# SERCA1 attenuates diaphragm relaxation and uptake rate of SERCA in rats with acute sepsis

JIANYOU ZHANG[1*], HUI LIU[2*], SHITONG LI[3], JIN WU[3] and JIANHONG SUN[1]

Departments of [1]Anesthesiology and [2]Endocrinology, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, Jiangsu 225000; [3]Department of Anesthesiology, Shanghai General Hospital of Nanjing Medical University, Shanghai 200080, P.R. China

Received October 27, 2016; Accepted June 15, 2017

DOI: 10.3892/mmr.2017.7134

**Abstract.** The present study aimed to investigate the effects of acute sepsis on diaphragm contractility and relaxation, via examining the $Ca^{2+}$-uptake function of sarco/endoplasmic reticulum $Ca^{2+}$ adenosine triphosphatase (SERCA), and the protein levels of SERCA1, SERCA2 and the ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR). A sepsis rat model was established through cecal ligation and puncture (CLP). A total of 6 and 12 h following CLP, the isometric contractile and relaxation parameters of the diaphragm were measured. In addition, $Ca^{2+}$ uptake and release from the SR, and the protein expression levels of SERCA1, SERCA2 and RyR in diaphragm muscle tissue were investigated. At 6 and 12 h post-CLP, the diaphragm half-relaxation time was prolonged and the maximum rate of tension decline was decreased and the $Ca^{2+}$-uptake function of SERCA was markedly reduced. The maximum rate of twitch force development, the maximal twitch and tetanic tension, and the release function of SR were decreased at 12 h post-CLP. A total of 12 h following CLP, the protein expression levels of SERCA1 were significantly downregulated, and its activity was significantly reduced; conversely, the protein levels of SERCA2 remained unaltered. The present findings indicated that at the acute stage of sepsis induced by CLP the contractile and relaxation functions of the diaphragm were significantly compromised. The impairments in relaxation may be a result of the impaired uptake function of the SR and the downregulation in SERCA1 protein expression. Conversely, the compromised contractility may be a result of the impaired release function of the SR and the downregulation in RyR protein levels. This could provide some new insights into the treatment of sepsis. In acute stages of sepsis, the improvement of SERCA function could reduce the disequilibrium of calcium homeostasis to improve the critical illness myopathy and respiratory failure.

*Correspondence to:* Professor Jianhong Sun, Department of Anesthesiology, The Affiliated Hospital of Yangzhou University, Yangzhou University, 368 Hanjiang Road, Yangzhou, Jiangsu 225000, P.R. China
E-mail: jianyouzh@qq.com

*Contributed equally

*Key words:* sepsis, diaphragm, muscle relaxation, uptake of calcium, sarco/endoplasmic reticulum $Ca^{2+}$ adenosine triphosphatase

## Introduction

Severe sepsis commonly leads to muscle dysfunction and multiple organ failure (1). The diaphragm is one of the most important respiratory muscles, and diaphragm injury may result in respiratory failure, thus prolonging the need for maintenance in intensive care and under mechanical ventilation (2-4). Prolonged stay in intensive care units during the recovery from critical illness may be accompanied by myopathy and muscle weakness (4); this may impair the function of the diaphragm, thus contributing to the development of respiratory failure following sepsis. In muscle tissue, sepsis results in dysfunctions in energy metabolism and oxidative damage of the mitochondria; it also triggers the production of inflammatory mediators and results in a disruption in intracellular $Ca^{2+}$ homeostasis (5,6).

$Ca^{2+}$ signaling is essential for physiological muscle function. The intracellular concentration of $Ca^{2+}$ in myocytes is critical for muscle contraction and relaxation, and the regulation of intracellular $Ca^{2+}$ levels in skeletal muscle cells serves a central role in diaphragm function (7). In muscle cells, $Ca^{2+}$ is stored in the sarcoplasmic reticulum (SR). Upon its release from ryanodine receptors (RyRs) on the SR membrane into the cytoplasm, skeletal muscles contract; muscle relaxation is achieved following $Ca^{2+}$ reuptake into the SR, through the sarco/endoplasmic reticulum $Ca^{2+}$ adenosine triphosphatase (SERCA) (8).

Sepsis-induced impairments in diaphragm contractility and relaxation have been studied in the late phases of sepsis; however, during the acute phase of sepsis, the alterations in diaphragm function, and $Ca^{2+}$ release and uptake functions of the SR, have yet to be elucidated. In addition, the exact mechanisms underlying the sepsis-induced $Ca^{2+}$ overload and the compromise in diaphragm function remain to be explored, and previous studies have not focused on the $Ca^{2+}$ release and uptake functions of the SR which are essential for physiological muscle contraction. The present study aimed to

examine the alterations in diaphragm function during acute sepsis. Furthermore, it aimed to investigate the sepsis-induced compromise of $Ca^{2+}$ release and uptake, and explore the roles of RyR and SERCAs in the SR and the mechanisms underlying the dysfunction of diaphragm contraction and relaxation during the early phase of sepsis.

## Materials and methods

*Animals*. A total of 40 male Sprague-Dawley rats weighing 215-240 g and aged five weeks were purchased from the Shanghai Animal Research Centre (Shanghai, China). Upon arrival at the animal house, rats were maintained in cages (5 rats/cage) in an environmentally-controlled room (temperature, 21-24˚C; relative humidity, 40-60%), under a 12 h light/dark cycle. A 2-week acclimation period, during which standard rat food and water were offered *ad libitum*, preceded the experiment. Rats weighing 245±9.5 g were used for the experiments. All experimental procedures were approved in advance by the Ethics Committee of Shanghai General Hospital and Animal Care (Shanghai, China).

*Surgical procedure*. The cecal ligation and puncture (CLP) procedure, as described by Chaudry *et al* (9) was used for the induction of sepsis. A total of 36 rats were randomly assigned to 3 groups as follows (n=12 rats/group): Sham group, where rats underwent a sham operation; and 2 experimental sepsis groups: 6 h CLP, where rats underwent CLP and were sacrificed 6 h post-surgery; and 12 h CLP, where rats underwent CLP and were sacrificed 12 h post-surgery.

Rats were weighed and anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). A 2-cm lower midline abdominal incision was made, the cecum was exposed and ligated at a site distal to the ileocecal valve. The cecum was then punctured through both walls with an 18-gauge hollow needle, and was gently compressed until feces were extruded into the peritoneal cavity. Subsequently, the cecum was returned to the peritoneal cavity, and the peritoneal and skin incisions were closed. Rats were resuscitated via a subcutaneous bolus injection of sterile normal saline (30 ml/kg body weight) in the back. Following recovery from anesthesia, rats moved around freely in the cages. Rats in the sham group underwent the laparotomy procedure without ligation and puncture. As septic rats exhibit a decrease in food intake, feeding was restrained following surgery for all rats, in order to minimize the variation between groups (10).

*Sample preparation*. The surviving rats in the sham and CLP groups were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) after 6 h (6 h CLP group) and 12 h (sham group and 12 h CLP group) following CLP. Subsequently, the chest and upper abdomen were opened through a median incision of 4-5 cm under sterile conditions, and the thoracic cavity was opened through the incision at the edges of the two clearly exposed costal arches. Strips from the left diaphragm were rapidly removed from each rat, for the assessment of isometric contractile characteristics, while leaving the central tendon and rib cage intact. The right hemidiaphragm was removed, instantly frozen in liquid nitrogen, and stored at -80˚C for subsequent use in western blot analysis. A small piece of tissue (~10x4 mm) was immediately obtained from the right hemidiaphragm and immediately homogenized as described below for the SR functional assays.

*Assessment of muscle contractility*. Isolated left diaphragm strips from the rats in each group (width, ~5 mm) were immediately immersed in temperature-controlled organ chambers (volume, 40 ml) at 37˚C filled with Krebs solution. Strips were fixed at the rib cage and, using a 3-0 silk suture, were suspended at the central tendon from a force displacement transducer (ALC-AF; Shanghai Alcott Biotech Co., Ltd., Shanghai, China). The organ bath was continuously perfused with a 95% $O_2$ and 5% $CO_2$ mixture. The modified Krebs solution contained 137 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $KH_2PO_4$, 12 mM $NaHCO_3$, and 6.5 mM glucose; the pH was adjusted to 7.4-7.5 through aeration with $CO_2$. Strips were placed between 2 platinum stimulating electrodes, and 12 $\mu$M d-tubocurarine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the bath to abolish neuromuscular transmission (11). Following equilibration for 20 min in the organ bath, the strips were placed at the optimal length (Lo), which was determined as when the maximal tension was produced in a fixed electrical stimulation and the micro-positioner and stimulation voltage were adjusted to obtain the maximum twitch force. The micropositioner and stimulation voltage (15 V) were adjusted to obtain the maximum twitch force. The MPA2000 m *in vitro* experiment system (Shanghai Alcott Biotech Co., Ltd., Shanghai, China) was used to supply the electrical stimuli and measure the isometric twitch tension. The length of the diaphragm strips was also measured. The measurements described below were assessed using the stimulation at Lo.

*Twitch characteristics*. A total of 2 twitches (frequency, 0.1 Hz; pulse duration, 0.2 msec) were delivered at Lo to determine the following parameters: Peak twitch tension, time to peak tension, maximum rate of twitch force development (+dF/dt), and maximum rate of tension decline (-dF/dt). Half-relaxation time was measured during the decline phase as the time required for the tension to reach 50% of maximal value. Average values of 3 experiments were calculated and used for further analysis.

*Maximal tetanic tension*. The maximal tetanic tension was recorded as the maximal tension of the diaphragm strips that was elicited following stimulation at 120 Hz.

*Force-frequency relationship*. Diaphragm strips were stimulated (pulse duration, 0.2 msec; train duration, 250 msec) at various frequencies, ranging from 10-120 Hz over 10 Hz increments using 2 min intervals, and the force-frequency relationship of the muscle was determined.

*Fatigue protocol*. The fatigue properties of the muscle were determined using 330-msec stimulations (pulse duration, 0.2 msec; train duration, 670 msec) repeated every sec at 25 Hz for 5 min. The ratio of the contraction force obtained following 1 sec over that obtained following 120 sec of stimulation was determined and defined as the fatigue index (FI).

Following the assessment of the aforementioned parameters, the strips were blotted dry and weighed. All force values were normalized to cross-sectional areas, calculated by dividing the muscle weight by the muscle length and specific density, as previously described (12).

*SR $Ca^{2+}$ release and uptake*. The functional characteristics of the SR were assessed using crude muscle homogenates. Following weighing, the right hemidiaphragm was placed in ice-cold homogenization buffer containing 250 mM sucrose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 0.2% sodium azide and 0.2 mM phenylmethylsulfonyl fluoride (pH 7.5). The tissue was minced and homogenized on ice using a rotor tissue homogenizer (Tissue-Tearor 985370; BioSpec Products, Inc., Bartlesville, OK, USA) with 15 sec bursts at 15,000 rpm repeated 3 times in 10 volumes of fresh buffer. The duration of tissue extraction and homogenization was typically 10 min. The homogenates were centrifuged at 1,600 x g for 15 min at 4˚C. The supernatants were harvested, rapidly frozen in liquid nitrogen and stored at -80˚C until use in the SR function assay. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay.

The kinetics of $Ca^{2+}$ uptake were evaluated by $Ca^{2+}$ imaging using the fluorescent $Ca^{2+}$-binding dye fura-2 (pentapotassium salt; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) on a Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described (12,13) and validated using SR vesicle preparations (14). The incubation buffer contained 100 mM KCl, 20 mM HEPES, 7.5 mM pyrophosphate, and 0.5 mM $Mg^{2+}$ (pH 7.0) and the temperature was maintained at 37˚C. Free $Ca^{2+}$ (2 $\mu$M) and fura-2 pentapotassium salt (1 $\mu$M) were added as the $Ca^{2+}$ indicator. Fura-2 was excited at a wavelength of 340 and 380 nm, and fluorescence emission was detected at 500 nm. A total of 10 $\mu$l protein homogenate and 190 $\mu$l incubation buffer were loaded onto black plates. $Ca^{2+}$ uptake was initiated following the addition of 20 mM $Na_2ATP$ (10 $\mu$l). $Ca^{2+}$ release was initiated 4 min following the addition of $Na_2ATP$, by adding 500 $\mu$M $AgNO_3$ (10 $\mu$l). $Na_2ATP$ and $AgNO_3$ were injected at a speed of 420 $\mu$l/sec into the wells using the instrument dispensers, and the plates were agitated. Between measurements, the dispensers were soaked in 70% alcohol for 30 min twice and rinsed in distilled water 10 times. The fluorescence signals were continuously recorded and the experiment was performed at 20-22˚C. The ratio of the fluorescence at the excitation wavelengths (340 and 380 nm), following background subtraction, was converted using SkanIt version 2.4.5 for Varioskan Flash (Thermo Fisher Scientific, Inc.) into free $Ca^{2+}$ concentration [$Ca^{2+}$], based on the ratiometric method of Grynkiewicz *et al* (15). Free [$Ca^{2+}$] levels were calculated according to the following formula: $[Ca^{2+}]=K_d x(S_{f2}/S_{b2})x(R-R_{min})/(R_{max}-R)$, where $K_d$ represents the equilibrium constant for the interaction between $Ca^{2+}$ and fura-2; R represents the ratio of fluorescence recorded at 340 and 380 nm; $R_{min}$ and $R_{max}$ are the ratios of fluorescence under $Ca^{2+}$-free and $Ca^{2+}$-saturating conditions, as determined at the completion of the assay following the addition of 3.5 mM EGTA and 5.0 mM $CaCl_2$, respectively (16) and $S_{f2}$ and $S_{b2}$ represent the fluorescence under $Ca^{2+}$-free and $Ca^{2+}$-saturating conditions, respectively. $R_{min}$, $R_{max}$, $S_{f2}$ and $S_{b2}$ were calculated following each run. The maximum rate of SR $Ca^{2+}$ uptake and release was calculated using the steepest negative and positive slope of the free [$Ca^{2+}$] vs. time curve, respectively, and normalized to the total protein concentration of the supernatants. Uptake and release assays were performed in triplicate for the 12 samples from each group.

*Western blot analysis*. Diaphragm tissue samples isolated at 6 and 12 h following the induction of sepsis or the sham operation were used for western blot analysis. Tissues were homogenized (Tissue-Tearor 985370; BioSpec Products, Inc., Bartlesville, OK, USA) on ice for 5 20-sec bursts at intervals of 20 sec in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 mM phenylmethylsulfonyl fluoride (Amresco, Solon, OH, USA). The obtained homogenate was centrifuged at 15,000 x g for 15 min at 4˚C. Protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts (50 $\mu$g) of extracted protein samples were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk for 1 h at room temperature with agitation, membranes were probed at 4˚C overnight with the following primary antibodies: Anti-RyR (ab2868; 1:5,000; Abcam, Cambridge, UK), anti-SERCA1 (ab2819; 1:1,000; Abcam), anti-SERCA2 (ab2861; 1:1,000; Abcam), and anti-GAPDH (KC-5G4; 1:5,000; KangChen Biotech, Shanghai, China). The membranes were then incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibodies (A0216; 1:5,000; Beyotime Institute of Biotechnology) and protein bands were visualized by enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA). Blots were semi-quantified (Image Pro Plus version 6.0, Media Cybernetics, USA) by densitometry and protein expression was normalized to GAPDH as an internal reference.

*Statistical analysis*. All data were obtained from three separate experiments and are expressed as the mean ± standard error of the mean. The statistical significance of the differences between groups was assessed using an unpaired Student's t-test for pair-wise comparisons or one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. The $Ca^{2+}$ curves were fitted smoothly by the values of the time points using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Physical examination*. No differences in body weight were detected between rats belonging to the sham and the CLP groups (data not shown). The width of the diaphragm strip that was used for the assessment of muscle contractility ranged between 4 and 6 mm for all groups. In addition, no differences were observed in total protein concentration in muscle tissue samples between groups (data not shown). Septic rats demonstrated symptoms including chills, hair erection, ocular exudates and diarrhea, which were not present

Table I. Contractile parameters of the diaphragm for rats in the sham, 6 h CLP and 12 h CLP groups.

| | Sham | 6 h CLP | 12 h CLP |
|---|---|---|---|
| Pt (mN $mm^{-2}$) | 3.49±0.66 | 3.38±0.81 | 2.45±0.65[b,c] |
| Po (mN $mm^{-2}$) | 8.98±1.87 | 8.87±1.51 | 6.75±1.54[b,c] |
| +dF/dt (mN $mm^{-2}$ $s^{-1}$) | 0.35±0.05 | 0.34±0.06 | 0.23±0.05[b,c] |
| -dF/dt (mN $mm^{-2}$ $s^{-1}$) | 0.31±0.05 | 0.26±0.07[a] | 0.17±0.04[b,c] |
| 1/2 RT (ms) | 8.41±0.46 | 9.12±0.74[a] | 10.05±0.84[b,c] |
| TPT (ms) | 18.15±0.58 | 18.18±0.76 | 22.49±1.12[b,c] |
| FI (%) | 83.1±5.9 | 79.2±9.2 | 81.7±10.5 |

Sham group (n=12), rats underwent a sham operation; 6 h CLP group (n=12), rats underwent CLP and were assessed 6 h post-surgery; 12 h CLP group (n=12), rats underwent CLP and were assessed 12 h post-surgery. Data are expressed as the mean ± standard error of the mean. [a]P<0.05, [b]P<0.01 vs. sham; [c]P<0.01 vs. 6 h CLP. CLP, cecal ligation and puncture; Pt, peak twitch tension; Po, peak tetanic tension; +dF/dt, maximum rate of twitch force development; -dF/dt, maximum rate of tension decline; 1/2 RT, half-relaxation time; TPT, time to peak tension; FI, fatigue index.

among sham-operated rats. Septic rats became lethargic and spontaneous activity was progressively reduced during the experiments. A single case of mortality occurred in the 12 h CLP group (mortality rate, 8.3%), and the rat was eliminated from the study; however, no cases of mortality occurred in the 6 h CLP and sham groups.

*Muscle contractile characteristics*. The contractile characteristics of diaphragm tissue samples isolated from surviving rats were investigated in the present study and are presented in Table I. Rats in the 12 h CLP group demonstrated significantly decreased peak twitch and tetanic tension, +dF/dt and -dF/dt compared with sham-operated rats (P<0.01; Table I). Rats in the 6 h CLP group exhibited a significant decrease in-dF/dt compared with the sham group, whereas +dF/dt remained unaffected. Conversely, the half-relaxation time in rats from the CLP groups was significantly increased compared with rats from the sham group, whereas the time to peak tension was significantly prolonged in rats from the 12 h CLP group (P<0.01; Table I). A statistically significant difference (P<0.01) was detected in all measured parameters between rats in the 6 and 12 h CLP groups, with the exception of the FI (Table I). No difference in FI was observed between rats belonging to the various groups.

Force-frequency curves were plotted for the diaphragm strips in response to stimuli of various frequencies, following normalization (Fig. 1). The contractile force of rat diaphragms from the 12 h CLP group was significantly lower compared with in the 6 h CLP and sham groups (P<0.05; Fig. 1). Conversely, the force of diaphragm contraction following stimulation was similar between rats in the 6 h CLP and sham groups (Fig. 1).

*SR $Ca^{2+}$ uptake and release*. A schematic representation of the $Ca^{2+}$ uptake and release imaging process is included in Fig. 2A. $Ca^{2+}$ imaging was conducted according to the fluorimetric method; both the initial fast phase and the second slower phase of $Ca^{2+}$ release were measured in the present study, as previously recommended (17). The fluctuation in free $Ca^{2+}$ concentration in diaphragm samples from all groups is presented in Fig. 2B. The maximum rate of SR $Ca^{2+}$ uptake induced by ATP was

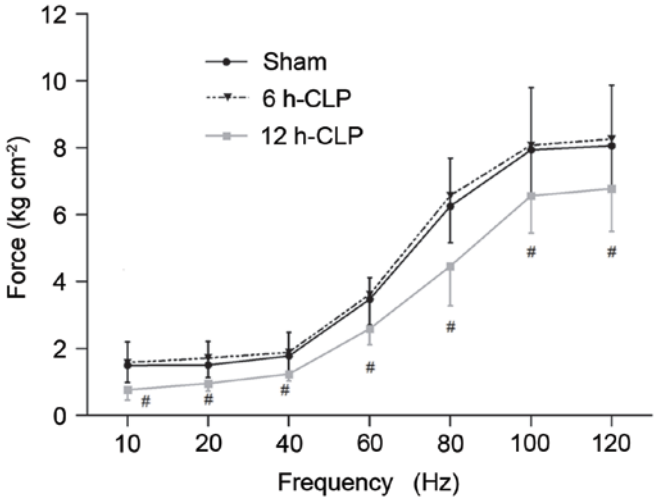


Figure 1. Force-frequency relationship of diaphragm skeletal muscle strips isolated from rats in the various treatment groups (n=12 rats/group). Sham group, rats underwent a sham operation; 6 h CLP group, rats underwent CLP and were assessed 6 h post-surgery; 12 h CLP group, rats underwent CLP and were assessed 12 h post-surgery. The force-frequency curve of 12 h CLP rats exhibited a significant downward shift compared with the sham group, indicating that the force of contraction following stimulation of varying frequency was significantly decreased. Data are presented as the mean ± standard error of the mean. #P<0.05 vs. sham group. CLP, cecal ligation and puncture.

significantly decreased in diaphragm muscle tissue of rats 6 and 12 h following CLP compared with the sham group (P<0.01; Fig. 2C). In addition, 12 h post-CLP, the maximum $AgNO_3$-induced $Ca^{2+}$ release rate of the SR was significantly decreased compared with the sham groups (P<0.01; Fig. 3). SERCA2 levels remained unaltered across the various groups. Representative SERCA1, SERCA2 and RyR blots for samples from the sham and CLP groups are presented in Fig. 3.

## Discussion

Sepsis is a severe systemic inflammatory response syndrome which is responsible for the high rates of mortality of patients

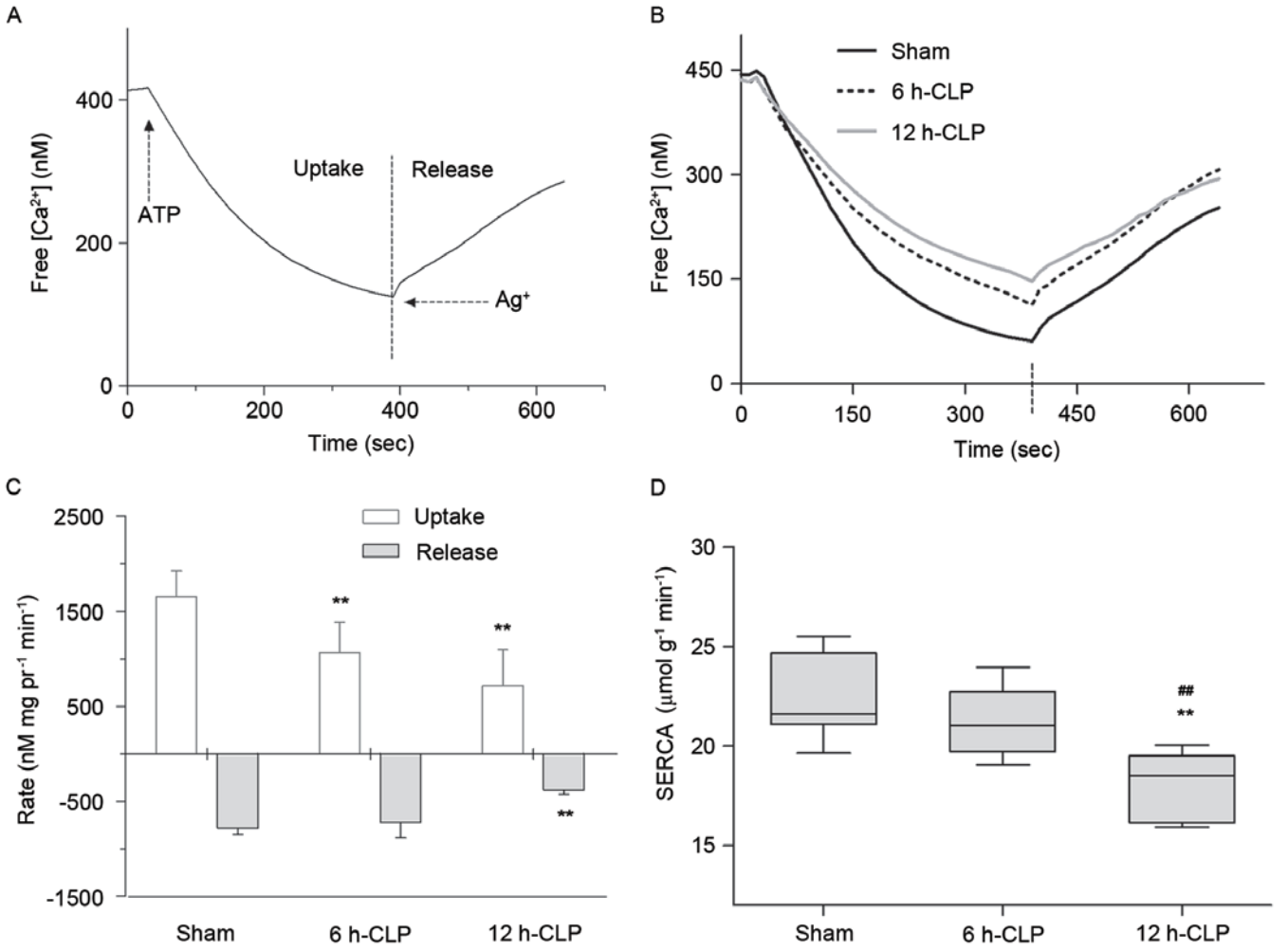


Figure 2. $Ca^{2+}$ kinetics in the SR of diaphragm skeletal muscle isolated from rats in the various treatment groups (n=12 rats/group). Sham group, rats underwent a sham operation; 6 h CLP group, rats underwent CLP and were assessed 6 h post-surgery; 12 h CLP group, rats underwent CLP and were assessed 12 h post-surgery. (A) Fluctuations in free $Ca^{2+}$ concentration in crude muscle homogenates measured with the $Ca^{2+}$-binding dye Fura-2. The maximal rate of SR $Ca^{2+}$ uptake and release were calculated using this curve. (B) Fitted curves demonstrating the kinetics of $Ca^{2+}$ release and uptake in the sham and CLP groups. (C) Peak rates of SR ATP-induced $Ca^{2+}$ uptake and $AgNO_3$-stimulated $Ca^{2+}$ release in diaphragm muscle tissue samples. (D) SERCA activity in diaphragm tissue. Data are presented as the mean ± standard error of the mean. $^{**}$P<0.01 vs. sham group; $^{\#\#}$P<0.01 vs. 6 h CLP group. SR, sarcoplasmic reticulum; CLP, cecal ligation and puncture; ATP, adenosine triphosphate; SERCA, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase; $[Ca^{2+}]$, $Ca^{2+}$ concentration.

in intensive care units. Critical illness myopathy (CIM) may occur in critically ill patients and may be a result of sepsis; its morbidity may reach 60-80% (18), and therapeutic strategies targeting the acute stage of CIM are currently unavailable. The compromising effects of sepsis are more pronounced in respiratory muscles compared with in peripheral muscles; among them, sepsis-induced diaphragm dysfunction may have deleterious consequences for patients (19), including pneumonia and respiratory failure (4). Therefore, understanding the mechanisms of sepsis-induced diaphragm dysfunction is critical for the development of effective therapeutic strategies targeting the acute stages of sepsis.

The CLP rat model has been extensively applied in sepsis-associated research, due to its high similarity to the pathophysiology of human sepsis. In the present study, CLP was performed according to standard procedures, as described by Rittirsch *et al* (20); the mid-grade CLP model (ligation of half of the cecum) was used to mimic sepsis.

The contractile characteristics of the diaphragm during sepsis have been previously investigated: Peritoneal cavity inflammation has been demonstrated to reduce the maximal force of diaphragm contraction (21). Conversely, Fujimura *et al* (22) revealed that the peak twitch tension did not decrease 4 h following CLP, in accordance with the present study; similar results were obtained regarding the twitch tension and contractile force of the septic diaphragm in response to various stimulation frequencies 10, 12 and 24 h following the induction of sepsis. Similarly, in the present study, the twitch and tetanic tension of the diaphragm were decreased 12 h following CLP. In addition, the time to peak tension was prolonged, +dF/dt was decreased, and the curve of the force-frequency association shifted downward in the 12 h CLP group. These results suggested that a compromise in the contractile abilities of the diaphragm occurred at 12, however not at 6 h post-CLP, thus indicating that diaphragm muscle force may deteriorate earlier than the previously reported 24 h post-CLP time point (23). Furthermore, 6 and 12 h following CLP, the half-relaxation time was significantly prolonged, whereas-dF/dt was decreased in septic rats compared with in the sham group. These findings suggested that during the

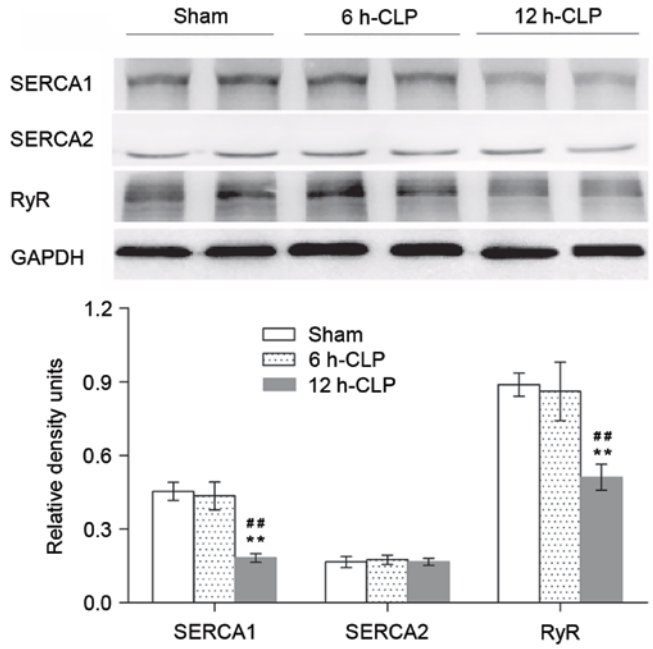


Figure 3. Expression of proteins associated with $Ca^{2+}$ release and uptake in diaphragm skeletal muscle isolated from rats in the various treatment groups (n=12 rats/group), as assessed using western blot analysis. Sham group, rats underwent a sham operation; 6 h CLP group, rats underwent CLP and were assessed 6 h post-surgery; 12 h CLP group, rats underwent CLP and were assessed 12 h post-surgery. Representative blots are included for SERCA1, SERCA2 and RyR. Densitometric analysis revealed that SERCA1 and RyR protein expression levels were significantly downregulated 12 h post-CLP. Data are presented as the mean ± standard error of the mean. $^{**}P<0.01$ vs. sham group; $^{\#\#}P<0.01$ vs. 6 h CLP group. CLP, cecal ligation and puncture; SERCA, sarco/endoplasmic reticulum $Ca^{2+}$-adenosine triphosphatase; RyR, ryanodine receptor.

acute phase of sepsis, the relaxation function of the diaphragm may be injured at a time point prior to the onset of contractile dysfunction.

Previous research has suggested several mechanisms underlying sepsis-induced diaphragm dysfunction, including increased cytokine release, oxygen free radical generation and mitochondrial damage (24,25). However, little has been revealed regarding the $Ca^{2+}$ release and uptake functions of the SR, which are critical for the maintenance of the contraction-relaxation cycle. Following T-tubule depolarization, $Ca^{2+}$ is released into the myoplasm from its SR stores to activate crossbridge cycling and produce the contractile force, via $Ca^{2+}$ channels. Subsequently, $Ca^{2+}$ reuptake into the SR via SERCA, which is an energy-dependent process, results in muscle relaxation (26). The mechanisms underlying the contractile dysfunction of the diaphragm during sepsis may involve the dysregulation of intracellular and SR $Ca^{2+}$ levels, an impairment in $Ca^{2+}$ release during the contraction sequence, and myofilament dysfunction (27).

In the present study, $Ca^{2+}$ imaging using a fluorescent dye was used to investigate SR function and SERCA activity, via measuring $Ca^{2+}$ release and uptake in diaphragm muscle tissue homogenates. The present results revealed that the maximum rate of $Ca^{2+}$ uptake was decreased 6 and 12 h following the induction of sepsis; however, the maximum rate of $Ca^{2+}$ release and SERCA activity were significantly suppressed only in the 12 h CLP group compared with in sham-operated rats. $Ca^{2+}$ release and uptake in the SR of myocytes may be reflected by twitch kinetics (28), whereas SERCA activity predominately regulates the duration of relaxation. Therefore, the decrease in twitch tension, contraction and relaxation speed, SERCA activity, and the prolonged half relaxation time that were observed in the present study, may be indicative of a dysfunction of the SR 6 or 12 h following induction of CLP. Due to impaired $Ca^{2+}$ SR reuptake, a dysfunction in diaphragm relaxation was observed 6 and 12 h post-CLP. In a previous study using a light sepsis model, $Ca^{2+}$ uptake in the rat myocardium was impaired at 18, however not 9 h following the induction of sepsis (29). The apparent inconsistency may be attributed to variations in sepsis severity, and the different local or systemic effects associated with the model. The different grades of sepsis can result in differing plasma levels of proinflammation factors, including HMGB1 and TNF-α, and the survival rates. Larger percentages of cecum ligation can lead to more local tissue necrosis and more severe local inflammatory reactions (20,30). During the development of sepsis, an increase in oxygen free radical generation has been revealed to result in diaphragm dysfunction and impair the $Ca^{2+}$ release capabilities of the SR (31). In addition, the function of intracellular organelles, including the SR, have been demonstrated to be compromised during sepsis (32).

The activation of RyRs, which are $Ca^{2+}$ release channels of the SR, lead to a massive $Ca^{2+}$ release that initiates muscle contraction. The primary type of RyR expressed in diaphragm muscle fibers is RyR1, which serves a critical role in excitation-contraction coupling; RyR2 is absent in skeletal muscle, and sepsis has not been revealed to affect RyR3 contents, which constitute only ~3% of the total RyRs (33). Therefore, RyR protein content in diaphragm fibers primarily reflects the RyR1 expression levels. In the present study, rats in the 12 h CLP group exhibited a decrease in diaphragm tension, which was accompanied by a decrease in the peak rate of $Ca^{2+}$ release. When RyR protein contents were assessed, the results indicated that the RyR protein expression levels were downregulated 12 h post-CLP; these findings were consistent with the author's previous study conducted at 24 h following sepsis (23). Therefore, it may be hypothesized that the reduced tension of the diaphragm muscle and the impairments in $Ca^{2+}$ release from the SR are associated with the reduced RyR protein levels in the tissue.

$Ca^{2+}$ released through RyRs is subsequently reuptaken into the SR via SERCA, upon which muscle relaxation occurs. In the adult rat diaphragm, the proportion of Type I (slow-twitching) and II (fast-twitching) muscle fibers are ~33 and 67%, respectively (34). SERCA1 is the predominant isoform expressed in Type II fibers, whereas SERCA2 is primarily expressed in Type I fibers in adult mammalian skeletal muscles (35); therefore, the overall expression levels of SERCA are reflected by the assessment of the 2 isoforms in the adult diaphragm (36). Low SERCA expression levels have been associated with dysfunctions in muscle relaxation (37). Previous studies have demonstrated that 24 h post-CLP, the expression of SERCA1 and SERCA2 was significantly downregulated in cardiac and diaphragm myofibers (23,38). In the present study, rats of the 12 h CLP group exhibited a significant decrease in SERCA1

protein expression; however, the protein levels of SERCA2 remained unaltered. The apparent discrepancy may be attributed to the high susceptibility of Type II fibers to several pathological conditions, due to the unique characteristics of the fast-twitch fibers (34,39). As the development of sepsis progresses, SERCA2 levels may be affected, as previously demonstrated at 24 h following sepsis induction (23). Furthermore, the observed impairments in diaphragm relaxation and the compromise in $Ca^{2+}$ SR uptake in 12 h CLP rats were consistent with the alterations in SERCA1 expression. However, in 6 h CLP rats, SERCA expression remained unaffected. In addition, the $Ca^{2+}$ release capabilities of the SR depend on the $Ca^{2+}$ stores uptaken from the cytoplasm via SERCA; therefore, the contractile function of the muscle may be affected by the $Ca^{2+}$ uptake function of the SR and the protein expression levels of SERCA.

In conclusion, the results of the present study indicated that during the acute stage of CLP-induced sepsis, the contractile and relaxation functions of the diaphragm appeared to decline. The dysfunctions in relaxation may be a result of the reduced $Ca^{2+}$ uptake function of the SR and the downregulation in SERCA1 expression, whereas the impairments in contractility may be associated with a compromise in the SR $Ca^{2+}$ release function and the decreased RyR expression levels in diaphragm muscle tissue.

## Acknowledgements

The authors would like to thank Dr Tao Lang and Dr Min Li for their excellent technical assistance and strong support. The present study was supported by the National Natural Science Foundation of China (grant no. 81171845) and the Shanghai Natural Science Foundation (grant no. 14ZR1438100).

## References

1. Bhatia M, He M, Zhang H and Moochhala S: Sepsis a model of SIRS. Front Biosei (Landmark Ed) 14: 4703-4711, 2009.
2. Maes K, Stamiris A, Thomas D, Cielen N, Smuder A, Powers SK, Leite FS, Hermans G, Decramer M, Hussain SN and Gayan-Ramirez G: Effects of controlled mechanical ventilation on sepsis-induced diaphragm dysfunction in rats. Crit Care Med 42: e772-e782, 2014.
3. De Jonghe B, Sharshar T, Lefaucheur JP, Authier FJ, Durand-Zaleski I, Boussarsar M, Cerf C, Renaud E, Mesrati F, Carlet J, *et al*: Paresis acquired in the intensive care unit: A prospective multicenter study. JAMA 288: 2859-2867, 2002.
4. Kress JP and Hall JB: ICU-acquired weakness and recovery from critical illness. N Engl J Med 370: 1626-1635, 2014.
5. Griffiths RD and Hall JB: Intensive care unit-acquired weakness. Crit Care Med 38: 779-787, 2010.
6. Latronico N and Bolton CF: Critical illness polyneuropathy and myopathy: A major cause of muscle weakness and paralysis. Lancet Neurol 10: 931-941, 2011.
7. Melzer W, Herrmann-Frank A and Lüttgau HC: The role of Ca2+ ions in excitation-contraction coupling of skeletal muscle fbres. Biochim Biophys Acta 1241: 59-116, 1995.
8. Weisleder N and Ma J: Altered Ca2+ sparks in aging skeletal and cardiac muscle. Ageing Res Rev 7: 177-188, 2008.
9. Chaudry IH, Wichterman KA and Baue AE: Effect of sepsis on tissue adenine nucleotide levels. Surgery 85: 205-211, 1979.
10. Mikawa K, Nishina K, Kodama S and Obara H: Propofol attenuates diaphragmatic dysfunction induced by septic peritonitis in hamsters. Anesthesiology 94: 652-660, 2001.
11. Narimatsu E, Niiya T, Kawamata M and Namiki A: Sepsis stage dependently and differentially attenuates the effects of nondepolarizing neuromuscular blockers on the rat diaphragm in vitro. Anesth Analg 100: 823-829, 2005.
12. Robinson JA, Jenkins NS, Holman NA, Roberts-Thomson SJ and Monteith GR: Ratiometric and nonratiometric Ca2+ indicators for the assessment of intracellular free Ca2+ in a breast cancer cell line using a fluorescence microplate reader. J Biochem Biophys Methods 58: 227-237, 2004.
13. Ingalls CP, Warren GL, Williams JH, Ward CW and Armstrong RB: E-C coupling failure in mouse EDL muscle after in vivo eccentric contractions. J Appl Physiol (1985) 85: 58-67, 1998.
14. Nishikawa K, Narimatsu E, Igarashi M and Namiki A: Effects of sepsis on the neuromuscular blocking actions of d-tubocurarine on rat adductor and abductor laryngeal muscles. J Anesth 23: 520-525, 2009.
15. Grynkiewicz G, Poenie M and Tsien RY: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440-3450, 1985.
16. Gigli I and Bussmann LE: Effects of exercise on muscle metabolites and sarcoplasmic reticulum function in ovariectomized rats. Physiol Res 51: 247-254, 2002.
17. Russ DW, Grandy JS, Toma K and Ward CW: Ageing, but not yet senescent, rats exhibit reduced muscle quality and sarcoplasmic reticulum function. Acta Physiol (Oxf) 201: 391-403, 2011.
18. Lacomis D, Zochodne DW and Bird SJ: Critical illness myopathy. Muscle Nerve 23: 1785-1788, 2000.
19. Maes K, Stamiris A, Thomas D, Cielen N, Smuder A, Powers SK, Leite FS, Hermans G, Decramer M, Hussain SN, *et al*: Effects of controlled mechanical ventilation on sepsis-induced diaphragm dysfunction in rats. Crit Care Med 42: e772-e782, 2014.
20. Rittirsch D, Huber-Lang MS, Flierl MA and Ward PA: Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc 4: 31-36, 2009.
21. Krause KM, Moody MR, Andrade FH, Taylor AA, Miller CC III, Kobzik L and Reid MB: Peritonitis causes diaphragm weakness in rats. Am J Respir Crit Care Med 157: 1277-1282, 1998.
22. Fujimura N, Sumita S and Narimatsu E: Alteration in diaphragmatic contractility during septic peritonitis in rats: Effect of polyethylene glycol-absorbed superoxide dismutase. Crit Care Med 28: 2406-2414, 2000.
23. Wu J, Zhang JY, Gong Y and Li ST: Slowed relaxation of diaphragm in septic rats is associated with reduced expression of sarco-endoplasmic reticulum Ca2+-ATPase genes SERCA1 and SERCA 2. Muscle Nerve 54: 1108-1113, 2016.
24. Shindoh C, Hida W, Ohkawara Y, Yamauchi K, Ohno I, Takishima T and Shirato K: TNF-alpha mRNA expression in diaphragm muscle after endotoxin administration. Am J Respir Crit Care Med 152: 1690-1696, 1995.
25. Kobzik L, Reid MB, Bredt DS and Stamler JS: Nitric oxide in skeletal muscle. Nature 372: 546-548, 1994.
26. MacLennan DH, Rice WJ and Green NM: The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca2-ATPases. J Biol Chem 272: 28815-28818, 1997.
27. Reid MB, Lännergren J and Westerblad H: Respiratory and limb muscle weakness induced by tumor necrosis factor-alpha: Involvement of muscle myofilaments. Am J Respir Crit Care Med 166: 479-484, 2002.
28. Nethery D, DiMarco A, Stofan D and Supinski G: Sepsis increases contraction-related generation of reactive oxygen species in the diaphragm. J Appl Physiol (1985) 87: 1279-1286, 1999.
29. Wu LL, Ji Y, Dong LW and Liu MS: Calcium uptake by sarcoplasmic reticulum is impaired during the hypodynamic phase of sepsis in the rat heart. Shock 15: 49-55, 2001.
30. Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M, Mackay CR, Zetoune FS, Gerard NP, Cianflone K, Köhl J, *et al*: Functional roles for C5a receptors in sepsis. Nat Med 14: 551-557, 2008.
31. Danjo W, Fujimura N and Ujike Y: Effect of pentoxifylline on diaphragmatic contractility in septic rats. Acta Med Okayama 62: 101-107, 2008.
32. Supinski G, Nethery D, Nosek TM, Callahan LA, Stofan D and DiMarco A: Endotoxin administration alters the force vs. pCa relationship of skeletal muscle fibers. Am J Physiol Regul Integr Comp Physiol 278: R891-R896, 2000.
33. Jiao GY, Hao LY, Gao CE, Chen L, Sun XF, Yang HL, Li Y and Dai YN: Reduced DHPRα1S and RyR1 expression levels are associated with diaphragm contractile dysfunction during sepsis. Muscle Nerve 48: 745-751, 2013.
34. Qiu YW, Chen D, Xu MY and Li ST: Beneficial effects of dantrolene on sepsis-induced diaphragmatic dysfunction are associated with downregulation of high-mobility group box 1 and calpain-caspase-3 proteolytic pathway. J Surg Res 200: 637-647, 2016.

35. Lamboley CR, Murphy RM, McKenna MJ and Lamb GD: Endogenous and maximal sarcoplasmic reticulum calcium content and calsequestrin expression in type I and type II human skeletal muscle fibres. J Physiol 591: 6053-6068, 2013.
36. Shortt CM, Fredsted A, Bradford A and O'Halloran KD: Diaphragm muscle remodeling in a rat model of chronic intermittent hypoxia. J Histochem Cytochem 61: 487-499, 2013.
37. Guglielmi V, Vattemi G, Gualandi F, Voermans NC, Marini M, Scotton C, Pegoraro E, Oosterhof A, Kósa M, Zádor E, *et al*: SERCA1 protein expression in muscle of patients with Brody disease and Brody syndrome and in cultured human muscle fibers. Mol Genet Metab 110: 162-169, 2013.
38. Akasaki Y, Ouchi N, Izumiya Y, Bernardo BL, Lebrasseur NK and Walsh K: Glycolytic fast-twitch muscle fiber restoration counters adverse age-related changes in body composition and metabolism. Aging Cell 13: 80-91, 2014.
39. Wang Y and Pessin JE: Mechanisms for fiber-type specificity of skeletal muscle atrophy. Curr Opin Clin Nutr Metab Care 16: 243-250, 2013.