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Distinct myocardial mechanisms underlie cardiac dysfunction in endotoxemic male and female mice

Ion A. Hobai^{1,2}, Kanwal Aziz¹, Emmanuel S. Buys², Peter Brouckaert³, Deborah A. Siwik¹, and Wilson S. Colucci.¹

¹Cardiovascular Medicine, Department of Medicine, Boston University Medical Center, Boston, MA

²Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard University, Boston, MA

³Department of Biomedical Molecular Biology, Ghent University, and Inflammation Research Center, Flanders Institute for Biotechnology (VIB), Ghent, Belgium

Abstract

In male mice, Sepsis-Induced Cardiomyopathy develops as a result of dysregulation of myocardial calcium (Ca^{2+}) handling, leading to depressed cellular Ca^{2+} transients (Ca_i). Ca_i depression is partially due to inhibition of sarcoplasmic reticulum Ca^{2+} ATP-ase (SERCA) via oxidative modifications, which are partially opposed by cGMP generated by the enzyme soluble guanylyl cyclase (sGC). Whether similar mechanisms underlie Sepsis-Induced Cardiomyopathy in female mice is unknown.

Male and female C57Bl/6J mice (WT), and mice deficient in the sGC α_1 subunit activity ($\text{sGC}\alpha_1^{-/-}$) were challenged with lipopolysaccharide (LPS, ip). LPS-induced mouse death and cardiomyopathy (manifested as the depression of left ventricular ejection fraction, LVEF, by echocardiography) to a similar degree in WT male, WT female and $\text{sGC}\alpha_1^{-/-}$ male mice, but significantly less in $\text{sGC}\alpha_1^{-/-}$ female mice. We measured Sarcomere Shortening and Ca_i in isolated, externally paced cardiomyocytes, at 37 °C. LPS depressed Sarcomere Shortening in both WT male and female mice. Consistent with previous findings, in male mice, LPS induced a decrease in Ca_i (to $30 \pm 2\%$ of baseline) and SERCA inhibition (manifested as the prolongation of the time constant of Ca^{2+} decay, τ_{Ca} , to $150 \pm 5\%$ of baseline). In contrast, in female mice, the depression of Sarcomere Shortening induced by LPS occurred in the absence of any change in

Ca_i , or SERCA activity. This suggested that, in female mice, the causative mechanism lies downstream of the Ca^{2+} transients, such as a decrease in myofilament sensitivity for Ca^{2+} . The depression Sarcomere Shortening after LPS was less severe in female $\text{sGC}\alpha_1^{-/-}$ mice showed (to $69 \pm 7\%$ of baseline) than in WT female mice, indicating that cGMP partially mediates cardiomyocyte dysfunction.

Contact Information: Ion Hobai, M.D., Ph.D. Boston University Medical Center, Evans Basic Research Building, 650 Albany Street, X740, Boston MA 02118, Tel: 617 638 8059; Fax: 617 638 8081; ihobai@partners.org.

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These results suggest, therefore, that LPS-induced cardiomyopathy develops through distinct sex-specific myocardial mechanisms. While in males LPS induces sGC-independent decrease in Ca_i , in female mice LPS acts downstream of Ca_i , possibly via sGC-dependent myofilament dysfunction.

Introduction

In 2014, a commanding call to action from the National Institutes of Health [1] exposed a troubling sex inequity in today's biomedical research. Despite adequate inclusion of female subjects in clinical trials, pre-clinical research lags behind. The over-reliance on male animals and cells appears wide spread and may be one of the factors behind the disconnect between the pre-clinical promise and ultimate failure of many novel treatment strategies [2].

Research in Sepsis-Induced Cardiomyopathy is not immune to this bias. In a recent review [3], we identified 27 studies that focused on myocardial calcium (Ca^{2+}) homeostasis in animal models of sepsis. Of these 27 studies, 23 used solely male animals [3]. Among the 19 studies that used isolated cardiomyocytes, all 19 used solely male animals [3].

These studies have amassed impressive evidence that, in male mice, Sepsis-Induced Cardiomyopathy develops as the result of dysregulation of myocardial Ca^{2+} handling, leading to a decrease in the amplitude of the cellular Ca_i transient (Ca_i). Underlying Ca_i depression, a number of Ca^{2+} transporters are dysfunctional, such as the L-type Ca^{2+} channel, the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) and ryanodine receptors [3]. As an exception to this rule, only a few animal models are associated with a decrease in myofilament Ca^{2+} sensitivity and preserved Ca_i [4]. Whether the same mechanisms underlie Sepsis-Induced Cardiomyopathy in female mice is yet unknown.

Upstream of Ca^{2+} handling, the mechanisms underlying the dysfunction of the Ca^{2+} transporters are poorly understood. In male mice, we have shown [5] that SERCA inhibition occurs as the result of oxidative modifications (sulphonylation) of one cysteine residue ($\text{Cys}^{674}\text{-SO}_3\text{H}$). Interestingly, SERCA oxidation was more pronounced in mice that were genetically engineered to be deficient in the activity of soluble guanylyl cyclase (sGC) $\alpha_1^{-/-}$ subunit, and have impaired cGMP production, suggesting that, in male mice, cGMP plays a protective, antioxidant effect. This contrasts with a number of previous studies in which sGC-generated cGMP has been suggested to play a pathological role in Sepsis-Induced Cardiomyopathy [6, 7]. No data is yet available about the role of cGMP in Sepsis-Induced Cardiomyopathy in female mice.

As a first step towards correcting this sex bias, we undertook here the first head-to-head comparison of male and female mice in a model of Sepsis-Induced Cardiomyopathy. To facilitate this comparison, we used a similar model as in our previous study in male mice [5], based on exogenous administration of bacterial endotoxin (lipopolysaccharide LPS). Without any previous information available in female mice, we hypothesized that LPS-induced cardiomyopathy develops through similar mechanisms as in male mice, including Ca_i depression and SERCA inhibition. Unexpectedly, the results revealed that, in female mice, LPS-induced cardiomyopathy develops in the absence of Ca_i depression, most likely

as the result of a dysfunction of the myofilaments. In addition, we found that female $sGC\alpha_1^{-/-}$ mice were partially protected against LPS-induced cardiomyopathy and death, which is contrary to the male phenotype [5]. This indicated that, in female mice, cGMP plays critical contributory roles in the development of LPS-induced cardiomyopathy, opposite to the protective roles exerted in male mice [5].

Materials and Methods

Animals

Male and female C57Bl/6J (WT) and $sGC\alpha_1^{-/-}$ mice were studied at the age of 15-35 weeks (20-35g). WT mice were purchased from Jackson Labs., ME. $sGC\alpha_1^{-/-}$ mice were bred in house. $sGC\alpha_1^{-/-}$ mice carry a targeted deletion of the sixth exon of the $sGC\alpha_1$ gene, resulting in the expression of a catalytically inactive protein [8]. $sGC\alpha_1^{-/-}$ mice were backcrossed 7 generations to C57Bl/6J background. As in our previous study [5], the $sGC\alpha_1^{-/-}$ and WT mice used in this study were not littermates. We are aware of known limitations of this design (mostly the remote possibility of spontaneous mutations that may have appeared in the $sGC\alpha_1^{-/-}$ mouse colony), however, we employed it in order to avoid producing 50% excess heterozygote mice.

The WT and $sGC\alpha_1^{-/-}$ female mice studied here were not selected on the basis of the estrus cycle (see Limitations section in Discussion).

All 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 by the *Institutional Animal Care and Use Committee* (IACUC) of *Boston University School of Medicine*.

Sepsis model

To allow comparison with our previous study [5], we used a model of Sepsis-Induced Cardiomyopathy based on intraperitoneal (ip) administration of LPS (from *E. Colli*, 0111:84, Sigma) at doses between 4 and 100 $\mu\text{g/g}$ body weight. LPS administration induced an inflammatory shock syndrome, with lethargy, piloerection and hypothermia. In surviving mice, the symptoms usually resolved by 72h after LPS administration.

Cardiomyocyte studies

Mice were euthanized at baseline or $14 \pm 2\text{h}$ after LPS (25 $\mu\text{g/g}$) challenge. Euthanasia was achieved using general anesthesia with isoflurane, followed by heart removal. LV cardiomyocytes were isolated enzymatically, as we described [5, 9]. Cardiomyocytes were placed in a physiological Tyrode solution (containing, in mM: NaCl 137, KCl 5.4, CaCl_2 1.2, MgCl_2 0.5, HEPES 10, glucose 5 and probenecid 0.5, pH 7.40) and externally paced at 2 and 5 Hz at 37°C . Experiments shown here were performed at a pacing frequency of 5Hz, with similar data being obtained at 2Hz.

Cardiomyocytes sarcomere length and intracellular Ca^{2+} (Ca_i) levels (using fura-2 AM, Molecular Probes) were measured simultaneously using an integrated system (IonOptix,

MA, featuring a HyperSwitch dual 340 / 380 nm excitation light source). Probenecid was added to the superfusing solution to increase fura-2 retention.

To verify the sensitivity of the Ca^{2+} imaging system we performed an *in vitro* calibration using fura 2 K salt (Molecular Probes). R_{min} (measured using droplets of the Tyrode solution given above, Ca^{2+} -free, and with 1mM EGTA) was 0.93 ± 0.01 . R_{max} (Tyrode solution with 3 mM Ca^{2+}) was 4.27 ± 0.58 . The beta coefficient (380 signal in Ca^{2+} -free / Ca^{2+} -bound) [10] was 2.10 ± 0.32 ($n=5$ measurements). Fura ratios recorded in cardiomyocyte are shown as raw signals, without attempting a calibration for free Ca^{2+} . We did not extrapolate the *in vitro* values to cardiomyocyte experiments, because of the known modulation of fura characteristics in the intracellular milieu [11].

The primary outcomes of the cardiomyocyte experiments were Sarcomere Shortening and Ca_i . Sarcomere Shortening was expressed as percent of Diastolic Sarcomere Length. Ca_i was measured as the difference between peak fura ratio and fura ratio at rest. Sarcomere departure velocity (Dep. V.) and return velocity (Ret. V.) were measured as the maximal rate of sarcomere shortening and relaxation, respectively. Ca^{2+} departure velocity (Ca_i/dt) was measured as the maximal rate of rise of the fura ratio. The decrease in fura ratio in diastole was fitted with a monoexponential curve, whose time constant (τ_{Ca}) represents an inverse measure of SERCA activity [5]. The Time-To-Peak of Sarcomere Shortening and of the Ca^{2+} transient were measured from the time of the pacing stimulus. Diastolic Sarcomere Length and diastolic Ca_i levels were measured just before the following Ca_i transient.

These parameters quantify different aspects of cardiomyocyte physiology. Sarcomere Shortening and Ca_i are the ultimate output of cardiomyocyte contractile function, and those most closely related to myocardial contractility *in vivo*. Sarcomere Shortening Departure Velocity and Ca_i/dt are a better estimate of the rate of the underlying biophysical processes, the myofilament crossbridge cycling and Ca^{2+} release through the ryanodine receptors, respectively. The Time-To-Peak of Sarcomere Shortening and Ca_i is another determinant of peak values, and is largely dictated by the opening time of the ryanodine receptors. Sarcomere Shortening Return Velocity and τ_{Ca} measure the diastolic relaxation properties of the cardiomyocytes, which are ultimately reflected in the diastolic levels of Ca^{2+} and Diastolic Sarcomere Length.

Echocardiography

Two-dimensional and M-Mode echocardiography of LV function was performed at baseline and 14 ± 2 h after challenge with 25 $\mu\text{g/g}$ LPS. Echocardiography was performed in isoflurane sedated mice using a VisualSonics Vevo 770 high-resolution machine and a 30-MHz transducer as previously described [12]. The primary outcome was LV ejection fraction (LVEF), which was calculated using the single dimension method. LV internal diameter (LVID) was measured at the maximum relaxation in diastole. LV total wall thickness (TWT) was calculated as the sum of the LV anterior and posterior walls thickness. Echocardiography assessments were performed at comparable heart rates between groups.

Survival assessments

Survival rate was assessed twice daily. Per *IACUC* 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 euthanasia.

To prevent hypovolemia, mice were administered 0.5 mL of warm, sterile Normal Saline solution ip at the time of LPS administration. After LPS administration, Normal Saline boluses were also administered at the time of health checks (twice daily) to mice that showed diminished mobility that would impede access to drinking water. Mice that displayed normal mobility and could easily access drinking water were assumed to be euvolemic and did not receive additional ip hydration.

All mice groups were initially challenged with a dose of 7 $\mu\text{g/g}$ LPS. Depending on the initial mortality results, the LPS dose was increased or decreased accordingly. Because of different sensitivity of male vs. female and $\text{sGC}\alpha_1^{-/-}$ vs. WT mice, the range of LPS doses used is not the same in all the groups. For example, male mice received a challenge with 4 $\mu\text{g/g}$, which was not used for female mice, in which a dose of 7 $\mu\text{g/g}$ LPS induced no mortality. Similarly, $\text{sGC}\alpha_1^{-/-}$ female mice were challenged with higher doses of LPS than female WT mice and male WT and $\text{sGC}\alpha_1^{-/-}$ mice, because of their reduced sensitivity to LPS.

Statistics

We compared 8 groups of mice: WT male, WT female, $\text{sGC}\alpha_1^{-/-}$ male and $\text{sGC}\alpha_1^{-/-}$ female mice, both at baseline and after LPS challenge. All values are shown as means $\pm$ SEM. $P < 0.05$ was considered significant.

Cardiomyocyte experiments (Figures 1-4) were performed using a sample size of 25 cells from 4 mice, which prior studies indicated as sufficient to observe a meaningful 20% difference. The only exception to this rule was the WT male LPS group, which aimed to confirm previously published results [5], and was performed in a reduced group of 2 mice.

We used 2 different approaches to analyze cardiomyocyte data. The first approach employed a two-way ANOVA test followed by a one sided, unpaired Student's *t*-test, with a Bonferroni correction for multiple comparisons (GraphPad Prism 6.00 for Windows). For this analysis, we considered each cell to be an independent observation (n =number of cells). Because this assumption was not unequivocally confirmed by an Interclass Correlation Coefficient test in each group studied, we used a second statistical method that applied a random intercepts model with post hoc tests using Tukey multiple comparisons (R Studio statistical software). The main conclusions of the study were the same with either approach. The statistical values shown in the Figures were obtained using the Tukey test.

Echocardiography experiments were performed before and after LPS challenge in the same mice, using a paired approach. 6-10 echocardiography stills were recorded and measurements averaged for each heart. Groups were compared using two -way ANOVA, followed by a paired, one sided *t*-test, with a Bonferroni correction (GraphPad Prism 6.00 for Windows).

Survival analysis in Figure 7 was performed using a Mantel-Cox test (GraphPad Prism 6.00 for Windows).

Results

The main goal of this study was to identify and compare the cellular mechanisms responsible for LPS-induced cardiomyopathy in male vs. female C57Bl/6J mice. For this, we isolated LV cardiomyocytes from male and female mice, at baseline and 14 ± 2h after LPS challenge. We measured Sarcomere Shortening and Ca_i in cardiomyocytes externally paced at 5 Hz and 37° C. Figure 1A shows typical experimental recordings from cardiomyocytes isolated from male and female mice, at baseline and after LPS challenge.

1. Sarcomere Shortening is depressed after LPS challenge in both male and female WT mice (Figure 1)

At baseline, cardiomyocytes isolated from female mice showed Sarcomere Shortening (Figure 1B), Departure Velocity (Figure 1C) and Return Velocity (Figure 1D) that were not significantly different than in male mice. Sarcomere Shortening Time-To-Peak was also similar in male and female mice (Figure 1E) at baseline, whereas Diastolic Sarcomere Length was slightly larger in female vs. male mice (Figure 1F).

Consistent with our previous findings [5], in male mice, LPS challenge induced a profound depression of cardiomyocyte Sarcomere Shortening (Figure 1B), Departure Velocity (Figure 1C) and Return Velocity (Figure 1D). The Time-To-Peak of Sarcomere Shortening was not changed (Figure 1E), and neither was Diastolic Sarcomere Length (Figure 1F).

In female WT mice, LPS challenge decreased Sarcomere Shortening (Figure 1B) and Departure velocity (Figure 1C), to a level not significantly different than in male mice. LPS also decreased Return Velocity, with borderline significance ($p = 0.10$, Figure 1D). There was no change in Sarcomere Shortening Time-To-Peak (Figure 1E) or Diastolic Sarcomere Length after LPS challenge in female mice (Figure 1F).

2. LPS depresses Ca_i and SERCA in WT male, but not in female WT mice (Figure 2)

At baseline, Ca_i (Figure 2A), Ca_i/dt (Figure 2B), T_{Ca} (Figure 2C), Ca^{2+} transient Time-To-Peak (Figure 2D) and diastolic Ca^{2+} level (Figure 2E) were similar in male and female mice.

In male mice, LPS administration induced a depression of Ca_i (Figure 2A), and Ca_i/dt (Figure 2B). T_{Ca} was prolonged after LPS (Figure 2C), indicating that SERCA function is inhibited, consistent with our previous findings [5]. Ca^{2+} transient Time-To-Peak was prolonged after LPS (Figure 2D), while diastolic Ca^{2+} level was unchanged (Figure 2E).

Importantly, in female mice, LPS challenge did not induce a significant decrease in Ca_i and Ca_i/dt , as it did in male mice (Figure 2A). As a result, Ca_i and Ca_i/dt were significantly greater in WT female vs. WT male mice after LPS challenge (Figure 2A). T_{Ca} was not changed, indicating that SERCA function was intact in female WT mice, as opposed to male

mice (Figure 2C). Ca^{2+} transient Time-To-Peak and diastolic Ca^{2+} level were unchanged after LPS (Figure 2D-E).

3. In female mice, $\text{sGC}\alpha_1$ deficiency protects against the depression of cardiomyocytes Sarcomere Shortening after LPS (Figure 3)

Figure 3A shows typical Sarcomere Shortening and Ca^{2+} transients of cardiomyocytes isolated from WT and $\text{sGC}\alpha_1^{-/-}$ female mice, at baseline and after LPS challenge. At baseline, Sarcomere Shortening (Figure 3B), Departure Velocity (Figure 3C), Return Velocity (Figure 3D) and Sarcomere Shortening Time-To-Peak (Figure 3E), and diastolic Sarcomere Length (Figure 3F) were similar in female $\text{sGC}\alpha_1^{-/-}$ and WT mice.

After LPS, Sarcomere Shortening (Figure 3B). Departure Velocity (Figure 3C) and Return Velocity (Figure 3D) were not significantly decreased in female $\text{sGC}\alpha_1^{-/-}$ mice (although a nonsignificant trend towards a decrease was evident). Sarcomere Shortening Time-To-Peak and Diastolic Sarcomere Length were unchanged after LPS in $\text{sGC}\alpha_1^{-/-}$ female mice (Figure 3E-F).

4. In female $\text{sGC}\alpha_1^{-/-}$ mice, LPS challenge does not change Ca_i (Figure 4)

At baseline, Ca_i was not significantly different in $\text{sGC}\alpha_1^{-/-}$ and WT female mice at both at baseline and after LPS (although a trend towards larger values in $\text{sGC}\alpha_1^{-/-}$ mice was evident, with borderline significance, $p=0.06$, Figure 4A-B). τ_{Ca} was similar, indicating that SERCA function is not changed (Figure 4C). Diastolic Ca^{2+} levels and Ca^{2+} transient Time-To-Peak were also unchanged (Figure 4D-E)

LPS challenge did not induce any change in Ca_i , Ca_i/dt , τ_{Ca} , diastolic Ca^{2+} levels or Ca^{2+} transient Time-To-Peak in $\text{sGC}\alpha_1^{-/-}$ female mice (Figure 4A-E). This was identical to the findings in WT female mice. There was a borderline trend ($p=0.09$) towards an increased Ca_i in LPS-challenged $\text{sGC}\alpha_1^{-/-}$ vs. WT female mice (Figure 4A)

5. LPS depresses LV EF to a similar degree in WT male and female mice (Figures 5-6)

Next we studied cardiac performance by echocardiography. We compared mice at baseline and 14 ± 2 h after challenge with $25 \mu\text{g/g}$ LPS (same protocol as for cardiomyocyte studies). To preserve euvolemia, all LPS-challenged mice received a bolus of warm Normal Saline (0.5 ml, ip) 20 min before the beginning of the echocardiography study. Figure 5 illustrates typical echocardiography stills recorded in male and female, WT and $\text{sGC}\alpha_1^{-/-}$ mice, at baseline and after LPS challenge.

LV EF (Figure 6A) was similar at baseline in male and female mice of both genotypes. After LPS administration, LVEF was decreased in both sexes and genotypes, signifying the development of cardiomyopathy. LVEF decrease after LPS was similar in WT male, WT female and $\text{sGC}\alpha_1^{-/-}$ male mice, but was less pronounced in $\text{sGC}\alpha_1^{-/-}$ female mice. The partial protection of $\text{sGC}\alpha_1^{-/-}$ female mice to LPS was consistent with the less pronounced decrease in cardiomyocyte Sarcomere Shortening (Figure 3).

LV TWT (Figure 6B) was similar at baseline in male and female mice, either WT or $\text{sGC}\alpha_1^{-/-}$. LPS challenge did not change TWT in either groups. LV ID (Figure 6C) was

similar at baseline in the 4 groups. LPS induced a slight decrease in LV ID in male WT mice, (and a similar trend in female WT mice), signifying the persistence of a slight degree of hypovolemia after LPS challenge, despite ip resuscitation. LV ID was not changed after LPS challenge in sGC $\alpha_1^{-/-}$ mice, either male or female.

At baseline, heart rate (Figure 6D) was similar in the 4 groups. LPS challenge was associated with a slight decrease in heart rate, in both male and female WT mice and in sGC $\alpha_1^{-/-}$ female mice (with a similar trend in sGC $\alpha_1^{-/-}$ male mice).

6. In female mice, sGC α_1 deficiency confers protection against LPS-induced mortality (Figure 7)

Next, we aimed to determine whether the differences in cardiomyocyte function and cardiac EF identified above play a role in determining mouse mortality after LPS challenge. For this, we used a range of LPS doses between 4 and 100 $\mu\text{g/g}$ body weight.

In WT male mice (Figure 7A), mortality increased with increasing doses of LPS, from zero mortality after a dose of 4 $\mu\text{g/g}$ LPS to 100% mortality after a dose of 20 $\mu\text{g/g}$ LPS.

In WT female mice (Figure 7B), mortality was zero after challenge with 7 or 10 $\mu\text{g/g}$ LPS, which was significantly less than in WT males. Challenge with 20 $\mu\text{g/g}$ LPS, however, induced 100% mortality, similar to WT males. Female protection against LPS-induced mortality was, therefore, manifested only at lower doses (that induce a mild disease state), but not at higher doses.

In male sGC $\alpha_1^{-/-}$ mice (Figure 7C), LPS induced mortality was not significantly different than in WT males, although a trend towards a worsening was seen for 4 $\mu\text{g/g}$ LPS ($p=0.070$).

Female sGC $\alpha_1^{-/-}$ mice (Figure 7D) showed an improved survival after LPS challenge, as compared to WT females (most clearly seen for a dose of 20 $\mu\text{g/g}$) and sGC $\alpha_1^{-/-}$ males (for doses between 7-20 $\mu\text{g/g}$). The improved survival of female sGC $\alpha_1^{-/-}$ mice, compared to female WT or male sGC $\alpha_1^{-/-}$ mice was consistent with the less severe depression in LV EF (Figure 5-6) and cardiomyocyte Sarcomere Shortening (Figure 3) found above.

Discussion

This is the first head-to-head comparison of female vs. male C57Bl/6J mice from the point of view of cardiomyocyte Ca^{2+} handling, at baseline and after challenge with LPS. Extending our previous studies in male mice [5, 9], we found that LPS depresses cardiomyocyte Sarcomere Shortening in female mice also, to a level comparable with that observed in male mice. Importantly, cardiomyocyte dysfunction was the result of different cellular mechanisms in male and female mice. In male mice, LPS acts by inhibiting SERCA function, which contributes to a decrease in Ca_i [5, 9]. In contrast, in female mice, Ca_i and SERCA were not changed after LPS, and the depression of cardiomyocyte Sarcomere Shortening was due to mechanisms downstream of the Ca^{2+} transient, possibly represented by an impairment of the myofilaments function. Upstream of Ca^{2+} handling, sGC α_1 – synthesized cGMP played opposite roles in male and female mice. We have previously found that, in male mice, cGMP plays protective roles [5, 9], by opposing SERCA redox-

dependent inhibition [5]. On the contrary, in female mice, cGMP plays a pathological role, by mediating the development of contractile dysfunction.

1. LPS depressed cardiomyocytes Sarcomere Shortening in male and female WT mice

At baseline, cardiomyocytes isolated from male and female mice had similar Sarcomere Shortening, Ca_i and T_{Ca} . LPS challenge depressed SS in both male and female mice, to comparable levels. However, the underlying mechanisms underlying SS depression were different in male and female mice (see below).

2. Distinct Ca^{2+} handling abnormalities underlie the development of LPS induced cardiomyopathy in male and female mice

In male mice, Sarcomere Shortening depression induced by LPS was associated with a profound decrease in Ca_i and SERCA inhibition. These results were consistent with our previous findings [5] that, in male C57Bl/4 mice, LPS induced SERCA dysregulation, as a results oxidative modifications (sulphonylation) of one reactive Cys residue (Cys⁶⁷⁴-SO₃H). Downstream of the Ca^{2+} transient, we have previously shown that there were no changes in myofilament function after LPS, and the decrease in Sarcomere Shortening occurred proportionally to the decrease in Ca_i [5].

In contrast, in female mice, the depression of cardiomyocytes Sarcomere Shortening after LPS occurred in the absence of changes in Ca_i and SERCA. This indicated the presence of deficits in mechanisms downstream of the Ca_i transients, whose exact nature remains to be determined. The most likely hypothesis is a decrease in myofilament sensitivity for Ca^{2+} , following troponin I hyperphosphorylation, as previously demonstrated in male mice after challenge with lower doses (6 µg/g) of LPS [4]. Alternative mechanisms include a decrease in myofilament maximal tension [13] or an increase in the passive resistance of the cell, such as internal cell viscosity (mainly determined by the density of microtubules) [14] or the myofilament (titin) elastic resistance [15].

3. Differential roles for cGMP in male and female hearts

A second important observation was that sGC $\alpha_1^{-/-}$ deficiency modulated cardiomyocyte response to LPS differently in male and female mice.

In male mice, we have previously reported that sGC α_1 deficiency is associated with increased SERCA Cys⁶⁷⁴-SO₃H formation and SERCA inhibition, and a more profound decrease in Ca_i and Sarcomere Shortening after LPS challenge [5, 9]. This indicated that, in male mice, cGMP generated by sGC plays a protective, antioxidant role [5] after LPS challenge.

In the current study, in female mice, sGC α_1 deficiency was associated with improved survival rates, less severe LVEF depression and less severe depression of cardiomyocyte Sarcomere Shortening, as compared to WT female mice. This indicated, therefore, that in female mice, the depression of cardiomyocyte Sarcomere Shortening after LPS is partially mediated by the release of cGMP from sGC α_1 . Mechanistically, one possible pathway involved could be, for example, the inhibition of the enzyme phosphodiesterase 3 (PDE3) by

cGMP[16], which would lead to an increase in cAMP levels and subsequent phosphokinase A –dependent phosphorylation of TnI. TnI hyperphosphorylation has been suggested as the causative factor leading to the decrease in myofilament sensitivity for Ca^{2+} in some models of Sepsis-Induced Cardiomyopathy [4]. Whether this is indeed the causative pathway in LPS-challenged female mice remains to be determined.

4. Female $\text{sGC}\alpha_1^{-/-}$ mice have increased survival rates, and less severe depression of LVEF than WT female or male WT and $\text{sGC}\alpha_1^{-/-}$ mice

In WT mice, female mice had preserved survival rates after challenge with low doses of LPS (7-10 $\mu\text{g/g}$). This is in accord with a previously published observation that challenge with 4 $\mu\text{g/g}$ LPS induces a less severe depression of LVEF in female vs. male mice [17] and with the general belief that female sex confers a relative protection against sepsis [18]. However, challenge with higher doses of LPS (15-20 $\mu\text{g/g}$) induced similar mortality rates in male and female mice, associated with a similar decrease in LVEF. It seems likely, therefore, that the relative protection conferred by female sex is limited to less severe forms of disease.

Importantly, $\text{sGC}\alpha_1^{-/-}$ female mice showed better survival and a less pronounced decrease in LV EF than WT female and $\text{sGC}\alpha_1^{-/-}$ male mice. This was consistent with the less pronounced decrease in cardiomyocyte Sarcomere Shortening observed, and strongly suggested that in female mice, sGC-released cGMP plays a significant pathophysiological role in LPS-induced cardiomyopathy and mortality. In contrast, after challenge with high doses of LPS, male $\text{sGC}\alpha_1^{-/-}$ mice had similar mortality rates and degree of LV EF depression as male WT mice (see Limitations).

5. Pathophysiological implications

A unitary hypothesis (Figure 8) can explain how redox- and cGMP mechanisms may mediate LPS-induced cardiomyopathy separately in male and female mice. We can assume that both mechanisms are initially activated by nitric oxide (NO) released by the inducible NO synthase (NOS2) in both male and female myocardium. In male mice, oxidative stress dominates, and cause SERCA oxidation and Ca_i depression, while it prevents cGMP formation by inhibiting sGC [19]. Female mice, however, are known to have more potent myocardial antioxidant defenses, as the result of higher levels of antioxidant enzymes (Mn-superoxide dismutase and glutathione peroxidase), and decreased mitochondrial ROS production [20]. Therefore, in the female myocardium, it is possible that LPS does not increase oxidative stress sufficiently to depress SERCA and Ca_i . In the same time, redox dependent sGC inhibition [19] is also absent, and the levels of cGMP increase, triggering myofilaments dysfunction downstream. Clearly, future experiments are needed to explore this hypothesis, which, if confirmed, may have significant therapeutic implications.

6. Clinical implications

Significant attention has been devoted to the concept of sepsis “endotypes [21]; subclasses of patients that fall under the diagnosis of septic shock but differ significantly (as the result of different genetic background, sepsis etiology or co-morbidities) to the result that they require different therapeutic strategies. Here we suggest, for the first time, that distinct etiological endotypes may exist within the diagnosis of Sepsis-Induced Cardiomyopathy. If

the sex differences found here also apply to humans, it is possible that male and female patients may require different therapeutic strategies. Women with Sepsis-Induced Cardiomyopathy may benefit from therapeutic strategies that increase myofilament sensitivity [22] and/or inhibit sGC activity [23]. In contrast, male patients may benefit from drugs that potentiate antioxidant defenses [24, 25] as well as any approaches that stimulate SERCA and/or L-type Ca^{2+} channels and accelerate Ca^{2+} handling.

It is important to realize that attempting to treat Sepsis-Induced Cardiomyopathy without knowing the exact cause of cardiomyocyte dysfunction may do more harm than good. For example, attempting to compensate a decrease in Ca_i by increasing myofilament sensitivity to Ca^{2+} [22] may improve the contractility, but at the expense of worsening cardiac diastolic compliance. On the reverse, increasing Ca_i (usually positive inotropic drugs such as dobutamine or digoxin) in patients in which the deficit lies at the level of myofilaments may induce life threatening ventricular arrhythmias.

7. Limitations and future directions

The current experiments were designed to reproduce those used in our previous study in male mice [5]. We used the same WT mice (from same vendor), and $\text{sGC}\alpha_1^{-/-}$ mice (from our own colony) as before [5]. We used the same LPS type (from *E. Colli*, 0111:84, Sigma), dose (25 $\mu\text{g/g}$) and incubation time (14 ± 2 h). In these conditions, we replicated our main previous findings [5], such as the observation that, in male mice, LPS induces a profound decrease in cardiomyocyte Ca_i , SERCA function and Sarcomere Shortening, associated with a decrease in LVEF and significant mortality. However, we acknowledge a number of minor differences between our current and previous results. For example, mortality rate was generally less in our previous study [9] (50% after a dose of 25 $\mu\text{g/g}$ LPS) than here (100% after a dose of 20 $\mu\text{g/g}$ LPS), for WT male mice. Also, the decrease in LVEF after LPS was more pronounced in $\text{sGC}\alpha_1^{-/-}$ vs. WT male mice in our previous [9], but was similar in $\text{sGC}\alpha_1^{-/-}$ and WT male mice in this study. These inconsistencies could be attributed to differences in animal care and housing, or batch of LPS, and do not influence our main conclusions.

The female mice used in this study were not selected on the basis of the estrus cycle. Given the short duration of the estrus cycle in mice (4-5 days), we did not expect any phenotypical changes at the level of the cardiomyocytes to occur cyclically so rapidly. However this may be possible, and it remains for the future to determine whether the sex differences identified here apply across the female estrus cycle or are cycle phase dependent.

We also acknowledge that the current results will have to be replicated in other Sepsis-Induced Cardiomyopathy models [26] and species, before its main conclusions could be translated in clinical research.

8. Summary and conclusions

The present study is the first investigation of myocardial Ca^{2+} handling in endotoxemic female mice. Consistent with a wealth of previous data [18], we show that that female sex is associated with improved survival rates after challenge with lower doses of LPS. However, higher doses of LPS were associated with similar survival rates and a similar decrease in

LVEF in male and female WT mice. LPS induces the depression of cardiomyocyte Sarcomere Shortening in both female than in male mice. Importantly, cardiomyocyte dysfunction occurs through different mechanisms in the two sexes. While in male mice, LPS induces SERCA inhibition and depressed Ca_i , in female mice Ca^{2+} handling is preserved, and the deficits appear primarily confined downstream of the Ca_i transient, possibly at the level of the myofilaments. Upstream of Ca^{2+} handling, cGMP generated by sGC also plays opposite roles in the two sexes, being protective in males and pathological in females. These sex differences suggest that different therapeutic strategies may be required for cardiac protection in septic men and women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LPS challenge decreases cardiomyocyte shortening in WT male and female mice

A. LV cardiomyocytes were isolated and externally paced at 5 Hz at 37 °C. Typical recordings of cell shortening and Ca^{2+} transients (measured with fura2 AM) in cells isolated from male and female WT mice at baseline and after LPS challenge.

B-F. Average Sarcomere Shortening (B), departure velocity (C), return velocity (D), sarcomere time to peak (E) and diastolic sarcomere length (F) in male and female WT mice, at baseline and after LPS challenge. N= 33 cells from 5 mice (33/5) for WT male BL, 27/2 for WT male LPS, 49/4 for WT female BL and 47/4 for WT female after LPS. For this and Figure 5, * denotes $p < 0.05$ for LPS vs. BL; #: denotes $p < 0.05$ for male vs. female for either baseline or LPS.

Figure 2. LPS challenge inhibits SERCA and decrease cardiomyocyte Ca^{2+} transients in male but not female WT mice

A. Average Ca_i (A), Ca_i/dt (B), τ_{Ca} (C), Ca_i transients Time-To-Peak (D) and diastolic Ca_i levels (E) in male and female WT mice, at baseline and after LPS. N's as in Figure 1.

Figure 3. The decrease in Sarcomere Shortening induced by LPS is partially prevented in female sGCα₁^{-/-} mice

A. Typical recordings of cell shortening and Ca²⁺ transients in cells isolated from female WT and sGCα₁^{-/-} mice at baseline and after LPS.

B-F. Average Sarcomere Shortening (B), departure velocity (C), return velocity (D), sarcomere time to peak (E) and diastolic sarcomere length (F) in female WT and sGCα₁^{-/-} mice, at baseline and after LPS challenge. WT data are the same as in Figure 1. N= 49/4 for WT female BL, 47/4 for WT female after LPS, 32/5 for sGCα₁^{-/-} BL and 36/4 for sGCα₁^{-/-} LPS. For this and Figure 7, * denotes p<0.05 for LPS vs. BL; #; denotes p<0.05 for WT vs. sGCα₁^{-/-}.

Figure 4. LPS challenge does not decrease cardiomyocyte Ca^{2+} transients in sGC $\alpha_1^{-/-}$ female mice

Average ΔCa_i (A), $\Delta Ca_i/dt$ (B), τ_{Ca} (C), ΔCa_i transients Time-To-Peak (D) and diastolic ΔCa_i levels (E) in female WT and sGC $\alpha_1^{-/-}$ mice, at baseline and after LPS. WT data are the same as in Figure 1. N's as in Figure 3

Figure 5. M modes echocardiography stills in mice at baseline (left panel) and after challenge with 25 μg/g LPS (right panel) in male and female, WT and sGCα₁^{-/-} mice at baseline and after LPS

A. Typical echocardiography still illustrating the main parameters measured. LV anterior wall is shown on top, and the posterior wall on the bottom. The anterior wall thickness (AW), posterior wall thickness (PW) and LV internal diameter (ID) were measured in systole (AW_S, PW_S, and ID_S) and diastole (AW_D, PW_D, and ID_D), respectively. Total wall thickness (TWT) was calculated as AW_D+PW_D. Heart filling was quantified by measuring the LV internal diameter in diastole (ID_D). LV EF was calculated from the ID_S and ID_D measurements, using VisualSonics software. B-E: Typical echocardiography stills WT male (B), WT female (C), sGCα₁^{-/-} male (D) and sGCα₁^{-/-} female mice (E) at baseline (left) and after LPS (right).

Figure 6. Echocardiography parameters at baseline and after LPS challenge, in male and female WT and sGC $\alpha_1^{-/-}$ mice

LV EF (A), TWT (B), LV ID (C) and HR (D), before and after LPS challenge in the 4 groups. N= 6 mice/group. *: $p < 0.05$ for LPS vs. BL; \$: $p < 0.05$ for M vs. F and #: $p < 0.05$ for sGC $\alpha_1^{-/-}$ vs. WT.

Figure 7. Kaplan-Meier diagrams illustrating survival after escalating doses of LPS in WT male (A) and female (B) mice, and sGC α_1 ^{-/-} male (C) and female (D)

LPS doses (in $\mu\text{g/g}$) are shown for each experiment on the right side. For easier identification, survival curves are color coded, using same color for each LPS dose and curve.

M: male; F: female. \$: $p < 0.05$ for male vs. female of the same genotype, for either BL and LPS; #: $p < 0.05$ for sGC α_1 ^{-/-} vs. WT, in either sex, for either BL or LPS. N=6-17 mice for each group.

Figure 8. A unitary theory can explain the different myocardial mechanisms in male and female mice after LPS challenge

We can assume that, in Sepsis-Induced Cardiomyopathy, NO acts by 2 pathways. First, NO activates sGC to release cGMP which may mediate myofilament dysfunction by inhibiting PDE3 [16], leading to an increase in cAMP –dependent hyperphosphorylation [4]. In the same time, NO can combine with ROS (such as superoxide, which is generated in excess in sepsis by uncoupled NOS, NADPH-dependent oxidases (Nox), cyclooxygenase (COX), xanthine oxidase (XO), as well as mitochondria [27, 28]) to form RONS (such as peroxynitrite, ONOO⁻). Both ROS and RONS can induce SERCA inhibition via formation of Cys⁶⁷⁴-SO₃H [5, 29] and Ca_i depression. Importantly, the two pathways inhibit each other. RONS are known to inhibit sGC, following thiol oxidative modifications of Cys residues [19, 30, 31]. On the other hand, we have shown that cGMP plays an antioxidant role in LPS-challenged myocardium, limiting SERCA Cys⁶⁷⁴-SO₃H formation (by a yet unknown mechanism) [5]. As such we propose that, in the male myocardium, RONS cause SERCA Cys⁶⁷⁴-SO₃H formation and Ca_i depression, while they prevent cGMP formation though sGC oxidation [19]. In female mice, RONS stress is prevented, as a result of a naturally increased antioxidant capability [20]. As such, in the female myocardium, RONS

induced SERCA depression is prevented, but so is sGC inhibition, which leaves NO free to activate cGMP synthesis from sGC, and cGMP-dependent myofilament dysfunction.

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