Calcium and Calmodulin-Dependent Protein Kinase II-Dependent Ryanodine Receptor Phosphorylation Mediates Cardiac Contractile Dysfunction Associated With Sepsis

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Objectives: Sepsis is associated with cardiac contractile dysfunction attributed to alterations in Ca^{2+} handling. We examined the subcellular mechanisms involved in sarcoplasmic reticulum Ca^{2+} loss that mediate altered Ca^{2+} handling and contractile dysfunction associated with sepsis.

Design: Randomized controlled trial.

Setting: Research laboratory

Subjects: Male wild type and transgenic mice

Interventions: We induced sepsis in mice using the colon ascendens stent peritonitis model.

Measurements and Main Results: Twenty-four hours after colon ascendens stent peritonitis surgery, we observed that wild type mice had significantly elevated proinflammatory cytokine levels, reduced ejection fraction, and fractional shortening (ejection fraction %, 54.76±0.67; fractional shortening %, 27.53±0.50) compared with sham controls (ejection fraction %, 73.57±0.20; fractional shortening %, 46.75±0.38). At the cardiac myocyte level, colon ascendens stent peritonitis cells showed reduced cell shortening, Ca^{2+} transient amplitude and sarcoplasmic reticulum Ca^{2+} content compared with sham cardiomyocytes. Colon ascendens stent peritonitis hearts showed a significant increase in oxidation-dependent calcium and calmodulin-dependent protein kinase II activity, which could be prevented by pretreating animals with the antioxidant tempol. Pharmacologic inhibition of calcium and calmodulin-dependent protein kinase II with 2.5 µM of KN93 prevented the decrease in cell shortening, Ca^{2+} transient amplitude, and sarcoplasmic reticulum Ca^{2+} content in colon ascendens stent peritonitis myocytes. Contractile function was also preserved in colon ascendens stent peritonitis myocytes isolated from transgenic mice expressing a calcium and calmodulin-dependent protein kinase II inhibitory peptide (AC3-I) and in colon ascendens stent peritonitis myocytes isolated from mutant mice that have the ryanodine receptor 2 calcium and calmodulin-dependent protein kinase II-dependent phosphorylation site (serine 2814) mutated to alanine (S2814A). Furthermore, colon ascendens stent peritonitis S2814A mice showed preserved ejection fraction and fractional shortening (ejection fraction %, 73.06±6.31; fractional shortening %, 42.33±5.70) compared with sham S2814A mice (ejection fraction %, 71.60±4.02; fractional shortening %, 39.63±3.23).

Conclusions: Results indicate that oxidation and subsequent activation of calcium and calmodulin-dependent protein kinase II has a causal role in the contractile dysfunction associated with sepsis. Calcium and calmodulin-dependent protein kinase II, through phosphorylation of the ryanodine receptor would lead to Ca^{2+} leak from the sarcoplasmic reticulum, reducing sarcoplasmic reticulum Ca^{2+} content, Ca^{2+} transient amplitude and contractility. Development of organ-specific calcium and calmodulin-dependent protein kinase II inhibitors may result in a beneficial therapeutic strategy to ameliorate contractile dysfunction associated with sepsis. (*Crit Care Med* 2016; XX:00–00)

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Contractile dysfunction has been well documented both in humans and animal models of sepsis (1, 2) and several lines of evidence suggest that alterations in intracellular Ca^{2+} handling are responsible (3–6). In cardiac muscle, contraction is maintained by the process termed excitation-contraction coupling (ECC) (7). During ECC, cardiomyocyte plasma membrane depolarization promotes the opening of voltage-dependent Ca^{2+} channels that results in the influx of a small amount of Ca^{2+}, which induces the opening of ryanodine receptors (ryanodine receptor 2 [RyR2]) on the sarcoplasmic reticulum (SR). The opening of RyR2 leads to the release of a large amount of Ca^{2+} from the SR toward the cytosol that activates contractile proteins and induces contraction. Thus, force developed by the contractile proteins is directly proportional to the amount of Ca^{2+} released from the SR. Given the critical role played by RyR2 in ECC, alterations in Ca^{2+} release by RyR2 have been proposed as one of the principal causes of the reduction in the Ca^{2+} transient amplitude and contractility in sepsis (8, 9). Indeed, reduced SR Ca^{2+} content and systolic Ca^{2+} release in sepsis have been attributed to an increase in diastolic leakage of Ca^{2+} from the SR (10, 11).

Contractile dysfunction associated with heart failure has been attributed, at least in part, to chronic Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) activation resulting in hyperphosphorylation of the RyR2 with the consequent increase in SR Ca^{2+} leak (12–15). CaMKII is a ubiquitous kinase that is activated by a sustained elevation of intracellular Ca^{2+} (16). However, CaMKII activity has also been shown to be modulated by reactive oxygen species (ROS)-dependent oxidation (17, 18). Interestingly, increases in intracellular ROS have been described in the septic heart (19, 20) and antioxidant therapy has been shown to preserve contractile function of the septic heart (21, 22). In the present study, we tested the hypothesis that ROS-dependent CaMKII activation and subsequent RyR2 phosphorylation mediates the alterations in Ca^{2+} handling that results in reduced SR Ca^{2+} content and contractile dysfunction of the septic heart. For this purpose, we first characterized contractile function and Ca^{2+} handling in sham-operated and colon ascendens stent peritonitis (CASP)-operated/septic mice. As contractile function and Ca^{2+} handling was severely disturbed in CASP-operated/septic mice, we examined whether CaMKII inhibitors and transgenic mice expressing a CaMKII inhibitory peptide (AC3-I) had improved contractile function and Ca^{2+} handling compared with sham. Additionally, using myocytes from CASP-operated mutant mice that have the RyR2 CaMKII-dependent phosphorylation site (serine 2814 [Ser2814]) mutated to alanine (S2814A), we tested the causal role of this site on Ca^{2+} mishandling and contractile dysfunction associated with sepsis.

METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication number: 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University and Federal University of Rio de Janeiro. Three-month-old male wild type (WT) C57BL6 mice and transgenic mice with cardiomyocyte-delimited transgenic expression of either a CaMKII inhibitory peptide (AC3-I) or a scrambled control peptide (AC3-C) or mutant mice where the CaMKII-dependent phosphorylation site on the RyR2 (site Ser2814) is mutated to alanine (S2814A) were used. All genetically modified mice were generated in the C57BL6 background.

Mouse Model of Sepsis

CASP surgery was performed to induce sepsis as previously described (23). In brief, a small stent is inserted into the ascending colon of mice leading to the continuous leakage of intestinal bacteria into the peritoneal cavity resulting in peritonitis and systemic bacteremia. Sham surgery was carried out using the identical surgical procedure, but without stent implantation. Details are provided in the **online supplemental material** (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Flow Cytometry Analysis of Cytokine Secretion

Flow cytometry was used for cytokine quantification. A cytokine bead array was used according to the manufacturer's instructions (CBA: BD Biosciences, San Jose, CA). Details are provided in the online supplemental material (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Western Blotting

Homogenates, cytosolic fractions, and SR membranes were prepared from the pulverized ventricular tissue from Langendorff perfused rat hearts as previously described (24). Proteins were electrophoresed and transferred to polyvinnylidene fluoride membranes. Blots were probed with antibodies raised against, oxidized CaMKII (OxiCaMKII), phosphorylated CaMKII (p-CaMKII), CaMKII, glyceraldehyde-3-phosphate dehydrogenase, RyR2-pS2815, RyR2, phosphorylated threonine-17 (pThr17) of phospholamban (PLN), and PLN. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Immobilon Western Millipore) using a Chemidoc Imaging System (Biorad, Hercules, CA). Details are provided in the supplemental material (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Cardiomyocyte Isolation

Myocytes were isolated by enzymatic digestion (24). Details are provided in the supplemental material (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Ca^{2+} and Cell Shortening Measurements

Isolated myocytes were loaded with the Ca^{2+} fluorophore Fura-2/AM. Fura-2 fluorescence signal was measured by epifluorescence and cell shortening using a video-based motion detector provided by Ionoptix hardware (IonOptix LLC, Wetwood, MA) (25). Details of Fura-2 fluorescence and shortening methods are provided in the supplemental material (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Detection of Spontaneous Ca^{2+} Release by Confocal Microscopy

Myocytes were loaded with the Ca^{2+} indicator, Fluo-3 AM and mounted on a Zeiss 410 inverted confocal microscope (LSMTech, Etters, PA) (25). Confocal images were acquired by exciting the fluorophore with an argon laser at 488 nm and collecting emission at greater than 515 nm. Details for confocal imaging are given in the supplemental material (Supplemental Digital Content 1, http://links.lww.com/CCM/C175).

Statistical Analysis

Unpaired Student *t* test was used for statistical comparisons when appropriate. Data are expressed as means ± SEM. Differences were considered significant at *p* value less than or equal to 0.05. * equal to *p* value less than or equal to 0.05 versus sham.

RESULTS

Polymicrobial Model of Sepsis

Several models of sepsis are available among which, the model of CASP has been shown to closely mimic the clinical course of sepsis (26). We examined whether this model, 24 hours after surgery, showed characteristic signs of sepsis and contractile dysfunction. In contrast to WT-sham mice that showed full recovery after surgery, WT-CASP mice showed clinical signs of sepsis including reduced mobility, decreased food intake, piloerection, and exudates around the nose. In addition, as shown in **Figure 1*A***, WT-CASP mice showed severe contractile dysfunction assessed by echocardiographic recordings. We observed that WT-CASP mice had a significant decrease in both ejection fraction (EF) and fractional shortening (FS) when compared with WT-sham. Supporting that 24 hours after surgery sepsis is clearly established, at this time we examined bacterial load and inflammatory cytokine levels in WT-sham and WT-CASP-operated mice. **Figure 1*B*** shows typical examples indicating no bacterial growth in WT-sham samples. In contrast, all mice subjected to WT-CASP surgery exhibited significant amounts of colony forming units after 24 hours of incubation. In addition, **Figure 1*C*** further shows that pro- and anti-inflammatory cytokine levels are significantly elevated in WT-CASP mice compared with WT-sham.

Contractile Dysfunction Is Associated With Reduced Ca^{2+} Transient and SR Ca^{2+} Content

Figure 2*A* shows overall results and representative traces of twitch contractions and the associated Ca^{2+} transients of WT-CASP and WT-sham cardiomyocytes stimulated to contract at 0.5 Hz. Consistent with previous reports (3–6, 8, 9), 24 hours after surgery, myocytes isolated from WT-CASP mice had a significant reduction in contraction associated with a reduction in the amplitude and prolongation of the time to half relaxation ($t_{1/2}$) of the Ca^{2+} transient, compared with myocytes isolated from WT-sham mice (n = 26 and 17 myocytes from 10 hearts, respectively). To examine whether sepsis-induced contractile dysfunction was due to alterations in SR Ca^{2+} handling, we measured SR Ca^{2+} content and spontaneous RyR2 Ca^{2+} release. **Figure 2*B*** depicts typical tracings of Fura-2 fluorescence from WT-CASP and WT-sham cardiomyocytes superfused with 15 mM caffeine to evaluate SR Ca^{2+} content. WT-CASP cardiomyocytes showed a significant reduction in amplitude of the caffeine-induced Ca^{2+} transient compared with WT-sham myocytes (n = 16 and nine myocytes from 10 hearts, respectively). We next examined spontaneous SR Ca^{2+} release by monitoring Ca^{2+} wave frequency as an indicator of RyR2 function (25). **Figure 2*C*** depicts overall results and typical confocal linescan images of cytosolic Ca^{2+} from WT-CASP and WT-sham myocytes showing that, after 2 minutes of 1.5 Hz stimulation to load the SR, WT-CASP myocytes have significantly enhanced Ca^{2+} wave frequency compared with WT-sham cells (n = 9–13 myocytes from three hearts). Collectively, these results suggest that spontaneous SR Ca^{2+} release is enhanced in WT-CASP myocytes.

CaMKII Mediates Contractile Dysfunction Associated With Sepsis

Figure 3*A* shows typical blots and overall results of the effect of sepsis on CaMKII activity in WT mice. Homogenates from WT-CASP hearts showed a significant increase in CaMKII activity (p-CaMKII) (n = 9) and a significant increase in the phosphorylation of CaMKII specific substrates such as threonine-17 (Thr17) of PLN and Ser2814 of the RyR2. Using an antibody that specifically recognizes the oxiCaMKII site, methionine 281/282 (Met281/282), we assessed oxiCaMKII. Figure 3*A*, right one, shows that the oxidation of site Met281/282 of CaMKII is significantly increased in WT-CASP heart homogenates (n = 7) compared with those from control WT-sham hearts (n = 7). To investigate whether oxiCaMKII is related to the enhanced CaMKII activity observed in WT-CASP hearts, we treated WT-sham and WT-CASP animals for 7 days before the day of surgery with 1 mM of the intracellular ROS scavenger, tempol, according to protocol previously used by Sommese et al (27) 2015. **Figure 3*B*** depicts typical blots and overall results showing that tempol treatment prevented the increase in CaMKII activity (p-CaMKII) and the phosphorylation of its specific substrates (Thr17 of PLN and Ser2814 of RyR2) as well as the enhanced oxidation of the kinase (OxiCaMKII) observed in homogenates from WT-CASP hearts (n = 4).

To test the involvement of CaMKII in altered Ca^{2+} handling and contractile dysfunction associated with sepsis, WT-sham and WT-CASP cardiomyocytes were pretreated for 1 hour with either the CaMKII inhibitor, KN93, or with its inactive analog, KN92. **Figure 4*A*** depicts overall results showing that the inhibition of CaMKII with 2.5μM of KN93 is able to prevent the reduction in cell shortening observed in WT-CASP cells as well as the reduction in Ca^{2+} transient amplitude (n = 16 myocytes from seven hearts) and of the caffeine-induced Ca^{2+} transient (n = 13 myocytes from seven hearts). In contrast, 2.5 μM of KN92 failed to prevent the sepsis-induced contractile dysfunction and the alterations in Ca^{2+} handling (n = 7 myocytes from three hearts). Confirming the results obtained using KN93, **Figure 4*B*** shows that transgenic myocytes expressing the CaMKII inhibitory peptide (AC3-I) were protected from the

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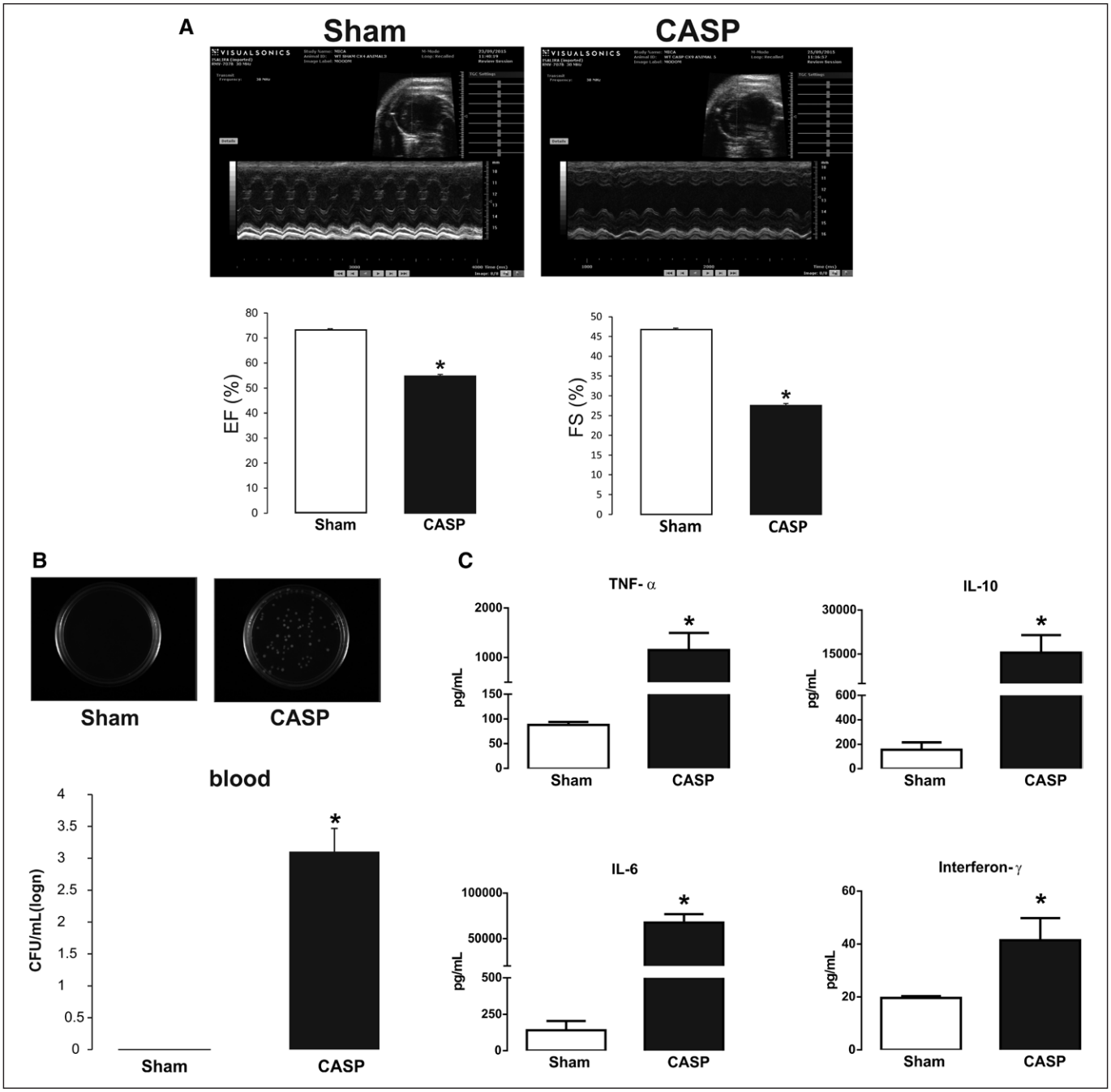

Figure 1. Polymicrobial model of sepsis is associated with cardiac contractile dysfunction. **A**, Evaluation of cardiac function by echocardiography. Representative and average M-mode echocardiography recordings of sham and colon ascendens stent peritonitis (CASP)-operated mice 24 hr after surgery. Results are expressed as mean ± SEM ($n = 10$ for each group). **B**, Typical images and overall results of bacterial cultures from blood samples of CASP and sham mice. Eleven single experiments were performed in each group and values were expressed as colony forming units (CFU) per milliliter. **C**, Serum levels of pro- (tumor necrosis factor [TNF]-α and interleukin [IL]-6) and anti- (IL-10 and interferon-γ) inflammatory cytokines. EF = left ventricular ejection fraction, FS = fractional shortening.

contractile dysfunction associated with CASP surgery ($n = 11$ myocytes from two hearts) when compared with myocytes isolated from CASP mice expressing the scrambled peptide (AC3-C) ($n = 9$ myocytes from two hearts). Furthermore, FS and EF was not significantly reduced in CASP AC3-I mice compared with sham AC3-I mice ($n = 4$). In contrast, FS and EF were significantly reduced in CASP-operated AC3-C mice ($n = 5$) compared with sham-operated AC3-C mice ($n = 5$) (**Fig. 4*C***).

Mechanisms Underlying CaMKII-Dependent Contractile Dysfunction in Sepsis

Using transgenic mice lacking the RyR2 CaMKII-dependent phosphorylation site, Ser2814 (S2812A), we examined the role of this phosphorylation in the contractile dysfunction associated with sepsis. **Figure 5*A*** shows typical blots and overall data indicating that, similar to WT-CASP mice, S2818A-CASP mice have a significant increase in OxiCaMKII ($n = 7$),

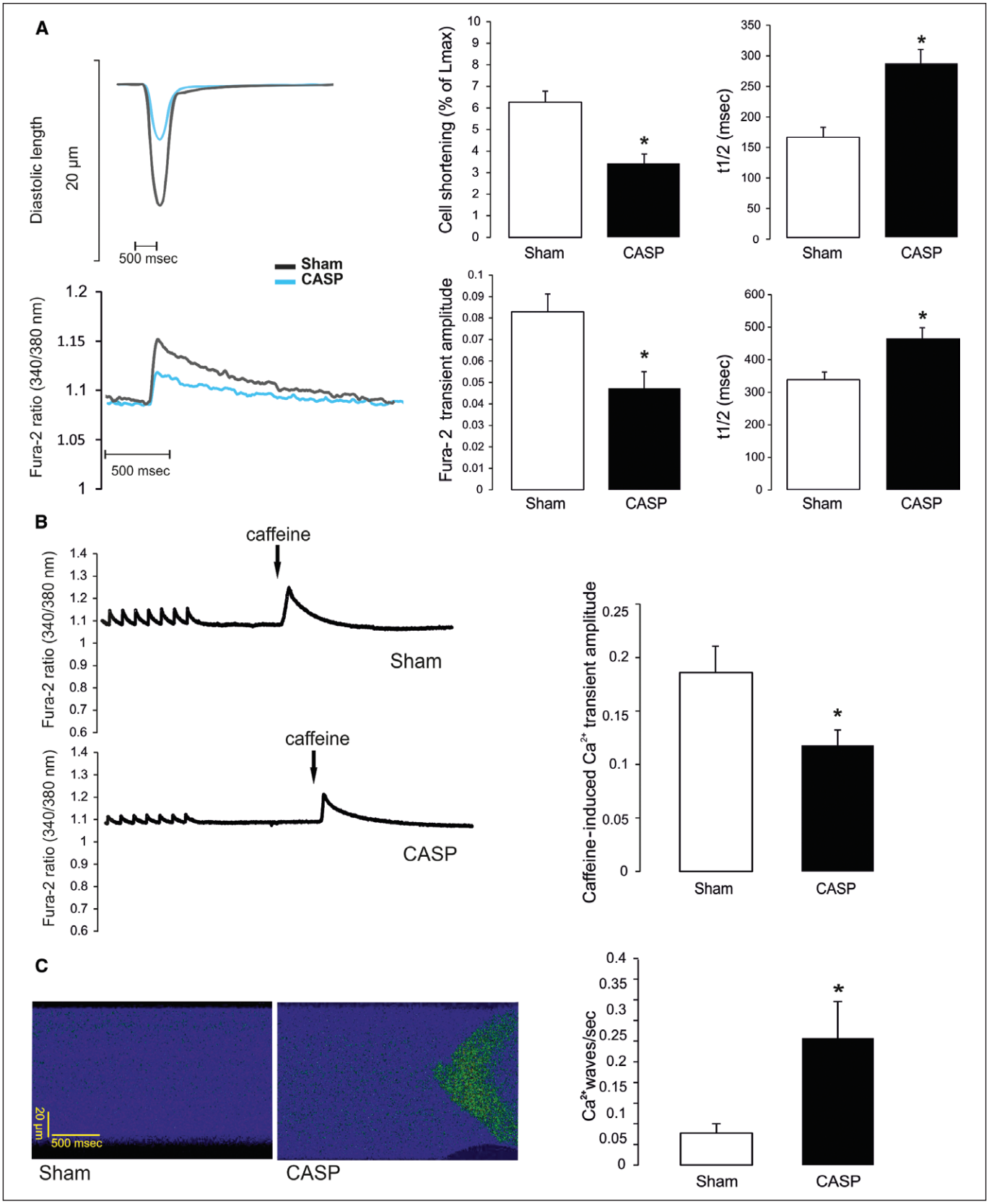

Figure 2. Sepsis affects Ca^{2+} handling at the cardiac myocyte level leading to reduced contractility. **A**, representative traces and average results of cell length (shortening and time to half relaxation [$t_{1/2}$]) and Fura-2 ratio (transient amplitude and decay [$t_{1/2}$]). **B**, Representative traces and average results, showing Fura-2 Ca^{2+} transients promoted by the application of caffeine 15 mM. **C**, Representative line scan images of Fluo-3 emitted fluorescence and average results of spontaneous Ca^{2+} wave frequency in myocytes stimulated at 1.5 Hz during 2 min to load the sarcoplasmic reticulum. Data are expressed as means ± SEM. CASP = colon ascendens stent peritonitis.

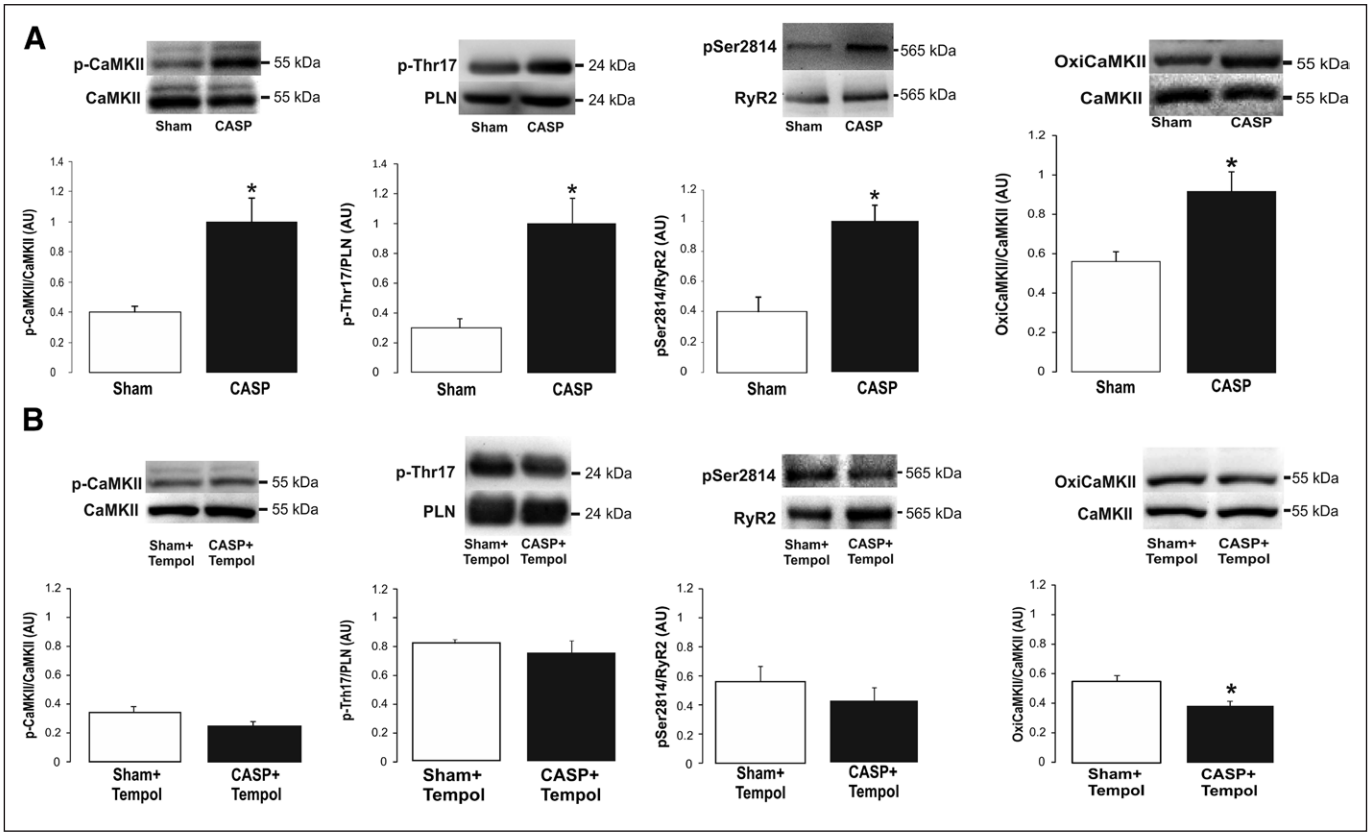

Figure 3. Oxidation-dependent calcium and calmodulin-dependent protein kinase II (CaMKII) activity is increased in septic hearts. **A**, Representative blots and overall results from homogenates of sham and colon ascendens stent peritonitis (CASP) hearts probed with antibodies for phosphorylated CaMKII (p-CaMKII), the CaMKII-specific phosphorylation sites, threonine-17 (Thr17) (phosphorylated Thr17 [pThr17]) of phospholamban (PLN) and phosphorylated serine 2814 (pSer2814) of the ryanodine receptor 2 (RyR2) as well as for the oxidation site of CaMKII, Methionine 281/282 (oxidized CaMKII [oxiCaMKII]). Results are expressed as mean ± SEM ($n = 7$ for each group). **B**, Representative blots and overall results showing the effect of tempol (1 mM) on CaMKII activity (p-CaMKII) as well as its oxidation (oxiCaMKII) and the phosphorylation of its downstream targets Thr17 of PLN and Ser2814 of the RyR2. The results are expressed as mean ± SEM ($n = 4$ for each group).

CaMKII activity (p-CaMKII) ($n = 7$), and phosphorylation of the CaMKII-specific residue of PLN, Thr17 ($n = 7$). However, echocardiographic recordings of S2814A-CASP mice show that they have preserved contractile function as assessed by the percentage of both EF and FS compared with S2814A-sham mice ($n = 5$) (**Fig. 5*B***). Consistently, at the cardiac myocyte level, S2814A-CASP myocytes have preserved cell shortening and Ca^{2+} handling when compared with S2814A-sham myocytes (**Fig. 5*C***).

DISCUSSION

The present study demonstrates, for the first time, that ROS-dependent activation of CaMKII mediates altered Ca^{2+} handling and contractile dysfunction observed in the setting of sepsis. In addition, results suggest that the underlying mechanism for the altered Ca^{2+} handling and contractile dysfunction involves CaMKII-dependent phosphorylation of the RyR2, known to enhance spontaneous diastolic Ca^{2+} release (25) and SR Ca^{2+} depletion. Our results using both pharmacologic and genetic tools show that contractile dysfunction associated with sepsis can be prevented by CaMKII inhibition, raising the possibility that CaMKII inhibitors could be a putative therapeutic strategy to ameliorate the cardiac symptoms of sepsis.

Mechanisms Underlying Contractile Dysfunction in Sepsis

The mechanisms responsible for contractile dysfunction associated with sepsis are not completely understood. The general consensus indicates that a decrease in myofilament responsiveness to Ca^{2+} (28–30) and a decrease in the intracellular Ca^{2+} transient (3–6) are among the most relevant mechanisms. The decrease in the Ca^{2+} transient has been attributed to a reduction of SR Ca^{2+} content (10, 11) resulting from diastolic Ca^{2+} leak from the SR via the RyR2 (9, 31, 32). Indeed, using a polymicrobial rat model of sepsis, Zhu et al (10) showed that RyR2 elementary Ca^{2+} release events, termed Ca^{2+} sparks, are enhanced in sepsis suggesting that RyR2 function is altered and contributes significantly to depressed myocyte shortening in sepsis. Consistent with the critical role of Ca^{2+} leak from the SR, Hassoun et al (11) showed that dantrolene, an SR Ca^{2+} leak inhibitor, is able to prevent contractile dysfunction associated with sepsis. In agreement with these results, we showed that the mouse model of sepsis used in this study was associated with severe myocardial dysfunction (Fig. 1) and altered Ca^{2+} handling including enhanced diastolic Ca^{2+} release as evidenced by the increase in spontaneous Ca^{2+} wave frequency (Fig. 2).

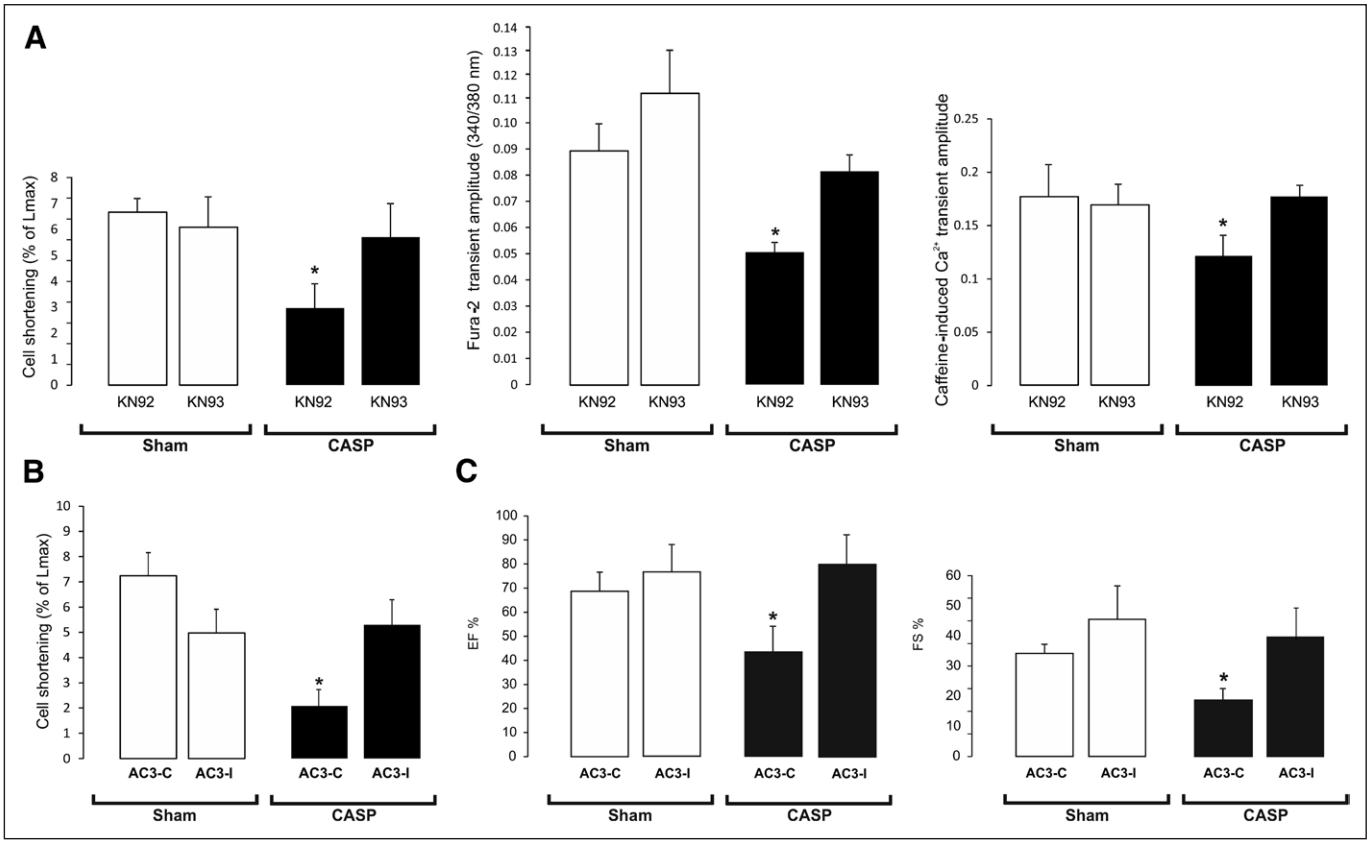

Figure 4. Pharmacologic and genetic calcium and calmodulin-dependent protein kinase II (CaMKII) inhibition prevents contractile dysfunction associated with sepsis. **A**, Average results showing the effect of 2.5 μM of the CaMKII inhibitor, KN93, or 2.5 μM of its inactive analog, KN92, on sham and colon ascendens stent peritonitis (CASP) myocyte cell shortening, Ca^{2+} transient amplitude and sarcoplasmic reticulum Ca^{2+} load. **B**, Overall results of myocyte shortening of sham and CASP cells isolated from transgenic mice overexpressing a CaMKII inhibitory peptide (AC3-I) or from mice expressing the scrambled control peptide (AC3-C) ($n = 4$ for each group). Data are expressed as means ± SEM. **C**, Overall results of echocardiographic recordings showing left ventricular ejection fraction (EF) and fractional shortening (FS) in sham and CASP AC3-C and AC3-I mice ($n = 4$ per group).

CaMKII Mediates Sepsis-Induced Alterations in Ca^{2+} Handling and Contractile Dysfunction

CaMKII is a serine/threonine kinase canonically activated by Ca^{2+} (16). Recent evidence has demonstrated that CaMKII can also be activated by oxidation of specific methionine residues on the regulatory domain (17). Since oxidation-dependent activation of CaMKII was demonstrated, this mechanism has been shown to be involved in multiple pathologic situations. In this scenario, we showed that angiotensin II-induced oxidative stress activates CaMKII and promotes apoptosis of cardiac myocytes (18). More recently, elevated levels of circulating aldosterone were shown to enhance oxiCaMKII, leading to apoptosis and impaired cardiac function (33, 34) and redox-dependent CaMKII activation has also been shown to contribute to prediabetic arrhythmogenic mechanisms (27). Although several reports have examined CaMKII activity in sepsis, the results are controversial. Aversa et al (35) showed that CaMKII activity was decreased or not changed in skeletal muscle during sepsis, whereas Wang et al (36) showed that CaMKII was activated in a cardiomyocyte model of sepsis. Similarly, Singh et al (37) showed that CaMKII was oxidized in neonatal cardiac myocytes treated with lipopolysaccharide. Nevertheless, none of these studies examined the role of CaMKII in sepsis-induced contractile dysfunction. Taking into account that multiple reports indicate that Ca^{2+} is depressed (3–6) but ROS production is increased (19, 20) in sepsis and that a large body of evidence indicates that increased ROS is involved in contractile dysfunction associated with sepsis (21, 22), we hypothesized that during sepsis, increased ROS production results in oxiCaMKII, which in turn alters normal Ca^{2+} handling and leads to contractile dysfunction.

Consistent with this proposition, we found that CaMKII was oxidized and its activity was increased in WT-CASP hearts (Fig. 2*A*). Furthermore, treating WT-CASP mice with antioxidant therapy (tempol) abrogated oxiCaMKII and activation in WT-CASP hearts (Fig. 3*B*), confirming that CaMKII is activated by oxidation in sepsis. Interestingly, we observed a tendency for CaMKII activity (p-CaMKII) and the phosphorylation of its substrates (pThr17 and phosphorylated Ser2814) to be lower in tempol-treated CASP hearts compared with tempol-treated sham hearts. This difference was significant at the level of oxiCaMKII. These results may suggest that in CASP mice, basal CaMKII activity could be more dependent on oxidation than in sham mice where basal activity could be more Ca^{2+}-dependent.

In the present study, we further observed that altered Ca^{2+} handling and contractile dysfunction of septic myocytes were significantly reduced by the CaMKII inhibitor KN93 (Fig.

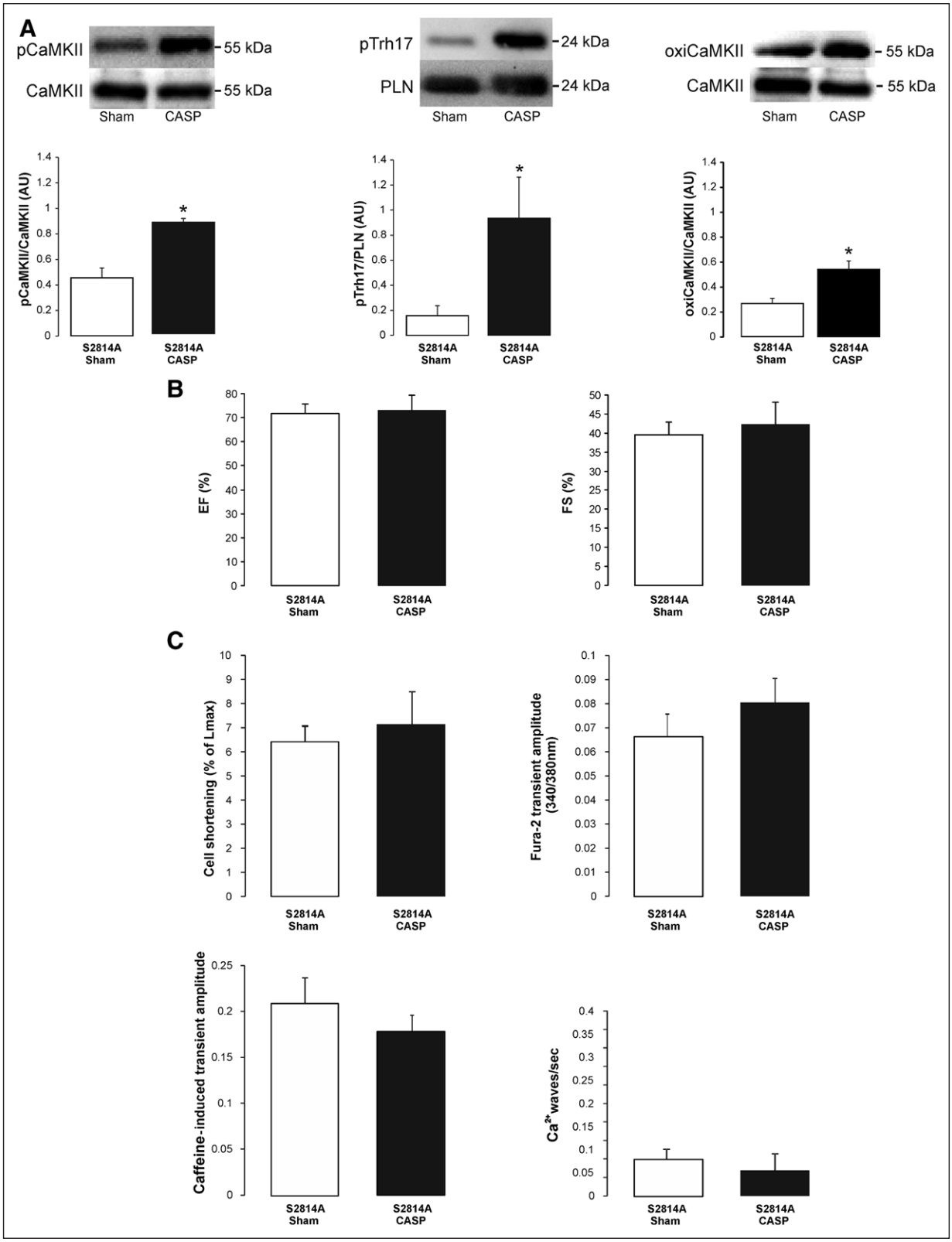

Figure 5. Calcium and calmodulin-dependent protein kinase II (CaMKII)-dependent ryanodine receptor phosphorylation mediates altered Ca^{2+} handling and contractile dysfunction associated with sepsis. **A**, typical Western blots and average results from cardiac homogenates of sham and colon ascendens stent peritonitis (CASP)-operated S2814A mice probed with antibodies for phosphorylated CaMKII (p-CaMKII), phosphorylated threonine-17 (pThr17) of phospholamban (PLN), and for the oxidation of CaMKII (oxiCaMKII). Data are expressed as means ± SEM ($n = 7$). **B**, Overall data of cardiac function from sham and CASP S2814A mice evaluated by echocardiography ($n = 5$ per group). **C**, Average data of cell shortening, Ca^{2+} transient amplitude, caffeine-induced Ca^{2+} transient amplitude, and Ca^{2+} wave frequency of myocytes isolated from sham- and CASP-operated transgenic S2814A mice. Data are expressed as means ± SEM. EF = left ventricular ejection fraction, FS = fractional shortening.

4*A*) but not by the inactive analog of KN93, KN92, excluding unspecific effects of KN93. Further confirming the involvement of CaMKII in sepsis-induced contractile dysfunction, we observed that cardiac myocytes isolated from CASP mice expressing the CaMKII inhibitory peptide (AC3-I) had preserved cell shortening compared with CASP mice expressing the scramble peptide (AC3-C) (Fig. 4*B*). Furthermore, CASP AC3-I mice had preserved EF and FS compared with sham AC3-I mice (Fig. 4*C*). Interestingly, CASP-operated AC3-I mice had a higher survival rate than CASP-AC3-C mice (results are available in the supplemental material, Supplemental Digital Content 1, http://links.lww.com/CCM/C175). These findings strongly suggest that CaMKII is mechanistically involved in altered Ca^{2+} handling and contractile dysfunction associated with sepsis.

Mechanisms Underlying CaMKII-Dependent Ca^{2+} Mishandling and Contractile Dysfunction

In sepsis, SR Ca^{2+} leak has been shown to contribute to contractile dysfunction because it depletes the SR of Ca^{2+} resulting in the reduction of Ca^{2+} transient amplitude. Consistently, we showed that myocytes isolated from CASP-operated mice have enhanced Ca^{2+} wave frequency, indicative of spontaneous SR Ca^{2+} release, and reduced SR Ca^{2+} content (Fig. 2).

The magnitude of SR Ca^{2+} leak or of Ca^{2+} wave frequency depends on two main factors: 1) SR Ca^{2+} load and 2) RyR2 open probability. CaMKII is known to phosphorylate de L-Type Ca^{2+} channel increasing Ca^{2+} influx, and phopholamban, whose phosphorylation increases SR Ca^{2+} uptake via the Ca^{2+} pump, SERCA2A. Both phosphorylations would not be consistent with the decrease in SR Ca^{2+} load observed in sepsis. In contrast, CaMKII-dependent RyR2 phosphorylation has been shown by us and others to increase RyR2 open probability (25, 38, 39). Thus, using myocytes from transgenic mice with site Ser2814 mutated to alanine and, therefore, not phosphorylatable by CaMKII, we examined the role of this phosphorylation on altered Ca^{2+} handling and contractile dysfunction associated with sepsis. We observed that S2814A-CASP mice, in spite of having elevated CaMKII activity had preserved EF and FS and at the cardiac myocyte level, they showed preserved cell shortening and Ca^{2+} transient amplitude as well as SR Ca^{2+} content and Ca^{2+} wave frequency (Fig. 5). Additionally, CASP-operated S2814A mice had a higher survival rate than CASP-operated WT mice (results are available in the supplemental material, Supplemental Digital Content 1, http://links.lww.com/CCM/C175). These results suggest that in sepsis, CaMKII-dependent RyR2 phosphorylation leads to an increase in RyR2 open probability and Ca^{2+} leak from the SR, which would result in Ca^{2+} depletion of the SR, reduced Ca^{2+} transient and myocyte contractility. RyR2 open probability can also be enhanced by oxidation (40), and we have previously shown that activation of CaMKII can lead to enhanced ROS production (24). Thus, in sepsis, enhanced RyR2 open probability could result from both CaMKII-dependent RyR2 phosphorylation and oxidation. However, our results showing that contractile function and Ca^{2+} handling was not different between sham and CASP myocytes from S2814A mice whose RyR2s can be oxidized but not phosphorylated would suggest that RyR2 oxidation does not contribute to enhanced RyR2 open probability in sepsis. Taken together, these results highlight CaMKII and RyR2s as promising targets for the treatment of cardiac dysfunction associated with sepsis. Indeed, CaMKII inhibition has been shown to protect against structural heart disease (41) and a recent report has shown that dantrolene, a therapeutic agent used to treat malignant hyperthermia, can reduce Ca^{2+} leak through the cardiac RyR2 and prevent cardiac mitochondrial and contractile dysfunction in sepsis (42), suggesting the potential use of this compound to ameliorate the cardiac symptoms of sepsis (11).

In summary, our results show for the first time that OxiCaMKII plays a pivotal role in altered Ca^{2+} handling and contractile dysfunction associated with sepsis. In addition, our results shed mechanistic insight indicating that CaMKII-dependent RyR2 phosphorylation could underlie the reduction in SR Ca^{2+} load that leads to reduced Ca^{2+} transient amplitude and contractility of the septic myocyte. Taken together, these finding suggest CaMKII as a new therapeutic target whose inhibition could serve to ameliorate the cardiac symptoms of sepsis.

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