The Trend of β_3 -Adrenergic Receptor in the Development of Septic Myocardial Depression: A Lipopolysaccharide-Induced Rat Septic Shock Model

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Keywords

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Abstract

Septic shock with low cardiac output is very common in children. However, the mechanism underlying myocardial depression is unclear. The role of β_3 -AR in the development of myocardial depression in sepsis is unknown. In the present study, we generated an adolescent rat model of hypodynamic septic shock induced by lipopolysaccharide (LPS). Neonatal cardiomyocytes were also treated with LPS to mimic myocardial depression in sepsis, which was confirmed via an in vivo left ventricular hemodynamic study, and measurements of contractility and the Ca^{2+} transient in isolated adolescent and neonatal cardiomyocytes. After 16 h of LPS treatment, cultured neonatal cardiomyocytes showed a diminished Ca^{2+} transient amplitude associated with an increase in the β_3 -AR level. With the addition of a β_3 -AR agonist, the Ca^{2+} transient in LPS-treated neonatal rat cardiomyocytes gradually decreased over time; such a change was absent in cells treated with nitric oxide synthase (NOS) inhibitors prior to treatment with a β_3 -AR agonist. In adoles-

cent rats with septic myocardial depression, cardiac function declined as indicated by decreased MAP, $\text{dP/dt}_{\text{max}}$, and $\text{dP/dt}_{\text{mix}}$ for 6 h after LPS injection; however, the β_3 -AR level first increased 2 h after LPS treatment and then decreased 6 h after LPS treatment in the absence of exogenous catecholamines. The results indicate that, in vitro, at the cellular level β_3 -AR may be involved in the development of myocardial depression (Ca^{2+} transient depression) in sepsis through NOS signaling pathways; however, in vivo, a complicated mechanism for modulating β_3 -AR may exist.

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Introduction

Nowadays, sepsis has become a major public health problem, and is the main cause of death among patients admitted to intensive care units. Notably, septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality [1], and is a common cause of death in pediatric intensive care units [2]. Unlike that in adults, “cold shock” with low cardiac output and high peripheral vascular resistance is very common in

children. Cardiovascular dysfunction occurs in about 70% of children with severe sepsis [3]; also called septic myocardial depression or septic cardiomyopathy, it increases the mortality of septic shock [4, 5]. However, the underlying mechanism of septic myocardial depression remains unclear.

β -Adrenergic receptors (β -ARs) and their signaling pathways play crucial roles in regulating cardiac function; they are also closely associated with the development of septic myocardial depression. Compared to β_1 -AR or β_2 -AR, the expression level of β_3 -AR is much lower under physiological conditions and increases in pathological conditions [6–9]. Additionally, β_3 -AR activation also requires a higher level of catecholamines. Patients with severe sepsis and septic shock have significantly elevated catecholamines in their serum, resulting from the production of endogenous catecholamines, as well as the medical administration of catecholamines that doubles the increase in adrenaline or norepinephrine [10, 11]. Although published in vitro studies suggested that β_3 -AR activation may contribute to myocardial depression in sepsis [8], its role in the pathogenesis of septic myocardial depression remains unclear.

In the present study, we investigated the dynamic changes of β_3 -AR expression in conjunction with the development of myocardial depression in an adolescent rat model of septic shock induced by lipopolysaccharide (LPS) and in primary cultured rat cardiomyocytes treated with LPS. We aimed to better understand the effect of β_3 -AR on the development of myocardial depression, and therefore provide new insights into therapeutic targets of myocardial depression in septic shock.

Materials and Methods

Animal Model of Septic Shock

An adolescent rat model of septic shock induced by intravenous injection of LPS was generated as previously described [12]. Male pathogen-free Wistar rats (Charles River, Beijing, China), 6–8 weeks old, 190–210 g, were first anesthetized by 20% urethane (1 g/kg i.p.). The left femoral artery was cannulated for continuous monitoring of mean arterial blood pressure (MAP) and a PE50 catheter was inserted into the left ventricle through the right common carotid artery in order to monitor left ventricular (LV) hemodynamic changes (Biopac MP150 Biopac Systems, Goleta, CA, USA). The right femoral vein was exposed for the injection of *Escherichia coli* 055:B5 (L-2880, Sigma-Aldrich, St Louis, MI, USA; 15 mg/kg, 5 mg LPS dissolved in 1 mL 0.9% saline, i.v., 0.1 mL/min). Septic shock was established when MAP decreased to 25–30% of the baseline value, which was about 1.5 h after the injection of LPS. For controls, saline (0.9%, 3 mL/kg, 0.1 mL/min) was injected through the right femoral vein of control rats.

Blood (3–4 mL) was collected from the abdominal aorta at 2, 4, and 6 h after LPS administration and centrifuged (3,000 rpm, 10 min) after sitting at room temperature for 15 min. The supernatant was collected and stored at -80°C for ELISA. The heart was excised within 6 h of LPS administration and stored at -80°C for real-time PCR and immunoblotting.

Adolescent Rat Cardiomyocyte Isolation

Adult rat cardiomyocytes were isolated as previously described [13]. Four hours after an LPS injection, the hearts were isolated from LPS-treated rats, washed, and retroperfused through the aorta using a Langendorff apparatus. The perfusion solution (Tyrode) contained (in mM): 140 NaCl, 5.4 KCl, 1 MgCl₂, 1.2 CaCl₂, 10 HEPES, and 10 D-Glucose (pH set to 7.4 with NaOH). After 3 min of perfusion with the Ca²⁺-containing Tyrode, the hearts were further perfused with Ca²⁺-free Tyrode for 5 min and then subjected to digestion with high-K⁺ Kraftbrühe (KB) solution containing (in mM): 80 KOH, 40 KCl, 25 KH₂PO₄, 25.0, 3 MgSO₄, 50 L-glutamic, 20 taurine, 10 HEPES, 1 EGTA, 10 D-glucose, and 0.03 Ca²⁺ (pH set to 7.2 with KOH), with the addition of 0.6% collagenase type II and 0.6% BSA. The left ventricles were then removed and chopped into small pieces followed by gentle pipetting in fresh KB solution to completely isolate cardiomyocytes. The resultant, isolated cardiomyocytes were transferred to fresh Tyrode solution and left at room temperature for 1 h. Ca²⁺ was restored gradually (0.3, 0.6, 1.2 mM Ca²⁺) over the following period of 30 min. Rats subjected to 4 h of 0.9% saline treatment were used as controls.

Measurement of Contractility and Ca²⁺ Transient in Isolated Adolescent Rat Cardiomyocytes

Mechanical properties and intracellular Ca²⁺ transients were measured simultaneously using an IonOptix Myocam system (IonOptix Inc., Milton, MA, USA). Freshly isolated rat cardiomyocytes were loaded with 5 μM of Fura-2 AM (Molecular Probes, Eugene, OR, USA) at room temperature for 20 min, placed into a perfusion chamber mounted on a microscope stage, and perfused (1.5 mL/min, 37°C) with Tyrode solution containing (in mM): 131 NaCl, 4 KCl, 1.2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, at pH 7.4, with 95% O₂. Under a field stimulation of 5 V (1 Hz), cells were excited by UV light (360 and 380 nm, alternately) for Ca²⁺ transient measurement. The Fura-2 emission at 510 nm was collected with a photomultiplier tube. Only rod-shaped cells with clear edges and a well-defined sarcomere structure and without spontaneous contractions were used for data collection. The kinetics of Ca²⁺ transients were analyzed in conjunction with myocyte mechanical measurements using the software, IonWizard 6.0 (IonOptix Inc.).

Neonatal Rat Cardiomyocyte Isolation and Culture

One-day-old rats were anesthetized with isoflurane and whole bodies were dipped in 75% ethanol for 5 s. The heart was quickly excised and washed 3 times with PBS. The ventricles were dissected and minced into 1-mm³ pieces followed by a series of digestions in a solution containing 0.06% trypsin and 0.08% collagenase with stirring at 36.8°C . After each digestion, the cell suspension was quickly placed in cell culture medium containing neonatal calf serum and then centrifuged at 800 rpm for 5 min. The resultant cell pellet was resuspended and preplated in a cell culture flask. After sitting in an incubator (37°C , 5% CO₂) for 1 h to eliminate fibroblasts, the cardiomyocyte-rich suspension was then plated in 6-well cell culture dishes with a cell intensity of $3 \times 10^5/\text{cm}^2$ for

myocyte functional studies and $6 \times 10^5/\text{cm}^2$ for immunoblotting and real-time PCR. Bromodeoxyuridine (0.1 mM) was added to the culture medium to inhibit fibroblast growth. Cells were ready for experiments within 72 h of culture.

Measurement of Ca^{2+} Transient in Cultured Neonatal Cardiomyocytes

Neonatal rat cardiomyocytes were treated with LPS (1 $\mu\text{g}/\text{mL}$) for 8, 12, and 16 h, respectively. LPS-treated and control cells were loaded with Fluo-4 acetoxymethyl ester (2 μM) for 20 min at room temperature and then placed in a perfusion chamber mounted on a microscope stage. The perfusion solution contained 1.8 mM Ca^{2+} . The intracellular Ca^{2+} transient was measured by a customized Ca^{2+} imaging system consisting of an excitation source supplied by an IonOptix Hyperwitness xenon lamp system, which consisted of an excitation filter EX470/40, emission filter EM525/50, dichroic mirror DM495, Olympus IX71 inverted fluorescence microscope with an objective lens (UPLSAPO10X), and an Andor IXon X3 DU897 EMCCD calcium signal acquisition system (binning 4×4 , sampling frequency 134 Hz). Available myocytes were chosen as a fluorescence region of interest (ROI). The following equation was applied: $\text{Ca}_{\text{transient}} = (\text{F}_{\text{peak}} - \text{F}_0)/(\text{F}_0 - \text{B})$, where B represents background fluorescence intensity.

Quantitative Real-Time PCR

Total RNA was isolated from heart tissue or cultured cells according to the manufacturer's instructions supplied with the TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and then reverse transcribed with a Thermo First Synthesis Kit. The expression levels of β_1 -AR, β_2 -AR, and β_3 -AR were then determined by real-time PCR. Each targeted cDNA was amplified using SYBR Green via the StepOnePLUS Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). The primers for β_1 -AR were forward 5'-CCA-TCA-CGC-TGC-CCT-TTC-G-3' and reverse 5'-CGC-CAC-CAG-TGC-ATG-AGG-AT-3'; for β_2 -AR they were forward 5'-GCAATTTCTGGTGCAGTT-3' and reverse 5'-TGAAGGCGCATGTGATAG-3'; for β_3 -AR they were forward 5'-TTCCGTCGTCTTCTGTGC-3' and reverse 5'-CCT-GTTGAGCGGTGAGTT-3'. GAPDH was used as an internal control.

Western Blot Analysis

Harvested rat heart tissue and cultured rat cardiomyocytes were homogenized in RIPA buffer. Proteins from the homogenate (30 $\mu\text{g}/\text{well}$) were separated on a one-dimensional 4–10% SDS-polyacrylamide gel and transferred to a polyvinylidene membrane (Bio-Rad Laboratories, Hercules, CA, USA) with a semidry electrotransfer apparatus for 1.5 h at 300 mA. The expression levels of β_1 -AR, β_2 -AR, and β_3 -AR proteins were determined by the monoclonal antibodies: anti- β_1 -AR (1:1,000; Abcam, Cambridge, UK), anti- β_2 -AR (1:1,000; Abcam), and anti- β_3 -AR (1:1,000; Santa Cruz, Dallas, TX, USA), respectively. The immunoblots were visualized by enhanced chemiluminescence (ECL detection kit from GE Healthcare, Chicago, IL, USA).

Statistical Analysis

All experiments were repeated at least 3 times. Data of a normal distribution were presented as the mean $\pm$ standard error of the mean (SEM). The Student *t* test for unpaired data was used to compare between 2 groups. Two-way ANOVA with repeated measures

was used to analyze the data of LV function and Ca^{2+} transient changes with the administration of LPS. The statistical analysis was done using SPSS13.0 software (IBM Corporation, Armonk, NY, USA). $p < 0.05$ indicated significance.

Results

Depressed Left Ventricular Function in Rats with Septic Shock

LV function with/without LPS treatment was monitored in situ throughout the entire LPS treatment period of 6 h. The LV functional data collected at 0, 2, 4, and 6 h time points are presented in Figure 1. MAP started to decline 1.5 h after the intravenous administration of LPS to rats. Septic shock was defined as once the rats' MAP decrease was about 25–30% of the baseline value, which occurred about 2 h after the LPS injection, and lasted for the subsequent 4 h of the observation period. Controls with a saline injection displayed an unchanged MAP throughout the entire observation period of 6 h (Fig. 1a). Compared to control rats, LPS-treated rats showed a gradual decline in cardiac function as evidenced by significant decreases in heart rate, LV peak rate of a pressure rise, and an LV peak rate of pressure decay, as well as a prolonged relaxation time constant, though the LV end-diastolic pressure remained unaltered (Fig. 1b–f). These data indicate a successful rat model of septic shock with more and more depressed cardiac function when LPS treatment lasted for the 6-h period.

Unaltered Serum Cardiac Troponin I and Creatine Kinase Isoenzyme in Rats with Septic Shock

Serum cardiac troponin I (cTnI) and creatine kinase isoenzyme (CK-MB) in LPS-treated rats was detected with an ELISA at 2, 4, and 6 h after treatment, and did not show significant changes in comparison to controls (Fig. 2). The results indicate that cell death or tissue damage did not occur during 6 h of LPS treatment in septic rats even though their overall cardiac function was significantly depressed.

Depressed Function of Cardiomyocytes Isolated from Rats with Septic Shock

Cardiomyocytes were isolated from the hearts of septic rats euthanized 4 h after LPS treatment. As shown in Figure 3, compared to cardiomyocytes from the control group, cardiomyocytes from septic rats showed significantly lower peak shortening, slower peak rate shortening ($+dL/dt$), and a peak rate of relengthening (dL/dt). They

Fig. 1. a-f In vivo hemodynamic studies. Septic shock was defined as MAP decreased by 25–30% of its baseline value. Septic shock in rats was induced by intravenous injection of LPS (*E. coli* 055:B5, 15 mg/kg, 0.1 mL/min) and rats injected (i.v.) with saline were used as controls. LV function was monitored over the entire 6 h of LPS/saline injection. ** $p < 0.01$, * $p < 0.05$ versus saline; ## $p < 0.01$ versus the respective baseline. $n = 6$.

Fig. 2. Serum cTnI and CK-MB levels in LPS-treated rats. Blood was collected from the rats at 2, 4, and 6 h after LPS or saline administration. Serum cTnI and CK-MB was detected by ELISA. $n = 8$.

also showed a longer time to 90% peak shortening (TPS₉₀) and 90% relengthening (TR₉₀), indicating an overall depression of contractile function in cardiomyocytes in septic rats in the absence of neurohumoral factors.

The intracellular Ca²⁺ transient was also detected in conjunction with cell shortening in isolated cardiomyocytes. In agreement with the contractile data, cardiomyo-

cytes from LPS-treated rats presented significantly lowered baseline and peak Ca²⁺ concentrations, a decreased amplitude of the Ca²⁺ transient (delta FFI), prolonged time to 90% Ca²⁺ decay, and an increased time constant of Ca²⁺ decay in comparison to controls (Fig. 4). Such data indicated that intracellular Ca²⁺ handling was negatively altered in cardiomyocytes of rats with septic shock.

Fig. 3. Contractile function of single cardiomyocytes. Cardiomyocytes were isolated from the hearts of the septic rats euthanized at 4 h after LPS treatment. **a** Peak shortening (PS). **b** Peak rate of shortening (+dL/dt). **c** Peak rate of relengthening (-dL/dt). **d** Time to 90% peak shortening (TPS₉₀). **e** Time to 90% of relengthening (TR₉₀). **f** Representative traces of cellular sarcomere shortening in rat LV myocytes paced at 1 Hz. $n = 88$ from 5 LPS-treated mice; $n = 35$ from 5 control mice. ** $p < 0.01$ compared to the control.

Fig. 4. Measurements of intracellular Ca^{2+} transients in single cardiomyocytes. Cardiomyocytes were isolated from the hearts of the septic rats euthanized at 4 h after LPS treatment. **a** Baseline Ca^{2+} concentration (baseline FFI). **b** Peak Ca^{2+} concentration during cell shortening (peak FFI). **c** Amplitude of Ca^{2+} transient (delta FFI). **d** Time to 90% Ca^{2+} decay (TP₉₀). **e** Time constant of Ca^{2+} decay (Tau). **f** Representative traces of cellular calcium transient in rat LV myocytes paced at 1 Hz. $n = 88$ from 5 LPS-treated mice; $n = 35$ from 5 control mice. ** $p < 0.01$ compared to the control.

Fig. 5. Measurements of intracellular Ca^{2+} transients in neonatal cardiomyocytes. The cells were exposed to LPS for 8 or 16 h. **a** A demonstration of Ca^{2+} transient of multiple cells (left) and a representative view of cultured rat neonatal cardiomyocytes under $\times 40$ magnification (right). **b** Amplitude of Ca^{2+} transient ($(F_{peak} - F_0)/(F_0 - B)$). **c** Time to 50% Ca^{2+} decay (TR_{50}). **d** Time constant of Ca^{2+} decay (τ). $n = 130-150$. * $p < 0.05$ compared to the control.

Altered Ca^{2+} Transient in Rat Neonatal Cardiomyocytes Treated with LPS

Primarily cultured rat neonatal cardiomyocytes were treated with LPS (1 $\mu\text{g}/\text{mL}$) for 8, 16, and 24 h. Significantly fewer beating cells were present in wells treated with LPS for 24 h than those with a shorter LPS exposure time. Only cells with 8 or 16 h of LPS treatment were used for Ca^{2+} transient measurements. Compared to cells without LPS treatment, LPS-treated cells decreased the amplitude of the Ca^{2+} transient and prolonged Ca^{2+} decay. Such negative changes were only observed in cells exposed to LPS for 16 rather than 8 h. Such data suggest that prolonged LPS exposure depresses the cardiac function of neonatal cardiomyocytes (Fig. 5).

The Expression Level of β -ARs in Rat Neonatal Cardiomyocytes Treated with LPS

Since neonatal cardiomyocytes showed functional changes after 16 h of LPS treatment, β -AR expression levels were detected at the corresponding time point. As shown in Figure 6a and b, β_3 -AR expression in LPS-treated cells was increased approximately 2-fold at both mRNA and protein levels, while β_1 -AR and β_2 -AR mRNA expressions remained normal.

The Effect of β_3 -AR Agonist on LPS-Treated Neonatal Cardiomyocytes

In order to test whether β_3 -AR activation caused myocardial depression in sepsis, a β_3 -AR agonist, BRL 37344

Fig. 6. The expression of β -ARs in rat neonatal cardiomyocytes with 16 h of LPS treatment. **a** The expression level of β_3 -AR protein detected by immunoblotting. A representative immunoblotting image (left) and the quantitative data (right). $n = 4$. **b** The expression levels of β_1 -, β_2 -, and β_3 -AR mRNA. $n = 4$. **c** The amplitude of Ca^{2+} transients in the LPS-treated cardiomyocytes with addition of the following compounds. BRL 37344 (0.1 μ M) is a β_3 -AR agonist. CGP 20712 (0.3 μ M) is a specific β_1 -AR antagonist. ICI 118551 (0.1 μ M) is a specific β_2 -AR antagonist. L-NAME (10 μ M) is a NOS inhibitor. The Ca^{2+} transient was measured at 0, 5, 10, 15, 20, and 30 min after the addition of the β_3 -AR agonist. $n = 40$ –45. * $p < 0.05$ versus the control.

(0.1 μ M), was added to the culture medium after 16 h of LPS treatment. Because of the imperfect selectivity of BRL37344, specific β_1 - and β_2 -AR antagonists (CGP 20712, 0.3 μ M and ICI 118551, 0.1 μ M) were added to block the activation of the 2 receptors. The Ca^{2+} transient was measured at 0, 5, 10, 15, 20, and 30 min after the addition of the β_3 -AR agonist. LPS-treated neonatal cardiomyocytes showed a gradual and significant decline in the amplitude of the Ca^{2+} transient over the time of exposure to the β_3 -AR agonist (Fig. 6c), while Ca^{2+} transients in non-LPS-treated cells were not affected by the same treatment (data not shown). The aforementioned negative effect of the β_3 -AR agonist on LPS-treated cardiomyocytes was totally absent in cells that were treated with a nitric oxide synthase (NOS) inhibitor (L-NAME, 10 μ M) 30 min ahead of β_3 -AR agonist treatment.

The Expression Level of β -ARs in Hearts of Septic Shock Rats

As the rats had developed and maintained septic shock-associated myocardial depression after 2 h of LPS treatment, expression levels of β -ARs in the left ventricle were detected 2, 4, and 6 h after LPS exposure. The expression level of β_3 -AR protein increased significantly at first at an early stage of septic shock (2 h of LPS treatment) and then decreased to a level significantly lower than the control group after 6 h of LPS treatment (Fig. 7a, b). The expression level of β_3 -AR mRNA changed over the period of LPS treatment in an identical pattern to its protein level (Fig. 7c). In contrast to β_3 -AR, the expression of β_1 - and β_2 -AR did not show a significant change after LPS treatment at either the mRNA or protein level (Fig. 7b, c).

Fig. 7. The expression of β -ARs in rats with myocardial depression in septic shock. Heart tissue was harvested from the septic shock rats at 2, 4, and 6 h after LPS treatment. **a** Representative immunoblotting image of β_3 -AR expression. **b** Quantitative data of the expression levels of β_1 -, β_2 -, and β_3 -AR proteins detected by immunoblotting. **c** Quantitative data of the expression levels of β_1 -, β_2 -, and β_3 -AR mRNA detected by real-time PCR. $n = 4$. * $p < 0.05$, ** $p < 0.01$ versus the control.

Discussion

By using an adolescent rat model of LPS-induced septic shock and isolated adolescent and neonatal rat cardiomyocytes, the present study demonstrated sepsis-associated intrinsic myocardial systolic and diastolic dysfunction both in vivo, at the intact animal level, and in vitro, at the cellular level. Furthermore, for the first time, we studied the expression level of β_3 -AR when myocardial depression manifested and developed without exogenous catecholamine. In vitro, the increase in β_3 -AR expression associates with the contractile depression of LPS-treated cultured rat neonatal cardiomyocytes, which involves NOS pathways. However, we did not observe a persistent

increasing trend of β_3 -AR without exogenous catecholamines during the development of septic myocardial depression; the change in β_3 -AR expression in vivo is likely a protective compensatory mechanism against septic myocardial depression.

Septic shock is a subset of sepsis with circulatory and cellular/metabolic dysfunction associated with a higher risk of mortality. Unlike “warm shock” with high cardiac output and low peripheral vascular resistance in adults, “cold shock” with low cardiac output and high peripheral vascular resistance is very common in children. We used an animal model of septic shock through the intravenous injection of endotoxin, which showed more significant myocardial depression causing low cardiac output and

decreased blood pressure, indicating this to be a good animal model for studying childhood septic shock.

Septic shock associated with myocardial dysfunction or septic cardiomyopathy leads to high mortality, yet the underlying mechanisms remain unclear. However, it is hard to measure intrinsic myocardial function in such patients [14]. Studies exist roughly describing septic myocardial depression by using echocardiography and cardiac biomarkers, such as troponin and brain natriuretic peptide [15–17]. As early as 2 decades ago, Robotham et al. [18] pointed out that the ejection fraction (EF) reflects the net effect of LV contractile function and its responses to afterload; in other words, when a decrease in peripheral arterial resistance lowers afterload, EF may be within the normal range, even with myocardial dysfunction. In septic myocardial depression, peripheral vascular dilation is common; therefore, EF is not an ideal diagnostic criterion for cardiac contractile function. Zaky et al. [15] also pointed out the limitation and insensitivity of using echocardiography to evaluate myocardial function in sepsis. An invasive hemodynamic study is a better way to study intrinsic cardiac contractile function in vivo, as employed in the present study to evaluate myocardial function in vivo in a rat model of LPS-induced sepsis with invasive hemodynamic measurements. Together with in vitro functional studies in isolated adolescent and neonatal cardiomyocytes, our work provides solid evidence of the presence of intrinsic myocardial depression in septic shock induced by LPS.

Previous reports have demonstrated the existence of cardiomyocyte ultrastructural damage in sepsis, yet without significant cardiomyocyte death [19]. Recently, Takasu et al. [20] studied myocardial tissue from patients with sepsis and found a lack of significant necrosis, apoptosis, or autophagy compared to control donor hearts. Zaky et al. [15] also suggested that an increased serum troponin level in sepsis is likely due to increased permeability of the cardiomyocyte plasma membrane caused by inflammatory factors rather than cardiac cell death. However, we did not detect any significant change in serum CK-MB or troponin, a specific marker of myocardial damage, in the rat model of septic shock, even after the development of myocardial depression. Therefore, severe myocardial necrosis or damage is not a characteristic of this rat model.

Recently, accumulating evidence has suggested the importance of β_3 -AR in regulating cardiac function and its potential as a therapeutic target [21, 22]; however, whether or not β_3 -AR activation correlates with myocardial depression in sepsis remains unclear. Moniotte et al.

[8] first reported that cardiac β_3 -AR is elevated in patients with sepsis. The human β_3 -AR gene is located on the eighth chromosome and has a 79% similarity to that in the rat, in which the transmembrane region shares the highest homology, while the C-terminus and the third intramembranous region share the lowest [21]. Therefore, we used the rat as the animal model in this study. In contrast to β_1/β_2 -AR that has a positive inotropic effect, β_3 -AR has a negative inotropic effect on myocardium in humans and several other animal models. β_3 -AR activation leads to the inhibition of L-type Ca^{2+} [23]; the NOS inhibitor, L-NAME, can partially block the inhibition [24]. β_3 -AR activation also decreases the intracellular Na^+ concentration via activating the Na^+/K^+ pump and, in turn, leads to an increase in Ca^{2+} efflux through the $\text{Na}^+-\text{Ca}^{2+}$ exchanger, which eventually lowers Ca^{2+} uptake by the sarcolemma reticulum [25]. However, such a functional change in the Na^+/K^+ pump leading to myocardial depression was observed in wild-type animals, but not in animal models of heart failure [26].

In the present study, the observed depression in the Ca^{2+} transient in rat neonatal cardiomyocytes reflects the development of myocardial dysfunction after LPS treatment; at the same time both β_3 -AR mRNA and protein expression levels increased significantly. Furthermore, the addition of BRL37344, a β_3 -AR agonist, exaggerated the Ca^{2+} transient depression over time, in the absence of β_1 - and β_2 -AR activation. Such an effect was completely blocked in cells pretreated with an NOS inhibitor, which contrasts with the study by Moniotte et al. [8], where the NOS inhibitor was found to incompletely block the negative inotropic contractility effect of β_3 -AR activation in culture medium conditioned with LPS-activated macrophage-conditioned medium. These results indicate that β_3 -AR activation may be involved in the development of myocardial Ca^{2+} transient dysfunction through NOS signaling in sepsis in vitro.

Our results showed that, without the intervention of exogenous catecholamines, a significant change in blood catecholamine concentration between septic shock and control rats was not observed (data not shown). With the decline in cardiac function and heart rate, the expression level of β_3 -AR firstly increased and then decreased rather than continuing to increase. A previous study indicated that the activation of β_3 -AR required more catecholamines than that of β_1/β_2 -AR [27]. Indeed, the concentration of catecholamines increased significantly in patients with severe sepsis; however, such an increase could have both an endogenous and exogenous origin because catecholamines are commonly used to treat patients with se-

vere sepsis or septic shock. Therefore, it is unclear whether β_3 -AR activation is a risk factor that contributed to septic myocardial depression, although Moniotte et al. [8] detected a significantly high β_3 -AR level in myocardium from patients who died of severe sepsis. In our septic shock model, myocardial β_3 -AR increased and then decreased, despite an ever-declining cardiac function. In contrast, β_3 -AR was slightly increased in the control group without any cardiac dysfunction, which may be related to long-time anesthesia without food or water administration and later operation stresses. A lasting high catecholamine level or stress often triggers a high level of oxygen consumption in the myocardium, Ca^{2+} overload, and toxin-related myocardium damage. Thus, activation of β_3 -AR in vivo is more likely to be a compensation mechanism to protect the heart from a high metabolic state during septic shock, which agrees with reported in vivo studies on β_3 -AR in heart failure and hypertrophic cardiomyopathy models [26, 28, 29]. With the activation of β_3 -AR, the third generation β -AR blocker, nebivolol, is more effective in inhibiting cardiac remodeling than the β_1 -AR blocker, metoprolol [30]. The aforementioned studies were performed in chronic disease models while septic myocardial depression is an acute condition. Therefore, β_3 -AR may play a more complicated role in septic myocardium depression in vivo, although β_3 -AR activation may be involved in the development of myocardial depression in isolated neonatal cardiomyocytes. Further studies are required to address questions, such as how β_3 -AR changes and how it affects myocardial function in the administration of exogenous catecholamines during septic shock, and what is the time window for treatment with an β_3 -AR agonist or antagonist.

In addition to β_3 -AR, we simultaneously detected changes in cardiac β_1 -AR and β_2 -AR at various time points of septic shock and did not find any significant alteration. Early reports indicated that the density of dominant β -AR decreased or stayed normal in sepsis [31, 32]; however, another study found a decrease in stimulatory G protein and an increase in inhibitory G protein [33, 34], all of which may lead to a decrease in intracellular cAMP and thus affect myocardium function.

Our study has several limitations. Unlike isolated adult cardiomyocytes with an elongated shape and clear edge, primary cultured neonatal cardiomyocytes have an irregular shape and thus lack any widely used commercialized measurement of contractile function, so there is no widely accepted method for the accurate measurement of primary cultured neonatal cardiomyocytes. Since cardiac contractile function is coupled with cyclic cytosolic Ca^{2+}

transient change, we indirectly evaluated the contractile function of neonatal cardiomyocytes by measuring Ca^{2+} transients. Compared to the conventional method, which only measures a cell at a time, our customized system is able to measure multiple cells simultaneously, which improves efficiency and minimizes the variance between each measurement.

Conclusion

Our present study is an investigation of the role of β_3 -AR in the development of myocardial depression in hypodynamic septic shock induced by LPS. It demonstrates septic myocardial depression in both the intact animal and in isolated adolescent and neonatal cardiomyocytes treated with LPS. Although β_3 -AR activation may be involved in the development of myocardial dysfunction through NOS signaling in isolated neonatal cardiomyocytes, a complicated mechanism may exist in vivo of septic shock considering a lack of a persistent increasing trend of β_3 -AR without exogenous catecholamines during the development of septic myocardial depression. Our findings shed light on an understanding of the role of β_3 -AR and its related signaling pathways in children with septic myocardial depression, which deserves further exploration.

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Statement of Ethics

This study was approved by the Medicine Ethics Committee of Shengjing Hospital of the China Medical University (2017PS232K). The animal experiments conformed to institutional standards.

Conflict of Interest

The authors declare no conflicts of interest.

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