SERCA Cys\(^{674}\) sulphonylation and inhibition of L-type Ca\(^{2+}\) influx contribute to cardiac dysfunction in endotoxemic mice, independent of cGMP synthesis

Ion A. Hobai,\(^{1,2}\) Emmanuel S. Buys,\(^2\) Justin C. Morse,\(^1\) Jessica Edgecomb,\(^1\) Eric H. Weiss,\(^3\) Antonis A. Armoundas,\(^2\) Xiuyun Hou,\(^4\) Alok R. Khandelwal,\(^4\) Deborah A. Siwik,\(^1\) Peter Brouckaert,\(^5\) Richard A. Cohen,\(^4\) and Wilson S. Colucci\(^3\)

\(^{1}\)Cardiovascular Medicine Section, Department of Medicine, Boston University Medical Center, Boston, Massachusetts; \(^{2}\)Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts; \(^{3}\)Cardiovascular Research Center, Massachusetts General Hospital, Boston, Massachusetts; \(^{4}\)Vascular Biology Section, Department of Medicine, Boston University Medical Center, Boston, Massachusetts; and \(^{5}\)Department of Biomedical Molecular Biology, Ghent University, and Department for Molecular Biomedical Research, Flanders Institute for Biotechnology, Ghent, Belgium

Submitted 21 May 2012; accepted in final form 30 July 2013

Hobai IA, Buys ES, Morse JC, Edgecomb J, Weiss EH, Armoundas AA, Hou X, Khandelwal AR, Siwik DA, Brouckaert P, Cohen RA, Colucci WS. SERCA Cys\(^{674}\) sulphonylation and inhibition of L-type Ca\(^{2+}\) influx contribute to cardiac dysfunction in endotoxemic mice, independent of cGMP synthesis. Am J Physiol Heart Circ Physiol 305: H1189–H1200, 2013. First published August 9, 2013; doi:10.1152/ajpheart.00392.2012.—The goal of this study was to identify the cellular mechanisms responsible for cardiac dysfunction in endotoxemic mice. We aimed to differentiate the roles of cGMP [produced by soluble guanylyl cyclase (sGC)] versus oxidative posttranslational modifications of Ca\(^{2+}\) transporters. C57BL/6 mice [wild-type (WT) mice] were administered lipopolysaccharide (LPS; 25 \(\mu\)g/g ip) and euthanized 12 h later. Cardiomyocyte sarcomerne shortening and Ca\(^{2+}\) transients (\(\Delta\text{Ca}_\text{trans}\)) were depressed in LPS-challenged mice versus baseline. The time constant of Ca\(^{2+}\) decay (\(\tau\text{Ca}_\text{dec}\)) was prolonged, and sarcoplasmic reticulum Ca\(^{2+}\) load (\(\text{Ca}_{\text{SR}}\)) was depressed in LPS-challenged mice (vs. baseline), indicating decreased activity of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). L-type Ca\(^{2+}\) channel current (\(I_{\text{Ca,L}}\)) was also decreased after LPS challenge, whereas Na\(^+/\)Ca\(^{2+}\) exchange activity, ryanodine receptors leak flux, or myofilament sensitivity for Ca\(^{2+}\) were unchanged. All Ca\(^{2+}\)-handling abnormalities induced by LPS (the decrease in sarcomerne shortening, \(\Delta\text{Ca}_\text{trans},\;\text{Ca}_{\text{SR}},\;I_{\text{Ca,L}},\;\text{and} \;\tau\text{Ca}_\text{dec}\)) were also more pronounced in pronuclei deficient in the cGMP (sGC\(^{\text{A}+/\text{C}}\)) mice versus WT mice. LPS did not alter the protein expression of SERCA and phospholamban in either genotype. After LPS, phospholamban phosphorylation at Ser\(^{16}\) and Thr\(^{17}\) was unchanged in WT mice and was increased in sGC\(^{\text{A}+/\text{C}}\) mice. LPS caused sulphonylation of SERCA Cys\(^{674}\) (as measured immunohistochemically and supported by iodoacetamide labeling), which was greater in sGC\(^{\text{A}+/\text{C}}\) versus WT mice. Taken together, these results suggest that cardiac Ca\(^{2+}\) dysregulation in endotoxemic mice is mediated by a decrease in L-type Ca\(^{2+}\) channel function and oxidative posttranslational modifications of SERCA Cys\(^{674}\), with the latter (at least) being opposed by sGC-released cGMP.

Cardiomyopathy; sepsis; sarcoplasmic reticulum; contractility; oxidative stress; sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; cysteine

Patients with sepsis and septic shock may develop a specific cardiomyopathy, which complicates their management and worsens prognosis (12). It is generally accepted that sepsis-induced cardiomyopathy (SIC) is due, at least in part, to the large amounts of nitric oxide (NO) released by inducible NO synthase (NOS2) (28). Substantial controversy exists, however, regarding the mechanisms downstream of NO. In principle, NO can act through two distinct pathways: 1) by activating the enzyme soluble guanylyl cyclase (sGC) to synthesize cGMP and 2) by causing oxidative posttranslational modifications (OPTMs) of proteins. However, the distinct roles of these two pathways in SIC are unclear.

Cardiac dysfunction induced by sepsis and sepsis mediators is associated with increases both in cGMP levels (18, 39) and radical oxygen/nitrogen species (RONS) (16) and can be prevented or reversed by both (nonselective) SGC inhibitors (31, 34) and RONS scavengers (20). Recently, it has been shown that transgenic mice that overexpress the antioxidant enzyme catalase were protected against endotoxemic cardiomyopathy (33), whereas mice deficient in the major (\(\alpha_1\beta_1\)) sGC isofrom (sGC\(^{\text{A}+/\text{C}}\)) mice were not (6). Thus, while there is a general agreement that oxidative stress is an important mediator of SIC, the exact cellular mechanisms involved are not known.

This study aimed to distinguish the roles of sGC-derived cGMP and OPTMs in SIC and, in particular, to examine the role of OPTMs of Ca\(^{2+}\) transporters. Myocytes from lipopolysaccharide (LPS)-challenged mice were examined for contractile function, intracellular Ca\(^{2+}\) fluxes, and signaling pathways, including evidence of OPTMs of Ca\(^{2+}\) transporters. We further tested whether sGC affected LPS-induced OPTMs in sGC\(^{\text{A}+/\text{C}}\)-deficient (sGC\(^{\text{A}+/\text{C}}\)) mice, since previous studies have suggested that sGC may oppose the development of SIC.

MATERIALS AND METHODS

LPS challenge. LPS (Sigma, 25 \(\mu\)g/g body wt) was administered intraperitoneally (with 0.5 ml normal saline) to male C57BL/6 mice [wild-type (WT) mice, purchased from Jackson Labs] and sGC\(^{\text{A}+/\text{C}}\)-deficient (sGC\(^{\text{A}+/\text{C}}\)) mice (body weight: 22–32 g, age: 15–25 wk old). In WT and sGC\(^{\text{A}+/\text{C}}\)-deficient (sGC\(^{\text{A}+/\text{C}}\)) mice, LPS induced an inflammatory shock syndrome, with lethargy, hypothermia, and Bradycardia (5). Twelve hours after LPS administration, mice were euthanized with a pentobarbital overdose (10 mg ip together with 200 U heparin) (5). After euthanasia, hearts were removed and used for cardiomyocyte isolation or biochemical assays.

All animal procedures were conducted in accordance with guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital and Boston University School of Medicine.

Address for reprint requests and other correspondence: I. Hobai, Boston Univ. Medical Center, Evans Basic Research Bldg., 650 Albany St., X740, Boston, MA 02118 (e-mail: ihobai@partners.org).

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sGCα1−/− mice. sGCα1−/− mice carry a targeted deletion of the sixth exon of the sGCα1 gene, resulting in the expression of a catalytically inactive protein. The sGCα1−/− mice were backcrossed seven generations to C57BL6 mice. The sGCα1−/− and WT mice used in this study were not littersmates. We are aware of known limitations of this design (mostly the remote possibility of spontaneous mutations that may have appeared in the sGCα1−/− mouse colony); however, we chose it to avoid producing 50% excess heterozygote mice.

**Isolated myocyte experiments.** Isolation of cardiomyocytes, measurement of cell contractility, and Ca2+ handling were performed as previously described (5). Briefly, left ventricular (LV) cardiomyocytes were isolated enzymatically, placed in physiological Tyrode solution [containing (in mM) 137 NaCl, 5.4 KCl, 1.2 CaCl2, 0.5 MgCl2, 10 HEPES, 5 glucose, and 0.5 probenecid; pH 7.40] and externally paced between 1 and 6 Hz at 37°C. Cardiomyocyte sarcomere length and intracellular Ca2+ (Ca) levels (using fura-2 AM, Molecular Probes) were measured simultaneously using an integrated system (IonOptix, featuring a HyperSwitch dual 340- to 380-nm excitation light source). Probenecid was added to the superfusing solution to increase fura-2 retention. Cardiomyocyte sarcomere shortening was triggered by external pacing and expressed as a percentage of the resting sarcomere length. The amplitude of the Ca transient (ΔCa) was measured as the difference between the peak fura ratio at various pacing frequencies and the fura ratio at rest.

**Protein expression.** Immunoblot analysis was performed as previously described (5). Briefly, LV tissue was homogenized in RIPA buffer (Boston BioProducts) supplemented with 1% protease inhibitor cocktail (Sigma) and microcentrifuged for 20 min at 20,000 g. Supernatant proteins (15–30 μg) were fractionated on SDS polyacrylamide gels and transferred to polyvinylidene difluoride or nitrocellulose membranes. Membranes were blocked (Odyssey blocking buffer, LI-COR) and incubated overnight (at 4°C) with primary antibodies as indicated for each experiment. Bound antibody was detected with secondary antibodies using an Odyssey infrared imaging system (LI-COR).

**Immunohistochemical detection of sarcoplasmic reticulum Ca2+-ATPase OPTMs.** LV samples were snap frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek, Sakura), and then sectioned (5 μm). Sections were fixed with aldehyde, blocked with 10% normal goat serum, and subsequently incubated with primary antibody raised against a peptide containing the sulpfonylated Cys residues, whose specificity has been previously proven (38). Slides pretreated with nonspecific rabbit IgG acted as negative controls. A goat biotin-conjugated anti-rabbit secondary antibody and diaminobenzidine peroxide substrate (Vector Laboratory) were used to visualize positive immunoreactivity. Slides were counterstained with hematoxylin (to visualize cell nuclei) and examined under an Olympus BX 40 microscope. For all sections (4 sections/group), the degree of staining was assigned a value between 1 and 4 by three blinded, independent examiners and averaged.

**Biotinylated iodoacetamide labeling.** Biotinylated iodoacetamide (BIAM) labeling was used to detect the presence of OPTMs of SERCA Cys674 (19). BIAM binds to native (i.e., reduced) reactive cysteine residues; as such, the presence of SERCA Cys674 oxidation is apparent as a decrease in the ratio of BIAM-labeled signal versus the total SERCA signal. Briefly, LV tissue samples were homogenized in a buffer containing 150 mM NaCl, 50 mM Tris, and 5 mM MgCl2 with 50 μM 1,1,4,7,7-diethyltriaminepentaaetic acid (DETPA), 5 μM Triton (pH 8.5), added protease inhibitor cocktail (1:100, Sigma), and 2 mM PMSF (a serine protease inhibitor) and 10 mM N-ethylmaleimide (pH 8.5). At pH 8.5, the addition of N-ethylmaleimide to the homogenization buffer allows the subsequent BIAM labeling to be specific for reactive cysteine residues, which, in the case of SERCA, are represented in the largest proportion (>75%) by Cys674 (38). After centrifugation (15,000 rpm), supernatants were washed through a desalting column (GE Healthcare) into a second buffer (containing 150 mM NaCl, 5 mM MgCl2, and 50 mM MES with 50 μM DETPA and 10 μM Triton; pH 6.5) and incubated with 100 μM BIAM for 30 min at room temperature. The reaction was terminated by the addition of 2-mercaptoethanol (50 mM). Subsequently, BIAM-bound proteins were immunoprecipitated (with streptavidin-agarose beads, GE Healthcare, overnight at 4°C) and then eluted in a third buffer (containing 500 mM NaCl, 150 mM Tris, 5 mM MgCl2, and 20 μM SDS; pH 7.4). Samples were then denatured by heating at 55°C for 30 min in Laemmli buffer (Bio-Rad; containing 5 M urea) and separated by electrophoresis alongside non-BIAM-treated homogenates (representing total SERCA) and quantified by immunoblot analysis. For each LV sample, we calculated the ratio of BIAM-precipitated versus total SERCA using data from the same blot. The ratios obtained were normalized to WT-baseline (BL) values, averaged, and compared using statistical methods.

**Patch-clamp experiments.** L-type Ca2+ channel (LTCC) current (ICa,L) was measured in isolated cardiomyocytes using the whole cell voltage-clamp technique, as previously described (14). The pipette solution was designed to allow selective measurements of ICa,L and contained (in mM) 110 CsCl, 5 MgATP, 10 HEPES, 0.4 MgCl2, 5 glucose, 20 tetaethylammonium, and 5 BAPTA (pH 7.2). Cells were superfused with the physiological solution shown above at 37°C.

To account for potential differences in cell size, ICa,L was normalized to cell capacitance, as measured by integrating the capacitative current elicited by a pulse from −80 to −100 mV. Cell capacitance was 149 ± 10 pF in WT-BL cells and was unchanged in WT-LPS, sGCα1−/−-BL, and sGCα1−/−-LPS cells (n values as in Fig. 6B).

The voltage protocol used to measure ICa,L (14) started from a holding membrane potential of −80 mV, from which a 100-ms pretest depolarization to −40 mV (to inactivate Na+ channels) was followed by a 500-ms test depolarization between −30 and +50 mV (test potential) to activate ICa,L (at 0.5 Hz). ICa,L amplitude was measured as the difference between the peak inward current and the current at the end of the test depolarization. To determine ICa,L inactivation parameters, the double-pulse protocol (as described above) at a test potential of 0 mV was preceded by a series of 1-s inactivating steps to various membrane potentials (inactivation potentials).

**Statistical methods.** Multiple comparisons were performed using a two-way ANOVA test followed by an unpaired Student’s t-test (with a Bonferroni correction for multiple comparisons). Values are shown as means ± SE. P values of <0.05 were considered significant.

**RESULTS**

**LPS depresses cardiomyocyte shortening and Ca transients.** We (5) previously reported that administration of LPS (25 μg/g ip) to sGCα1−/− and WT mice induces an inflammatory shock syndrome, associated with cardiomyopathy. LPS-induced cardiomyopathy was more severe in sGCα1−/− mice than in WT mice, as evidenced by the more pronounced depression in LV ejection fraction and developed pressure (5).

To identify the cellular Ca2+ transporters responsible for the cardiac dysfunction induced by LPS, we studied LV myocytes isolated from WT and sGCα1−/− mice at BL and 12 h after the administration of LPS. We first measured sarcomere shortening and ΔCa in isolated cardiomyocytes externally paced at 1–6 Hz (see MATERIALS AND METHODS; Fig. 1A).
At BL, sarcomere shortening and ΔCai were similar in sGCα1−/− (sGCα1−/−-BL) and WT (WT-BL) mice at 6-Hz pacing; however, at lower pacing rates (1–4 Hz), sarcomere shortening and ΔCai were smaller in sGCα1−/−-BL mice than in WT-BL mice (Fig. 1, B and C). For both genotypes, cells isolated from LPS-challenged mice showed smaller sarcomere shortening and ΔCai than cells isolated from unchallenged mice (Fig. 1, A–C). The decrease in sarcomere shortening and ΔCai induced by LPS administration was more pronounced in sGCα1−/− mice (sGCα1−/−-LPS) than in WT mice (WT-LPS), which was consistent with the more pronounced decrease in ejection fraction previously reported (5).

Diastolic Cai levels were similar in WT and sGCα1−/− mice at BL and decreased slightly after LPS to a similar extent in both genotypes (Fig. 1D).

**LPS inhibits SERCA activity.** Since the decay of Cai transient under the conditions of this study is largely due to SERCA-mediated sarcoplasmic reticulum (SR) refilling (see DISCUSSION), we next examined the effects of LPS on the time constant of Cai decay (τCai). At BL, τCai was similar in WT and sGCα1−/− mice (Fig. 1E). In both genotypes, LPS administration caused a prolongation of τCai, which was greater in sGCα1−/− versus WT mice (Fig. 1E). This indicated that LPS administration inhibits SERCA activity and, furthermore, that SERCA inhibition is opposed by sGCα1.

**LPS decreases SR Cai2+ content and fractional release.** SERCA inhibition after LPS administration (Fig. 1E) would be expected to cause a decrease in steady-state SR Cai2+ load. We measured SR Cai2+ content (CasR) as the rise in Cai induced by rapid applications of caffeine (Fig. 2A) (3). In the same experiments, we also measured the SR fractional release (FR) function (i.e., how much of the available SR Cai2+ is released during a paced action potential) as the ratio of pacing-induced ΔCai to CasR (Fig. 2A). In unchallenged mice, CasR and FR were similar in WT and sGCα1−/− mice (Fig. 2, B–D). In both genotypes, cells isolated from LPS-challenged mice had smaller CasR and FR than cells isolated from unchallenged mice (Fig. 2, B–D). The decreases in CasR and FR induced by LPS were both more pronounced in sGCα1−/− (vs. WT) mice (Fig. 2, B–D).

The time constant of Cai2+ decay during caffeine application (τCaf) measures the combined activity of all Cai2+ extrusion mechanisms other than SERCA, since during caffeine application, the SR release channels [ryanodine receptors (RyRs)] are kept open and SERCA cannot effectively refill cytosolic Cai2+ (3). The mechanisms that contribute to τCaf include...
primarily the sarcolemmal Na⁺/Ca²⁺ exchanger, with a more minor contribution from the plasmalemmal Ca²⁺ pump, mitochondrial transporters (3), and probably others. τ_Ca did not differ in WT and sGCα₁⁻/⁻ mice at BL and was not affected by LPS administration (Fig. 3E), indicating that the Na⁺/Ca²⁺ exchanger and other Ca²⁺ extrusion mechanisms were not affected by LPS in our experimental model.

RyR leak is not changed by LPS in either group. To exclude other possible mechanisms that may contribute (beside SERCA) to the decrease in CaSR and ΔCa, after LPS, we measured the diastolic Ca²⁺ “leak” flux through the RyR as the decrease in the diastolic fura-2 ratio induced by rapid applications of the RyR blocker tetracaine (1 mM; Fig. 3A) (29). In all groups, RyR leak was found to be minimal (although statistically different from zero) and not different from the WT-BL group (Fig. 3B). This is consistent with the observation that diastolic Ca²⁺ levels were not increased after LPS challenge in both groups (Fig. 1D) and argues that RyR dysfunction (16) is not a primary mechanism in our model.

LPS depresses SERCA activity in a dose-dependent manner. After excluding the possibility of an increase in RyR leak (Fig. 3B) and the involvement of other (non-SERCA) Ca²⁺ extrusion mechanisms (including sarcolemmal Na⁺/Ca²⁺ exchange; Fig. 2E), SERCA inhibition remained the only possible mechanism that could account for the decrease in CaSR (Fig. 3B) and prolongation of τ_Ca (Fig. 1E) after LPS.

To confirm that SERCA inhibition is a consistent and reproducible effect of LPS, we challenged WT mice with an increased dose (50 μg/g) of LPS (due to the severity of symptoms, these mice were killed at 7 h, as opposed to 12 h, for the 25 μg/g dose). Increasing the dose of LPS induced a more pronounced inhibition of sarcomere shortening (Fig. 3C) and ΔCa (Fig. 3D) and a more pronounced prolongation of τ_Ca (reflecting a dose-dependent inhibition of SERCA function; Fig. 3E).

The effect of LPS is not mediated by changes in SERCA or phospholamban expression or phospholamban phosphorylation. We next aimed to identify the cause of the decreased SERCA activity in LPS-challenged mice. SERCA protein expression (measured by immunoblot analysis) was similar in WT and sGCα₁⁻/⁻ mice and was not affected by LPS administration in either genotype (Fig. 4, A and B).

The expression of phospholamban (PLB; the main regulatory protein of SERCA) was also similar before and after LPS challenge in both genotypes (Fig. 4C).
PLB regulation of SERCA is modulated by its phosphorylation (37) at two sites, Ser16 and Thr17, which were quantified using specific phosphoantibodies. The expression of both Ser16 and Thr17 sites in sGC/H9251 induced a significant increase in PLB phosphorylation (for both Ser16 and Thr17 sites) in sGC/H9251 mice at BL (Fig. 4, D and E). At BL, Cys674 sulphonylation was similar in WT and sGCα1−/− mice (Fig. 5A). After LPS, SERCA Cys674 sulphonylation in sGCα1−/− mice showed a marked decrease in the ratio of BIAM-labeled versus total SERCA (Fig. 5B). After LPS, SERCA Cys674 sulphonylation increased in both genotypes and increased to a greater extent in sGCα1−/− versus WT mice (Fig. 5B).

We then used a complementary method to quantify OPTMs of SERCA. The BIAM-labeling technique (21) is based on the reaction of NO with superoxide) is a hallmark of endotoxemia and sepsis (26) and has been demonstrated in our model of murine LPS-induced cardiomyopathy (15). Therefore, we hypothesized that SERCA inhibition in LPS-challenged mice may be caused by Cys674 sulphonylation.

We quantified Cys674 sulphonylation immunohistochemically using a site-directed antibody in LV sections from WT and sGCα1−/− mice at BL (Fig. 4, D and E). LPS administration induced a significant increase in PLB phosphorylation (for both Ser16 and Thr17 sites) in sGCα1−/− mice only, whereas PLB phosphorylation was unchanged in WT mice after LPS (Fig. 4, D and E). Of note, the increased PLB phosphorylation seen in sGCα1−/− mice after LPS would activate SERCA and therefore cannot be responsible for the SERCA inhibition observed in LPS-challenged mice.

**LPS causes oxidative modifications of SERCA.** Recently, SERCA modulation by OPTMs, and specifically SERCA inhibition after the sulphonylation of a specific cysteine residue, Cys674, has been recognized (1) and implicated in cardiac pathology and dysfunction (27). Increased oxidative stress (e.g., via the formation of peroxinitrite, a potent oxidant formed by the reaction of NO with superoxide) is a hallmark of endotoxemia and sepsis (26) and has been demonstrated in our model of murine LPS-induced cardiomyopathy (15). Therefore, we hypothesized that SERCA inhibition in LPS-challenged mice may be caused by Cys674 sulphonylation.

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labeled SERCA, which was more severe in sGCα1−/−-LPS mice than in WT-LPS mice, is thus consistent with the increased levels of SERCA Cys674 sulphonation described above. Interestingly, in sGCα1−/− mice at BL, SERCA BIAM labeling was also markedly decreased versus WT-BL mice (see discussion; Fig. 5D).

As shown in Fig. 5E, we plotted the degree of SERCA inhibition (calculated as the inverse of Cai, using the data shown in Fig. 1E, and expressed as a percentage of the WT-BL group) against the degree of Cys674 sulphonation (data shown in Fig. 6B) for the four groups. There was a very strong correlation between the degree of Cys674 sulphonation and SERCA transport inhibition induced by LPS in both WT and sGCα1−/− mice, consistent with the hypothesis that SERCA inhibition after LPS is mediated by Cys674 sulphonation.

Cardiac LTCC current is decreased in endotoxemic WT and sGCα1−/− mice. In addition to SERCA (Fig. 1E), the Na+/Ca2+ exchanger (Fig. 2E), and the RyR (Fig. 3B), the LTCC is another major Ca2+ transporter in the cardiac cell. The fact that LPS administration induced a decrease in SR FR [which is dependent on the amplitude of Ca2+ entry (Cafr) via LTCCs; Fig. 2D] suggested that LPS may induce an inhibition of LTCC function in mice. Therefore, we next measured Ica,l selectively in voltage-clamped myocytes from WT and sGCα1−/− mice at BL and after LPS administration (see materials and methods).

At BL, Ica,l (normalized for cell capacitance) was similar in sGCα1−/− and WT cells at all membrane potentials (Fig. 6, A and B). Administration of LPS caused a decrease in Ica,l that was similar in sGCα1−/− and WT mice (Fig. 6, A and B).

To investigate whether the decrease in Ica,l induced by LPS is associated with changes in the voltage-dependent gating of the channel, we measured the activation and inactivation parameters of LTCC, as previously described (14). In WT-BL cells, LTCC activation (Fig. 6C) occurred with a half-maximal voltage of −15.5 ± 0.4 mV and slope of 5.7 ± 0.35 mV, whereas channel inactivation (Fig. 6D) occurred with a half-maximal voltage of −28.6 ± 0.2 mV and a slope of 5.6 ± 0.2 mV. For both activation and inactivation functions, half-maximal voltages and slopes were similar in WT and sGCα1−/− mice and were not changed after LPS administration (Fig. 5, C and D).

LPS decreases Cafr via LTCCs in both genotypes. As a complementary method of assessing LTCC function, we measured Cafr into the cell in intact myocytes as the amplitude of the first Ca2+ transient elicited by resuming external pacing after a caffeine application (as shown in Fig. 2A). Under these conditions, after caffeine washoff, with all SR Ca2+ having been released and removed from the cell (mostly via forward Na+/Ca2+ exchange) and thus in the absence of any Ca2+ release from the SR, the first Ca2+ transient recorded when pacing resumes was a reflection of the amount of Cafr during the triggered action potential (Fig. 6E).

At BL, Cafr was smaller in sGCα1−/− versus WT myocytes. Administration of LPS caused a decrease in Cafr in both groups, with a larger decrease observed in sGCα1−/− versus WT cells (Fig. 6F).

Administration of LPS is not associated with decreased myofilament Ca2+ sensitivity. Finally, we wanted to exclude other potential mechanisms that could contribute to the development of LPS-induced cardiomyopathy, such as a possible decrease in myofilament sensitivity (32). When we compared the results shown in Fig. 1, B and C, we found that, in both genotypes, LPS induced a parallel decrease in sarcomere shortening and ΔCa (Fig. 7A). It was reasonable to assume that the decrease in sarcomere shortening was (at least partially) the result of the decrease in ΔCa, but whether there was an additional contribution from a decrease in the Ca2+ sensitivity of the myofilaments (32) was unclear.

To answer this question, we measured sarcomere shortening at various ΔCa in WT-BL cells (building a sarcomere shortening-ΔCa dependence curve) and compared sarcomere shortening at similar ΔCa in WT-BL and WT-LPS as well as sGCα1−/−-BL and sGCα1−/−-LPS mice. For this, we used caffeine applications to unload the SR, and we analyzed sarcomere shortening and ΔCa during their gradual increase observed when pacing was resumed after caffeine washoff [a
phenomenon also known as “staircase” (Fig. 7B) and reflecting the refilling of the SR with Ca\textsuperscript{2+} (Fig. 7C). We then plotted sarcomere shortening versus \( \Delta \text{Ca}_9 \) for each paced Ca\textsuperscript{2+} transient (Fig. 7D) and averaged sarcomere shortening for \( \Delta \text{Ca}_9 \) bins equal to 0.1 fura ratiometric units (Fig. 7E). We then plotted sarcomere shortening versus \( \Delta \text{Ca}_9 \) in WT-BL cells and compared data obtained with the steady-state values measured in cells from WT and sGC\textsubscript{1-/-} mice before and after LPS.

As shown in Fig. 7E, the decrease in sarcomere shortening induced by LPS in WT and sGC\textsubscript{1-/-} mice was not different from what we would expect from the decrease in \( \Delta \text{Ca}_9 \) alone. We therefore concluded that, in this model, the decrease in sarcomere shortening after LPS is solely due to the decrease in \( \Delta \text{Ca}_9 \) without a significant change in myofilament sensitivity for Ca\textsuperscript{2+}.

DISCUSSION

This study aimed to identify the mechanisms responsible for the development of cardiac dysfunction in endotoxemic mice and, in particular, to differentiate between the roles of sGC-released cGMP and OPTMs of cardiac Ca\textsuperscript{2+}-handling proteins. We first identified SERCA and LTCC inhibition as the primary mechanisms responsible for SIC. We then demonstrated that SERCA inhibition in LPS-challenged mice is associated with OPTMs of SERCA and, specifically, the sulphonylation of Cys\textsubscript{674}, a modification that is known to inhibit SERCA activity. Experiments performed in sGC\textsubscript{1-/-} mice further revealed that, consistent with our previous findings (5) and in contrast to previously held notions (31, 34), sGC-released cGMP does not contribute to the contractile deficit and, in fact, appears to play a protective role in SIC, by limiting the degree of oxidative damage to SERCA.

SERCA dysregulation in LPS-induced cardiac dysfunction. In our model, the impairment of cardiac contractility after LPS challenge is due to a decrease in cell \( \Delta \text{Ca}_9 \) (Fig. 1) in the absence of any additional contribution that could have arisen from a decrease in myofilament sensitivity to Ca\textsuperscript{2+} (Fig. 7) (23).

The absence of a decrease in myofilament sensitivity is interesting, given that this is one of the first mechanisms that has been recognized and implicated in the pathology of sepsis-induced cardiac dysfunction (4) and has been demonstrated across species and disease models (17, 32). In particular, a decrease in myofilament sensitivity has been demonstrated in mice challenged with a low dose (5 \( \mu \)g/g) of LPS and was found to be secondary to increased PKA phosphorylation of troponin I (23). However, we found myofilament sensitivity to
be unchanged in mice challenged with 25 μg/g LPS. In another study (16), myofilament sensitivity was actually increased in mice challenged with 50 μg/g LPS, secondary to OPTM of the myofilaments. Putting together the data from these three laboratories (with the associated caveats), the emerging picture is that of a reversed dose dependency, with myofilament sensitivity being decreased after low-dose LPS challenge but increased at higher doses, reflecting the interplay between the inhibitory effects of troponin I phosphorylation (23) and activatory OPTMs (16).

In our model, the decrease in ΔCaₙᵢ was due to a concerted decrease in SR Ca²⁺ load, SR FR (Fig. 3), and Caₑ (Fig. 6), which, in turn, appeared secondary to the inhibition of SERCA and LTCC activity (Fig. 4). Among the other Ca²⁺ transporters, no change was found in the function of RyRs (Fig. 4), sarcolemmal Na⁺/Ca²⁺ exchange, or other Ca²⁺ removal mechanisms (Fig. 3D).

SERCA inhibition has been previously demonstrated in two different rat (7, 35, 41) and dog (36) models of SIC. From a methodological point of view, it is important to point out that, in our study, SERCA activity was measured in intact cells, as Ca²⁺ transient τCa (Fig. 1E). As such, SERCA inhibition after LPS was evidenced as τCa prolongation. This approach has the advantage of being performed in the same cells and the same conditions as the measurements of ΔCaᵢ and sarcomere shortening (Fig. 1, A–C), thus implicating SERCA inhibition as the primary mechanisms responsible for the decrease in sarcomere shortening without the need of additional assumptions regarding experimental conditions. We are aware that, from a methodological point of view, this approach is not perfectly specific since τCa is not solely determined by SERCA transport but includes also other extrusion mechanisms, such as sarcolemmal Na⁺/Ca²⁺ exchange, the sarcolemmal Ca²⁺ pump, the mitochondrial Ca²⁺ uniporter, and probably others. We therefore measured global Ca²⁺ transport through these “non-SERCA extrusion mechanisms” as τCaيف (Fig. 2E). τCaيف was unchanged after LPS administration in both WT and sGCα₁⁻/⁻ mice, indicating that there was no change in Ca²⁺ transport through any other extrusion mechanisms other than SERCA. Moreover, if one compares the absolute values of τCa (75 ms; Fig. 1E) with τCaиф (1,700 ms, i.e., ~23 times slower; Fig. 3D), it becomes apparent that SERCA reuptake is by far the largest contributor (~96%) for τCaиф with Na⁺/Ca²⁺ exchange and all other mechanisms contributing to only 4%, which is the
usual finding in mice at 37°C (3). As such, the only possible explanation for the τCₐ prolonged after LPS in both WT and sGCα₁⁻/⁻ mice was that SERCA transport was depressed.

**LPS induces inhibitory SERCA OPTMs.** Likewise, the only mechanisms that could explain the inhibition of SERCA transport induced by LPS is the oxidation of Cys⁶⁷⁴ demonstrated by both sulfonic acid present on the Cys⁶⁷⁴ thiol (Fig. 6, A and B) and by decreased BIAM labeling (Fig. 6, C and D). Although other irreversible oxidative modifications on Cys⁶⁷⁴ (as well as other amino acids in SERCA) are possible, our antibody and BIAM methods demonstrate conclusively that the most reactive thiol on the protein is oxidized. Neither SERCA nor PLB expression were changed in LPS-challenged mice. PLB phosphorylation at both Ser¹⁶ (Fig. 5D) and Thr¹⁷ (Fig. 5E) was unchanged after LPS in WT mice and actually increased in sGCα₁⁻/⁻ mice (which would have an activating effect on SERCA uptake; see below).

SERCA regulation by RONS has recently emerged as the underlying mechanism in a number of physiological and pathological processes. RONS-induced OPTMs are able to both activate and inhibit SERCA transport, depending on the specific RONS involved and its concentration. Reversible glutathiolation of SERCA Cys⁶⁷⁴ such as that induced by the NO donor nitroxy (21) increases SERCA transport, whereas irreversible sulfonation by H₂O₂ or peroxynitrite is associated with SERCA inhibition (19).

The present study is the first to provide direct evidence showing that oxidative modifications of SERCA play a role in the development of endotoxemic cardiomyopathy. Increased cellular RONS levels have been a hallmark of the pathology of sepsis and septic shock (26), including increased levels of superoxide anion (generated by uncoupled NOS and also by NADPH oxidases, xanthine oxidase, and mitochondria), peroxynitrite (formed by superoxide anion reaction with NO), and also hydroxyl radical and H₂O₂ (26). Peroxynitrite, in particular, has been previously postulated to play a role in the pathogenesis of endotoxemic cardiomyopathy (20), although the detailed mechanisms were still unclear at that time. The new data obtained here indicate that one possible mechanism involved is sulphonylation of SERCA Cys⁶⁷⁴.
another mechanism that underlies the decrease in ΔCa, CaSR, and FR in endotoxemic mice. LTCC dysfunction after LPS challenge was demonstrated here as both a decrease in Ica,L (Fig. 6, A and B) and a decrease in Cae (Fig. 6, C and D). As yet, the mechanisms underlying LTCC dysfunction are unclear. In particular, it is still unknown whether the decrease in Ica,L after LPS is due to LTCC downregulation or to an allosteric inhibitory effect. However, the fact that the decrease in Ica,L and Cae after LPS persisted in sGCα1−/− mice would indicate that LTCC dysregulation is also mediated by cGMP-independent mechanisms.

**LPS-induced Ca2+ dysfunction is more severe in sGCα1−/− (vs. WT) mice.** As far as our initial goal is concerned, an additional confirmation of the notion that the development of cardiac Ca2+ dysregulation in endotoxemic cardiomyopathy occurs through mechanisms independent of sGC-released cGMP was the fact that all the Ca2+-handling abnormalities induced by LPS in WT mice (the decrease in sarcomere shortening, ΔCa, Cae, FR, Ica,L, and Cae and the prolongation of τCa) were not prevented (and many were actually worsened) in sGCα1−/− mice. These observations are consistent with and extend our prior observation that LPS-induced cardiac dysfunction and death were not prevented in sGCα1−/− mice (5).

sGCα1−/− mice are genetically engineered to express a mutant, catalytically inactive sGC α1β1-isofrom, which leads to an almost complete inability to synthesize cGMP (<1% of WT mice) both at BL and after stimulation with NO donors (5) (although the minor sGC α2β1-isofrom is still present and at levels comparable with WT mice). However, since cGMP generated by various sources may act in distinct intracellular compartments (30), the possibility persisted that the effects of LPS in sGCα1−/− mice (5) are mediated by localized increases in cGMP originating from the sGC α2-isofrom.

As far as SERCA inhibition is concerned, if this was the case, then we would have expected to find evidence for underlying mechanisms compatible with the known effects of cGMP, such as, for example, a decrease in PLB phosphorylation after activation of phosphodiesterase (PDE)2 (25), similar to the effects described for the LTCC (11). Our experiments dismissed this possibility by demonstrating that PLB phosphorylation was unchanged after LPS in WT mice (and actually increased after LPS in sGCα1−/− mice; see below) and identified SERCA OPTMs as the causative mechanisms for SERCA inhibition in endotoxemic mice.

**Dual effects of cGMP in sepsis.** By comparing the effects of LPS on SERCA in sGCα1−/− and WT mice, we revealed that sGCα1−/− mice showed (vs. WT mice) a more severe inhibition of SERCA function (Fig. 1E), associated with two different modifications. On the one hand, sGCα1−/−-LPS mice showed higher levels of inhibitory Cys674 sulphonylation (Fig. 6), which most likely represents the mechanisms underlying SERCA inhibition. On the other hand, however, sGCα1−/−-LPS mice also showed higher levels of PLB phosphorylation (vs. WT-LPS mice; Fig. 5). PLB phosphorylation would activate SERCA and thus would represent a partially compensatory mechanism that opposes SERCA inhibition by OPTMs in endotoxemic cardiomyopathy.

Therefore, the emerging picture is that cGMP plays dual, opposite roles in what regards SERCA regulation in the endotoxemic myocardium. The principal effect of cGMP is protective, by limiting SERCA OPTMs. However, cGMP appears to also play a second, more minor role, in which, by limiting PLB phosphorylation, cGMP may limit a possibly compensatory PKA-dependent SERCA activation. The latter effect is compatible with cGMP-dependent activation of PDE2 (25), which would increase cAMP degradation and thus limit PKA-dependent phosphorylation.

**Antioxidant effect of cGMP.** It is important to emphasize that the overall effect of cGMP in SIC is a protective one, which is consistent with our previous study (5). This is supported by the finding that sGCα1−/− mice exhibited an increased susceptibility to LPS-induced Ca2+-handling abnormalities, such as the decrease in sarcomere shortening, ΔCa, Cae, and FR. Underlying these effects, SERCA transport inhibition (evidence here as the prolongation of τCa) and the degree of SERCA OPTMs (Fig. 6) were all more pronounced in sGCα1−/− mice versus WT-LPS mice. The latter finding suggests that sGC-released cGMP may play an antioxidant role (10) in sepsis and SIC, thus offering a possible explanation for the cardioprotective effects of SIC previously reported (5).

With respect to the possible interactions between sGC-released cGMP and RONS, it is important to note that, at BL, sGCα1−/− mice also showed evidence of SERCA OPTMs (as the decrease in SERCA BIAM labeling; Fig. 6, C and D) other than Cys674 sulphonylation (which was not increased; Fig. 6, A and B) and that were not associated with a change in the rate of SERCA transport (Fig. 1E). It is known that RONS-induced OPTMs are able to both activate and inhibit SERCA transport, with milder modifications [such as glutathionylation by low concentration peroxinitrite (2)] activating SERCA and more severe OPTMs [such as sulphonylation (19)] being inhibitory. Therefore, it is possible that sGCα1−/− mice at BL present either a combination of both activating and inhibitory OPTMs of SERCA or other, currently not described OPTMs that leave the overall transport rate unchanged. The exact answer of this question remains for the future, together with the identity and sources of RONS involved, as well as providing direct evidence for a causative relationship between cGMP deficiency and increased RONS stress.

**Clinical implications and future directions.** This study was partly motivated by the disappointing result of a clinical trial (24) in which administration of a NOS inhibitor (N-methyl-L-arginine) failed to provide benefits and actually increased the mortality of septic patients (24). In this trial (24), the deaths of patients in the N-methyl-L-arginine-treated group were largely due to the development of cardiac dysfunction (24), suggesting that some components of the NO pathway (which would have been interrupted by N-methyl-L-arginine) may play a critical cardioprotective role in sepsis. Subsequent experimental work indicated that such cardioprotective effects could be mediated by the constitutive NOS isoforms NOS1 (8) and/or NOS3 (16) and/or by mediators distal of NO, such as sGCα1β1 (5). Our data indicate that the adverse effects of N-methyl-L-arginine may be related to loss of the cardioprotective effects of sGC-released cGMP to decrease SERCA OPTMs.

Regardless of the exact explanation, given the pivotal role played by NOS2-released NO in sepsis and septic shock (22), it is likely that any effective therapeutic strategy must be able to interrupt the NOS2 pathway. If direct NOS inhibition is not beneficial (24), then it follows that the search for an effective therapy for septic shock requires the identification and target-
ing of the mediators that are downstream of NO. In this respect, the present experiments implicate the effects of cellular oxidative stress on SERCA and exonerate the sGC-cGMP axis. Therefore, our experiments indicate that the search for an effective therapeutic strategy in sepsis should focus on the selective inhibition of RONS-mediated OPTMs (9, 13) while sparing cardioprotective sGC-cGMP signaling.

ACKNOWLEDGMENTS

I. A. Hobai thanks Dr. Jeanine Wiener-Kronish, Dr. Lisa Leffert, and Dr. Keith Miller (Massachusetts General Hospital) for continuing support and encouragement.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants HL-061639 (to W. S. Colucci), HL-064750 (to W. S. Colucci), and HL-36107 (to R. A. Cohen) and by the NIH-sponsored Boston University Cardiovascular Proteomics Center (Contract No. N01-HV-28178, to W. S. Colucci). I. A. Hobai acknowledges support from National Institutes of Health Grants K08-GM-096082 and T32-GM-007592 and the Department of Anesthesiology, Critical Care and Pain Medicine, Massachusetts General Hospital. Other support includes American Heart Association Grants 10SDG2610313 (to E. S. Buys), 063512TN (to A. A. Armoundas), and 0815767D (to E. H. Weiss), the Deane Institute for Integrative Research in Atrial Fibrillation and Stroke (to A. A. Armoundas), the Cardiovascular Research Society (to A. A. Armoundas), and Boston University (Student Research Awards to J. C. Morse and J. Edgecomb).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


