Original Article

Accelerated Autophagy of Cecal Ligation and Puncture-Induced Myocardial Dysfunction and Its Correlation with Mammalian Target of Rapamycin Pathway in Rats

Hao Wang, Na Cui, Wen Han, Long-Xiang Su, Yun Long, Da-Wei Liu

Department of Critical Care Medicine, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Science, Beijing 100730, China

Abstract

Background: Recent studies have indicated that autophagy is involved in sepsis-induced myocardial dysfunction. This study aimed to investigate the change of autophagy in cecal ligation and puncture (CLP)-induced myocardium dysfunction and its relationship with mammalian target of rapamycin (mTOR) pathway.

Methods: Totally, 12 rats were randomly divided into CLP group or sham-operated (SHAM) group. Cardiac tissues were harvested 18 h after CLP or sham operation. Pathology was detected by hematoxylin and eosin staining, cardiac functions by echocardiography, distribution of microtubule-associated protein light chain 3 type II (LC3II) by immunohistochemical staining, and autophagic vacuoles by transmission electron microscopy. Moreover, phosphorylation of mTOR (*p*-mTOR), phosphorylation of S6 kinase-1 (PS6K1), and LC3II and p62 expression were measured by western blotting. Pearson's correlation coefficient was used to analyze the correlation of two parameters.

Results: The results by pathology and echocardiography revealed that there was obvious myocardial injury in CLP rats (left ventricle ejection fraction: SHAM 0.76 ± 0.06 vs. CLP 0.59 ± 0.11, $P < 0.01$; fractional shortening: SHAM 0.51 ± 0.09 vs. CLP 0.37 ± 0.06, $P < 0.05$). We also found that the autophagy process was elevated by CLP, the ratio of LC3II/LC3I was increased ($P < 0.05$) while the expression of p62 was decreased ($P < 0.05$) in the CLP rats, and there were also more autophagosomes and autolysosomes in the CLP rats. Furthermore, the mTOR pathway in CLP myocardium was inhibited when compared with the sham-operated rats; *p*-mTOR ($P < 0.01$) and PS6K1 ($P < 0.05$) were both significantly suppressed following CLP challenge. Interestingly, we found that the mTOR pathway was closely correlated with the autophagy processes. In our study, while *p*-mTOR in the myocardium was significantly correlated with p62 ($r = 0.66$, $P = 0.02$), PS6K1 was significantly positively correlated with p62 ($r = 0.70$, $P = 0.01$) and negatively correlated with LC3II ($r = -0.71$, $P = 0.01$).

Conclusions: The autophagy process in the myocardium was accelerated in CLP rats, which was closely correlated with the inhibition of the mTOR pathway.

Key words: Autophagy; Cecal Ligation and Puncture; Mammalian Target of Rapamycin; Myocardial Dysfunction; Sepsis

Introduction

Septic is a devastating clinical condition that is a leading cause of mortality in Intensive Care Units.[1,2] Previous studies have indicated that sepsis leads to compromised cardiac structure and impaired cardiac dysfunction, which contributes to hypovolemic or cardiogenic shock.[3-5] The mortality rate of people suffered from severe heart failure increased by 50%.[6]

Autophagy is a dynamic process in which damaged organelles and long-lived proteins are delivered to the lysosome for degradation and recycling.[7] During this dynamic process, a double-membrane vesicle called autophagosome fuses with lysosomal to form an autolysosome. Light chain 3 type II (LC3II) and p62 are two major proteins in autophagy. LC3II functions at an early stage of phagophore expansion,

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Address for correspondence: Dr. Da-Wei Liu, Department of Critical Care Medicine, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China
E-Mail: dwliu98@163.com

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while p62 has an adaptor function to recognize ubiquitinated proteins that need to be removed from the cytoplasm during autophagy, and its amount is generally considered to inversely correlate with autophagic activity.[8-10] As a housekeeping process, autophagy is vital for the normal structure and functions of the heart.[11] It also plays an important role ischemia-reperfusion (I/R) injury.[12] Evidence suggests that autophagy protects against I/R injury. Recent studies have found that autophagy also plays an important role in septic myocardial depression. There is evidence showing that autophagic activity in cardiomyocytes changes during sepsis, but the results are still unclear.[13,14]

Mammalian target of rapamycin (mTOR) is a master sensor of energy status and will be inhibited in the state of energy depletion.[15] Myocardial energy depletion plays a major role in myocardial dysfunction during sepsis.[16,17] There are little data describing the role of the mTOR pathway during sepsis in the heart.[18] Furthermore, among various pathways known to regulate autophagy in mammalian cells, mTOR complex 1 (mTORC1)/S6 kinase-1 (S6K1) signaling pathway is one of the best-understood pathways.[19] Accumulating evidence has indicated that the mTORC1/S6K1 pathway negatively regulates autophagy, so the relationship between the mTOR pathway and the change of autophagy process in cecal ligation and puncture (CLP)-induced myocardial dysfunction was investigated in this study.

Methods

Animal model preparation

All procedures used in this study were in accordance with our institutional guidelines that complied with the international ethics and humane standards for animal use. Every effort was made to minimize the number of animals and reduce their suffering.

Healthy Wistar rats, male, aged 7–8 weeks, and weight of 220 ± 20 g were obtained from the Animal Facility Center, Peking Union Medical College Hospital. All animals were housed in a pathogen-free facility and used according to the protocols approved by the IACUC of Peking Union Medical College Hospital. Totally, 12 rats were randomly divided into sham-operated (SHAM) group or CLP group. CLP was performed as previously described to establish a mid-grade sepsis model.[20] In brief, the cecum was ligated at half the distance between the distal pole and the base of the cecum. Two cecal punctures were made with a 22G needle, and a droplet of feces was forced out from both the mesenteric and antimesenteric penetration holes. Sham-operated rats were subjected to the same laparotomy without CLP. After surgery, all animals immediately received a subcutaneous injection of sterile saline (0.9% NaCl, 5 ml per 100 g body weight) for resuscitation.

Hematoxylin and eosin staining

The left ventricle (LV) hearts of rats were transversely cut at a 2 mm-thickness, immediately fixed in 4% paraformaldehyde, and embedded in paraffin. Sections of 3-μm thickness were affixed to slides, deparaffinized, and stained with hematoxylin and eosin (H and E) to evaluate the morphological changes of the heart.

Transmission electron microscopy analysis

Transmission electron microscopy was performed as described previously.[21] The samples after pretreatment were cut into thin sections (90 nm) that were viewed at 120 kV with a H7650 transmission electron microscope (HITACHI, Tokyo, Japan). Micrographs were obtained using a Philips CM12 (10–15 per sample) by random sampling.

Western blotting analysis

LV tissues were homogenized in lysis buffer. Tissue lysates were centrifuged at 17,000 ×*g* for 10 min. An aliquot of the supernatant was used to determine the protein concentration. Protein samples were mixed with ×4 lithium dodecyl sulfate sample buffer, electrophoresed on SDS-polyacrylamide gels, and then transferred electrophoretically onto nitrocellulose. The membranes were immunoblotted with antibodies against LC3 (ab48394, Abcam, 1:1000 dilution), p62 (#9234, Cell Signaling Technology, 1:1000 dilution), phosphorylation of mTOR (*p*-mTOR) (Ser2448) (#5536, Cell Signaling Technology, 1:8500 dilution), phosphorylation of S6K1 (PS6K1) (Thr389) (#9234, Cell Signaling Technology, 1:500 dilution), and actin. A horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was used. After the final wash, membranes were developed using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) and autoradiographed. Actin was used as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of the rat hearts were subjected to immunohistochemical analysis as described previously.[22] The heart sections were immune with anti-LC3 antibody (ab48394, Abcam, 1:1000 dilution). All slides were viewed using a DP70 Olympus digital microscope camera at ×200 and ×400. Images were captured with Olympus DP-Soft823 version 3.2 acquisition software (Olympus Corporation, Tokyo, Japan).

Echocardiography examination

An Ultrasonic Machine (M-Turbo SonoSite, USA) equipped with a 15-MHz transducer was used for noninvasive transthoracic echocardiography. Rats were anaesthetized intraperitoneally with pentobarbital (70–80 mg/kg) at 18 h after CLP and situated in the supine position on a warming pad. LV end diastolic and systolic dimensions were measured. The LV ejection fraction (LVEF) and fractional shortening (FS) were also calculated from M-mode echocardiograms. Data from three consecutive selected cardiac cycles were analyzed and averaged.

Statistical analysis

Data were analyzed by SPSS 18.0 software (SPSS Inc., IBM Corp., Armonk, NY, USA). All data for continuous variables in this study had normal distributions and were shown as mean ± standard deviation. Differences were assessed using

analysis of independent-samples *t*-test. Pearson's correlation coefficient was used to analyze the correlation of two parameters. A $P < 0.05$ was considered statistically significant.

Results

Cardiomyopathy and cardiac dysfunction in cecal ligation and puncture rats

H and E staining revealed that there were pathological changes in CLP rats. The myocardium of SHAM rats had a normal architecture and clear myocyte boundaries, whereas CLP rats after 18 h of sepsis showed marked myocardial injury with myocardial necrosis and interstitial edema adjacent to localized extravasation of red blood cells. These results indicated cardiomyopathy of the sepsis model established by CLP [Figure 1a]. As shown in Figure 1b, LVEF and FS were measured at 18 h after treatment. Compared with the sham-operated rats, the LVEF and FS were both significantly reduced in the CLP group (LVEF: SHAM 0.76 ± 0.06 vs. CLP 0.59 ± 0.11, $P < 0.01$; FS: SHAM 0.51 ± 0.09 vs. CLP 0.37 ± 0.06, $P < 0.05$).

Increased expression of light chain 3 type II and decreased expression of p62 in cecal ligation and puncture rats

Autophagy is under the control of the gene of LC3. LC3II is a main mediator in autophagy and functions at an early stage of phagophore expansion. We found that LC3II/LC3I in the LV was significantly increased at 18 h after CLP compared with sham-operated rats [$P < 0.05$, Figure 2a]. We also evaluated LC3II in the LV by immunohistochemical staining. In immunohistochemical staining experiments, autophagosome was evaluated by examining the punctate form (type II) of LC3. As shown in Figure 2b, LV sections from CLP rats showed an increase in LC3II expression compared with sham-operated rats; we even observed LC3B globular structures in some sections from CLP rats. Since p62 has an adaptor function to recognize the ubiquitinylated proteins and to be removed from the cytoplasm during autophagy, its amount is generally considered to inversely correlate with autophagic activity. As shown in Figure 2c, the expression of p62 in the heart from CLP rats was lower than that from sham-operated rats ($P < 0.05$).

More autophagosomes and autolysosomes in cecal ligation and puncture rats

As shown in Figure 3, LV tissues from the sham-operated rats showed normal structure with proper mitochondria distribution, while CLP rats revealed cellular disorganization and myofibrillar disarray. Autophagic vacuoles were also observed through transmission electron microscopy; we found that there were more larger autophagosomes and more autolysosomes compared with sham-operated rats.

Inhibited mammalian target of rapamycin pathway in myocardium of cecal ligation and puncture rats

To investigate the role of mTOR pathway in sepsis-induced myocardial dysfunction, we detected

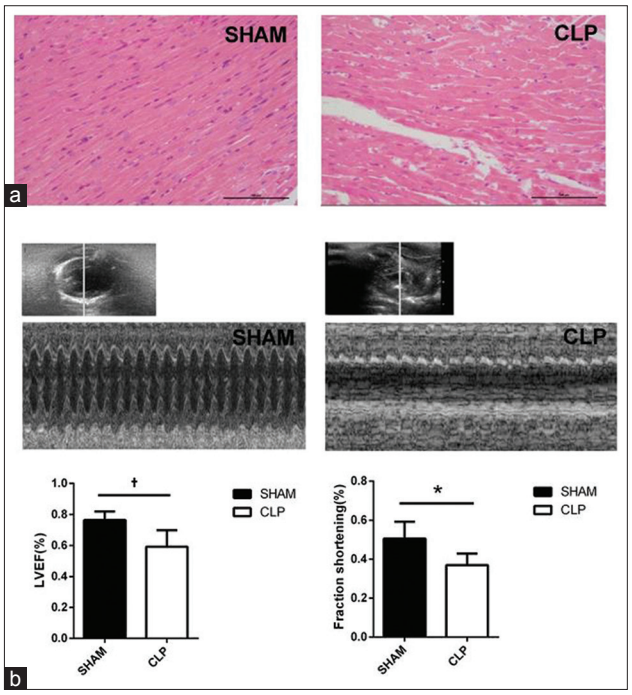

Figure 1: Cardiomyopathy and cardiac dysfunction in CLP rats. Representative hematoxylin and eosin staining of left ventricle sections (a). Cardiac functions examined by echocardiography, representative echocardiographic recordings from the two groups, LVEF and FS were measured (b). Original magnification, ×200; mean ± standard deviation, six rats per group, *$P < 0.05$; †$P < 0.01$. SHAM: Sham operated; CLP: Cecal ligation and puncture; LVEF: Left ventricle ejection fraction; FS: Fraction shortening.

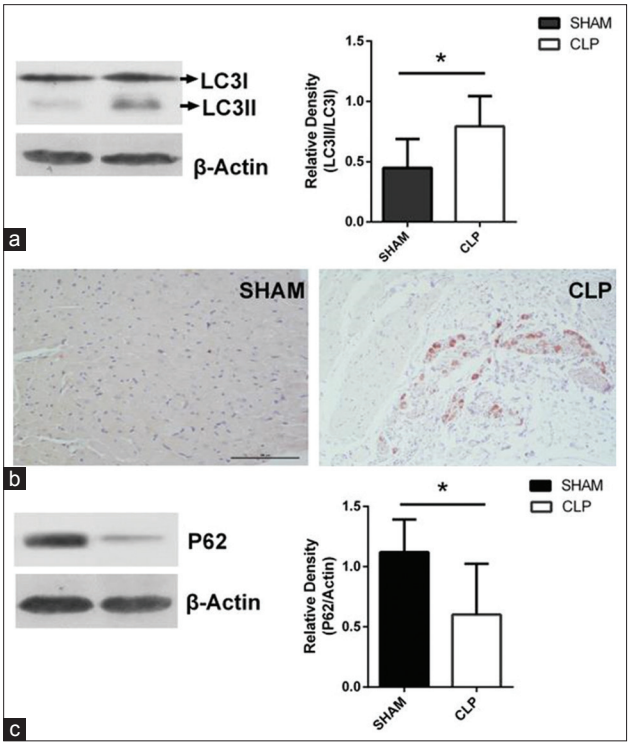

Figure 2: The expression of LC3II and p62 in left ventricle. The protein levels of LC3II and p62 were quantified by Western blotting in the methods (a and c). Left ventricle sections stained with an anti-LC3 antibody (b). Original magnification: ×200. Bar, 100 μm. Mean ± standard deviation, six rats per group, *$P < 0.05$.

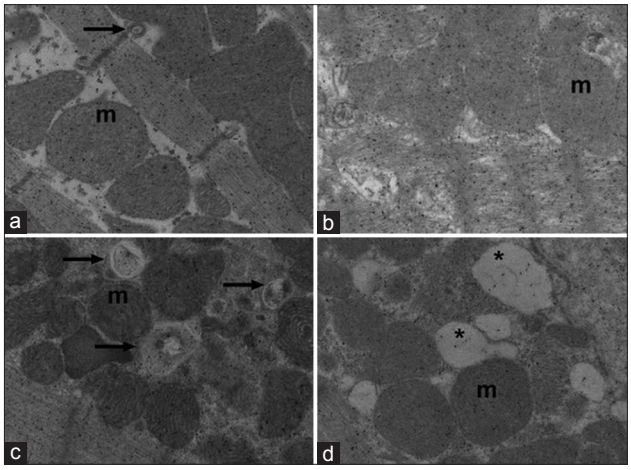

Figure 3: Ultrastructural features of autophagic vacuoles in the left ventricle harvested 18 h after CLP. Myocardium was normal in appearance with proper mitochondria distribution in sham-operated rats (a); myofibrillar disarray can be seen in CLP rats (b); there were more larger autophagosomes and more autolysosomes in CLP rats (c and d). The autophagosome appears as a double-membrane vesicle (arrows in [a and c]), the autolysosome is a single-membrane structure containing cytoplasmic materials at various stages of degradation (asterisks in [d]). Mitochondria (m) can be seen throughout the cytoplasm (×10,000). CLP: Cecal ligation and puncture.

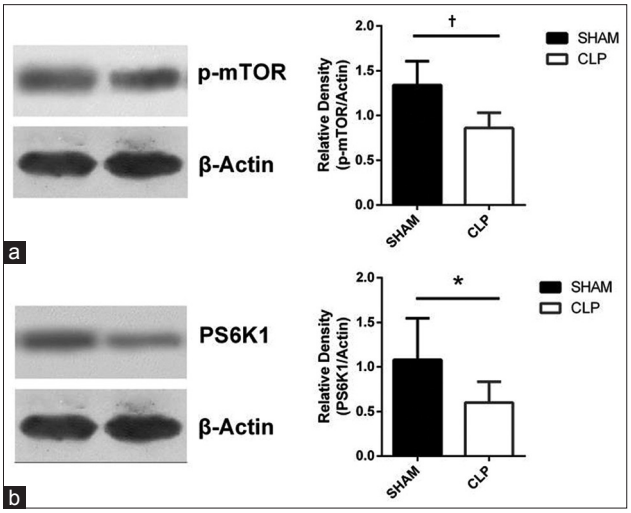

Figure 4: The expression of *p*-mTOR (a) and PS6K1 (b) in left ventricle, left ventricle was harvested 18 h after CLP, and the protein levels of *p*-mTOR and PS6K1 were quantified by Western blotting in the methods. Mean ± standard deviation, six rats per group, $^{*}P < 0.05$; $^{\dagger}P < 0.01$. *p*-mTOR: Phosphorylation of mammalian target of rapamycin; CLP: Cecal ligation and puncture; PS6K1: Phosphorylation of S6 kinase-1.

the level of *p*-mTOR (phosphorylation at Ser2448) and PS6K1 (phosphorylation at Thr389), and we found that the level of *p*-mTOR and PS6K1 was both decreased in the myocardium of CLP rats ($P < 0.01$; $P < 0.05$, Figure 4].

Correlation between mammalian target of rapamycin or S6 kinase-1 and light chain 3 type II or p62

Pearson's correlation analysis indicated that *p*-mTOR in the myocardium was significantly positively correlated with p62 ($r = 0.66$, $P = 0.02$) but not with LC3II [Figure 5a and 5b]. Similarly, PS6K1 was positively correlated with p62 ($r = 0.70$, $P = 0.01$) and negatively correlated with LC3B [$r = -0.71$, $P = 0.01$, Figure 5c and 5d].

Discussion

The autophagy process has been indicated correlated with sepsis-induced myocardial dysfunction; however, the role of autophagy in the heart has been controversial, and its relationship with mTOR pathway is not well known. In this study, we found that compared with sham-operated rats, the autophagy process in myocardium was accelerated in CLP rats, and mTOR pathway was inhibited and closely correlated with the change of autophagic protein, indicating that the change of autophagy process in CLP rats may be mediated by mTOR pathway.

Autophagy is a dynamic process in which damaged organelles and long-lived proteins are delivered to the lysosome for degradation and recycling.[7] During this dynamic process, the outer membrane of the autophagosomes (a double-membrane vesicle) fuses with the lysosomal membrane forming an autolysosome. Within the lysosome, the inner autophagosomal membrane and autophagosomal compartment are degraded and the resulting molecules are recycled. As a housekeeping process, autophagy is vital for the normal structure and functions of the heart.[11] In addition, autophagy plays a critical role in the maintenance of cardiac functions by removing damaged proteins and subcellular organelles under stress conditions.[23] It also plays an important role in the modulation of I/R injury.[12] However, there was little evidence showing the change of entire process of autophagy in cardiomyocytes during sepsis. In our study, we found that 18 h after CLP, cardiomyocytes show a considerably higher LC3II/LC3I ratio (a marker of autophagy induction), accompanied by an increased number of autophagosomes. The autolysosomal (the product of an autophagosome fusing with a lysosome) numbers were also increased accompanied by a decrease in the expression of SQSTM1/p62 receptor proteins. These results indicate that the autophagy process was complete and accelerated in CLP rats. However, in a study by Hsieh *et al*.,[13] they found that the process of autophagy in myocardial cells was not complete at 24 h after CLP. They found that localization of LC3 and LAMP1 (a lysosome marker) was decreased 24 h after CLP. They also observed that there were few autolysosomes in CLP mice. The reason why we got different results may be that we obtained myocardial tissues at different stages of sepsis. A recent study demonstrated that autophagy was activated initially in sepsis, followed by a subsequent phase of incompletion in the liver,[24] which may be similar in the heart. Even though the timing chosen was almost same, we ligated the cecum at half the distance between the distal pole and base of the cecum to establish the mid-grade sepsis model, whereas they ligated the cecum just below the ileocecal junction for high-grade sepsis. As a result, the results of our study may indicate the process of autophagy process in the heart during early stage of sepsis, which is a critical stage to detect the

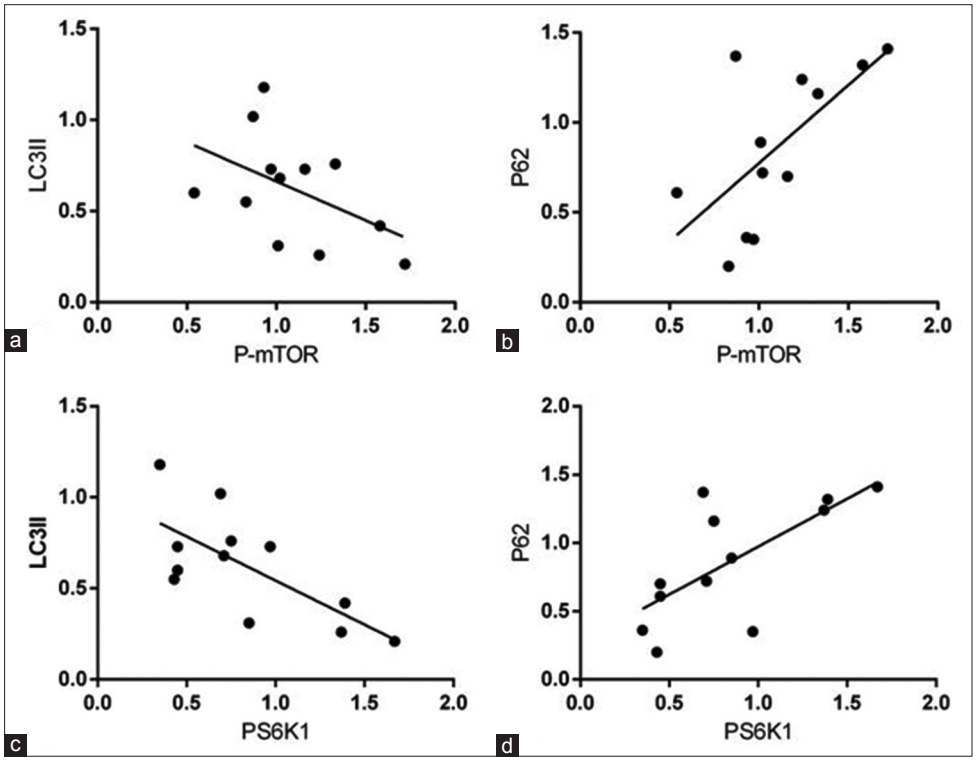

Figure 5: Correlation between *p*-mTOR, PS6K1, LC3II, p62. (a) mTOR and LC3II ($n = 12$, *p*-mTOR in the myocardium was not correlated with LC3II). (b) mTOR and p62 ($n = 12$, $r = 0.66$). (c) PS6K1 and LC3II ($n = 12$, $r = -0.71$). (d) PS6K1 and p62 ($n = 12$, $r = 0.70$). mTOR: Mammalian target of rapamycin; *p*-mTOR: Phosphorylation of mTOR; LC3II: Light chain 3 type II; PS6K1: Phosphorylation of S6 kinase-1.

myocardial dysfunction and repair timely. Furthermore, in this study, we investigated the formation and degradation of autophagosomes during autophagic process in the LV in CLP-induced septic mice. We found that the formation and the degradation of autophagosomes were both increased in the LV at 18 h after CLP, which confirmed the above dynamic change of autophagy process in myocardium of CLP-induced septic mice.

Furthermore, we found accompanied with the accelerated process of autophagy that the mTOR pathway was inhibited in CLP rats. *p*-mTOR and PS6K1, downstream targets of mTORC1, were significantly suppressed following CLP challenge. mTOR is a master sensor of energy status and will be inhibited in the state of energy depletion. Under nutrient-rich growth conditions, mTOR supports cellular growth and suppresses autophagy. On the contrary, in response to nutrient starvation, mTOR pathway is inhibited and autophagy is induced to provide energy source. Recent studies showed that in myocardial ischemia, the mTOR signaling pathway is inhibited by AMPK.[25] Energy depletion also plays a major role in septic cardiac dysfunction induced by mitochondrial dysfunction and reduced cardiac fatty acid oxidation.[26] Our previous studies have found that the mTOR pathway in CD8(+) T-cell was changed during the sepsis.[27] However, there is little evidence to prove the relevance of mTOR in sepsis-induced cardiac dysfunction, and nobody has confirmed the relationship between the mTOR pathway and the change of autophagy process in CLP-induced myocardial dysfunction.

Most importantly, we found that *p*-mTOR was significantly positively correlated with p62, PS6K1 was significantly positively correlated with p62 and negatively correlated with LC3B, which means that the activity of mTOR pathway was closely correlated with the change of autophagic protein, indicating that the change of autophagy process in CLP rats may be mediated by mTOR pathway. However, there was a limitation in our inference. To reveal mTOR pathway mediated the change of autophagy process in sepsis-induced myocardial dysfunction, it is necessary for us to inhibit or accelerate the mTOR pathway using the methods of pharmacology or gene knockout. In consideration of this, we will use mTOR myocardial-specific knockout mice to confirm our inference in our next step experiment.

In conclusion, the autophagy process in myocardium was accelerated in CLP rats, which was correlated with the inhibition of the mTOR pathway. Investigation the dynamic process of autophagy could help us clear out the controversial role of autophagy in CLP-induced myocardial dysfunction. Whether mTOR pathway could be used as a target of protection of sepsis-induced myocardial dysfunction in the future requires further study.

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Conflicts of interest

There are no conflicts of interest.

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CLP诱导的大鼠心肌功能障碍中的加速自噬与mTOR信号通路的关系

摘要

背景：最近的研究表明，自噬参与脓毒症引起的心肌功能障碍。本研究旨在探讨在盲肠结扎穿孔（CLP）诱导的心肌功能障碍中自噬的变化，以及与哺乳动物雷帕霉素靶蛋白（mTOR）信号通路的关系。

方法：12只大鼠随机分为CLP组和假手术组。在CLP或假手术后18小时收集心脏组织。苏木精和伊红染色（H&E）检测病理，超声心动图检查心脏功能，免疫组织化学染色检测II型微管相关蛋白轻链3（LC-3）的分布，II型，透射电子显微镜检测自噬泡。此外，应用蛋白质印迹法检测磷酸化mTOR（p-mTOR），磷酸化核糖体S6蛋白激酶（ps6k1），LC3II和p62的表达。皮尔森相关系数用于分析两个参数之间的相关性。

结果：病理和超声心动图显示，CLP大鼠心肌明显损伤。我们还发现CLP大鼠中自噬进程的升高，LC3II/LC3I比值升高（P＜0.05），p62的表达明显降低（P＜0.05），CLP大鼠有更多的自噬体和自噬溶酶体。此外，在CLP组中，与假手术组相比，心肌mTOR信号通路被抑制，CLP后p-mTOR（P＜0.01）和ps6k1（P＜0.05）均显著下降。有趣的是，我们发现mTOR信号通路与细胞自噬过程密切相关。在我们的研究中发现，心肌组织中p-mTOR和P62显著相关（r = 0.66，P = 0.02），ps6k1与p62呈显著正相关（r = 0.70， P = 0.01），与LC3II负相关（r = -0.71，P = 0.01）。

结论：CLP大鼠心肌自噬的过程加快与mTOR信号通路的抑制密切相关。