Based on AMPK/mTOR/ULK1 pathway to explore the molecular mechanism of autophagy and the intervention effect of esmolol in septic myocardial injury

Maoxia Liu (cherry_liumaoxia@163.com)  
First Affiliated Hospital of Soochow University  
https://orcid.org/0000-0002-3113-0460

Yan Qin  
Soochow University Affiliated No 1 People's Hospital: First Affiliated Hospital of Soochow University

Zheng-da Li  
Soochow University Affiliated No 1 People's Hospital: First Affiliated Hospital of Soochow University

Jun Jin  
Soochow University Affiliated No 1 People's Hospital: First Affiliated Hospital of Soochow University

Yan-bing Zhang  
Soochow University Affiliated No 1 People's Hospital: First Affiliated Hospital of Soochow University

Xin-jing Yang  
Soochow University Affiliated No 1 People's Hospital: First Affiliated Hospital of Soochow University  
https://orcid.org/0000-0003-2990-2828

Research Article

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Abstract

Purpose

Autophagy is a double-edged sword. The purpose of this study was to investigate the signal transduction pathway of esmolol (ES) interfered with lipopolysaccharide (LPS)-induced cardiomyocyte autophagy.

Methods

Sepsis was induced by intraperitoneal injection of LPS (10mg/kg) in male Sprague-Dawley rats (250–300) g, which were treated with ES (15mg/kg-h), 3-methyladenine (3-MA, 15mg/kg) and rapamycin (RAP, 4mg/kg) respectively for twelve hours. The severity of myocardial necrosis was analyzed by hematoxylin-eosin (HE) staining. The expression quantity of autophagy protein in myocardial tissue was detected by Western blotting.

Results

LPS-induced increase in the expression of p- mTOR as well as decrease in the expression of LC3-II, Beclin-1, p-AMPK and p-ULK1 was also inhibited by pretreatment with ES or rapamycin (agonists of autophagy). On the contrary, 3-MA didn’t play a role in enhancing LPS-induced autophagy inhibition.

Conclusion

This study suggests that ES may provide a new strategy for treatment of sepsis cardiomyopathy through activating the AMPK/mTOR/ULK1 signal pathway-regulated autophagy.

Introduction

Autophagy is related to several factors, including nutritional status, energy status, oxidative stress, ischemia and hypoxia. The induction and regulation of Autophagy is such a complex and precise process. The mechanisms of autophagy include mammalian target of rapamycin (mTOR) signaling pathway[1], AMP activated protein kinase (AMPK) pathway and Toll Like receptor, autophagy related gene (ATG), p53 pathway, etc[2]. ULK1 (UNC-51-like kinase 1) protein is a key protein in the initiation of autophagy, and can form ULK1 complex with Atg13, Atg101 and FIP200 to induce the initiation of autophagy[3]. ULK1 complex activates downstream TYPE III PI3K-Beclin-1 complex to initiate autophagy formation, and then TYPE III PI3K-Beclin-1 complex recruits Atg12, Atg5, Atg16, LC3 and other substances to participate in the extension and expansion of autophagy[4]. Eventually, autophagosomes are formed[5].
At the initial stage, Beclin-1 mainly recruits autophagy-related proteins to promote the initiation of autophagy. There are two subtypes of LC3, namely LC3-I and LC3-II[6]. The conversion of LC3-I to LC3-II is generally regarded as a marker of autophagosome formation. LC3-II always binds to the membrane of intracellular autophagosomes, and the content of LC3-II is proportional to the number of autophagosomes[7]. Moreover, vital signal molecules downstream of ULK1 include Beclin1 and LC3, and simultaneous detection of both index can reflect the integrity of autophagy flow. Upriver nutrient receptor mTOR or energy receptor AMPK can selectively regulate ULK1. The cell growth, proliferation and survival are regulated by mTOR, and mTOR negatively regulated most signal transduction pathways of autophagy[8].

Sepsis causes multiple organ failure. Many clinical trials have also shown ES to be effective in patients with sepsis, particularly in reducing heart rate and mortality[9]. Septic shock, a common syndrome[10] in which infection leads to potentially fatal disruption of homeostasis, accounts for 10% of all intensive care unit (ICU) admissions and 30% of all ICU mortality[11]. Despite recent improvements in mortality for sepsis[12], hospital mortality for patients with septic shock remains 22–50%[13].

β-blockers are classic drugs for cardiovascular diseases, and have been gradually applied in the study of sepsis due to their effects on myocardial oxygen metabolism, immune regulation, inflammatory response and metabolism. ES, a selective β1 blocker, was found to improve hemodynamics and reduce mortality in patients with septic shock[14]. β1-receptor blockers can counteract the hypercatabolic state of sepsis patients, weaken metabolic disorders, relieve excessive inflammatory response, and regulate immune function[15]. They are also used to treat septic-related damage to the heart, lungs, kidneys and other organs. Studies have shown that low-dose β1-blockers can reduce inflammatory response and improve cardiac function and vascular reactivity in septic rats[16]. β1-blocker ES can inhibit apoptosis by down-regulating autophagy and mitochondrial autophagy in cardiomyocytes after resuscitation from cardiac arrest in rats[17].

Based on these reports, our hypothesis is that ES induces enhanced autophagy by activating AMPK pathway and inhibiting mTOR pathway to treat myocardial injury caused by autophagy inhibition due to LPS-induced sepsis in Sprague-Dawley (SD) rats.

**Methods**

**Experimental animals**

One hundred and sixty male rats were provided by Zhejiang Vital River Laboratory Animal Technology Co., Ltd. (Zhejiang, China), weighing 250–300 g. Presently, one hundred and sixty rats were anesthetized by intraperitoneal injection of 4% chloral hydrate (10ml/kg), and randomly divided into five groups (each group containing 32 rats) as follows: Sham (Sham operation); LPS (LPS intraperitoneal injection ); LPS + ES (LPS intraperitoneal injection + ES treated); LPS + 3-MA (LPS intraperitoneal injection + 3-methyladenine (3-MA) treated), and LPS + RAP (LPS intraperitoneal injection + rapamycin(RAP) treated).
Subsequently, each group was randomly divided into four subgroups (n = 8), which included 3hS, 6hS, 12hS, and 24hS (3, 6, 12, and 24h subgroups, respectively), according to the ES, 3-MA or RAP infusion time after the operation.

This study was approved by the experimental animal ethics committee of the First Affiliated Hospital of Soochow University. LPS (Sigma-Aldrich Co., Ltd. Shandong Province, China), 3-MA (MedChemExpress Pharmaceutical Co., Ltd. Shanghai, China), RAP (MedChemExpress Pharmaceutical Co., Ltd. Shanghai, China), and normal saline (NS) (Kelum Pharmaceutical Co., Ltd. Sichuan Province, China) were generously donated by the pharmacy at the First Affiliated Hospital of Soochow University. The approval number of animal experiment of this study was SCXK (Zhe) 2019-0001.

**Animal model establishment**

After inducing general anesthesia under 4% chloral hydrate, the sepsis model of the rats was established via the intraperitoneal injection method, in which LPS (10mg/kg) was injected in each group except Sham group. In the LPS + 3-MA/RAP groups, we just administrated the 3-MA (15mg/kg) or RAP (4mg/kg) by IP. In ES group, ES dilution (15mg·kg⁻¹·h⁻¹) was continuously pumped through left internal jugular vein for 24 h. We settled the infusion tube in the internal jugular vein by catheterization in the ES groups. Subsequently, the ES solution (ES injection was diluted to 10mg/mL with NS) was continuously injected into the internal jugular vein of the rats at a flow velocity of 0.5 mL/h in different subgroups of the LPS + ES group, lasting for 3h, 6h, 12h and 24h respectively. An infusion pump (Beijing Slgo Medical instrument Co., Ltd., China) was used for continuous pumping in this experiment, in order to maintain the liquid input at an even speed. After the liquid was infused, the neck incision of the rats was sutured, and the rats were feed separately. All rats were treated according to the time points of subgroups, and then were sacrificed at the end of treatment, and heart specimens were taken immediately.

**Measurement of ratio of autophagy proteins’ expression in myocardial tissues**

The myocardiac tissues, which were obtained from each subgroup, were ground into tissue homogenates and lysed for 30 min on ice in the radio immunoprecipitation assay (RIPA) buffer. After that, the supernatants were used for the western blot analysis of p-AMPK, p-ULK1, p-mTOR, Berlin-1 and LC3-II proteins. Equal amounts of protein from each sample were resolved by 10% SDS-PAGE and the proteins were transferred onto PVDF membranes (Hybond TM-ECL; Amersham Pharmacia Biotech). The membranes were blocked for 2 h at room temperature with 5% skimmed milk in PBS and 0.1% Tween20. The blots were incubated overnight with a 1:1,000 dilution of the following primary antibodies: anti-LC3-II (Abcam Corporation, abc62721), anti-Beclin-1 (Epitomics, Inc. 2026-1), anti-p-AMPK (Cell Signaling tech, [Thr172][40H9]), anti-p-ULK1 (Cell signaling tech, [Ser757][D7O6U]), anti-p-mTOR(Cell signaling tech, [Ser2448][D9C2]), and β-actin (SantaCruz Biotech, AP0063) followed by incubation for 2 h with a secondary antibody (HRP-conjugated anti-rabbit IgG; 1:2,000, Abgent, LP1001a). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and quantified.
by NIH image software. Data were normalized to β-actin. All of the data were presented as fold change of the control group.

**HE staining**

To observe the morphological and inflammatory changes under the binocular optical microscope, the fresh cardiac tissues of the rats were washed with NS 3 times, fixed with 10% neutral formaldehyde buffer, dehydrated, treated with paraffin, sliced with microtome, roasted, dewaxed, and stained with HE.

**Statistical analysis**

All data were presented as the mean ± SEM. Statistical significance between two or more groups was tested using two-way ANOVA followed by the Newman-Keel test or an unpaired two-tail Student's t test. P values of < 0.05 were considered statistically significant.

**Results**

**Effects of LPS on the expression of LC3-II, Beclin-1**

The increase or decrease of LPS is time dependent. Sepsis rats were inferenced with LPS (10mg/kg) for 3, 6, 12 and 24 h. LPS dependently increased the LC3 II and beclin-1 expression at 3h. However, from 6 to 24 h, expression of LC3-II and beclin-1 significantly decreased. Statistical analysis showed that LPS stimulation reduced expression of Beclin-1 (Fig. 1a) and LC3-II (Fig. 1b) in a 6, 12, 24h, with the optimal dose of 10mg/kg, while the opposite results were observed at 3h . These data suggest that LPS-induced autophagy confirms the effect of LPS on myocardial autophagy.

**Effects of ES on LPS-induced decrease of LC3-II, Beclin-1, p-AMPK and p-ULK1 expression, and increase of p-mTOR expression**

When sepsis rats were intraperitoneally injected with LPS (10mg/L) for 12h, the AMPK/mTOR/ULK1 autophagy pathway was significantly suppressed. Moreover, 3-MA(15mg/kg) markedly enhanced LPS-induced inhibition of autophagy. Contrarily, ES (15mg/kg•h), and RAP (4mg/kg) also markedly reversed LPS-induced inhibition of autophagy.

Thirty minutes before LPS (10mg/kg) stimulation, LPS + ES subgroup was intravenous pumping of β1-blocker, ES (15mg/kg•h). The results showed that ES completely suppressed the LPS-induced decrease of LC3-II expression (Fig. 2c), Beclin-1(Fig. 2b), p-AMPK (Fig. 2a) and p-ULK1 (Fig. 2d) expression, and increase of p-mTOR(Fig. 2e) expression. These results revealed that ES promotes autophagy.

**Effects of RAP on LPS-induced decrease of LC3-II, Beclin-1, and p-ULK1 expression, and increase of p-mTOR expression.**

Similar results were also obtained in the RAP pretreatment experiments. A well-known autophagy inducer, rapamycin, markedly increased expression of LC3-II (Fig. 2c), Beclin-1 (Fig. 2b), and p-ULK1 (Fig. 2d)
expression, and decrease of p-mTOR (Fig. 2e) expression. RAP alone did not significantly affect the expression of p-AMPK (Fig. 2a).

No effects of 3-MA on LPS-induced autophagy inhibition

Besides, an autophagy inhibitor, 3-MA was intraperitoneally injected to the sepsis rats. The results showed that 3-MA had no effect on the LPS-induced autophagy process. Obviously, there was no significant difference between LPS subgroup and LPS + 3-MA subgroup.

Microscopic observation of cardiomyocytes of sepsis rats

LPS induced a series of reactions in rats, including inflammation, apoptosis, and necrosis. As was shown in the picture, myocardial fibers were obviously disordered, and myocardial cells were distinctly dissolved and necrotic, with visible vacuolation and degeneration. Myocardial interstitium was dilated and hyperemic with scattered inflammatory cells (Fig. 3a-e HE staining 10 ×).

In LPS + ES and LPS + RAP subgroups, myocardial fiber disorder, myocardial cell lysis, necrosis, myocardial interstitial dilatation and congestion were significantly reduced. However, there was no significant difference in LPS + 3-MA subgroup compared to the LPS subgroup. These microscopic changes were slightly presented (Fig. 4a-e HE staining 40 ×).

Discussion

Sepsis is still a common critical disease with high morbidity and mortality in intensive care unit[18]. Recently, a meta-analysis showed that reversible myocardial depression occurs early in severe sepsis and septic shock[19]. In other words, sepsis myocardial injury would be reversed with early intervention. The clinical evidence for ES for septic cardiomyopathy is still insufficient, and it is not a first-line drug[20]. The use of ES in the treatment of patients with sepsis has been debated for a long time. Presently, several studies have shown that heart rate control with selective beta-1 blockers in septic shock is safe[21]. Moreover, a meta-analysis suggested that ES may improve survival in patients with tachycardia sepsis[22]. Many clinical trials have also shown that ES can protect heart function by lowering heart rate at the early time and reducing the work of the heart muscle[23]. Our experiment broadly confirmed the protective effect of ES on myocardial injury.

The specific mechanism of ES on myocardial injury in sepsis remains unclear. A certain animal study confirmed that ES can suppress inflammation and apoptosis in the intestinal tissue via the overexpression of NF-kappa B-p65 in the early stage[24]. Early-stage use of ES might be an ideal treatment method for sepsis. The effect of esmolol on autophagy has been reported in the post-resuscitation model of cardiac arrest, but not in this model of septic myocardial injury. Our experiment is necessary for elucidating the significance of ES in the treatment of septic cardiomyopathy.

Autophagy pathways are complex and varied, and are closely related to apoptosis and inflammation. The molecules involved are very diverse and the regulatory mechanisms among them are complex. The
autophagic proteins preserve mitochondrial integrity\[25\]. AMPK plays a major regulatory role in cellular energy homeostasis by directly phosphorylating metabolic enzymes and nutrient transporters and indirectly promoting the transactivation of nuclear genes involved in mitochondrial biogenesis and function\[26\]. AMPK-ULK1 signaling is important in skeletal muscle, and ULK1 activation is dependent on AMPK\[27\]. Beclin-1 may promote mitochondrial biogenesis through up-regulation of AMPK/ULK1, in addition to removing damaged mitochondria through mitochondrial autophagy\[28\]. The amount of LC3-II is correlated with the extent of autophagosome formation\[29\]. The results showed that the autophagy pathway was significantly inhibited at 6, 12, 24h under LPS induction, leading to increased myocardial injury in rats. In the group pretreated with ES, myocardial injury improved significantly. According to the expression of autophagic protein, ES may protect myocardium by promoting the autophagy of AMPK-ULK1 single signaling pathway.

In the experiment, we added rapamycin and 3-MA treatment groups to confirm the mechanism of ES on autophagy. Rapamycin is well known as an undisputed autophagy activator\[30–32\]. Rapamycin has been shown to be cardioprotective in pressure-overloaded and ischemic heart diseases by regulating the mechanistic target of rapamycin (mTOR) signaling network\[33, 34\]. Our results showed that although rapamycin is cardioprotective in septic myocardial damage, its myocardial protection is not as good as ES. Furthermore, 3-MA, an inhibitor of PI3K, plays a vital role in forming and developing autophagosomes\[7\]. We also found that 3-MA showed no significant difference in LPS-induced autophagy protein expression. It is still unclear which part of the autophagy pathway ES participates in and which targets it applies.

Therefore, whether this pathway exists in the myocardium becomes the entry point of our experiment. By measuring the protein expression of p-AMPK, p-mTOR and p-ULK1, we found that ES can activate the expression of p-AMPK, which leads to the opening of energy regulation pathway, thus achieving the effect of autophagy promotion, and playing a positive protective role in the myocardium of septic rats.

During the experiment, we wrapped the front end of the catheter with aluminum skin to effectively prevent rats from biting the catheter during intravenous pumping. The single-cage pumping mode was used in each rat. However, due to the stenosis of blood vessels and catheters in rats as well as the complexity of time points, we failed to find the blocking of pumping drugs in some rats in time, which ultimately led to treatment failure. Moreover, rats may be affected by the experimental environment and batch, and many confounding factors are inevitable.

In conclusion, our results are still problematic and need to be verified by more experiments. It has to be said that ES may protect myocardial injury by promoting autophagy, which provides a new idea for the subsequent treatment of myocardial injury in sepsis.

**Conclusions**

In summary, we have shown that ES can help restore myocardial function in septic rats through activating the AMPK/mTOR/ULK1 signal pathway-regulated autophagy. These findings provide a novel
target for the subsequent sepsis cardiomyopathy therapies in humans in clinical.

**Declarations**

**Author contribution** X.-J.Yang and Y.-B.Zhang conceptualized and designed this study. Material (instruments and solutions) preparation, specimen collection, and data analysis were performed by all authors. Animal experiments were performed M.-X.Liu and Y.-B.Zhang. Blinded analysis of data was performed by M.-X.Liu, Y.Qin, Z.-D,Li, and J.Jun. The first draft of the manuscript was prepared by M.-X.Liu and X.-J.Yang. X.-J.Yang prepared the resubmitted manuscripts. All authors gave final approval of this version to be published and all agreed to be responsible for all aspects of the work.

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**Data availability** Request for data used in this manuscript should be addressed to the corresponding author.

**Code availability** Not applicable.

**Ethical approval and consent to participate** All animal procedures were approved by the Medical Ethics Committee of The First Affiliated Hospital of Soochow University with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

**References**


Figures

a. Beclin-1
   β-actin
   Time (hrs) sham 3 6 12 24

b. LC3-II
   β-actin
   Time (hrs) sham 3 6 12 24

*P<0.05 vs. sham group, n=6

Figure 1

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Figure 2

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**Figure 3**

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Figure 4

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Scientific Hypothesis:

Figure 5

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