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LPS and cytokines inhibit rat cardiomyocyte contractility in vitro

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Abstract

Background—Sepsis-induced cardiomyopathy (SIC) is thought to be the result of detrimental effects of inflammatory mediators on cardiac muscle. Here we studied the effects of prolonged (24 ± 4 h) exposure of adult rat ventricular myocytes (ARVM) to bacterial lipopolysaccharide (LPS) and inflammatory cytokines tumor necrosis factor (TNF) and interleukins-1 (IL-1) and -6 (IL-6).

Materials and methods—We measured sarcomere shortening (SS) and cellular calcium (Ca^{2+}) transients (Ca_i , with fura-2AM) in isolated cardiomyocytes externally paced at 5Hz at 37 °C.

Results—SS decreased after incubation with LPS (100 µg/ml), IL-1 (100 ng/ml) and IL-6 (30 ng/ml), but not with lesser doses of these mediators, or TNF (10–100 ng/ml). A combination of LPS (100 µg/ml), TNF, IL-1 and IL-6 (each 100 ng/ml; i.e. “Cytomix-100”) induced a maximal decrease in SS and Ca_i . Sarcoplasmic reticulum (SR) Ca^{2+} load (Ca_{SR} , measured with caffeine) was unchanged by Cytomix-100, however, SR fractional release ($\text{Ca}_i/\text{Ca}_{\text{SR}}$) was decreased. Underlying these effects, Ca^{2+} influx into the cell (via L-type Ca^{2+} channels) and Ca^{2+} extrusion

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Authors contributions

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conception and design	x	x	x	x
data collection	x	x		
analysis and interpretation	x	x	x	x
writing the article	x	x		
critical revision of the article	x	x	x	x
obtaining funding	x	x		x

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via $\text{Na}^+/\text{Ca}^{2+}$ exchange were decreased by Cytomix-100. SR Ca^{2+} pump (SERCA) was not affected.

Conclusions—Prolonged exposure of ARVM to a mixture of LPS and inflammatory cytokines inhibits cell contractility. The effect is mediated by the inhibition of Ca^{2+} influx via LTCC, and partially opposed by the inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange. Since both mechanisms are commonly seen in animal models of SIC, we conclude that prolonged challenge with Cytomix-100 of ARVM may represent an accurate *in vitro* model for SIC.

Keywords

Sepsis; sepsis –induced cardiomyopathy; calcium; excitation-contraction coupling; L-type calcium channel; SERCA; $\text{Na}^+/\text{Ca}^{2+}$ exchange

1. Introduction

Patients with sepsis and septic shock may develop a specific cardiomyopathy, which complicates their management and worsens prognosis (1). It is generally thought that sepsis-induced cardiomyopathy (SIC) is mediated, at least in part, by circulating pathological factors. Among these are bacterial components (such a bacterial lipopolysaccharide, LPS) and inflammatory cytokines, such as tumor necrosis factor (TNF), interleukine-1 (IL-1), IL-6, and others. In a larger sense, inflammation- and cytokine-induced cardiac dysfunction also impacts patients after prolonged surgery, trauma and burns, and post-cardiopulmonary bypass (2) as well as in chronic congestive heart failure (3).

However, the evidence supporting a causative role for systemic inflammatory mediators in the genesis of SIC is controversial. It has been shown that cardiac cells isolated from healthy animals can develop contractile dysfunction when challenged with serum from septic patients (4). However, this was not the case when cells were challenged with media containing circulatory factors released from activated immune cells (5, 6). Moreover, cells exposed specifically to LPS, IL-1, IL-6 or TNF *in vitro* can show either decreased (7–11), unchanged (12) or increased (10, 13, 14) contractility.

One potential explanation for these divergent results could lie in the experimental conditions used. Many studies (5, 7, 11, 13) used cells isolated from neonatal animals, in which the cellular calcium (Ca^{2+}) handling and excitation-contraction coupling is different from adult cells (see *Discussion*). In addition, with few exceptions (13, 15, 16), almost all available studies investigated the effects of short exposures to inflammatory mediators, between 5min (8) and 4h (12). This short exposure may be insufficient to induce the activation of the enzyme nitric oxide synthase 2, NOS2 (the main downstream mediator) (17) and thus may not replicate accurately the effects present *in vivo*.

Therefore, here we aimed to determine whether *prolonged* (24 ± 4 h) exposure of *adult* cardiac cells to LPS and inflammatory cytokines is capable of inducing a contractile deficit. In particular, we wanted to determine whether a combination of LPS and cytokines exerts an inhibitory effect, since these mediators are present concomitantly *in vivo*, and may exert synergic effects (7). Furthermore, we aimed to identify the specific Ca^{2+} transporters (18)

whose dysfunction is responsible for the inhibition of contractility induced by LPS and/or cytokines.

Apart from providing direct evidence for the causative role of circulating inflammatory mediators in the pathophysiology of SIC, we aimed to establish an *in vitro* model of SIC, which can be used to identify the signaling pathways responsible and to test novel drugs and therapeutic strategies.

2. Materials and Methods

2.1. Cell isolation and culture

Cells were isolated from the hearts of adult (200–220 g) male Sprague-Dawley rats as previously described (19).

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 Committees* of Boston University School of Medicine.

Cells were plated at a non-confluent density of 30–50 cells/mm² on 100-mm plastic culture dishes (Fisher) precoated with laminin (1 µg/cm², Becton-Dickinson) and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) also containing bovine serum albumin (BSA, 2 mg/ml), L-carnitine (2 mM, Sigma), creatinine (5 mM, Sigma), taurine (5 mM, Sigma), penicillin (100 IU/ml) and streptomycin (10 µg/ml). LPS (Sigma), TNF, IL-1 and IL-6 (R&D systems) were diluted according to manufacturer's instructions in sterile PBS containing 1 mg/ml BSA, and added to the media. Control cells had an equivalent volume of vehicle solution added. Cells were maintained for >20 h in a 5% CO₂ incubator at 37 °C before measurements.

2.2. Measurements of cell shortening and Ca_i levels

Cardiomyocytes were superfused with a physiological Tyrode solution, containing, in mM: NaCl 137, KCl 5.4, CaCl₂ 1.2, MgCl₂ 0.5, HEPES 10, glucose 5 and probenecid 0.5, pH 7.40. Probenecid was added to increase fura-2 retention (see below). Cells were externally paced at 2 and 5 Hz. The data shown are obtained using a 5Hz pacing protocol, and identical results were obtained at 2Hz (not shown). All experiments were performed at 37°C.

Cardiomyocytes sarcomere shortening (SS) and intracellular Ca²⁺ (Ca_i) levels was measured simultaneously using an integrated system (IonOptix, MA, featuring a µStep Light Source). In order to be considered for data collection, myocytes had to show distinct striations, a diastolic sarcomere length of >1.65 µm, and no arrhythmic behavior.

SS was expressed as percent of resting sarcomere length. We also measured the maximal rates of shortening (departure velocity, DV) and relaxation (return velocity, RV) and diastolic sarcomere length (DSL). To measure Ca_i, cells were incubated with the fluorescent dye fura-2 AM (Molecular Probes, 1 µM) for 25 min at room temperature. The amplitude of

the intracellular Ca^{2+} transient (Ca_i) was measured as the difference between peak fura ratio and fura ratio at rest.

2.3. Rapid applications of caffeine

In some experiments (Figures 7–8), rapid application of different experimental solutions containing caffeine was performed using a rapid solution exchanger. The home-made device included a 8-channel, valve-controlled gravity perfusion system (VC3-8xG, ALA Scientific Instruments, NY) connected to a multi-tube in-line heater (MPRE8, Cell MicroControls, VA), whose tip was brought close to the individual cell studied. The solution exchanger allowed for the rapid (<100 msec) change in the superfusing solution, while maintaining the temperature at 37°C.

2.4. Measurement of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity

In order to measure $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (Figure 8), we measured the rate of Ca^{2+} decay during caffeine application. In addition, we measured Ca^{2+} decay during rapid application of a caffeine-containing solution designed to inhibit the $\text{Na}^+/\text{Ca}^{2+}$ exchange, containing LiCl (137mM, instead of NaCl), with no CaCl_2 added, and also including K-EGTA (100 μM) and NiCl_2 (5mM, a $\text{Na}^+/\text{Ca}^{2+}$ exchange blocker (20)).

2.5. Statistical Analysis

Comparisons among experimental groups were analyzed by two-way ANOVA tests. Where applicable, unpaired Student's t-test was also performed. P-values less than 0.05 ($p < 0.05$) were considered significant. All values are shown as means $\pm$ SEM.

3. Results

3.1. Administration of LPS decreases cardiac myocyte contractile function (Figure 1)

After 24 ± 4 h in culture, control cardiomyocytes (C) showed SS (Figure 1A–B), DV (Figure 1C), RV (Figure 1D) and DSL (Figure 1E) that were not different from freshly isolated cells (Day 0, D0).

Compared to C, incubation with a dose of 100 $\mu\text{g}/\text{ml}$ LPS induced a profound decrease in SS (Figure 1B), DV (Figure 1C) and RV (Figure 1D), while DSL was unchanged (Figure 1E). Lower concentrations of LPS (10 and 30 $\mu\text{g}/\text{ml}$) did not induce a significant change in cell contractile parameters, although 10 $\mu\text{g}/\text{ml}$ LPS induced a trend towards an increase in SS, DV and RV.

3.2. TNF (10–100 ng/ml) does not decrease cardiomyocyte contractility (Figure 2)

Cardiomyocyte incubation with TNF (between 10 and 100 ng/ml, Figure 2A) did not induce a significant change of SS (Figure 2B), DV (Figure 2C) and RV (Figure 2D), or DSL (Figure 2E).

3.3. Incubation with IL-1 (100 ng/ml) decreases cardiomyocyte contractility (Figure 3)

Incubation with 100 ng/ml IL-1 (Figure 3A) induced a profound decrease in SS (Figure 3B), DV (Figure 3C) and RV (Figure 3D), while DSL was unchanged (Figure 3E). Lower

concentrations of IL-1 (4 and 40 ng/ml) did not induce a significant change in cell contractile parameters.

3.4. Incubation with IL-6 (30 ng/ml) decreased cardiomyocyte contractility (Figure 4)

Incubation with 30 ng/ml IL-6 (Figure 4A) induced a profound decrease in SS (Figure 4B), DV (Figure 4C) and RV (Figure 4D), while DSL was unchanged (Figure 4E). Lower concentrations of IL-6 (10 ng/ml) did not induce a significant decrease of cell contractile parameters. Increasing IL-6 dose to 100 ng/ml did not augment the degree of inhibition induced in SS, DV and RV and induced a small but significant increase in DSL (Figure 4E).

3.5. A mixture of LPS and cytokines inhibits cell contractility (Figure 5)

We next aimed to determine whether LPS, TNF, IL-1 and IL-6 together exert an additive effect (7). For this we used a combination of all 4 mediators studied (LPS, TNF, IL-1 and IL-6) and gradually increased the doses used.

A combination of LPS (10 µg/ml) + TNF, IL-1 and IL-6 (each 10 ng/ml; i.e. “Cytomix-10”, Figure 5A) induced a moderate decrease in SS (Figure 5B), DV (Figure 5C) and RV (Figure 5D), without a change in DSL (Figure 5E).

Increasing the doses used to 30 µg/ml LPS + 30 ng/ml TNF + 30 ng/ml IL-1 + 30 ng/ml IL-6 (“Cytomix-30”) induced a similar decrease in SS (Figure 5A), slightly less inhibition of DV and RV (Figure 5C–D), and no change in DSL (Figure 5E).

A further increase in the doses used, to 100 µg/ml LPS + 100 ng/ml TNF + 100 ng/ml IL-1 + 100 ng/ml IL-6 (“Cytomix-100”) induced a profound decrease in SS (Figure 5B), DV (Figure 5C), and RV (Figure 5D), again, without a change in DSL (Figure 5E).

3.6. Cytomix-100 decreases Ca_i (Figure 6)

For the rest of the study, we aimed to identify the Ca^{2+} handling mechanisms responsible for the decrease in SS induced by Cytomix-100. First, we measured Ca_i in isolated, externally paced cells (Figure 6A–B). Compared to D0, C cells showed a $27 \pm 5\%$ decrease in Ca_i (Figure 6B). Incubation with Cytomix-100 induced a further decrease in Ca_i of $20 \pm 4\%$, as compared to C (Figure 6B). The level of diastolic Ca^{2+} was similar in D0 and C cells, and not changed by incubation with Cytomix-100 (Figure 6C).

Therefore, the contractile deficit induced by Cytomix-100 was associated with (and likely the result of) the dysregulation of cellular Ca^{2+} handling, manifested as a decrease in Ca_i .

3.7. SERCA function is preserved after incubation with Cytomix-100 (Figure 6D)

Since, under the conditions used here, the decay of the intracellular Ca^{2+} transient is largely due to Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) via the SR Ca^{2+} ATP-ase (SERCA, see Discussion), we next examined the effects of Cytomix-100 on the time constant of Ca^{2+} decay (τ_{Ca}), to quantify SERCA function.

As compared to D0 cells, τ_{Ca} was prolonged in C cells, signifying a modest ($9 \pm 3\%$) decrease in SERCA activity after 24 ± 4 h in culture. Compared to C, cells incubated with

Cytomix-100 showed no further change in τ_{Ca} , indicating that SERCA function is unchanged by Cytomix-100.

3.8. Ca_{SR} is not changed by Cytomix-100 (Figure 7A–C)

Ca_{SR} was measured in intact cells using rapid applications of caffeine (10mM), a ryanodine receptor (RyR) opener. Cells were paced until steady state, then pacing was stopped and caffeine was rapidly applied, which led to the opening of RyR and SR Ca^{2+} release in the cytosol (Figure 7A). As such, the amplitude of the rise in Ca^{2+} induced by caffeine (Figure 7B) is a measure of Ca_{SR} . Ca_{SR} was unchanged in C cells as compared to D0 (Figure 7C). Incubation with Cytomix-100 did not induce a change in Ca_{SR} , as compared to C. This finding is consistent with the lack of effect of Cytomix-100 on SERCA function (which is one of the major determinants of Ca_{SR}).

3.9. SR fractional release (FR) is decreased after incubation with Cytomix-100 (Figure 7D)

SR FR was measured as the ratio between the steady state Ca_i immediately preceding each caffeine application and Ca_{SR} . SR FR was similar in freshly isolated and control cells (Figure 7D). Importantly, incubation with Cytomix-100 decreased SR FR with $19 \pm 4\%$ of control (Figure 7D). Since the major determinant of SR FR is the amount of trigger Ca^{2+} that enters the cell via the L-type Ca^{2+} channel, the decrease in FR induced by Cytomix-100 suggested that LTCC function may be inhibited after Cytomix-100 (see below).

3.10 Na^+/Ca^{2+} exchange function is inhibited by Cytomix-100 (Figure 7E and 8A–B)

During caffeine application, RyR are kept open and SERCA cannot effectively reuptake cytosolic Ca^{2+} (21). As such, Ca^{2+} decay during caffeine application is the result of Ca^{2+} extrusion from the cell, primarily through the sarcolemmal Na^+/Ca^{2+} exchanger, with minor contribution from other transporters, such as the plasmalemmal Ca^{2+} pump, mitochondrial transporters (21) and probably others. Thus, the time constant of Ca^{2+} decay during caffeine application (τ_{Caff}) measures primarily the activity of the Na^+/Ca^{2+} exchange.

τ_{Caff} was similar in C cells as compared to D0 cells (Figure 7E). Incubation with Cytomix-100 induced a significant increase in τ_{Caff} , indicating an inhibition of the Na^+/Ca^{2+} exchange of $16 \pm 6\%$. Note that in all groups, τ_{Caff} is ~ 8 – 10 times longer than τ_{Ca} (in D0 cells, for example, average τ_{Ca} was 92 msec and τ_{Caff} was 791 msec; similar ratios were for C and Cytomix-100 groups). This indicates that the combined non-SERCA mechanisms (measured by τ_{Caff}) are responsible for only ~ 10 – 13% of total Ca^{2+} extrusion (as measured by τ_{Ca}). Therefore, in all the groups studied, SERCA represents the largest contributor to diastolic Ca^{2+} extrusion, being responsible for 87–90% of τ_{Ca} , which is the usual finding in rat myocardium in these conditions (21).

To ascertain that τ_{Caff} prolongation with Cytomix-100 is the result of Na^+/Ca^{2+} exchange inhibition, we measured the time constant of Ca^{2+} decay during caffeine applications in a Tyrode solution that was Na^+ - (with Na^+ replaced with Li^+) and Ca^{2+} -free, and also contained 5 mM Ni, to inhibit Na^+/Ca^{2+} exchange. The time constant of Ca^{2+} decay in these conditions ($\tau_{Caff-Li}$) measured the combined activity of all the “minor extrusion mechanisms”, such as the plasmalemmal Ca^{2+} pump, mitochondrial transporters, and

probably others (21). $\tau_{\text{Caff-LI}}$ (measuring between 3.4 and 5.2 sec) was much slower than τ_{Ca} or τ_{Caff} , and thus measured extrusion rates that represented only 2–3% of the total extrusion rate (measured by τ_{Ca}). Importantly, $\tau_{\text{Caff-LI}}$ was shorter in C vs. D0 cells, and similar in control cells and cells incubated with Cytomix-100.

Taken together, these findings lead us to conclude that Cytomix-100 induces an inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchange, which is manifested as a prolongation of τ_{Caff} (see Discussion).

3.11. Ca^{2+} entry via LTCC is inhibited by Cytomix-100 (Figure 8C–D)

After caffeine wash-off (as shown in Figure 7A), with the SR Ca^{2+} having been released into the cytosol and removed from the cell (mostly via forward $\text{Na}^+/\text{Ca}^{2+}$ exchange), the first Ca^{2+} transient recorded when pacing resumes (Figure 8C) is a reflection of the amount of Ca_E via LTCC during the action potential, and thus an assay of LTCC function. In previous experiments, this method gave identical results when compared to measuring the L-type Ca^{2+} current by patch-clamp (22).

Ca_E was similar in C and D0 cells. Incubation with Cytomix-100 induced a profound decrease in Ca_E , with to $37 \pm 7\%$ of control. The decrease in Ca_E with Cytomix-100, is consistent with the decrease in SR FR (Figure 7D) and indicate that LTCC channel activity is inhibited by Cytomix-100.

4. Discussion

The goal of this study was to establish an *in vitro* model of SIC, which can be used subsequently to identify the signaling pathways involved, to test novel drugs and therapeutic strategies. Two important aspects distinguish it from previous work: the use of adult cardiomyocytes and the prolonged time of application of inflammatory mediators. As such, here we report, for the first time, that prolonged (24 ± 4 h) challenge with LPS and inflammatory cytokines is able to inhibit the contractile function of adult cardiomyocytes, by inducing the dysregulation of cellular Ca^{2+} handling, and specifically, the inhibition of Ca^{2+} entry into the cell via LTCC.

4.1. Adult vs. neonatal cardiomyocytes

The current experiments were performed in adult rat cardiomyocytes kept in culture for 24 ± 4 h. C cells show the same excitation-contraction coupling mechanism as D0, based on Ca^{2+} -induced Ca^{2+} release (CICR) from the SR (23). Only minor quantitative differences exist between D0 and C cells, that concern mostly the modulation of CICR mechanisms. Compared to D0 cells, C cells show a slightly decreased Ca_i (Figure 6B), less active SERCA (Figure 6D), and a decrease in the activity of the minor Ca^{2+} extrusion mechanisms (Figure 8B).

By comparison, a number of published studies (5, 7, 11, 13) used cells isolated from neonatal animals. In neonatal cells, the contractile mechanism is different from adult cells. Neonatal cells have no t-tubules, no coupling between LTCC and SR ryanodine receptors and no CICR (24, 25). The contraction of neonatal cells relies on Ca_E through the LTCC, that directly activates the myofilaments, with little or no role for the SR and ryanodine

receptors (24, 25). This is an important concern when one attempts to determine whether inflammatory mediators exert inhibitory effects on cardiac contractile function. Obviously, any mediators that would inhibit contractility by inhibiting SERCA (22, 26), or RyR (26, 27), for example, would not affect the contractile function of neonatal cells, and thus be reported as false negatives.

4.2. Prolonged vs. immediate effects of inflammatory mediators

Another important aspect is that we studied the effects of prolonged (24 ± 4 h) applications of LPS and/or cytokines. In contrast, most of the previously published studies (with few exceptions (13, 15, 16)) have tested only short lived applications, between 5min (8) and 4h (12).

For example, Taverner, et al, (12) challenged mouse cells with LPS (at an unspecified dose) for 4h, and recorded no effect on cell contractility, despite the presence of LPS-specific TLR4 receptors. The authors concluded, therefore, that LPS does not act directly on cardiomyocytes. However, a 4h exposure to LPS may be insufficient to generate the full induction of NOS2 (which may represent the main downstream effector) (17). NOS 2 induction after LPS challenge may take between 3–6h to develop (28). Therefore, a short-lived exposure to LPS may not be able to replicate the full mechanisms involved in vivo. In contrast, here we showed that LPS (100 μ g/ml) does induce cardiomyocyte contractile depression, if incubated for 24 ± 4 h.

Similarly, short-term (30 min) challenges with TNF (10) and IL-6 (14) were shown to increase cardiac contractility, as opposed to the inhibition (for IL-6) or lack of effect (for TNF) seen here after a 24 ± 4 h incubation. Again, these differences may be explained by the lack of involvement of NOS2-mediated effects after a 30 min challenge, but this remains to be ascertained in the future.

4.3. Prolonged exposure to LPS, IL-1 and IL-6 (but not TNF) inhibits cardiac cell contractility in vitro

Here we showed that 24 ± 4 h incubation with LPS (100 μ g/ml), IL-1 (100 ng/ml) and IL-6 (30 ng/ml) inhibits cardiac cell contractility (measured as SS, Figures 1–4). This is similar to the decrease in cardiac contractility that is commonly seen in animal models of SIC (22, 27, 29–31) and is consistent with the notion that cardiac dysfunction in sepsis is due to the effects of circulating mediators.

However, the doses of IL-1 and IL-6 necessary to achieve the inhibition of ARVM contractile function were 10–13 times higher than the serum concentrations (approx 1–3 ng/ml) reported for septic patients (without SIC (32), no data is available for patients with SIC, specifically). LPS required a 100,000-times higher dose than that measured in vivo (approx. 10 endotoxin units, or 1 ng/ml) in septic patients (again, without SIC) (32). As potential explanations, it is possible that the local concentration of inflammatory mediators (released within the myocardium) is much higher than the measured circulating levels. Also, there may be other mediators (some other of the >20 known cytokines not tested here, or other unknown factors) that contribute in a major way to the decrease in cardiac contractility

(either primarily or by potentiating the effects of LPS, IL-1, IL-6). Obviously, it is also possible that septic patients with SIC may show much higher levels of LPS and inflammatory cytokines than the general septic patients. Other explanations are also possible.

As concerns TNF, it is notable that doses up to 100 ng/ml were unable to significantly inhibit SS after a 24 ± 4 h exposure (while the serum levels of TNF in septic patients have been reported to be in the 3–5 ng/ml range). This is surprising, given that TNF is a inflammatory factor well known for its virulence (33), and has been shown to induce a significant depression of cardiac contractility after short lived exposures (12). The lack of inhibitory effect of TNF in our conditions warrants, thus, further study.

4.4. The effect of LPS, TNF, IL-1 and IL-6 is additive and redundant

At lower concentrations, a combination of LPS, TNF, IL-1 and IL-6 appear to induce an additive effect. Cytomix-10 induced a significant inhibition of SS, although none of the components (10 µg/ml LPS, and 10 ng/ml TNF, IL-1 and IL-6) did that when tested individually. This is consistent with previous observations that inflammatory mediators act in a cooperative fashion on the myocardium (7).

In contrast, at higher doses, the effect of the LPS and/or cytokines shows signs of saturation, since Cytomix-30 did not show a significantly greater effect than Cytomix-10 (Figure 5B). Moreover, Cytomix-100, although inducing the highest degree of inhibition from all the agents tested, has an overall effect that is less than the sum of the effects of the components. Assuming an independent effect of all 4 components, by multiplying the % inhibition achieved by 100 µg/ml LPS (to 46 % of C, Figure 1B), 100 ng/ml TNF (to 88 % of C, Figure 2B), 100 ng/ml IL-1 (41 % of C, Figure 3B) and 100 ng/ml IL-6 (43 % of C, Figure 4B) we could have expected a combined inhibition of 93 % of control. In contrast, Cytomix-100 achieved “only” 62% inhibition of SS vs. control (Figure 5B). This suggests that two or more of the components of Cytomix-100 act via a common pathway, in a redundant fashion, as is commonly the case for inflammatory cytokines (34).

4.5. Cytomix-100 inhibits Ca_i

The primary mechanism responsible for the decrease in ARVM SS by Cytomix is represented by a decrease in Ca_i , which indicated the dysregulation of cellular Ca^{2+} handling. A decrease in Ca_i is also the predominant mechanism underlying cardiac depression in many animal models of SIC (22, 27, 29–31) indicating that Cytomix incubation of ARVM does constitute a representative *in vitro* model for SIC. In contrast, the decrease in myofilament sensitivity, which has been reported to underlie exclusively (i.e. without a decrease in Ca_i) cardiac dysfunction in other models of SIC (35) was not evident in Cytomix-100 treated cells.

At a first analysis, Ca_i is determined by three partially inter-dependent parameters: Ca_E , Ca_{SR} and SR FR. Therefore, the remainder of the study aimed to identify which of these parameters, and which cellular Ca^{2+} transporters are responsible for the decrease in Ca_i after Cytomix.

4.6. Cytomix-100 inhibits CaE through LTCC

The primary mechanisms by which Cytomix-100 inhibits SS was the inhibition of Ca_E via LTCC (Figure 8D). This is also the only explanation for the decrease in SR FR (Figure 7D). The other theoretical alternative for the decrease in SR would be a decrease in RyR sensitivity for Ca²⁺ and in the gain of CICR, but this would have led to a compensatory increase in Ca_{SR} (36) and thus would be unable to induce a sustained decrease in Ca_i.

LTCC inhibition is a common finding in animal models of SIC (22, 37, 38).

Mechanistically, despite the paucity of data, LTCC inhibition in SIC appears to be due to channel downregulation (39, 40), but whether this is the case in Cytomix-100 challenged cells is currently unknown.

4.7. Cytomix-100 inhibits Na⁺/Ca²⁺ exchange function

Cytomix inhibited the Na⁺/Ca²⁺ exchange, as evidenced by the prolongation of Ca²⁺ decay during caffeine applications (Figure 7E), which was prevented in conditions when Na⁺/Ca²⁺ exchange was blocked by a Na⁺/Ca²⁺- free solution (Figure 8A–B). Similar to the LTCC, Na⁺/Ca²⁺ exchange inhibition is also commonly reported in animal models of SIC (27, 31).

From a functional point of view, it is important to realize that Na⁺/Ca²⁺ exchange inhibition will increase cardiac contractility, by decreasing the amount of Ca²⁺ extruded from the cell in diastole (41), Na⁺/Ca²⁺ exchange inhibition could, thus, be seen as a partially compensatory mechanism in SIC, a salutatory effect that co-exists and partially compensates for LTCC inhibition. Importantly, this phenomenon is present both in animal models of SIC (27, 31) and in Cytomix-100 challenged cells, and is therefore amenable to further study in our *in vitro* model.

4.8. SERCA and Ca_{SR} are not affected by Cytomix-100

Two other mechanisms that have been reported extensively in SIC models and are thought to play primary roles in the development of Ca²⁺ dysfunction in septic hearts are the inhibition of SERCA transport (22, 26, 27) and a decrease in Ca_{SR} (22, 26, 27). However, these mechanisms were not present in cells challenged with Cytomix-100, indicating that our *in vitro* model may reproduce some, but not all the cellular mechanisms that underlie SIC in animal models.

4.9. An integrative paradigm of signaling pathways in SIC (Figure 9)

The accepted paradigm is that SIC is the result of the detrimental effects exerted on cardiomyocytes by inflammatory mediators, such as LPS and various cytokines. A vast amount of evidence (18) exists supporting the notion that the decrease in cardiac contractility that defines SIC is the result of the dysregulation of intracellular Ca²⁺ handling, including the dysfunction of LTCC (22, 37), SERCA (22, 26), etc), and others. However, little is known about the signaling pathways that connect the LPS / cytokine receptors and Ca²⁺ handling. Addressing this gap in knowledge is a priority area for research. Without identifying the specific signaling molecules involved, it will be difficult, if not impossible to design and/or propose an effective therapeutic strategy for SIC.

The *in vitro* model described here offers distinct promises in this respect. Our model replicates the decrease in Ca_i and cell contractility seen in SIC, and implicates the dysfunction of LTCC and NCX as participating factors. It remains for the future to elucidate the signaling pathways involved and to propose corrective strategies. This will be achieved through experimental work that will commonly involve use of specific pathway inhibitors (together with other strategies). Therefore, these two main goals (elucidating of pathology and proposing of strategies) will likely develop largely in parallel.

4.10. Limitations and future directions Which circulating factors are the most potent depressors of cardiomyocyte contractility?

Apart from LPS, TNF, IL-1 and IL-6, numerous other circulating factors (bacterial components, cytokines, growth factors, complement factors, etc) are activated in sepsis and could potentially induce cardiomyocyte depression. The existence of additional depressant factors may explain the fact that the concentrations of LPS, IL-1 and IL-6 required to inhibit cardiomyocyte contractility *in vitro* were much higher than those measured in septic patients (as discussed above). Identifying other circulating mediators that may exert a cardiomyocyte depressant effect in sepsis is a priority area for investigation, that our novel *in vitro* model is ideally positioned to address.

Sex differences in SIC—The experiments presented here used exclusively cells isolated from male rats. It is an unfortunate reality that, with very few exceptions, all the studies that investigated intracellular Ca^{2+} handling dysregulation in animal models of sepsis have been performed in male animals (18). Such an unbalanced focus on male physiology is not unique to this field, and represents a priority area for future research (42). However, since our goal was to develop an *in vitro* model that could reproduce the Ca^{2+} dysregulation seen in animal models of SIC, we were forced to persist in using male animals in this study. We are aware that this will increase the existing knowledge gap regarding sex differences in SIC. However, this first *in vitro* study had to follow the mass of information already existing in *in vivo* models. Identifying sex differences (43) in SIC must remain for the future. For this goal, our *in vitro* model offers distinct promises, since it will allow the direct comparison of cells isolated from male vs. female rats after identical challenge with Cytomix *in vitro*, as well as studying the effects of sex hormones on modulating Ca^{2+} handling dysregulation induced by Cytomix.

4.11. Summary and conclusions

The present experiments show that prolonged (24 ± 4 h) exposure to LPS and cytokines (such as IL-1, IL-6, but not TNF) inhibits the contractile function of adult rat cardiomyocytes. A combination of LPS and cytokines induces a more pronounced effect, consistent with an additive mechanism. Cytomix-100 exerts its inhibitory effects by inhibiting Ca_E via LTCC, an effect that is opposed by the inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchange. Both effects are commonly described in animal models of SIC, making the current system a useful *in vitro* model of SIC. Further experiments are warranted to identify the signaling pathways leading to the Ca^{2+} handling deficits identified here, and to test various strategies and drugs that could prevent the cardiac dysfunction induced by systemic inflammation.

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Figure 1. Administration of LPS decreases cardiac myocyte contractile function

A. Representative experimental recordings of cardiomyocyte sarcomere shortening in response to external pacing at 5Hz, in D0 and C cells and cells incubated with 10, 30 and 100 $\mu\text{g}/\text{ml}$ LPS. B–E. Average SS (as % of diastolic length, B), departure velocity (C), return velocity (D) and diastolic sarcomere length (E) in the same groups. In this and the following, * signifies $p < 0.05$ vs. C. In this and following, the number of cells/rats is shown for each group in the first panel shown and is the same in the following panels in the same figure, unless stated otherwise.

Figure 2. TNF (10–100 ng/ml) does not decrease cardiomyocyte contractility

A. Representative recordings of sarcomere shortening in C cells and cells incubated with 10, 30 and 100 ng/ml TNF.

B–E. Average SS (as % of diastolic length, B), departure velocity (C), return velocity (D) and diastolic sarcomere length (E) in the same groups.

Figure 3. IL-1 (100 ng/ml) decreased cardiomyocyte contractility

A. Representative sarcomere shortening in C cells and cells incubated with 4, 40 and 100 ng/ml IL-1.

B–E. Average SS (as % of diastolic length, B), departure velocity (C), return velocity (D) and diastolic sarcomere length (E) in the same groups.

Figure 4. Incubation with IL-6 (30 ng/ml) decreased cardiomyocyte contractility

A. Representative sarcomere shortening in C cells and cells incubated with 10, 30 and 100 ng/ml IL-6.

B–E. Average SS (as % of diastolic length, B), departure velocity (C), return velocity (D) and diastolic sarcomere length (E) in the same groups.

Figure 5. A mixture of LPS and cytokines inhibits cell contractility

A. Representative sarcomere shortening in C cells and cells incubated with Cytomix-10, Cytomix-30 and Cytomix-100.

B–E. Average SS (as % of diastolic length, B), departure velocity (C), return velocity (D) and diastolic sarcomere length (E) in the same groups.

Figure 6. Cytomix-100 decreases Ca_i

A. Representative Ca_i^{2+} transients from D0, C cells and cells incubated with Cytomix-100 for 24 ± 4 h.

B–D. Average Ca_i (B), diastolic Ca_i levels (C) and τ_{Ca} (D) in D0, C and Cytomix-100 cells. r.u.: fura 2 ratiometric units

Figure 7. Cytomix-100 decreases SR fractional release, and inhibits Na⁺/Ca²⁺ exchange activity, without a change in Ca_{SR} or SERCA function

A. A representative experiment in which rapid applications of caffeine were used to measure Ca_{SR}, SR FR and Ca_E. See text for details.

B. Typical caffeine-induced rises in Ca_i in D0, C and Cytomix-100 cells.

C–E. Average Ca_{SR} (C), FR (D) and τ_{Caff} (E) in D0, C and Cytomix-100 cells.

Figure 8. Cytomix-100 does not affect the minor extrusion mechanisms and inhibits Ca_E into the cell

A. Typical experimental traces of Ca²⁺ fluxes when caffeine was applied in a Na⁺ / Ca²⁺-free Tyrode solution, containing 5 mM Ni²⁺, in D0, C and Cytomix-100 cells.

B. Average τ_{Caff-Li} in the 3 groups.

C. The amplitude of the first Ca_i when pacing is resumed after caffeine wash-off is a measure of Ca_E via LTCC. Typical traces showing the first and few subsequent Ca_i after caffeine in the three groups.

D. Average Ca_E in D0, C and Cytomix-100 cells.

Figure 9. An integrative paradigm of signaling pathways in SIC

SIC is generally thought to be initiated by the activation of the cardiomyocytes receptors for LPS (Toll-like receptor, TLR) and cytokines, such as TNF (TNF-R), IL-1 (IL-1R) and IL-6 (IL-6R), among others. At the other end of the chain of events, the decrease in cardiac contractile force is known to be largely due to the dysregulation of intracellular Ca^{2+} handling (18). This includes the dysfunction of LTCC (22, 37), SERCA (22, 26), SR ryanodine receptors, that together conspire to decrease Ca_{SR} and Ca_i . Added to the decrease in Ca_i , myofilament dysfunction (35, 44) contributes to the decrease in cardiac

contractile force. In contrast, inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchange (27, 31) may play a partially compensatory role, by increasing Ca_{SR} and Ca_i (41) in SIC.

Little is known however, about the signaling pathways that mediate Ca^{2+} handling dysregulation. A number of specific pathways have been incriminated, such as activation of the nitric oxide (NO) pathway (45), radical oxygen (ROS) and nitrogen (RONS) stress (46), increased (47) or decreased (48) cAMP-dependent phosphorylation, NO-induced activation of cGMP synthesis (16), changes in protein expression (40, 49), etc. However, little specific information currently exists. We know that SERCA inhibition may be due to RONS (22) and/or a decrease in PLB phosphorylation (48), and that myofilament desensitization is due to cAMP-dependent hyperphosphorylation of troponin I (47). Apart from this fragmentary information, practically nothing is known about what causes LTCC and RyR dysfunction, as well as $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibition.

The novel *in vitro* model described here would greatly aid in elucidating these remaining aspects of SIC patho-physiology, as well the screening and development of effective drugs.