Inhibition of mTORC1 renders cardiac protection against lipopolysaccharide

Xiang Li1,2*, Lijing Jiang1*, Shenghui Lin3*, Yunfen He4, Guofeng Shen1, Zhenlin Cai1, Meirong Ling1, Jindi Ni1, Hao Zhang1, Min Zhang2

1Department of ICU, Minhang Hospital, Fudan University, Minhang, Shanghai 201199, People’s Republic of China; 2Department of Pathology, Gansu Provincial Hospital, Lanzhou 730000, Gansu, People’s Republic of China; 3Department of Cardiology, Jinjiang Hospital of Quanzhou Medical College, Jinjiang 362200, Fujian, People’s Republic of China; 4Minhang District Maternal and Child Health Hospital of Shanghai, Minhang, Shanghai 201199, People’s Republic of China. *Equal contributors.

Received September 30, 2014; Accepted November 26, 2014; Epub December 1, 2014; Published December 15, 2014

Abstract: Sepsis-induced cardiac dysfunction is a severe clinical problem. It is evident that rapamycin can protect heart from pathological injuries. However, there are no data demonstrating rapamycin reverse cardiac dysfunction induced by sepsis. In this study, Lipopolysaccharide (LPS) was administrated to mice and H9c2 cells. After treatment, we further determined cardiac function by echocardiography, ANP, BNP and inflammatory markers by qPCR and apoptosis by TUNEL staining. Moreover, mTORC1 signaling pathway and Akt activity were measured by Western blots. We found that rapamycin attenuated cardiac dysfunction, increase in ANP and BNP as well as apoptosis induced by LPS both in mice and in H9c2 cells. Unexpectedly, LPS did not significantly affect the mRNA levels of TNF-α and IL-6. Furthermore, rapamycin further reduced the decrease in mTORC1 signaling and Akt activity induced by LPS. In conclusion, rapamycin can protect heart from LPS induced damages by inhibition mTORC1 signaling and elevation of Akt activity.

Keywords: LPS, cardiac dysfunction, rapamycin, mTORC1

Introduction

Sepsis-induced cardiac dysfunction is one of the severe clinical problems in intense care unit (ICU), which contributes the high mortality rate of patients with sepsis [1, 2]. Although early recognition of myocardial dysfunction is critical under this setting, it is also imperative to looking for more useful drugs to cure this condition [3].

Lipopolysaccharide (LPS) was found to play a key role in sepsis-induced cardiac injuries. Previous studies have showed that inflammation and apoptosis contribute LPS-induced cardiac dysfunction [1, 4]. TNF-α and IL-6, etc. are reported to be elevated early during sepsis, but return to normal levels within several days. However, the precise molecular mechanisms remain elusive. mTORC1 signaling activity can be inhibited with LPS in the skeletal muscle cells [5], but which is activated in LPS-treated liver cells and monocytes conversely [5, 6]. mTORC1 signaling pathway is demonstrated as a central regulator of cellular pathophysiological process such as energy metabolism, protein synthesis and apoptosis, and which is relative to cardiac pathological remodeling induced by myocardial infarction and hypertrophy [7]. Inhibition of mTORC1 activity renders cardiac protection against pathological remodeling. Rapamycin, as an inhibitor of mammalian target of rapamycin complex 1 (mTORC1), was reported to protect heart against pathological damages including myocardial infarction and hypertrophy, reversing cardiac dysfunction via inhibition of mTORC1 signaling pathway and activation of Akt [7-11]. However to the best of our knowledge there are no data describing LPS affects mTORC1 signaling in heart. Furthermore, the effects of rapamycin on LPS-induced cardiac dysfunction are also still unknown which this study was designed to investigate.
In the present study, we found rapamycin may render cardiac protection against LPS-induced heart damages, leading to alleviate of cardiac dysfunction through reduced mTORC1 signaling and elevated Akt activity.

Methods

Animal model

Mice on a C57BL/6 genetic background were housed in groups with 12 h dark/light cycles and with free access to food in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised in 1996) and the regulations on mouse welfare and ethics of Shanghai Fudan University. All procedures were approved by the Ethics Committee of Shanghai Fudan University, Medical Institution Animal Care and Research Advisory Committee (Shanghai, China). LPS and rapamycin were purchased from Sigma Aldrich (St. Louis, Mo, USA). In the separate experiment, LPS (20 mg/kg) or saline was injected intraperitoneally in LPS group and control group, respectively [12]. Rapamycin (2 mg/kg) was administrated intraperitoneally 1 hour after treatment with LPS, which was called LPS + Rap group. All detections were performed 12 hours later [10, 11].

Echocardiography assessment of cardiac function

Mice were anesthetized intraperitoneally with pentobarbital (70-80 mg/kg), monitored by evaluation of toe pinch reflex and breathing rate, and situated supine on a warming pad as described in JOVE [13]. A Vevo 770 (Visual Sonics), equipped with a 30-MHz transducer, was used for noninvasive transthoracic echocardiography. Two-dimensional guided M-mode tracings were recorded. The internal diameter of the LV in the short-axis plane was measured at end diastole and end systole from M-mode recordings just below the tips of the mitral valve leaflets. The interventricular and LV posterior wall thicknesses were measured at end diastole. LVFS (LV fractional shortening percentage) and LVEF (LV ejection fraction), as a index of LV systolic function, were calculated according to the guidelines accompanying the Vevo 770 UBM system. Echocardiography was performed without knowledge of the animal genotypes.

Cell lines and culture

The H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. The H9c2 cell line was obtained from Shanghai Institute of Biochemistry and Cell Biology. Rapamycin was purchased from Cell Signaling Technology, Inc. (Beverly, MA). H9c2 cells were treated with saline in control group and LPS (1 microg/mL) in LPS group for 4 hours, respectively [14]. In LPS + Rap group, rapamycin (10 nmol) was added treated H9c2 cells with LPS for 4 hours [15]. After treatment, H9c2 cells were collected to do further experiments.

Western blotting analysis

Heart lysates of mice and H9c2 cells lysates were prepared in lysis buffer (20 mM Tris, 150 mM NaCl, 10% glycerol, 20 mM glycerophosphate, 1% NP40, 5 mM EDTA, 0.5 mM EGTA, 1 mM Na3VO4, 0.5 mM PMSF, 1 mM benzamidine, 1 mM DTT, 50 mM NaF, 4 μM leupeptin, pH = 8.0). Samples were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.5 mM Tween-20, pH = 7.5) and then incubated with primary antibodies overnight. Antibodies used in this study were purchased from Cell Signaling Technology (CST), Bioworld: S6 (CST #2317), phosphor-S6 (S235/236) (CST #2211), 4E-BP1 (CST #9452), total Akt (CST #9272), phospho-Akt (Ser473) (CST #9271), mTOR (CST #2972), cleaved caspase 3 (CST #2922), GAPDH (#AP0063), Image J software (NIH) was used to perform densitometric analysis (http://rsb.info.nih.gov/ij/).

TUNEL assay

TUNEL assay was performed according to standard protocol. Briefly, the sections and coated H9c2 cells were treated with proteinase K (20 μg/ml) and incubated with terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP. Fluorescence microscopy images were obtained with a Research Fluorescence Microscope (Olympus, Japan) equipped with a digital camera. Images were collected and recorded using Adobe Photoshop® 5.0 on an IBM R52 computer.
Rapamycin and LPS-induced cardiac dysfunction

A

Control

LPS

LPS+Rap

B

C

D

E

LVIDs (mm)

LVIDd (mm)

Ejection fraction (%)

Fraction shortening (%)

ns

ns

ns

ns

n=6

n=6

n=6

n=6

n=6

n=6

n=6

n=6

n=6

Control

LPS

LPS+Rap

Control

LPS

LPS+Rap

Control

LPS

LPS+Rap

Control

LPS

LPS+Rap

***

*

***

*
Quantitative real-time PCR for metabolism relative genes and fetal genes

Total RNA was extracted from LV myocardium and H9c2 cells using TRIZOL reagent (Invitrogen), according to the manufacturer's protocol. One microgram of total RNA from each specimen was reverse transcribed to cDNA using SuperScript Reverse Transcriptase and random hexamers as primers (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed with an ABI Stepone plus instrument (Applied Biosystems) using 1 x TaQ SYBR green Supermix Kit (Bio-Bad, Reinach, Switzerland) and 300 nmol/l for forward and reverse primers in a total volume of 20 μl. The mRNA level was based on the critical threshold (Ct) value. Primer sequences for quantitative real-time PCR referred to literature [12]. Gapdh was used as internal control.

Results

Rapamycin alleviated LPS-induced cardiac dysfunction in mice

In order to explore the effects of rapamycin on LPS-induced cardiac dysfunction in mice, we treated mice with saline, LPS (20 mg/kg) or LPS (20 mg/kg) plus rapamycin (2 mg/kg), respectively. As shown in Figure 1A-E, ejection fraction (EF), fraction of shortness (FS), left
ventricular internal diameter in systole (LVIDs) and left ventricular internal diameter in diastole (LVIDd) were measured by echocardiography at 12 hour after treatment. LPS or and rapamycin...
Rapamycin and LPS-induced cardiac dysfunction

We further observed the effects of rapamycin on atrial natriuretic peptide (ANP), B-type brain natriuretic peptide (BNP) and inflammatory markers as indicated by tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS-treated and LPS plus rapamycin-treated mice, respectively. As Figure 2A and 2B shown, the mRNA levels of ANP and BNP were significantly increased in LPS group compared with control group. Furthermore, rapamycin attenuated LPS-induced increase in ANP and BNP in mice. Unexpectedly, the mRNA levels of TNF-α and IL-6 by qPCR were subtle but not significantly increased in LPS group compared with control group. Also, rapamycin did not affect TNF-α and IL-6 levels.

**Rapamycin attenuated LPS-induced cardiomyocyte apoptosis in mice**

In order to explore the effects of rapamycin and LPS on apoptosis of cardiomyocytes in mice, we further detected apoptotic cardiomyocytes by TUNEL staining and cleaved caspase 3 by Western blot. As shown in Figure 3A and 3B, there were more apoptotic cardiomyocytes in LPS group than that in control group and...
Rapamycin and LPS-induced cardiac dysfunction

Rapamycin reduced LPS-induced cardiomyocyte apoptosis via TUNEL staining analysis. Consistently, cleaved caspase 3, as an apoptosis marker, was significantly increased in LPS group compared with control group and significantly decreased in LPS plus rapamycin group compared with LPS group (Figure 3C and 3D).

Rapamycin attenuated LPS-induced increase of ANP, BNP and inflammatory markers in H9c2 cells

We further did in vitro experiments in H9c2 cell line. H9c2 cells were treated with PBS, LPS or LPS plus rapamycin, respectively, for 4 hours.
Then the relative mRNA levels of ANP, BNP, TNF-α, and IL-6 were measured by qPCR. In consistence with the data in mice, the mRNA levels were significantly increased by LPS treatment and rapamycin partially and significantly reduced LPS-induced increase in ANP and BNP (Figure 4A and 4B). In contrast, LPS treatment significantly increased the mRNA levels of TNF-α and IL-6 and rapamycin significantly reversed LPS-induced increase in TNF-α and IL-6 (Figure 4C and 4D).

Rapamycin attenuated LPS-induced apoptosis of H9c2 cells

Apoptosis detection was also performed in H9c2 cells. As shown in Figure 5A and 5B, LPS treatment significantly increased H9c2 cells apoptosis compared with control group and rapamycin significantly reduced H9c2 cells apoptosis compared with LPS group by TUNEL staining analysis. Consistently, cleaved caspase 3 detected by Western blot was also increased in LPS group compared with control group and reversed by rapamycin treatment in LPS plus rapamycin group compared with LPS group (Figure 5C and 5D).

LPS treatment led to reduced mTORC1 signaling and mTORC1 pathway decreased more after adding rapamycin both in mice and H9c2 cells

In order to explore the molecular mechanisms, we detected the mTORC1 signaling and Akt activity by Western blots. As shown in Figure 6A-I, LPS treatment significantly reduced mTORC1 signaling indicated by phosphorylated ribosome protein 6 (pS6) and elevated phosphorylated 4E-BP1, and Akt activity indicated by phosphorylated Akt (pAkt) both in mice and H9c2 cells. Expectedly, rapamycin treatment after LPS further reduced mTORC1 signaling and enhanced Akt activity significantly, which may prevent cardiomyocytes from LPS-induced injures.

Discussion

Cardiac dysfunction is common in patients with severe sepsis, and that is an important predictor of mortality [16]; however, there are still no effective drugs to cure the dysfunction in this condition [3, 4]. Rapamycin, as an mTORC1 inhibitor, was reported to inhibit cardiac pathological remodeling and then improve cardiac function [7, 11]. Accordingly, we explored the effects of rapamycin on LPS-induced cardiac dysfunction in this study. Intriguingly, we found rapamycin can alleviate LPS-induced cardiomyocyte apoptosis and cardiac dysfunction.

LPS was reported to be a major contributor to cardiac dysfunction in patients with sepsis [4]. Experimental evidence indicates that LPS can induce inflammatory factors such as TNF-α and IL-6, which further cause cardiomyocytes apoptosis and heart failure [1, 17]. In this study, we confirmed that LPS decreases cardiac function and increases TNF-α and IL-6 in H9c2 cells. In addition, ANP and BNP, as markers of heart failure, were also increased in LPS-treated mice and H9c2 cells [2]. In contrast, the mRNA levels of TNF-α and IL-6 slightly but not significantly elevated in LPS-injected mice, which is not consistent with previous reports [12, 14, 18]. This is an unexpected finding, the reason why comparable differences were not present in the mice is not clear, the mRNA of TNF-α and IL-6 that were detected at a relatively later time (12 hours) after LPS treatment in this study than that in others, this may be some of the reasons, and further study is warranted. Moreover, we found LPS also resulted in significant elevation of apoptosis both in mice heart and H9c2 cells, which is in line with previous reports [1, 4, 14]. Hence, we further confirm that LPS leads to cardiomyocyte apoptosis and cardiac dysfunction.

Mounting evidences have showed that mTORC1 signaling pathway regulates important cellular physiological process including energy metabolisms, protein synthesis, apoptosis and autophagy. Also, mTORC1 is closely relative to cardiac remodeling [7]. Genetic and pharmaceutical inhibition of mTORC1 signaling can protect heart against pathological remodeling in adult myocardial infarction animal model and transverse aortic constriction (TAC) animal model as well as ischemia/reperfusion animal model [7-10, 19, 20]. Rapamycin, as an mTORC1 inhibitor, showed promising in anti-pathological remodeling in the settings mentioned above [7-10, 19-21]. In this study, we evidenced that rapamycin can reverse LPS-induced cardiomyocyte apoptosis and cardiac dysfunction both in vivo and in vitro. There are no comparable data in the literature. In skeletal muscle, LPS can reduce mTORC1 signaling pathway; however, in
liver and monocytes, LPS can increase mTORC1 activity [5, 6, 22]. We demonstrated that LPS also can reduce mTORC1 signaling pathway in cardiomyocytes, and rapamycin further reduced mTORC1 signaling and elevated Akt activity in the sepsis model. Therefore, we believe that LPS-induced inhibition of mTORC1 signaling and elevation of Akt activity are ben-
Rapamycin and LPS-induced cardiac dysfunction

Official for cardiomyocyte survival, leading to improvement of cardiac function under sepsis setting.

In conclusion, the present study shows that rapamycin can protect cardiomyocytes against LPS-induced damages, reducing LPS-induced apoptosis, alleviating LPS-induced cardiac dysfunction, which maybe become a benefit therapeutic intervention for patients with sepsis in the future clinical practices.

Acknowledgements

This work was supported by the Natural Science Foundation of Shanghai (13ZR1436500) and Shanghai Municipal Commission of Health and Family Planning (20124063) grants to Xiang Li.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Min Zhang, Department of Pathology, Gansu Provincial Hospital, 204 Donggangxilu, Lanzhou 730000, China. Tel: +86-13893226738; Fax: +86-931-8281640; E-mail: sallyzhangmin126.com

References


Rapamycin and LPS-induced cardiac dysfunction


[22] Frost RA and Lang CH. mTor signaling in skeletal muscle during sepsis and inflammation: where does it all go wrong? Physiology (Bethesda) 2011; 26: 83-96.