

Competing interests The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.
Consent to publish All the co-authors agreed to publish the final version.

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RCAN1 deficiency promoted LPS-mediated cardiac dysfunction. (A-B) The endogenous protein expression of RCAN1 was examined and quantified in vitro cultured cardiomyocytes stimulated by LPS (1.0 μg/ml) for the indicated times (0, 2, 4, 6, 12, 24 hours). (C and I) RCAN1 protein level was also determined in the heart tissue of mice with LPS (10 mg/kg) induction. (D) Representative M-mode images of echocardiography of WT mice and RCAN1−/− mice after LPS (10 mg/kg) induction for 4 weeks. (E-H) Cardiac function parameters, quantitative data (n=5) of left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular internal dimensions at end systole (LVIDs) and left ventricular internal dimensions at end diastole (LVIDd). (J) Level of serum LDH (U/ml) in LPS-induced WT mice and RCAN1−/− mice (n=6). (K) Mean data of body weight (L) and heart to body weight ratio (mg/g) of LPS-induced WT mice and RCAN1−/− mice (n=5). Data were presented as mean ± SEM. * P<0.05 vs. LPS-induced WT mice; # P<0.05 vs. Control.
LPS-induced cardiac hypertrophy and perivascular fibrosis were enhanced in RCAN1⁻/⁻ mice. (A) Representative images of heart sections of mice with LPS or PBS control treatment were stained by hematoxylin-eosin (HE), Masson's trichrome staining or WGA (wheat germ agglutinin) staining (scale bar: 50 μm). (B-C) Quantitative data of the left ventricular (LV) perivascular and (D-E) interstitial fibrosis (n=5). (F-G) Statistical results of the cross sectional area (CSA) of cardiomyocytes stained with WGA (n=20 cells). (H) Protein levels of phosphorylated ERK1/2 and NFAT3 detected by Western blotting in the LV tissue of LPS (or PBS control)-treated WT mice and RCAN1⁻/⁻ mice. (I-J) Quantitative data of the protein expressions of p-ERK1/2 and NFAT3 (n=4). Data were presented as mean ± SEM. *P<0.05 vs. LPS-induced WT mice.
Figure 3

RCAN1 deficiency enhanced LPS-mediated mitochondrial dysfunction. (A) Mitochondrial morphology in LV tissue was detected by transmission electron microscope (TEM), and increased mitochondrial fission was observed in RCAN1−/− mice compared with that in WT mice (scale bar: 2 μm). (B) Levels of mitochondrial ROS in LPS-induced WT mice and RCAN1−/− mice were determined using the 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (n=4). (C) Quantification of mitochondria number per μm² that located in the sarcomere of cardiac muscle (n=5). (D) Mitochondrial membrane potential in isolated cardiomyocytes from WT mice and RCAN1−/− mice induced by LPS respectively was determined by the tetramethylrhodamine ethyl ester (TMRE) fluorescent probe (n=4). (E-F) Protein levels of total Drp1 and phosphorylated Drp1 (p-Drp1ser616) in LPS-treated cardiomyocytes were detected by Western blotting (n=4). Data were presented as mean ± SEM. *P<0.05 vs. LPS-induced WT mice; #P<0.05 vs. Control.

Figure 4

Effect of Mdivi-1, the fission inhibitor, on septic cardiac remodeling and dysfunction. (A-B) Representative heart section images of LPS-induced mice pretreated with or without Mdivi-1 (10 mg/kg) were stained by hematoxylin-eosin (HE), Masson's trichrome staining staining (scale bar: 50 μm). (C) Representative M-mode images of echocardiography from LPS-induced RCAN1−/− mice pretreated with or without Mdivi-1. (D-G) Statistical data of LVEF, LVFS, LVESDs and LVESDd (n=5). Data were presented as mean ± SEM. *P<0.05 vs. LPS-induced RCAN1−/− mice.
Figure 5

RCAN1 suppressed CAMKII phosphorylation and mitochondrial translocation. (A-B) The protein expression of phosphorylated CAMKII in cytoplasm or (C-D) in mitochondrial extract was determined and quantified by Western blotting (n=4). (E) Confocal microscopy showed images of LPS-induced adult cardiac myocytes isolated from WT mice and RCAN1⁻/⁻ mice, cells were stained by anti-Ryr2 antibody (green), anti-p-CAMKII antibody (red) and anti-p-Drp1 antibody (blue). Data were presented as mean ± SEM. *P<0.05 vs. LPS-induced WT mice; #P<0.05 vs. Control.

Figure 6

Effect of KN93, the CAMKII inhibitor, on sepsis-related cardiomyopathy and cardiac dysfunction caused by RCAN1 deficiency. RCAN1⁻/⁻ mice were treated with PBS, LPS (10 mg/kg) or LPS together with KN93 (5 mg/kg) for 2 weeks. (A) Representative images (scale bar: 2 μm) showed mitochondria morphology of cardiac tissues of mice. (B) Representative images (scale bar: 50 μm) of heart sections of mice stained by HE and (C) Masson's trichrome staining. (D) Representative images of M-mode echocardiography from LPS-treated RCAN1⁻/⁻ mice. (E-H) Statistical data of LVEF, LVFS, LVESDs and LVESDd (n=5). (I) Immunoblotting blots of anti-NFAT3, anti-p-ERK1/2 and anti-β-actin bands detected from cardiac LV tissues of mice. (J-K) Relative quantification of the protein levels of NFAT3 and p-ERK1/2 (n=4). (M) Level of LDH (U/ml) determined in serum samples of mice (n=6). Data were presented as mean ± SEM. *P<0.05 vs. PBS-induced RCAN1⁻/⁻ mice; #P<0.05 vs. LPS-induced RCAN1⁻/⁻ mice.

Figure 7
The carton shows a proposed mechanism for the role of RCAN1 in the regulation of sepsis-related mitochondrial dysfunction and cardiac remodeling. Sepsis activates calcineurin/CaMKII signaling pathway, which promotes mitochondrial fission and cardiac hypertrophy through CaMKII phosphorylation and mitochondrial translocation. RCAN1 expression rescues septic cardiac injury by antagonizing the calcineurin/CaMKII pathway and improving mitochondrial function.