Up-regulation of Intracellular Calcium Handling Underlies the Recovery of Endotoxemic Cardiomyopathy in Mice

Justin C. Morse, Joanne Huang, Natasha Khona, Edward J. Miller, M.D., Ph.D., Deborah A. Siwik, Ph.D., Wilson S. Colucci, M.D., Ion A. Hobai, M.D., Ph.D.

ABSTRACT

Background: In surviving patients, sepsis-induced cardiomyopathy is spontaneously reversible. In the absence of any experimental data, it is generally thought that cardiac recovery in sepsis simply follows the remission of systemic inflammation. Here the authors aimed to identify the myocardial mechanisms underlying cardiac recovery in endotoxemic mice.

Methods: Male C57BL/6 mice were challenged with lipopolysaccharide (7 μg/g, intraperitoneally) and followed for 12 days. The authors assessed survival, cardiac function by echocardiography, sarcomere shortening, and calcium transients (with fura-2-acetoxymethyl ester) in electrically paced cardiomyocytes (5 Hz, 37°C) and myocardial protein expression by immunoblotting.

Results: Left ventricular ejection fraction, cardiomyocyte sarcomere shortening, and calcium transients were depressed 12 h after lipopolysaccharide challenge, started to recover by 24 h (day 1), and were back to baseline at day 3. The recovery of calcium transients at day 3 was associated with the up-regulation of the sarcoplasmic reticulum calcium pump to 139 ± 19% (mean ± SD) of baseline and phospholamban down-regulation to 35 ± 20% of baseline. At day 6, calcium transients were increased to 123 ± 31% of baseline, associated with increased sarcoplasmic reticulum calcium load (to 126 ± 32% of baseline, as measured with caffeine) and inhibition of sodium/calcium exchange (to 48 ± 12% of baseline).

Conclusions: In mice surviving lipopolysaccharide challenge, the natural recovery of cardiac contractility was associated with the up-regulation of cardiomyocyte calcium handling above baseline levels, indicating the presence of an active myocardial recovery process, which included sarcoplasmic reticulum calcium pump activation, the down-regulation of phospholamban, and sodium/calcium exchange inhibition. **(Anesthesiology 2017; 126:1125-38)**

PATIENTS with sepsis and septic shock may develop cardiac dysfunction (also known as sepsis-induced cardiomyopathy) that complicates their management and aggravates prognosis.[1] In surviving patients, sepsis-induced cardiomyopathy is spontaneously reversible within 7 to 10 days from the onset of sepsis.[2,3] In the absence of any experimental data, it is generally thought that the recovery of the heart function during sepsis resolution is due to the remission of the inciting inflammatory triggers.

Sepsis-induced cardiomyopathy develops primarily as the result of the dysregulation of myocardial Ca^{2+} handling,[4] including the dysfunction of the L-type Ca^{2+} channel,[5,6] the sarcoplasmic reticulum Ca^{2+} pump (SERCA),[6,7] and the sarcoplasmic reticulum Ca^{2+} release channels (ryanodine receptors).[7,8] We were curious to see what happens with the dysfunctional Ca^{2+} handling mechanisms during the recovery phase of sepsis-induced cardiomyopathy. We hypothesized that, if the resolution of cardiomyopathy was due to

What We Already Know about This Topic

- Septic shock remains a major cause of morbidity and mortality and can be exacerbated by subsequent cardiomyopathy
- One remarkable feature of sepsis-induced cardiomyopathy is its spontaneous reversibility in surviving patients
- The molecular mechanisms related to the recovery phase of sepsis-induced cardiomyopathy are unknown

What This Article Tells Us That Is New

- The authors have performed a functional and molecular assessment of myocardial calcium handling in surviving mice in a preclinical model of sepsis
- The authors have found a supernormal augmentation of heart function and myocytes calcium handling during the recovery phase of sepsis-induced cardiomyopathy that was associated with distinct changes in the expression and function of calcium-handling proteins in the heart
- The work suggests the existence of an active myocardial recovery mechanism in sepsis, with significant implications toward possible future therapies

Preliminary results have been presented in abstract form at the annual meeting of the Biophysical Society in San Francisco, California, February 14 to 19, 2014.

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the remission of systemic inflammation, then the Ca^{2+} transporters should recover gradually, from their initial decline, back to baseline levels, in parallel with the resolution of the inflammatory shock state.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from Jackson Laboratory (USA) and studied at the age of 15 to 25 weeks (20 to 30 g).

Sepsis Model

Lipopolysaccharide (LPS; Sigma, USA) was administered intraperitoneally at doses of 4 and 7 μg/g of weight. A volume of 0.5 ml of warm (37°C), sterile normal saline was administered intraperitoneally with LPS to mitigate the ensuing hypovolemia. Mice were checked twice daily until the surviving mice recovered fully and showed normal mobility, feeding, and grooming behavior. Mice that showed diminished mobility were resuscitated with 0.5 ml of normal saline twice daily, intraperitoneally. At various times after LPS administration (from 12 h to 12 days), mice were euthanized with a pentobarbital overdose (10 mg intraperitoneally, together with 200 U of heparin).[9] After euthanasia, hearts were removed and used for cardiomyocyte isolation or immunoblotting. All of the animal procedures were conducted in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine (Boston, Massachusetts).

Mortality Assessments

Mortality was assessed twice daily. Per Institutional Animal Care and Use Committee recommendations, mice that were found to be *in extremis* (immobile and showing agonal breathing) were euthanized and included in the mortality count at the time of assessment.

Echocardiography

Two-dimensional and M-mode echocardiography of left ventricular function was performed in isoflurane anesthetized mice using a Vevo 770 high-resolution machine (VisualSonics, Canada) and a 30-MHz transducer, as described previously.[10] The primary outcome was left ventricle ejection fraction. Secondary outcomes included the total wall thickness of the left ventricle, measured as the sum of the anterior and posterior wall dimensions in diastole, and left ventricle internal diameter, which was also measured in diastole.

Isolated Myocyte Studies

Isolation of cardiomyocytes, measurement of cell contractility and Ca^{2+} handling were performed as described previously.[6] Briefly, left ventricle cardiomyocytes were isolated enzymatically, placed in a physiologic Tyrode solution (containing, in mM: NaCl 137.0, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 0.5, HEPES 10.0, glucose 5.0, and probenecid 0.5 [pH 7.40]) and externally paced at 37°C. Probenecid was added to the superfusing solution to increase fura-2 retention. Experiments shown here were performed at a pacing frequency of 5 Hz, with similar data being obtained at 2 Hz.

Cardiomyocyte sarcomere length and intracellular Ca^{2+} levels (using fura-2-acetoxymethyl ester, Molecular Probes, USA) were measured simultaneously using an integrated system (IonOptix, USA) featuring a HyperSwitch dual 340- to 380-nm excitation light source. Fura ratios recorded in cardiomyocyte are shown as raw signals, without attempting a calibration for free Ca^{2+}, due to uncertainties inherent to the calibration procedure.[11] We regularly perform an *in vitro* calibration using fura-2 K salt (Molecular Probes) to confirm the dynamic range of the Ca^{2+} imaging setup. Minimum R (measured using droplets of the Tyrode solution given above, Ca^{2+}-free, and with 1 mM EGTA) was 0.93 ± 0.01. Maximum R (Tyrode solution with 3 mM Ca^{2+}) was 4.27 ± 0.58. The β coefficient (380 signal in Ca^{2+}-free/Ca^{2+}-bound)[12] was 2.10 ± 0.32 (n = 5 measurements).

The primary outcomes of the cardiomyocyte experiments were the amplitude of sarcomere shortening and the amplitude of the Ca^{2+} transient (ΔCa_i). Sarcomere shortening was expressed as the percentage of diastolic sarcomere length. ΔCa_i was measured as the difference between peak fura ratio and fura ratio at rest. Secondary outcomes were other parameters of cardiomyocyte contractility that offer insight into the underlying mechanisms. Sarcomere departure velocity (ΔSS/dt) and return velocity were measured as the maximal rate of sarcomere shortening and relaxation, respectively. Ca^{2+} rise velocity (ΔCa_i/dt) was measured as the maximal rate of rise of the fura ratio. The time to peak of sarcomere shortening and of the Ca^{2+} transient were measured from the time of the pacing stimulus. Fura ratio decrease in diastole was fitted with a monoexponential curve, the time constant (τ_{Ca}) of which measures the activity of SERCA.[6] Diastolic sarcomere length and diastolic Ca_i levels were measured just before the following Ca_i transient. In some experiments, rapid application of 10 mM caffeine–containing Tyrode solution to individual cardiac cells was performed using a rapid solution exchanger, as described previously.[6]

Immunoblotting

The expression of SERCA and phospholamban was measured with specific antibodies (MA3-919 and MA3-922, respectively; Thermo Fisher Scientific, Inc., USA) and normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (ab8245, Abcam, United Kingdom), as loading control, as we described previously.[6]

Statistical Methods

Experimental Groups. We compared mice at baseline with mice challenged with LPS (either 7 or 4 μg/g) that were

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euthanized at different time points after LPS administration. Mice were assigned to the different experimental groups unsystematically but without a formal randomization protocol. All mice that were assigned to the study groups were included in the analysis, and there were no exclusions. The observers were not blinded.

Sample Size. An *a priori* power analysis was not performed for this study. Instead we used sample sizes that previous studies indicated as sufficient to identify biologically meaningful differences. Mortality, echocardiography, and immunoblotting studies were performed on samples of five or more mice. Cardiomyocyte experiments were performed on sample sizes of 12 or more cells from two or more mice.

Significance. Multiple comparisons were performed using ANOVA tests followed by an unpaired Student's *t* test between different time points and baseline, with a Bonferroni correction for multiple comparisons (Microsoft Excel, USA). $P < 0.05$ was considered significant. Survival analysis in figure 1 was performed using a Mantel–Cox test (GraphPad Prism 6.00 for Windows, USA). Values are shown as means ± SDs.

Results

Administration of LPS Induces Dose-dependent Mortality

To study the recovery phase of LPS-induced cardiomyopathy, we first needed to find a suitable dose of LPS. In order for our studies to remain clinically relevant, we aimed to find

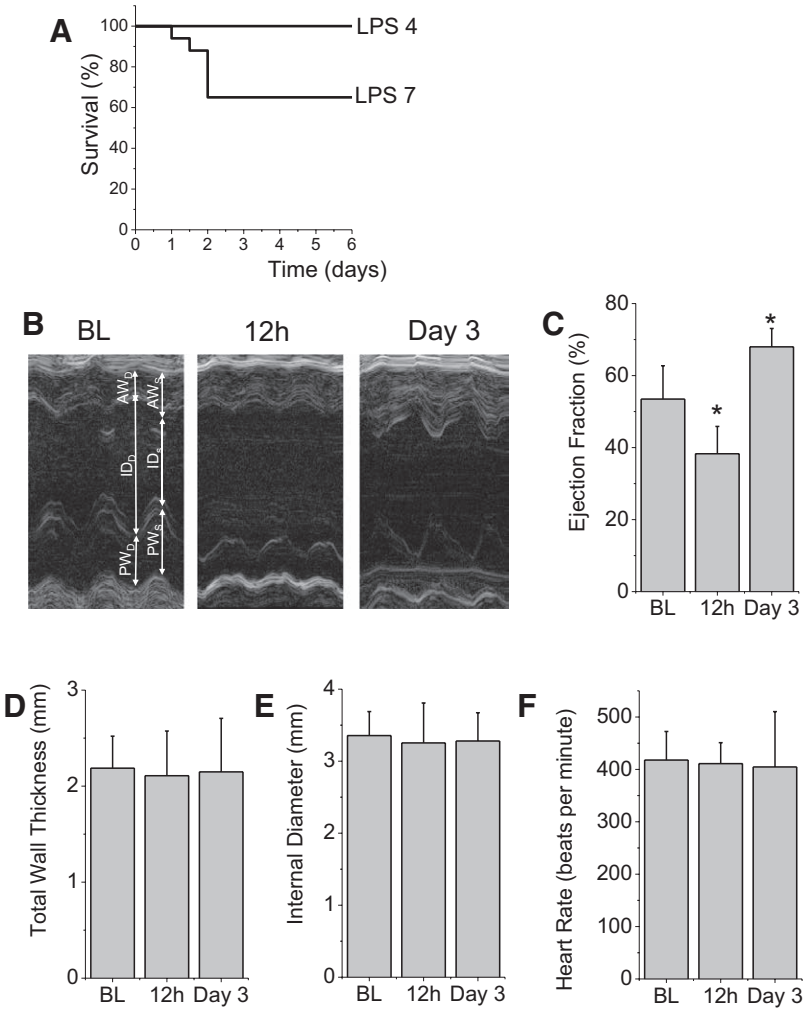

Fig. 1. Left ventricle ejection fraction is initially depressed and subsequently recovers after lipopolysaccharide (LPS) administration. (*A*) Kaplan–Meier diagram showing mice survival after challenge with LPS doses of 4 (n = 6 mice) and 7 μg/g (n = 17 mice). (*B*) M-mode echocardiography stills for mice at baseline (BL; *left*), 12 h (*middle*), and 3 days (*right*) after administration of 7 μg/g of LPS. *Arrows* show the main parameters measured: the systolic and diastolic anterior wall thickness (AW_S and AW_D, respectively), posterior wall thickness (PW_S and PW_D), and left ventricle internal diameter (ID_S and ID_D). Total wall thickness was calculated as $AW_D + PW_D$. Left ventricle ejection fraction was calculated from the ID_S and ID_D measurements, using VisualSonics (Canada) software. (*C–F*) Average results of a serial echocardiography study in a cohort of six mice that survived LPS challenge (7 μg/g). Studies were performed at baseline, 12 h after LPS administration, and at day 3. Data are shown as mean ± SD. Data from mice that died before day 3 are not included in this analysis and are shown in figure 6. Shown are left ventricle ejection fraction (*C*), total wall thickness (*D*), and internal diameter (*E*). Heart rate (*F*) was similar between groups. *$P < 0.05$ *versus* baseline.

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a dose of LPS that induced a sufficiently severe disease, as indicated by the associated mortality and cardiomyopathy, with cardiomyocyte dysfunction. At the same time, to allow the study of survivors, the dose used had to be less than 100% lethal. *A priori*, we decided to find a dose of LPS that induces 30 to 50% mortality.

Mice were administered LPS (in various doses) and followed for 6 days (fig. 1A). A dose of 4 μg/g of weight induced a mild inflammatory syndrome with no mortality. A dose of 7 μg/g induced lethargy, piloerection, and hypothermia,[6,9] as well as 35% mortality by day 6. Therefore, most of the experiments (figs. 1–6) used a dose of 7 μg/g of LPS.

Left Ventricle Ejection Fraction Is Initially Depressed and Subsequently Increased after LPS Challenge

Cardiac contractile function was assessed by echocardiography in lightly anesthetized and volume-resuscitated mice. Figure 1B shows representative stills of a serial echocardiography study in a cohort of six mice that were administered LPS and survived by day 3. (Note that, for this experiment, the baseline and 12-h values are not representative for all of the mice enrolled. A comparison between baseline echocardiography results of survivor *vs*. nonsurvivor mice is shown in fig. 6.) Twelve hours after LPS administration, left ventricle ejection fraction was decreased to 72% of baseline ($P = 0.028$; fig. 1C). By day 3, ejection fraction was not only restored but actually increased to 127% of baseline ($P = 0.006$).

The total wall thickness of the left ventricle was unchanged at both 12 h after LPS and day 3, as compared with baseline (fig. 1D), indicating that no myocardial edema developed. Left ventricle internal diameter was also unchanged (fig. 1E), confirming that our empirical resuscitation protocol was effective, and there was no change in cardiac filling after LPS administration. The unchanged internal diameter also indicated that no cardiac dilation developed after LPS administration. To allow meaningful comparison, heart rate was similar between the groups at the time of echocardiography examination (fig. 1F).

The increased ejection fraction at day 3 *versus* baseline could be the result of an increase in myocardial contractility or due to other factors, such as a persistent vasodilation or increased sympathetic tone. To assess myocardial contractile function directly, we next measured sarcomere shortening and ΔCa_i in isolated cardiomyocytes.

Sarcomere Shortening Depression and Recovery after LPS Challenge

Cardiomyocyte sarcomere shortening is the final output of the contractile process and the direct result of the activation of myofilament cross-bridge cycling by the Ca^{2+} transients (fig. 2A). Twelve hours after LPS administration, sarcomere shortening was decreased to 44% of baseline ($P = 0.0011$; figs. 2A and B), which was consistent with our previous results.[6] Sarcomere shortening started to recover as soon as 24 h after LPS administration, although it was still less than baseline levels ($P = 0.019$) at day 1. Sarcomere shortening continued to increase after day 1 and reached levels not significantly different from baseline between day 3 and day 12.

Sarcomere shortening is the product of ΔSS/dt and the sarcomere time to peak. ΔSS/dt (fig. 1C) was decreased at 12 h ($P < 0.001$) and day 1 ($P = 0.045$) after LPS administration and recovered to baseline values by day 3. Sarcomere time to peak (fig. 1D) was unchanged from baseline at all of the time points.

Sarcomere return velocity and diastolic sarcomere length are the cardiomyocyte determinants of the cardiac diastolic function and are result of the decay kinetics of the Ca^{2+} transient (measured directly by τ_{Ca}, as detailed below), the elastic recoil of the titin myofilaments,[13] and cardiomyocyte viscosity.[14] Sarcomere return velocity was depressed at 12 h after LPS administration ($P < 0.001$), recovered by day 1, and not significantly different from baseline between days 1 and 12. Diastolic sarcomere length was largely unchanged from baseline at any time after LPS administration, with the exception of a small increase (2%; $P = 0.010$) at day 6.

Cardiomyocyte ΔCa_i Depression, Recovery, and Subsequent Up-regulation after LPS Challenge

ΔCa_i is the final result of cardiomyocyte Ca^{2+} handling and an immediate determinant of the myocardial contractile force. Twelve hours after LPS administration, ΔCa_i was decreased to 76% of baseline ($P = 0.0036$; figs. 3A and B), which was consistent with previous findings.[6] By day 1, ΔCa_i started to recover and continued to increase gradually between days 1 and 6. By day 6, ΔCa_i reached levels that were significantly higher (124%) than baseline ($P = 0.038$). By day 12, ΔCa_i returned to baseline values.

ΔCa_i is the product of $\Delta Ca_i/dt$ and Ca^{2+} transient time to peak. $\Delta Ca_i/dt$ measures the rate of Ca^{2+} release from the sarcoplasmic reticulum. $\Delta Ca_i/dt$ was depressed at 12 h after LPS administration ($P < 0.001$) and day 1 ($P = 0.026$) and recovered to baseline levels at day 3 and beyond (fig. 3C).

The time to peak of the Ca_i transient contributes to peak ΔCa_i and is determined largely by the opening times of the ryanodine receptors. The time to peak was slightly prolonged at 12 h ($P = 0.0053$) and day 4 ($P < 0.001$) and unchanged from baseline at all of the other time points (fig. 3D).

SERCA Depression, Recovery, and Subsequent Up-regulation after LPS Challenge

In the conditions used here, the large majority (more than 95%) of diastolic Ca^{2+} decay is the result of Ca^{2+} reuptake into the sarcoplasmic reticulum by SERCA.[6] As such, τ_{Ca} is an accurate measure of SERCA function, one of the major Ca^{2+} transporters in the heart and a main determinant of cardiac systolic and diastolic properties. Twelve hours after LPS administration, τ_{Ca} was increased to 113% of baseline, signifying a 12% inhibition of SERCA function (with borderline significance, $P = 0.097$). This was consistent with previous findings in our laboratory

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that demonstrated that SERCA is inhibited after LPS challenge in male mice, in a dose-dependent fashion.[6]

Importantly, SERCA function started to recover soon after the 12-h time point, and, by day 3, τ_{Ca} was less than baseline, signifying an increase in SERCA function to 139% of baseline ($P < 0.001$). By day 4, τ_{Ca} was back to baseline values, where it remained between days 4 and 12.

The diastolic level of Ca_i is the direct consequence of Ca^{2+} decay kinetics and a direct determinant of the cardiac diastolic properties. The diastolic Ca^{2+} level was largely unchanged between groups, with the exception of a small decrease at day 3 ($P = 0.034$), consistent with the up-regulated SERCA at this time point, and a small increase at day 6 ($P = 0.034$), consistent with the Na^+/Ca^{2+} exchange inhibition observed at that time point (see below).

Phospholamban Down-regulation Underlies SERCA Up-regulation at Day 3

To identify the mechanisms responsible for SERCA up-regulation at day 3, we measured the expression levels of SERCA and phospholamban, the SERCA main regulatory subunit that exerts a tonic inhibitory effect.[15] Glyceraldehyde 3-phosphate dehydrogenase served as a loading control. We compared mice at baseline, 12 h after LPS administration (when SERCA function reached the nadir), and at day 3, when SERCA reached peak function. SERCA

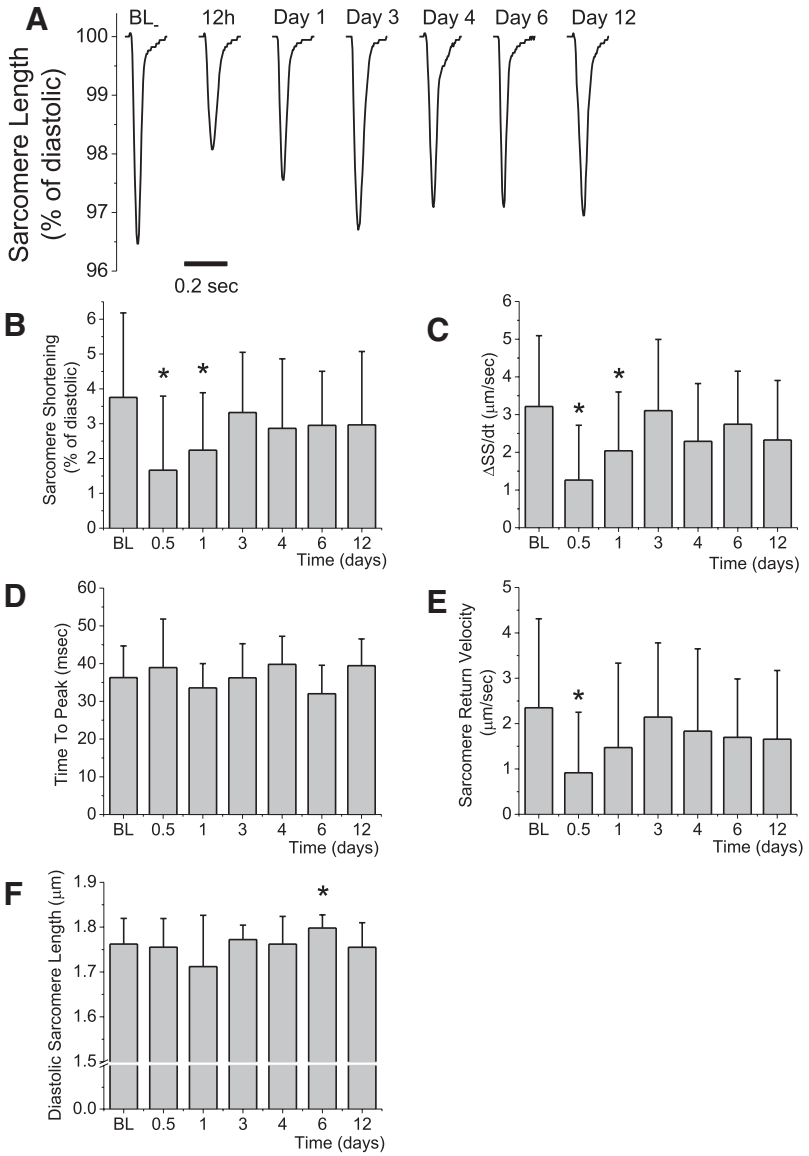

Fig. 2. The depression and recovery of sarcomere shortening after lipopolysaccharide (LPS) administration. (*A*) Typical experimental recordings of sarcomere shortening in isolated, externally paced cardiomyocytes at baseline and at different times after LPS administration (7 μg/g). (*B–E*) Average sarcomere shortening (*B*), sarcomere departure velocity (ΔSS/dt; *C*), sarcomere time to peak (*D*), return velocity (*E*), and diastolic sarcomere length (*F*) at baseline and at different times after LPS administration. Data are shown as mean ± SD. N = 42 cells from seven mice at baseline (42/7), 45/5 for 12 h, 18/2 for day 1, 38/4 for day 3, 19/2 for day 4, 17/3 for day 6, and 16/2 for day 12. BL = baseline. *$P < 0.05$ *versus* baseline.

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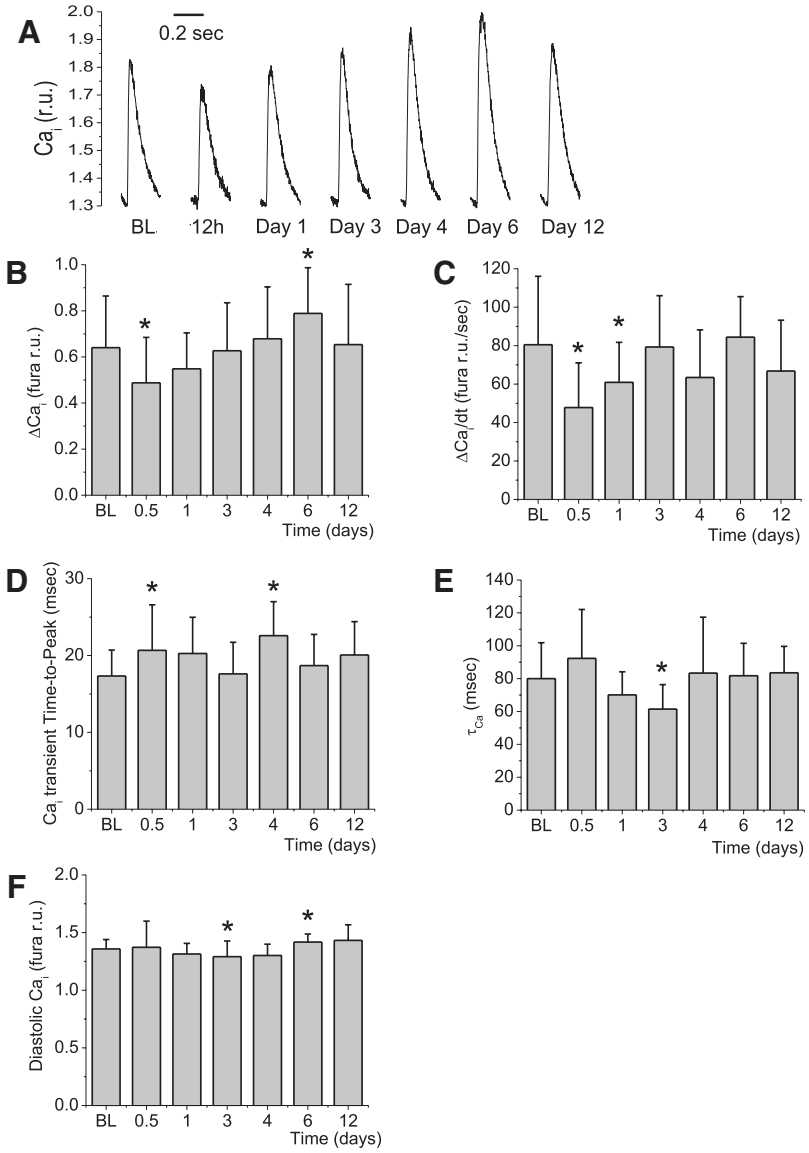

Fig. 3. The depression, recovery, and subsequent up-regulation of the Ca^{2+} transient (ΔCa_i) after lipopolysaccharide (LPS) administration. (*A*) Typical experimental recordings of Ca_i transients in isolated, externally paced cardiomyocytes at baseline and at different times after LPS administration (as shown). (*B–E*) Average ΔCa_i (*B*), Ca^{2+} rise velocity (ΔCa_i/dt; *C*), Ca_i transient time to peak (*D*), time constant of Ca^{2+} decay (τ_{Ca}; *E*), and diastolic Ca_i levels (*F*) in cells isolated from mice at baseline and at different times after LPS administration. Data are shown as mean ± SD. N = 42 cells from seven mice at baseline (42/7) at baseline, 45/5 for 12 h, 18/2 for day 1, 38/4 for day 3, 19/2 for day 4, 17/3 for day 6, and 16/2 for day 12. BL = baseline; r.u. = ratiometric units. **P* < 0.05 *versus* baseline.

and phospholamban expression were unchanged 12 h after LPS challenge, which is consistent with previous findings.[6] At day 3, SERCA expression was unchanged, but phospholamban was down-regulated to 35% of baseline ($P = 0.0019$). Therefore, we concluded that SERCA activation at day 3 is likely the result of phospholamban down-regulation.

Sarcoplasmic Reticulum Ca^{2+} Load (Ca_{SR}) Is Increased and the Na^+/Ca^{2+} Exchange Is Inhibited at Day 6

To gain additional insights into the mechanisms governing myocardial Ca^{2+} handling, we measured Ca_{SR}, sarcoplasmic reticulum fractional release function, as well as the activity of the sarcolemmal Na^+/Ca^{2+} exchange and L-type Ca^{2+} channel using rapid applications of caffeine. For this analysis, we focused on the most noteworthy time points after LPS administration: 12 h, when sarcomere shortening, ΔCa_i, and SERCA function reached their lowest points; day 3, when ΔCa_i was recovered to baseline and SERCA was maximally activated; and day 6, when ΔCa_i reached its supranormal levels as compared with baseline.

Cells were paced until steady state, then pacing was stopped and caffeine was rapidly applied using a home-built rapid solution exchanger (fig. 5A). Caffeine, a ryanodine receptor opener,[16] causes all Ca^{2+} ions stored in the sarcoplasmic

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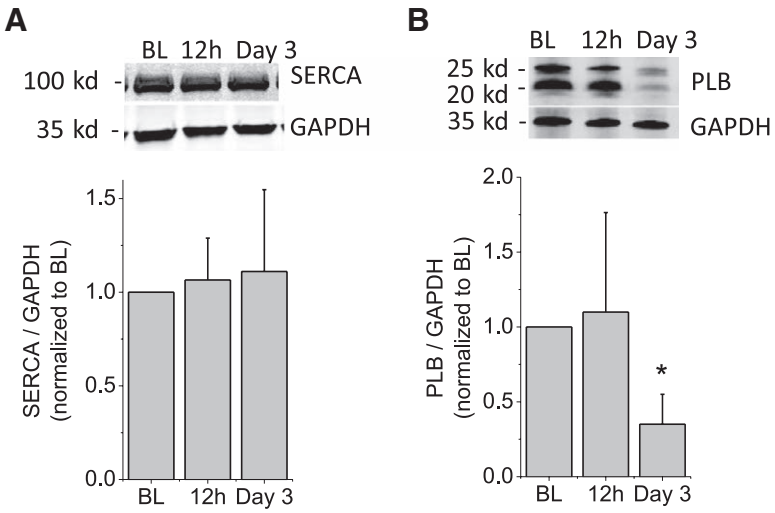

Fig. 4. Phospholamban is down-regulated at day 3. Sarcoplasmic reticulum Ca^{2+} pump (SERCA; *A*) and phospholamban (PLB; *B*) expression in mice at baseline, 12 h after lipopolysaccharide (LPS) administration, and at day 3. *Upper panels* show illustrative Western blots of SERCA, phospholamban, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in left ventricle extracts. *Lower panels* shown average data, normalized to GAPDH (loading control), and shown as a ratio of baseline. N = 6 hearts each. Phospholamban was visualized in the pentameric (25 kilodalton [kd]) and tetrameric forms (20 kd). Average data shown are for the predominant 20-kd isoform, and similar results were obtained for the 25-kd isoform. Data are shown as mean ± SD. BL = baseline. *$P < 0.05$ *versus* baseline.

reticulum to be released into the cytosol. As such, the amplitude of the caffeine-induced Ca_i transient is a measure of Ca_{SR}. Ca_{SR} was unchanged from baseline at 12 h and day 3 but was increased to 126% of baseline at day 6 ($P = 0.034$), the time point when ΔCa_i also reached maximal values (figs. 5B and C).

During caffeine application, the sarcoplasmic reticulum ryanodine receptors are kept open, SERCA cannot effectively reuptake cytosolic Ca^{2+}, and therefore Ca^{2+} removal is largely the result of trans-sarcolemmal extrusion *via* forward mode of the Na^+/Ca^{2+} exchange. As such, the time constant of Ca^{2+} decay during caffeine application (τ_{Caff}) measures the activity of the Na^+/Ca^{2+} exchange. τ_{Caff} was unchanged at 12 h after LPS administration (fig. 5E), which was consistent with previous findings.[6] τ_{Caff} was also unchanged at day 3 but profoundly increased at day 6 ($P < 0.001$), signifying an inhibition of Na^+/Ca^{2+} exchange activity to 49% of baseline at this time point. Na^+/Ca^{2+} exchange inhibition is likely the mechanism underlying the increase in Ca_{SR} observed at day 6 (see Discussion).

Sarcoplasmic Reticulum Fractional Release and L-type Ca^{2+} Channel Function Are Depressed 12 h after LPS Administration and Recovered by Day 3

The fractional release function of the sarcoplasmic reticulum represents how much of the available Ca^{2+} is released during a paced action potential. Fractional release was measured as the ratio of pacing-induced $\Delta Ca_i/Ca_{SR}$, as illustrated in figure 5A. At 12 h after LPS administration, fractional release was decreased by 23% of baseline ($P < 0.001$), which is consistent with our previous results.[6] Fractional release was fully recovered by day 3 and also similar to baseline at day 6 (fig. 5E).

Mechanistically, fractional release is determined primarily by the amount of Ca^{2+} that enters the cell *via* the L-type Ca^{2+} channels during the depolarization phase of the action potential (ΔCa_{entry}) and triggers the opening of the ryanodine receptors and Ca^{2+}-induced Ca^{2+} release.[17] ΔCa_{entry} was measured as the amplitude of the first ΔCa_i elicited by resuming external pacing after a caffeine application (figs. 5A and F). In these conditions, after caffeine wash-off, all of the Ca^{2+} stored in the sarcoplasmic reticulum has been released and removed from the cell *via* forward Na^+/Ca^{2+} exchange. In the absence of any more Ca^{2+} release from the sarcoplasmic reticulum, the first Ca^{2+} transient recorded when pacing resumes is a reflection of the amount of Ca^{2+} ions that enter the cell *via* L-type Ca^{2+} channels during the triggered action potential (fig. 5F). In a previous study, we validated this assay of L-type Ca^{2+} channel function against the commonly used patch clamp method.[6] As compared with patch clamp, however, measuring ΔCa_{entry} offers two distinct advantages: first it can be used in intact cells, and, second, it measures directly the amount of Ca^{2+} entering the cell during the action potential, which represents the physiologic trigger of sarcoplasmic reticulum Ca^{2+} release. In contrast, the patch clamp method measures the ionic current carried during imposed square voltage pulses, in cells in which the intracellular milieu has been exchanged by the pipette solution.

Consistent with previous studies,[6] ΔCa_{entry} was decreased to 54% at 12 h after LPS administration ($P < 0.001$). ΔCa_{entry} was fully recovered at day 3 but was decreased again at day 6 ($P < 0.0022$).

Baseline Left Ventricle Ejection Fraction Is Not Different between Mice That Survived LPS Challenge and Mice That Died

One unavoidable caveat of our study design was the fact that 35% of mice enrolled died between 12 h and day 2 (fig. 1A). As such, the groups of mice studied after 12 h are different

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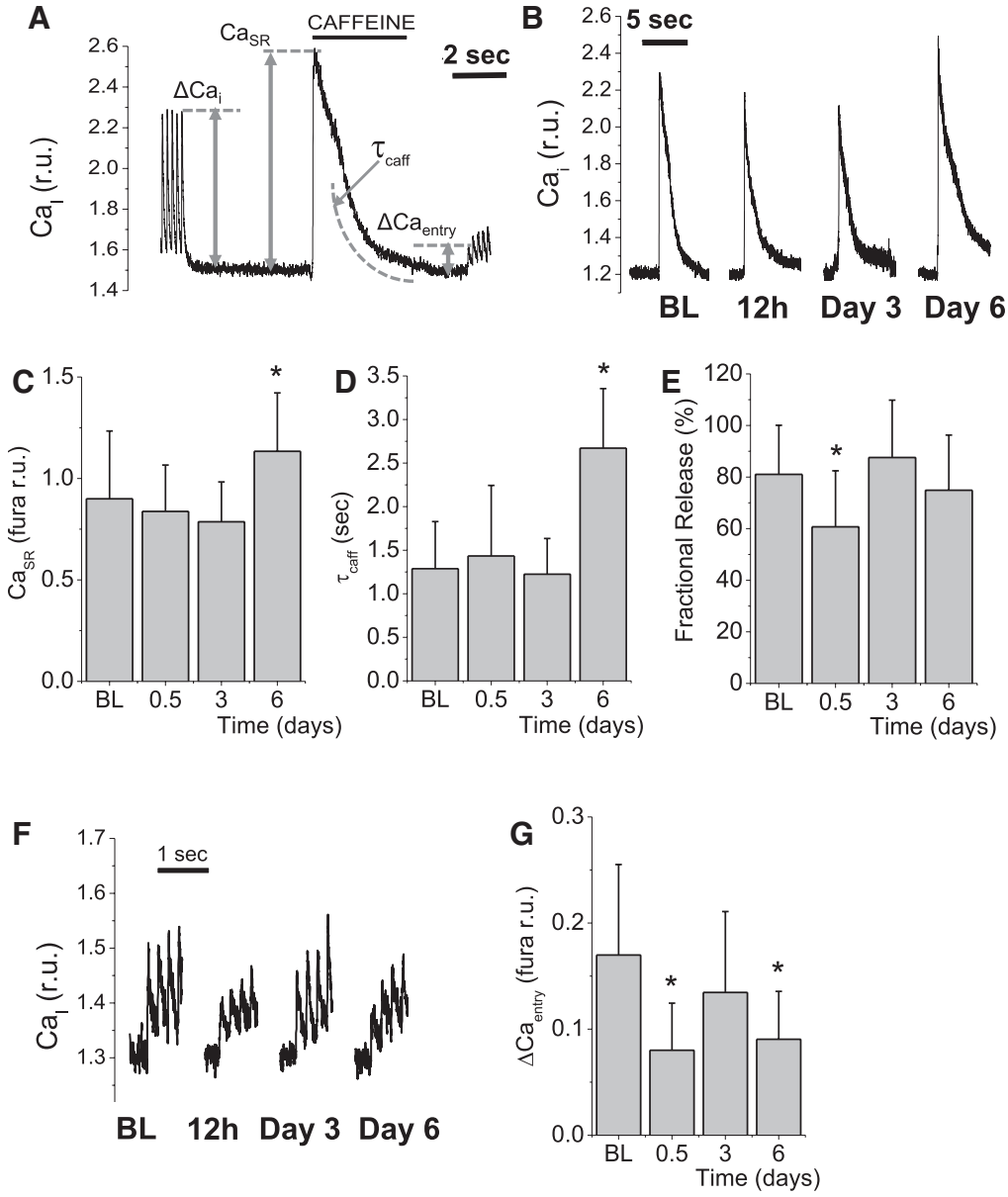

Fig. 5. Sarcoplasmic reticulum, Na^+/Ca^{2+} exchange and L-type Ca^{2+} channel function at baseline and after lipopolysaccharide (LPS) administration. (*A*) Typical experimental recording of caffeine application, at rest, after a train of external pacing, and the quantification of the main parameters measured. ΔCa_i is the steady-state amplitude of triggered Ca^{2+} transients. Sarcoplasmic reticulum Ca^{2+} load (Ca_{SR}) was measured as the amplitude of the caffeine-induced Ca^{2+} transient. The time constant of Ca^{2+} decay during caffeine application (τ_{Caff}) is a measure of Na^+/Ca^{2+} exchange activity. Sarcoplasmic reticulum fractional release was calculated as $\Delta Ca_i/Ca_{SR}$. Ca^{2+} entry *via* the L-type Ca^{2+} channels (ΔCa_{entry}) was measured as the amplitude of the first Ca^{2+} transient elicited by external pacing after caffeine wash-off. (*B*) Typical caffeine-induced Ca_i transients at baseline, 12 h after LPS administration, at day 3, and at day 6. (*C–E*) Average Ca_{SR} (*C*), τ_{Caff} (*D*), and sarcoplasmic reticulum fractional release (*E*) at baseline and at different times after LPS. Data are shown as mean ± SD. N = 25 cells from five mice at baseline (25/5), 28/5 for 12 h, 21/4 for day 3, and 16/3 for day 6. (*F*) Representative traces of first (measuring ΔCa_{entry}) and subsequent Ca_i transients elicited by external pacing after caffeine wash-off. (*G*) Average ΔCa_{entry} at baseline, 12 h after LPS administration, at day 3, and at day 6. N = 22 cells from six mice at baseline (22/6), 24/5 for 12 h, 16/4 for day 3, and 12/3 for day 6. BL = baseline; r.u. = ratiometric units. *$P < 0.05$ *versus* baseline.

from the mice studied at baseline. Could it be possible that the differences that we recorded at days 3 and 6 were the result of a survival bias? In other words, could cardiac contractile function be different in mice that survived and mice that died after LPS challenge? The following two experiments (figs. 6–8) were performed to answer this question.

First we compared baseline echocardiography parameters between mice that survived LPS challenge and mice that died (fig. 6). For this experiment, a cohort of 12 mice were studied by echocardiography (fig. 6A), then challenged with LPS and followed for 6 days. Five mice died and seven mice survived. When we compared the baseline echocardiography

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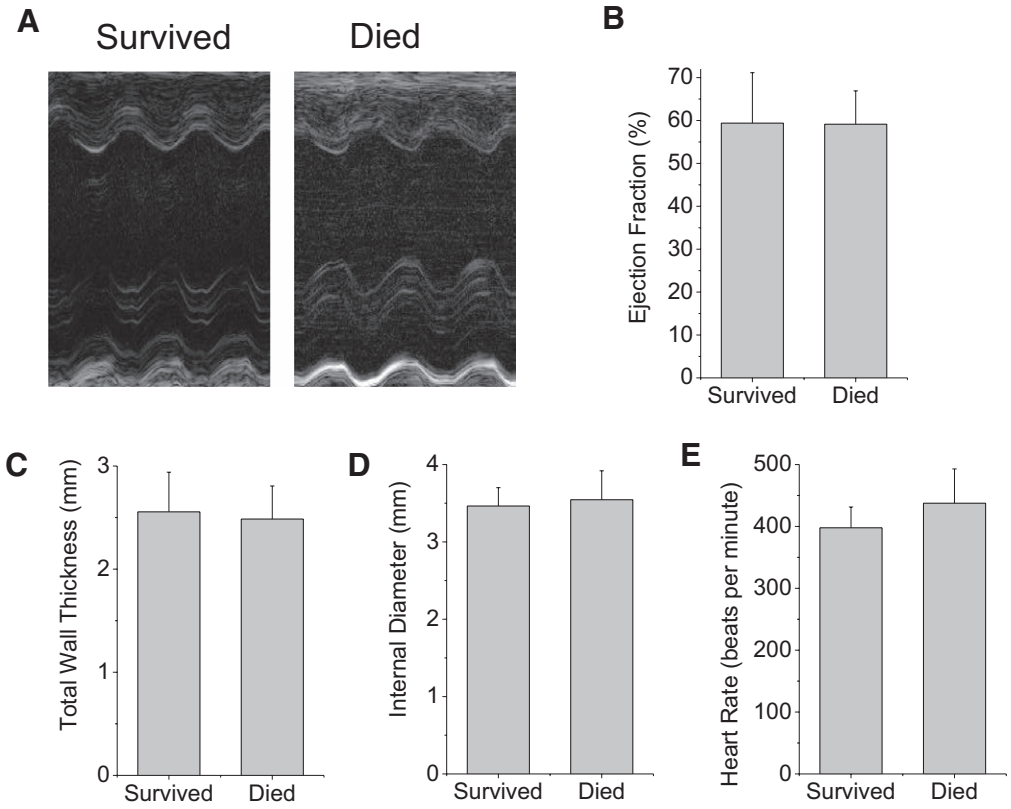

Fig. 6. Baseline left ventricle ejection fraction was not different between survivor and nonsurvivor mice. A cohort of 12 mice had echocardiography studies at baseline, and then were administered lipopolysaccharide (LPS) and followed for 6 days. We compared baseline echocardiography parameters between mice that survived LPS challenge *versus* mice that died. (*A*) Typical echocardiography stills of a mouse that survived *versus* a mouse that died. (*B–E*) Average baseline left ventricle ejection fraction (*B*), total wall thickness (*C*), internal diameter (*D*), and heart rate (*E*) in mice that survived (n = 7) *versus* mice that died (n = 5).

characteristics, left ventricle ejection fraction (fig. 6B), total wall thickness (fig. 6C), and internal diameter (fig. 6D) were similar in mice that died and mice that survived the LPS challenge. For a meaningful comparison, heart rate was similar between the groups at the time of the echocardiography examination (fig. 6E). Therefore, we concluded that baseline cardiac function was similar in mice that survived and mice that died after LPS challenge, at least as far as echocardiography can indicate.

Cardiomyocyte Sarcomere Shortening and ΔCa_i Up-regulation after Challenge with 4 μg/g of LPS

Next we studied cardiomyocyte contractile function (fig. 7A) after challenge with a dose of 4 μg/g of LPS, which does not induce mortality (as shown in fig. 1A). *A priori*, we chose to study the most noteworthy time points from the previous experiment (that used 7 μ/g of LPS; figs. 1–4) and compared them to baseline: (1) at 12 h after LPS administration, when sarcomere shortening, ΔCa_i, and SERCA function reached their lowest points; (2) day 3, when SERCA was maximally activated; and (3) day 6, when ΔCa_i reached its supranormal levels.

Cardiomyocyte sarcomere shortening (fig. 7B) was depressed at 12 h after LPS administration ($P < 0.001$; fig. 7B). Importantly, sarcomere shortening was increased to 138% of baseline at day 3 ($P = 0.031$). At day 6, sarcomere shortening was not significantly different than baseline. ΔSS/dt and sarcomere return velocity showed the same time course as sarcomere shortening, being depressed at 12 h after LPS ($P < 0.001$ for both), augmented to above baseline levels by day 3 ($P = 0.014$ and 0.020, respectively), and not significantly different from baseline at day 6 (fig. 7C). Sarcomere time to peak was shorter than baseline at 12 h ($P < 0.001$) and unchanged from baseline at days 3 and 6 (fig. 7E). Diastolic sarcomere length was similar to baseline at 12 h and day 6 and showed a small (2%; $P = 0.003$) increase at day 3.

Cardiomyocyte ΔCa_i was not decreased 12 h after challenge with LPS (fig. 8A), which is consistent with previously published studies[18] that indicated that, after this dose, the contractile deficit induced by low doses of LPS is due to a decrease in myofilament sensitivity for Ca^{2+}, in the absence of changes in intracellular Ca^{2+} fluxes. Despite that, at day 3, ΔCa_i was up-regulated to 120% of baseline ($P = 0.028$). At day 6, ΔCa_i was again similar to baseline (fig. 8A). $\Delta Ca_i/dt$ (fig. 8B), τ_{Ca} (fig. 8C), Ca_i transient time to peak (fig. 8D), and the level of diastolic Ca^{2+} (fig. 8E) were unchanged from baseline at all of the time points studied.

In conclusion, the up-regulation of Ca^{2+} handling during the recovery phase of LPS-induced cardiomyopathy was also

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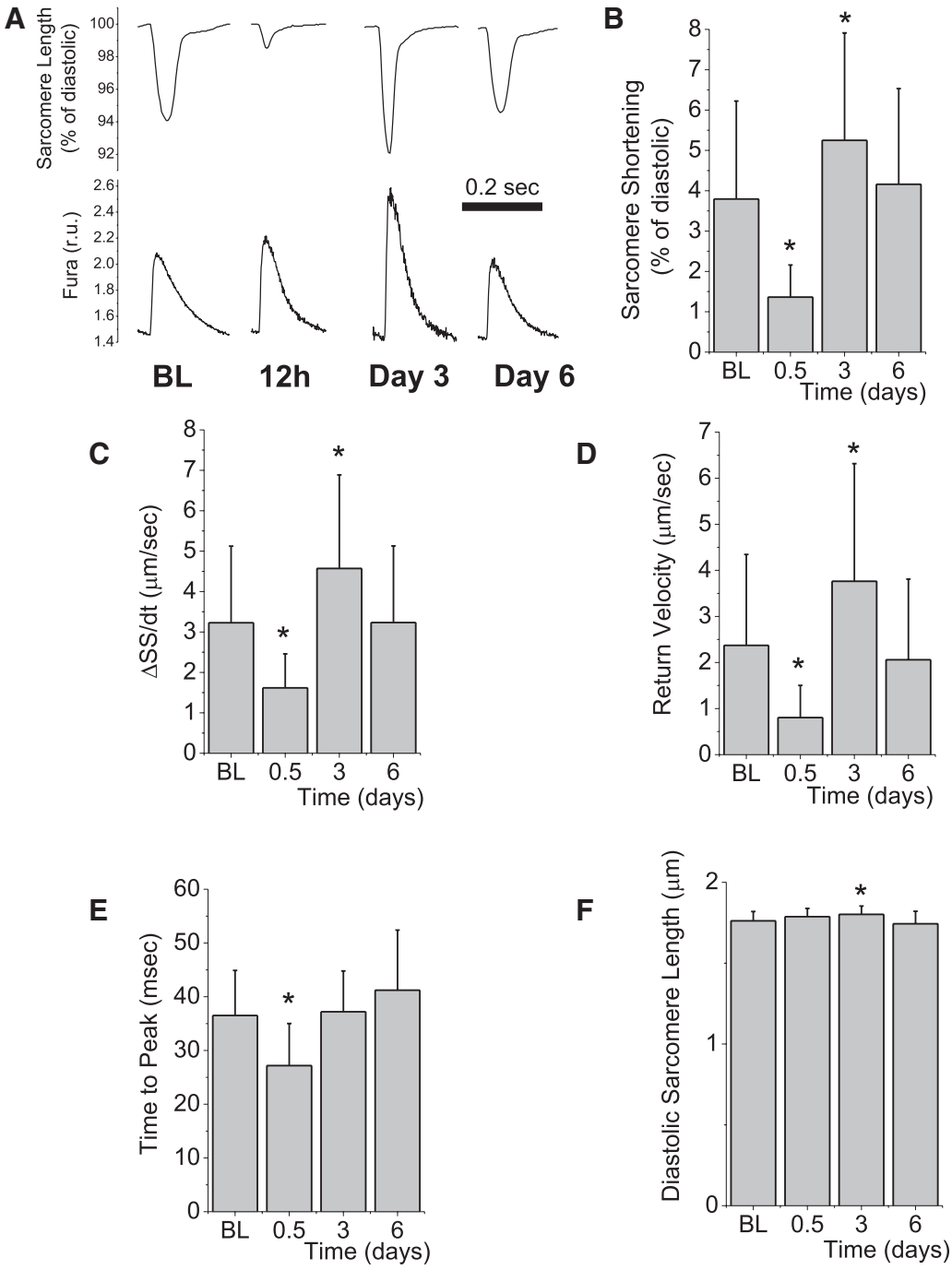

Fig. 7. Cardiomyocyte sarcomere shortening depression and subsequent up-regulation after challenge with 4 μg/g of lipopolysaccharide (LPS). (*A*) Typical experimental recordings of sarcomere shortening and Ca_i transients in cardiomyocytes isolated at baseline and at different times (as shown) after challenge with 4 μg/g of LPS. (*B–E*) Average sarcomere shortening (*B*), sarcomere departure velocity (ΔSS/dt; *C*), return velocity (*D*), sarcomere time to peak (*E*), and diastolic sarcomere length (*F*) at baseline, 12 h after LPS administration, at day 3, and at day 6. Data are shown as mean ± SD. N = 37 cells from seven mice at baseline (37/7), 17/2 for 12 h, 39/3 for day 3, and 14/2 for day 6. BL = baseline; r.u. = ratiometric units. *$P < 0.05$ *versus* baseline.

observed after a dose of 4 μg/g, which induced no mortality. Compared to the challenge with 7 μg/g, ΔCa_i up-regulation occurred earlier (day 3 as compared with day 6) in the absence of SERCA up-regulation and was associated with a significant increase in sarcomere shortening.

Discussion

This is the first investigation of the mechanisms underlying the spontaneous recovery of sepsis-induced cardiomyopathy. Currently, it is generally believed that the resolution of cardiac dysfunction in sepsis is simply due to

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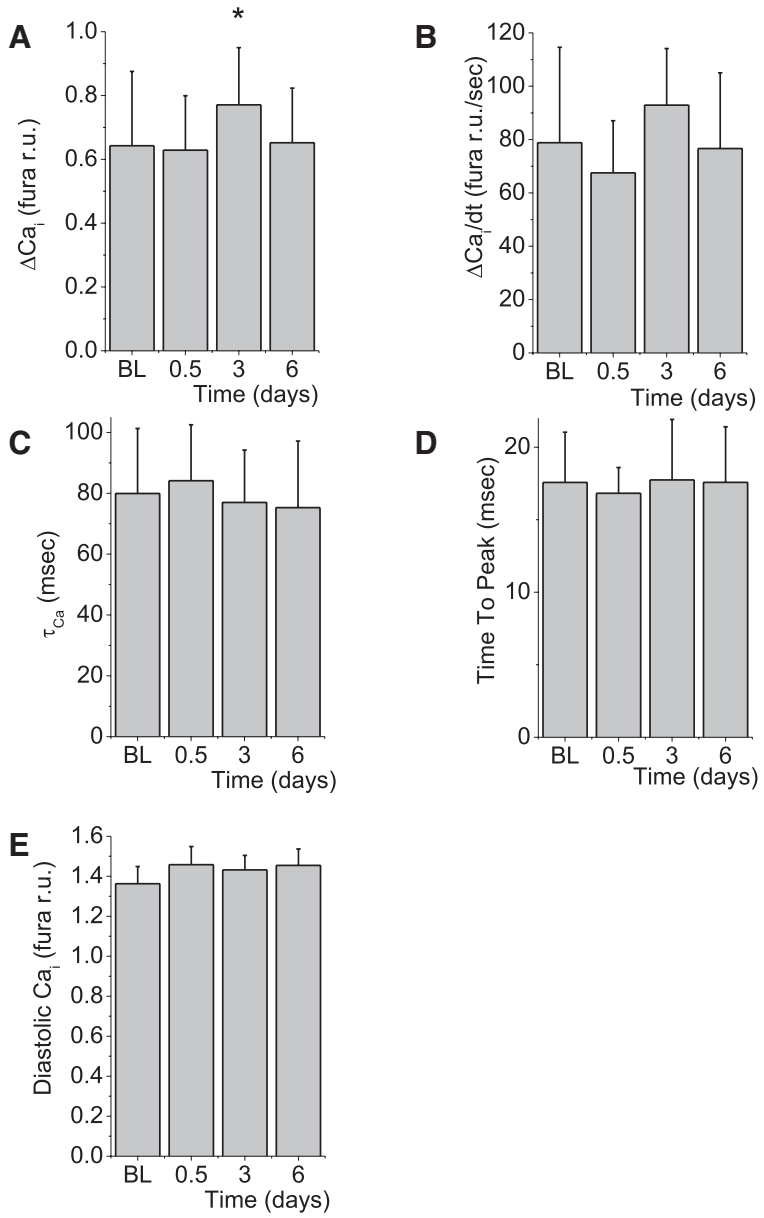

Fig. 8. Cardiomyocyte Ca^{2+} transient (ΔCa_i) depression and subsequent up-regulation after challenge with 4 μg/g of lipopolysaccharide (LPS). Average ΔCa_i (*A*), Ca^{2+} rise velocity ($\Delta Ca_i/dt$; *B*), time constant of Ca^{2+} decay (τ_{Ca}; *C*), Ca_i transient time to peak (*D*), and diastolic Ca_i levels (*E*) at baseline, 12 h after LPS administration, at day 3, and at day 6. Data are shown as mean ± SD. N = 37 cells from seven mice at baseline (37/7), 17/2 for 12 h, 39/3 for day 3, and 14/2 for day 6. BL = baseline; r.u. = ratiometric units. *$P < 0.05$ *versus* baseline.

the remission of systemic inflammation. We hypothesized that, if this was the case, then the efficiency of intracellular Ca^{2+} handling should recover gradually, from its initial decline, back to baseline levels, in parallel with the resolution of the inflammatory shock. Instead, we found that, 6 days after the inception of sepsis-induced cardiomyopathy, ΔCa_i overshoots baseline, suggesting that ΔCa_i recovery is not a passive process but rather the result of active myocardial mechanisms. Three such mechanisms were found here: SERCA activation, as the result of phospholamban down-regulation, and the down-regulation of Na^+/Ca^{2+} exchange.

Myocardial Ca^{2+} Handling Is Up-regulated in the Recovery Phase of LPS-induced Cardiomyopathy

Before cardiac recovery began, at 12 h after administration of 7 μg/g of LPS,[6] the presence of cardiomyopathy was indicated by the depression of the left ventricle ejection fraction, cardiomyocyte sarcomere shortening (fig. 2B), and ΔCa_i (fig. 3B). In surviving mice, 3 days after LPS administration, cardiomyocyte sarcomere shortening and ΔCa_i were fully recovered, whereas left ventricle ejection fraction was increased to levels above normal. The apparent hypercontractility observed at day 3 is thus likely the result of extramyocardial factors,

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such as an increased sympathetic tone or a persistent degree of vasodilation, with the associated decrease in cardiac afterload.

Importantly, ΔCa_i continued to increase after day 3 and reached levels above normal at day 6. This up-regulation of myocardial Ca^{2+} handling was, in itself, inconsistent with the initial hypothesis that the recovery of cardiac function is simply due to the remission of systemic inflammation. Instead, it suggested the existence of distinct myocardial recovery mechanisms, which are different from those responsible for the initial depression of ΔCa_i.

Phospholamban Down-regulation and SERCA Up-regulation at Day 3 after LPS Challenge

Previously,[6] we found that challenge with 25 μg/g of LPS induces SERCA inhibition at 12 h, associated with a depression of Ca_{SR}. In these conditions, SERCA inhibition occurred as the result of oxidative modifications (sulphonylation) of one reactive thiol, at cysteine-674, and in the absence of any changes in SERCA expression or the expression levels and phosphorylation state of phospholamban.[6]

Here we used a lower dose of 7 μg/g of LPS and observed a similar trend toward SERCA inhibition and Ca_{SR} depression at 12 h. Importantly, the initial SERCA down-regulation reversed quickly and by day 3 generated a transport rate that was greater than baseline, as indicated by the shortened τ_{Ca}. SERCA up-regulation at day 3 was likely the result of a decrease in phospholamban expression, which would decrease SERCA tonic inhibition and induce a positive inotropic effect.[19,20] The signaling pathways underlying phospholamban down-regulation at day 3 are yet unknown.

Na^+/Ca^{2+} Exchange Down-regulation Additionally Supports ΔCa_i Recovery

The down-regulation of Na^+/Ca^{2+} exchange function at day 6 is consistent with previous findings in two other models of sepsis-induced cardiomyopathy.[8,21] It is important to realize that Na^+/Ca^{2+} exchange down-regulation would exert a positive inotropic effect in the diseased myocardium[22] by partially opposing SERCA inhibition and increasing Ca_{SR}.[22] In fact, one author (I.A.H.) has previously proposed Na^+/Ca^{2+} exchange inhibition as a novel therapeutic intervention in congestive heart failure.[22–24] It was exciting, therefore, to find that Na^+/Ca^{2+} exchange inhibition occurs spontaneously in the recovering septic hearts, contributing to the increase in Ca_{SR} and ΔCa_i above baseline levels by day 6. It is currently unknown whether the decrease in Na^+/Ca^{2+} exchange function is the result of a decrease in protein expression or an allosteric inhibition of the Na^+/Ca^{2+} exchange. The identity of the signaling pathways responsible is also unknown.

L-Type Ca^{2+} Channels and Sarcoplasmic Reticulum Fractional Release Are Initially Depressed after LPS and Subsequently Recover

Consistent with our previous findings,[6] ΔCa_{entry} *via* L-type Ca^{2+} channels was decreased at 12 h after LPS administration (fig. 5), indicating that L-type Ca^{2+} channels are down-regulated. L-Type Ca^{2+} channel down-regulation induced a decrease in sarcoplasmic reticulum fractional release (fig. 4D) at this time point and thus represented a major determinant of the initial decrease in ΔCa_i after LPS challenge.[6] Importantly, at day 3, both ΔCa_{entry} (fig. 5B) and fractional release recovered to baseline levels.

The mechanisms underlying L-type Ca^{2+} channel recovery are currently unknown. The initial depression in ΔCa_{entry} has been associated with a decrease in the expression of the main ($\alpha 1$) channel subunit.[25] As such, it is tempting to speculate that the rapid recovery of L-type Ca^{2+} channel function after LPS is due to a rapid recovery of channel expression.[26] Alternatively, L-type Ca channel recovery could also be due to allosteric activation by β-adrenergic[27] or redox[28] signaling.

At day 6, ΔCa_{entry} was again depressed, without a decrease in ΔCa_i. From the principle of autoregulation of cardiomyocyte contractility,[29] one could predict that inhibition of Na^+/Ca^{2+} exchange at day 6 would secondarily lead to a decrease in ΔCa_{entry}, because, at steady-state, ΔCa_{entry} needs to be equal to the amount of calcium extrusion through the Na^+/Ca^{2+} exchange. This is exactly what we found. Mechanistically, ΔCa_{entry} decrease at day 6 may be due to an enhanced Ca^{2+}-induced Ca^{2+} inactivation,[30] following an increase in Ca^{2+} release from the sarcoplasmic reticulum.

Could Ca^{2+} Up-regulation Be the Result of Survival Bias?

The majority of experiments presented here used a dose of 7 μg/g of LPS. We chose this dose to ensure that the disease model that we used is sufficiently severe to be clinically relevant, as indicated by the associated mortality. The obvious caveat was that the surviving mice studied were not representative for the entire group of mice enrolled. Could the increase in ΔCa_i at day 6 be explained by the fact that the mice that survive LPS challenge have an increased ΔCa_i at baseline compared with mice that died?

Three observations argue against this idea. First, all of the LPS-induced mouse death occurred between 12 h and day 2. Therefore, no mice died between day 3, when ΔCa_i was similar to baseline, and day 6, when ΔCa_i was increased from baseline. If the increased ΔCa_i at day 6 was due to a survival bias, then it should have been seen at day 3 as well. Instead, the ascending trend of ΔCa_i continued between days 3 and 6, in the absence of any additional mortality. Second, baseline cardiac contractility, measured by echocardiography, was similar between mice that survived LPS challenged and those that died (fig. 6). Third, up-regulation of ΔCa_i was also observed in a different series of experiments that used a reduced dose of LPS (4 μg/g) and induced no mortality (see figs. 7 and 8).

Ca^{2+} Handling Up-regulation after Challenge with a Dose of 4 μg/g of LPS

A dose of 4 μg/g of LPS induced a significant depression in cardiomyocyte sarcomere shortening, without any changes in ΔCa_i, indicating that, in these conditions, cardiomyocytes dysfunction is due solely to the dysfunction of myofilaments,

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whereas Ca^{2+} handling, *per se*, is intact.[18] Importantly, at day 3, both ΔCa_i and sarcomere shortening were again increased to levels higher than baseline. This observation indicated that myocardial Ca^{2+} handling is up-regulated during the recovery phase of sepsis-induced cardiomyopathy in these conditions too. The exact nature of the recovery mechanisms is currently unknown and is likely different from those acting after a dose of 7 μg/g of LPS. First, the peak in ΔCa_i occurs earlier, at day 3, as compared with day 6 after 7 μg/g of LPS. Second, there is no SERCA inhibition at 12 h, nor SERCA activation at day 3, as we found after a challenge with 7 μg/g of LPS. However, the fact that ΔCa_i is up-regulated in this model also indicates that an intrinsic myocardial recovery mechanism is still present after a dose of 4 μg/g, despite the fact that the initial cardiomyocyte depression occurs through different mechanisms than after challenge with 7 μg/g of LPS.

Clinical Implications

Currently, it is generally believed that cardiac recovery in surviving patients with sepsis is due to the remission of the inciting inflammatory triggers. A number of observations made here are inconsistent with this idea. First, if this was the case, then ΔCa_i should have recovered back to baseline levels after its initial decline after LPS administration but should not have overshot above baseline. Second, ΔCa_i started to recover at a time point between 12 h and day 1, a time window when clinical recovery had not become apparent yet, as evidenced by the ongoing mortality. Third, phospholamban down-regulation and Na^+/Ca^{2+} exchange inhibition are new mechanisms that appear at days 3 and 6, respectively, and do not represent the reversal of the mechanisms responsible for ΔCa_i depression at 12 h. Therefore, these observations forced us to postulate the existence of a myocardial recovery mechanism that underlies sepsis-induced cardiomyopathy resolution, independent of the evolution of systemic inflammation.

If this conclusion is also true for human sepsis, then therapeutic strategies of the future will need to be tailored to avoid interfering with the intrinsic myocardial recovery process in the subacute phase of sepsis and sepsis-induced cardiomyopathy. This task may prove difficult if the same signaling pathways are responsible for the initial ΔCa_i depression and its subsequent up-regulation.

It is also evident from our study that mouse mortality occurred between 12 h and day 2 after LPS administration, while cardiomyocyte function was already recovering. This observation served as a powerful reminder that mortality of septic patients is usually due to multiorgan failure, and the correction of any one deficit (the heart, in our study) is not sufficient to secure survival. Therapeutic strategies of the future will need, therefore, to address all or at least most organ dysfunctions (*e.g.*, the loss of vascular tone and lung and kidney failure) before expecting an improvement in survival rates.

Assumptions and Limitations

The current study used a simplified model of sepsis-induced cardiomyopathy based on direct challenge with LPS. This model has been shown previously not to correspond to all aspects of clinical sepsis.[31] Therefore, it will be important to confirm and extend these findings in the future using models of sepsis that more faithfully replicate human disease.[32]

In this study we only used male mice. We have found previously that sepsis-induced cardiomyopathy develops through different mechanisms in male and female mice.[33] It remains for the future, therefore, to expand these studies to include female mice.

Conclusions

This is the first investigation of the cardiac Ca^{2+} handling mechanisms during the recovery phase of endotoxemic cardiomyopathy in mice. We show that, after an initial depression, cardiac Ca^{2+} handling starts recovering quickly (within 24 h), reaches baseline levels by day 3, and is upregulated above baseline by day 6. This observation is inconsistent with the generally held notion that, in septic patients, cardiac dysfunction recovers as a result of the remission of systemic inflammation. Mechanistically, ΔCa_i recovery is based on phospholamban down-regulation, up-regulation of SERCA, a full recovery of L-type Ca^{2+} channel function, and the down-regulation of the Na^+/Ca^{2+} exchange. Identifying the signaling pathways responsible for cardiac recovery in sepsis and turning these insights into novel therapies remain exciting opportunities for the future.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Hobai: Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts

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