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β_1-Adrenergic cardiac contractility is increased during early endotoxemic shock: Involvement of cyclooxygenases

David Roul[a,1], Bertrand Rozec[b,*,1], Marine Ferron[a,2], Mortéza Erfanian[a], Antoine Persello[a], Leslie Audigane[a], Amandine Grabherr[a], Angelique Erraud[a], Nolwenn Merlet[a,2], Damien Guijarro[b], Ikunobu Muramatsu[c], Benjamin Lauzier[a], Chantal Gauthier[a]

[a] l'institut du thorax, INSERM, CNRS, UNIV Nantes, Nantes, France
[b] l'institut du thorax, INSERM, CNRS, UNIV Nantes, CHU Nantes, Nantes, France
[c] School of Medicine, University of Fukui, Fukui, Japan

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ABSTRACT

Aims: Endothelial dysfunction is one of the earliest symptoms in septic patients and plays an important role in the cardiovascular alterations. However, the endothelial mechanisms involved in the impaired sympathetic regulation of the cardiovascular system are not clear. This study aimed to determine the role of the endocardial endothelium (EE) in the cardiac β-adrenergic (β-AR) remodeling at the early phase of endotoxemic shock.
Main methods: Rats received either lipopolysaccharide (LPS) or saline (control) intravenously. Three hours later, β-AR cardiac contractility was evaluated on papillary muscles with or without a functional EE.
Key findings: Isoproterenol-induced contractility was strongly increased in papillary muscles from LPS rats. A similar increase was observed with a β_1-AR stimulation, whereas β_2-AR and β_3-AR produced similar contractility in control and LPS treatments. The removal of the EE did not modify β_1-AR-induced contractility in controls, whereas it abolished the increased β_1-AR response in LPS-treated muscles. In LPS-treated papillary muscle, the increased β_1-AR-induced contractility was not modified by pretreatment with a NOS inhibitor or an endothelin receptor antagonist. Conversely, the increased β_1-AR-induced contractility was abolished by indomethacin, a non-selective cyclooxygenase (COX) inhibitor, as well as by selective inhibitors of COX1 and COX2. An early treatment with indomethacin improved the survival of LPS rat.
Significance: Our results suggest that the EE is involved in the increased cardiac β_1-AR contractility in the early phase of endotoxemic shock. This effect is mediated through the activation of COX1 and COX2 and suggests these may be novel putative therapeutic targets during endotoxemic shock.

1. Introduction

Septic shock is a leading cause of death with an increasing incidence worldwide [1,2]. Guidelines from the Surviving Sepsis Campaign stated that "similar to polytrauma, acute myocardial infarction or stroke, the speed and appropriateness of therapy administered in the initial hours after severe sepsis develops are likely to influence outcome" [3]. Unfortunately, most studies aiming to identify new relevant therapeutic targets have not investigated the initial stage of this syndrome.

The initial hours of septic shock are mainly characterized by an important and expanded endothelial dysfunction [4]. This endothelial dysfunction is clearly established in vessels where it is involved in the significant hypotension and multi-organ failure syndrome observed in septic shock [5,6]. In the septic heart, only a few studies have demonstrated that endothelial dysfunction regulates cardiac function through an increased production of nitric oxide (NO), endothelin and prostaglandins [7–10]. Interestingly, these mediators are not only released by the vascular endothelium but also by the endocardial endothelium [7–9].

Besides endothelial dysfunction, early septic shock is also characterized by increased circulating catecholamine concentrations in the plasma of patients leading to a sympathetic overstimulation [11,12]. Such a phenomenon has already been described in the early stage of heart failure where it induced a remodeling of the β-adrenergic receptor

* Corresponding author at: 8 quai Moncousu, 44000 Nantes, France.
E-mail address: bertrand.rozec@chu-nantes.fr (B. Rozec).
[1] The first two authors contributed equally to this work.
[2] Present address: Montreal Heart Institute, Montreal, H1T 1C8 Québec, Canada.

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(β-AR) [13]. The three β-AR subtypes, β_1-, β_2- and β_3-AR, are expressed in the heart, with the first two inducing a positive inotropic effect and the last inducing a negative inotropic effect [14].

β-1AR is down-regulated in septic mice [15] whereas other studies in rats suggest an externalization of β-1AR during early sepsis [16], and its inhibition through the use of β-blockers like atenolol or esmolol produces an improvement in cardiac and vascular functions [17–19]. In parallel, a recruitment of β_2-AR was observed in hearts from acute endotoxemic rats [20], and an up-regulation of β_3-AR was reported in autopsied hearts from patients having died from septic shock [21].

Given the potential dysfunction of the endocardial endothelium and the probable alterations of cardiac β-AR in the initial stage of septic shock, we hypothesized that endocardial endothelium dysfunction observed in septic shock could be due to an alteration of the β-AR pathway. In this study, we used a well-known and reproducible rat model of sepsis (endotoxemic shock), and investigated cardiac β-AR remodeling of papillary muscles devoid of endothelium to assess the possible involvement of the endocardial endothelium in this remodeling process. Endothelial modulation focused on assessing the three previously described mediators; NO, endothelin and the prostaglandins.

2. Methods

2.1. Animals

Twelve-week-old male Sprague-Dawley rats (Janvier, Le Genest St, France) were housed under standard conditions of temperature (21–24 °C), humidity (40–60%) and 12 h light/dark cycle. Food and water were available *ad libitum*. The experiments were performed in compliance with the 2010 Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (CEEA-2012-231).

2.2. Endotoxemic rat model

Endotoxemia was induced by intra-venous injection of 5 $mg{\cdot}kg^{-1}$ of purified *Escherichia coli* LPS derived from *E. coli* O111:B4 (Sigma, St Quentin Fallavier, France) as previously described [22].

2.3. Echocardiographic measurements

Three hours after LPS or saline injection, animals were anesthetized with 2% volume of isoflurane at 1 $L{\cdot}min^{-1}$ O_2 flow to limit hemodynamic repercussion. Transthoracic echocardiography was performed using an ultrasound system VIVID7 (GE Healthcare, Horton, Norway) equipped with a 10 MHz sectorial probe as previously described [23]. Measurements were made on five cardiac cycles and averaged for each data value.

2.4. Hemodynamic measurements

After LPS or saline injection, anesthesia was maintained on spontaneously breathing rats using an inhalational. The right carotid artery was isolated, ligated at the proximal region and a 2F microtip pressure catheter inserted (Millar instruments Inc., Houston, Texas). Pressure and heart rate were continuously recorded during 3 h after LPS or saline injection using an A/D converter (EMKA Technologies, Paris, France) and stored and displayed on a computer by the IOX1.5.7 Software System (EMKA Technologies). Animals were sacrificed by pentobarbital overdose.

2.5. Tissue segment binding experiments

Tissue segment binding experiments were performed as previously described [23]. Briefly, the isolated left ventricle was cut into small pieces under a microscope. About 30 pieces were prepared for each rat. Each piece was incubated at 4 °C in 1 mL of Krebs incubation buffer (Sigma) with [3H]-CGP-12177 (a high affinity antagonist of both β_1 and β_2-AR and partial β_1-AR agonist) for 12 h.

In the saturation binding experiments, 50 pM–20 nM [3H]-CGP-12177 was used in the absence or presence of 1 μM propranolol (a non-selective β-AR antagonist) to define nonspecific binding. Competition binding experiments were conducted using 300 pM of [3H]-CGP-12177 and 1 pM–10 μM of ICI-89,406 (a β1-AR antagonist) in the absence or presence of 1 μM propranolol to define nonspecific binding. The pieces were then blotted and solubilized in 1 mL of 0.3 M NaOH (Sigma) to estimate the radioactivity and protein content. The specific binding was determined by subtracting the radioactivity bound per mg protein in the presence of propranolol from total radioactivity bound per mg protein. Experiments were performed in duplicate.

Radioactivity was counted using a liquid scintillation counter (LC-3500, Aloka Co Ltd, Tokyo, Japan) using water-miscible scintillation fluid (ULTIMA GOLDTM, Packard Bioscience, Groningen, Netherlands). The protein content of tissue segments in each tube was measured using the Bradford method using BSA as a standard.

2.6. Papillary muscle contractility measurement

Papillary muscles from the left ventricle were harvested and treated with Triton-X 100 to remove the endocardial endothelium (EE−) as previously described [14,24]. Untreated muscles (EE+) served as controls. Papillary muscles were then perfused at a flow rate of 5 mL/min with oxygenated (95% O_2, 5% CO_2) Tyrode's solution (37 ± 0.5 °C) composed as follows (in mmol/l): 116 NaCl, 5 KCl, 2.7 $CaCl_2$, 1.1 $MgCl_2$, 0.35 NaH_2PO_4, 27 $NaHCO_3$, 5 glucose, 5 pyruvic acid sodium salt, 0.15 fumaric acid and 5 L-glutamic acid. Papillary muscles were subjected to field stimulation at a frequency of 1 Hz using square-wave pulses of 1 to 2 ms duration, and amplitude was twice the diastolic threshold. Mechanical tension was recorded using a mechano-electric force transducer Akers AE 801 (SensoNor, Horton, Norway). After a 60 min equilibration period, papillary muscles were stretched stepwise (10 μm increments) to a length at which the contraction force was at 90% of the maximal tension. Cumulative concentration-response curves of isoproterenol, associated or not, with specific β_1- and/or β_2- and/or β_3-AR antagonists were then determined by superfusion with successive increasing concentrations of isoproterenol to evaluate non-specific and subtype specific β-AR responses. Tension was measured using a digital storage oscilloscope HMO1024 (HAMEG Instruments, Mainhausen, Germany) and a strip chart recorder model 8188 (Gould, Les Ullis, France), and a digital tape recorder (model DTR-1200, Biologic, Claix, France).

2.7. Electron microscopy

Papillary muscles were fixed in glutaraldehyde (1 h30) followed by postfixation in 2% osmium tetroxide (1 h). After dehydration in a graded ethanol series, the fragments were embedded in epoxy resin. 70 nm sections were stained using the Reynolds method and examined using a JEOL-JEM 1010 (Jeol Korea Ltd, Seoul, South Korea).

2.8. Western blotting

Western blotting experiments were performed using left ventricle samples as previously described [15] using antibodies against: β_1-AR (A-272, Sigma), β_2-AR (ab36956, AbCam Ltd, Cambridge Science Park, Cambridge, UK), β_3-AR (M-50, sc-50436, Santa-Cruz Biotechnology, Tebubio®, Le Peray en Yvelines, France), COX (ab133319, AbCam Ltd) and COX2 (ab133319, AbCam Ltd). Briefly, proteins were extracted in lysis buffer (RIPA + PMSF) and quantified using a BCA protein assay kit. 25 mg of each sample was separated on a SDS-PAGE gel and transfer to a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS-T and then incubated with primary antibodies (β_1-AR, 1:2000;

β_2-AR, 1:2000; β_3-AR, 1:5000; COX1, 1:800; COX2, 1:1330, GAPDH, 1:10,000) overnight at 4 °C. After 3 washes with TBS-T, membranes were incubated with a HRP conjugated secondary antibody (anti-rabbit, 1:50,000, sc-2054, Santa-Cruz Biotechnology). Densitometry analyses of bands were analyzed using Image Lab software and normalized to GAPDH.

2.9. Real time quantitative RT-PCR

Rat total RNA was extracted from left ventricle using TRIzol reagent (ThermoFischer). Reverse transcription was performed on 3 µg of total RNA using the high-capacity cDNA reverse transcription kit with random primers according to manufacturer's protocol (ThermoFischer). Real-time PCR was performed using an equivalent of 10 ng input DNA. The primers used were; Ptgs1 (Fwd: AGTACCAGGTGCTGGATGGA, Rev: GCTGCTCGTCATCCCATGTA, efficiency: 98.2%) and Ptgs2 (Fwd: CATTCTTTGCCCAGCACTTC, Rev: CTCTCCACCGATGACCTGAT, efficiency: 102.6%). Analysis was performed using the StepOne v2.3 software and gene expression was normalized to Gapdh (Fwd: TGATGGC ATGGACTGTGG, Rev: CAGCAATGCATCCTGCAC, efficiency: 98.9%) and Ywhaz (Fwd: CTTTGCTTTCTGGCTGCGAA, Rev: AGCTGAGGGAC ATCTGCAAC, efficiency: 102.5%). These housekeeping genes were previously determined according to the MIQE guidelines [25].

2.10. Survival curve

One hour after LPS injection, rats were randomly allocated for injection with either 1 $mg{\cdot}kg^{-1}$ of Indomethacin in 1 mL of saline (0.9%) or the same volume of saline 0.9% intravenously as the control group. Rats were allowed to move freely in cages after treatment with food and water *ad libitum*. Survival was evaluated every hour during the following 48 h.

2.11. Drugs

Isoproterenol (a non-specific β-AR agonist), Indomethacin (a non-specific COX inhibitor), Nadolol (a β_1-AR and β_2-AR antagonist) and Triton X-100 were purchased from Sigma. CGP 20712 (a specific β_1-AR antagonist), ICI 118,551 (a specific β_2-AR antagonist), L-748,337 (a specific β_3-AR antagonist), SC560 (a specific COX1 inhibitor) and NS398 (a specific COX2 inhibitor) were purchased from Tocris (Bristol, United Kingdom). NG-monomethyl-L-arginine, mono-acetate (L-NMMA) (a non-specific NOS inhibitor) was purchased from Calbiochem (La Jolla, California). Bosentan (a non-specific endothelin receptor antagonist) was purchased from Actelion (Allschwil, Switzerland).

2.12. Data and statistical analysis

Results are expressed as the mean ± SEM of n experiments. Significant differences in echocardiographic parameters and protein levels were determined using a Mann-Whitney *U* test. The comparison of hemodynamic parameters and concentration-response curves was performed by a two-way ANOVA (concentration, treatment) with repeated measures completed when appropriate by a Bonferroni test as *post hoc* analysis.

3. Results

3.1. Effect of endotoxemic shock on hemodynamic and echocardiographic parameters

Three hours after LPS injection animals presented a hypotension (60.1 ± 7.1 *vs* 87.9 ± 7.1 mm Hg; $P < 0.05$) associated with tachycardia (449 ± 21 *vs* 330 ± 17 $beats{\cdot}min^{-1}$; $P < 0.001$) (Table 1). An impaired systolic function, as shown by decreased ejection (74.5 ± 2.2 *vs* 83.8 ± 2.7%; $P < 0.05$) and shortening fractions (38.6 ± 1.8 *vs* 48.6 ± 3.0%; $P < 0.05$), was associated with an impaired diastolic function characterized by an increased isovolumic time of relaxation (29.6 ± 2.31 *vs* 24.1 ± 1.77 ms; $P < 0.05$) and a reduction in early diastolic peak flow velocity (0.579 ± 0.057 *vs* 0.978 ± 0.048 $m{\cdot}s^{-1}$; $P < 0.05$) (Table 1).

Table 1
Evaluation of hemodynamic and cardiac parameters in control and LPS-treated rats.

	Control (n = 6–8)	LPS (n = 6–10)
Mean arterial pressure (mm Hg)	87.9 ± 7.1	60.1 ± 7.1*
Heart rate (bpm)	330.2 ± 16.6	488.8 ± 23.4*
Ejection fraction (%)	83.8 ± 2.7	74.5 ± 2.2*
Shortening fraction (%)	48.6 ± 3.0	38.6 ± 1.8*
Isovolumic relaxation time (ms)	24.1 ± 1.8	29.3 ± 2.3*
Early diastolic peak flow velocity ($m{\cdot}s^{-1}$)	0.98 ± 0.05	0.58 ± 0.06***

* $P < 0.05$, after a Mann-Whitney *U* test.
*** $P < 0.001$ after a Mann-Whitney *U* test.

3.2. Effect of endotoxemic shock on cardiac β-AR function and expression

The isoproterenol concentration dependent papillary muscle contractility was increased by 105% in papillary muscles from LPS-treated rats compared to control papillary muscles (306 ± 31.4 *vs* 149 ± 8.4%; $P < 0.05$) (Fig. 1A).

Both β_1- and β_2-AR total protein levels were decreased in left ventricles (LV) from LPS-treated rats compared to saline-treated muscles, by 56 and 47%, respectively (Fig. 1E and F). β_1- and β_2-AR membrane protein levels were unchanged in the LV from LPS-treated rats compared to controls, as shown by the preserved radioligand [^{3}H]-CGP-12117 maximum binding capacity (Bmax) (Table 2). Moreover, neither the β_1-/β_2-AR ratio or β_1- and β_2-AR affinities for the CGP-12177, as shown by the equilibrium dissociation constant (K_D) of the CGP-12177, were modified in the LV from LPS compared to control treated rats (Table 2). We also demonstrated, as above for β_1- and β_2-AR, an ~20% reduced total β_3AR protein level in LV from LPS-treated rats compared to control-treated ones (Fig. 1G).

While β_2- and β_3-AR-induced effects on contractility (Fig. 1C and D, respectively) were not modified when the animals were pre-treated with LPS, we observed an increased β_1ARinduced contractility (Fig. 1B) of 94% in LPS-treated papillary muscle compared to controls (534 ± 83 *vs* 276 ± 35%; $P < 0.05$).

3.3. Endothelial involvement in the increased β_1-AR response

To determine the involvement of the EE in this increased β_1-AR-induced contractility the same experiments were performed on papillary muscles without EE. Compared to controls (Fig. 2A), papillary muscles pre-treated with Triton-X100 presented altered EE cells without alteration of the underneath cardiomyocytes. The viability of cardiomyocytes was measured by sarcomere and mitochondria integrities. The EE removal was confirmed by an accelerated relaxation in papillary muscles leading to a shortened contraction period (Fig. 2B).

The EE removal had no significant effect on the β_1-AR-induced contractility on papillary muscle from control rats, whereas it completely abolished the increased of β_1ARinduced contractility observed in LPS-treated rats (246 ± 29 *vs* 534 ± 83%; $P < 0.05$) (Fig. 2C).

3.4. Endocardial endothelial mediators involved in the increased β_1-AR response

To determine the cellular pathway(s) involved in the increased β_1-AR-induced contractility, concentration-response curves to β_1-AR stimulation were established in the presence of 1 µM of LNMMA or 10 µM of Bosentan. We show that neither L-NMMA nor Bosentan modified the

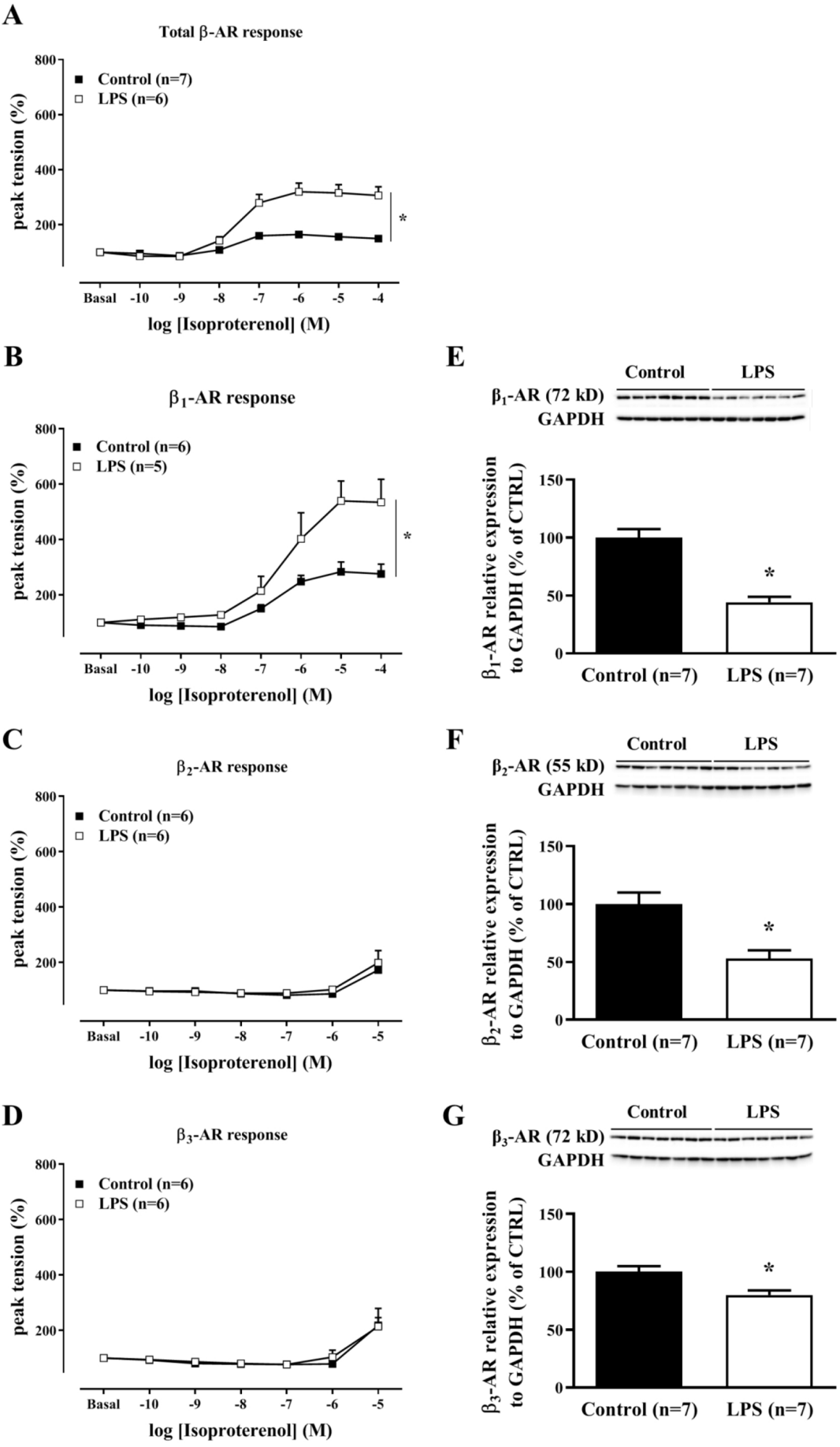

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Fig. 1. Effects of lipopolysaccharide (LPS) administration on left ventricle papillary muscle functional response and expression of (A) β-, (B, E) β_1-, (C, F) β_2-, and (D, G) β_3-AR subtypes. Papillary muscle results were expressed as the percentage of contraction from the basal contraction. Isoproterenol concentration-curves were performed in the presence of 1 μM of ICI 118,551 and L-748,337 to evaluate β_1-AR functional response, 1 μM of CGP 20712 and L-748,337 to evaluate β_2-AR functional response and 10 μM of Nadolol to evaluate β_3-AR functional response. Solid and open squares represent control and LPS-treated rats respectively. Values represent the mean ± SEM of a minimum of 5 rats per group, the results were considered significant when $P < 0.05$. *$P < 0.05$ after a Mann-Whitney *U* test or a *two-way* analysis of variance with repeated measures followed by Bonferroni test as *post hoc* analysis.

Table 2
Evaluation of binding site characteristics of [^{3}H]-CPG-12177 on left ventricles from control and LPS-treated rats. K_D: equilibrium dissociation constant; B_{max}: maximum binding capacity.

	Control (n = 4)	LPS (n = 4)
K_d (pM)	290.0 ± 28.0	218.3 ± 31.8
B_{max} (fmol·mg^{-1} total tissue protein)	41.7 ± 2.2	44.6 ± 3.2
β_1-AR (%)	65.5 ± 1.1	68.6 ± 1.5

β_1-AR enhanced contractility of papillary muscle from LPS-treated rats (Fig. 2D). These molecules had no more effects on the β_1-AR-induced contractility on papillary muscle from control rats (data not shown).

In another set of experiments, we assessed the involvement of the cyclooxygenases (COX) pathway in the increased β_1-AR contractility. These concentration-response curves to β_1-AR stimulation were constructed in the presence of 10 μM of indomethacin (Fig. 3A). While indomethacin had no significant effect on the β_1-AR-induced contractility of papillary muscle from control rats (data not shown), it totally blunted the potentiated β_1-AR-induced contractility observed on papillary muscle from LPS-treated rats (312 ± 59 *vs* 534 ± 83%; $P < 0.05$). To further explore the COX isoforms involved in this phenomenon, similar experiments were performed in the presence of 3 μM of SC560 or NS398 (Fig. 3A). It appeared that both drugs inhibited the potentiated β_1-AR-induced contractility observed on papillary muscles from LPS-treated rats (SC560: 261 ± 31 *vs* 534 ± 83%; NS; 398: 258 ± 41 *vs* 534 ± 83%; $P < 0.05$) without any effect on the β_1-AR-induced contractility recorded on papillary muscles from control rats (data not shown). COX1 and COX2 mRNA expression (Fig. 3B and C, respectively) and protein expression (Fig. 3D and E, respectively) were

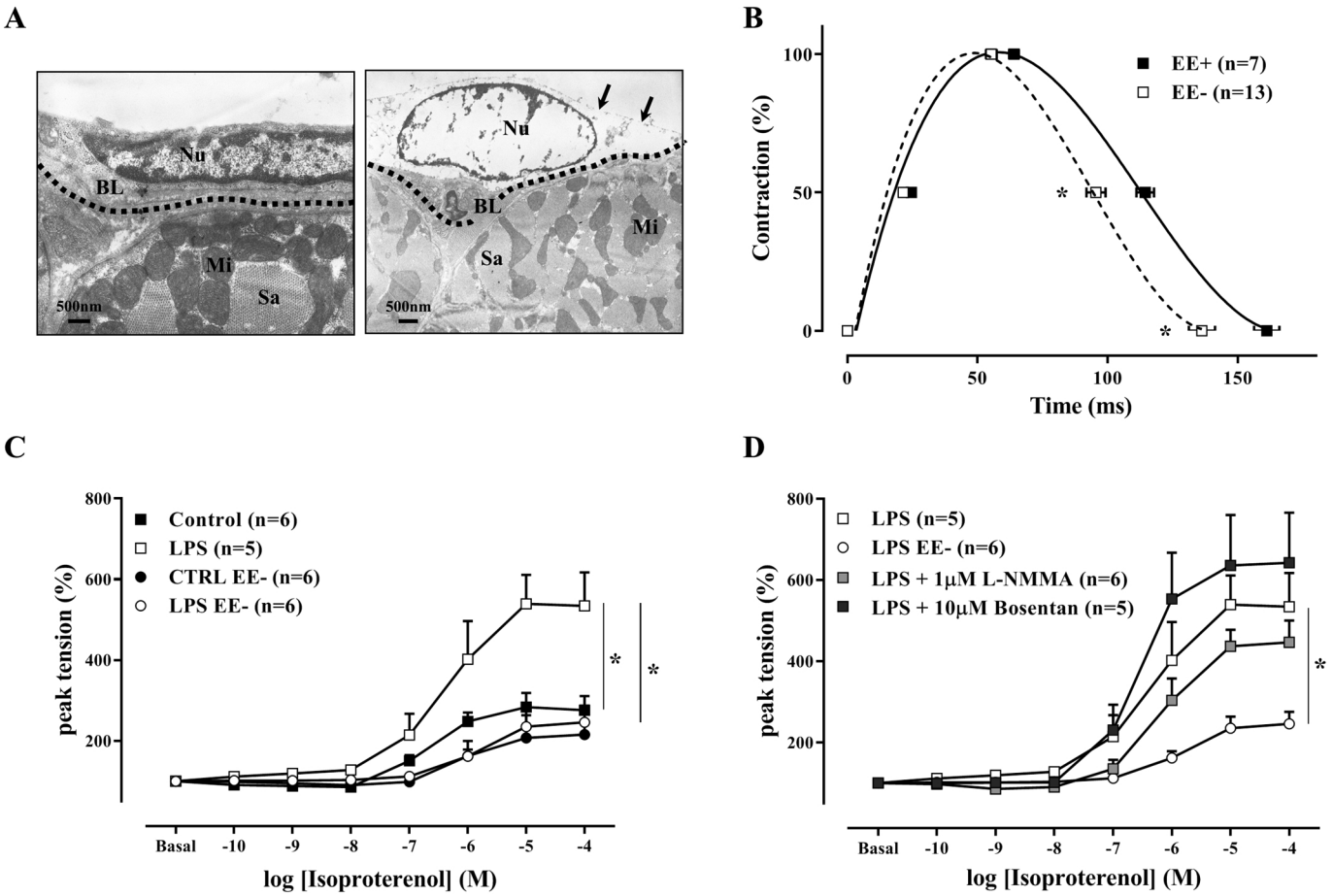

Fig. 2. Effects of Triton-X100 pretreatment (A-B) on endocardial endothelium (EE) integrity. Effects of endocardial endothelium (C) and NOS and endothelin signaling pathways (D) on the β_1-adrenergic-potentiate contractility in LPS-treated rats. (A) Cardiomyocyte mitochondria (Mi) and sarcomere (Sa) integrities were evaluated using electron microscopy of papillary muscles with preserved (left) or altered (right) EE. Arrow: disruption site of the plasmatic membrane of endothelial cells, BL: basal lamina, Nu: Nucleus. (B) Papillary muscle contractility was evaluated with preserved (solid square) or altered (open square) EE (C–D). Isoproterenol concentration-curves were performed in the presence of 1 μM of ICI 118,551 and L-748,337. β_1-AR-induced contractility was evaluated in control (solid symbol) and LPS (open symbol) with (square) or without endocardial endothelium (circle). In LPS papillary muscles, β_1-AR-induced contractility was evaluated in the presence of L-NMMA (light grey square) or Bosentan (dark grey square). Results are expressed as the percentage of contraction from the basal contraction. Values represent mean ± SEM of a minimum of 5 rats per group, and the results were considered significant when $P < 0.05$. *$P < 0.05$ after a *twoway* analysis of variance with repeated measures followed by Bonferroni test as *post hoc* analysis.

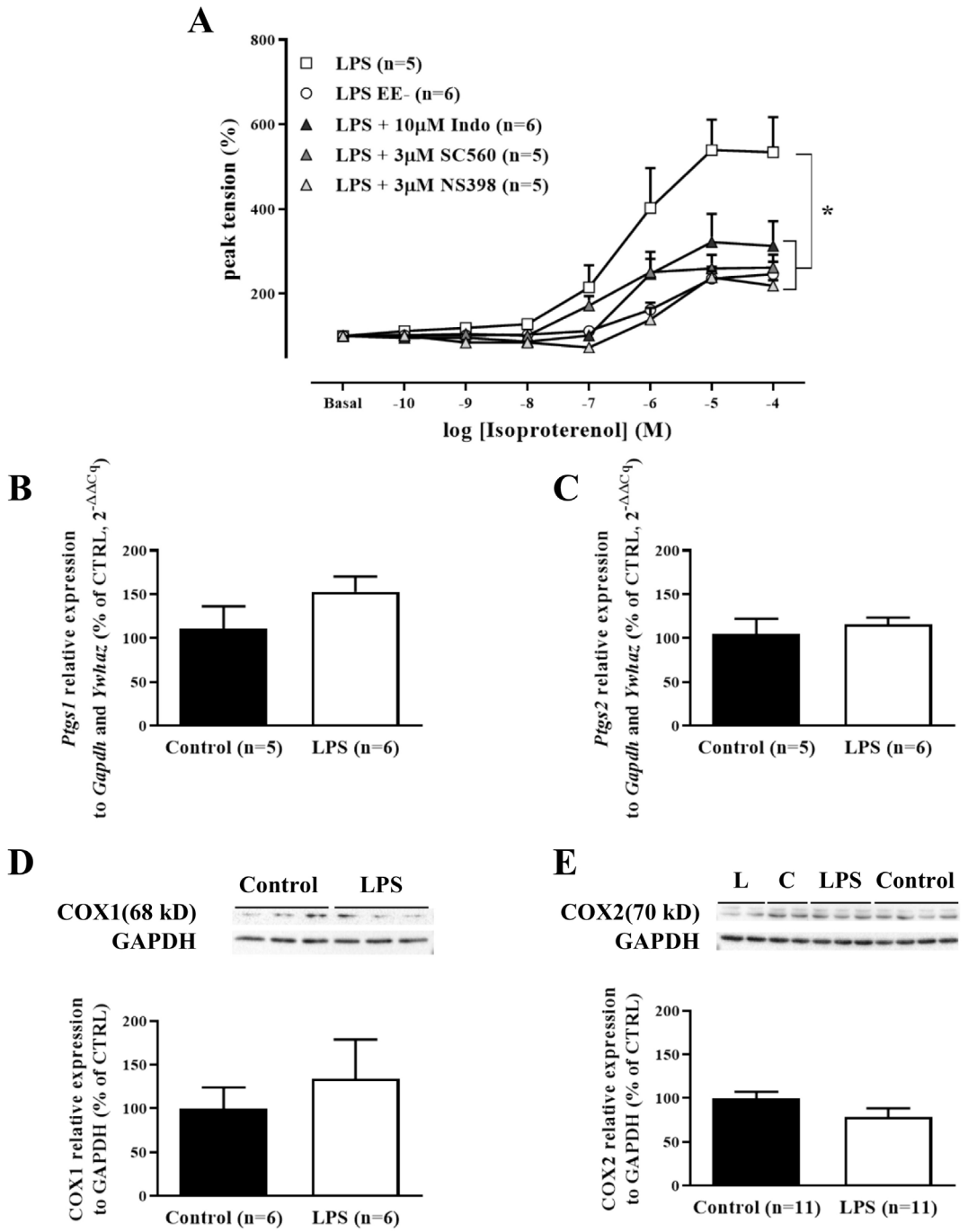

Fig. 3. Effects of (A) COX signaling pathway (LPS) on the β_1-adrenergic-potentiate contractility in LPS rats and left ventricle RNA and protein expression of (B and D) COX1 and (C and E) COX2 isoforms. Papillary muscle experimental results were expressed as the percentage of contraction from the basal contraction. Isoproterenol concentration-curves were performed in the presence of 1 µM ICI 118,551 and L-748,337. β_1-AR-induced contractility was evaluated for LPS papillary muscles with (open square) or without endocardial endothelium (open circle) and in the presence of indomethacin (dark grey triangle), SC560 (medium grey triangle) or NS398 (light grey triangle). Western blot experiments were corrected for protein levels using GAPDH. Values represent mean ± SEM of a minimum of 5 rats per group. The results were considered significant when $P < 0.05$. *$P < 0.05$ after a Mann-Whitney *U* test or a *two-way* analysis of variance with repeated measures followed by Bonferroni test as *post hoc* analysis.

not different in left ventricles from LPS compared to control-treated animals.

3.5. Survival experiments

COX pathway inhibition in our endotoxemic shock model was evaluated through survival experiments. While every control rat survived after NaCl injection (as expected), LPS rats began dying 9 h after the LPS injection, and all of them died by 18 h, with a median survival of 12 h. Rats treated with an intravenous injection of indomethacin (1 mg·kg^{-1}) 1 h after LPS injection began dying almost as quickly as the untreated rats but presented a significantly extended survival to 48 h after the LPS injection with a median survival of 19 h (Fig. 4).

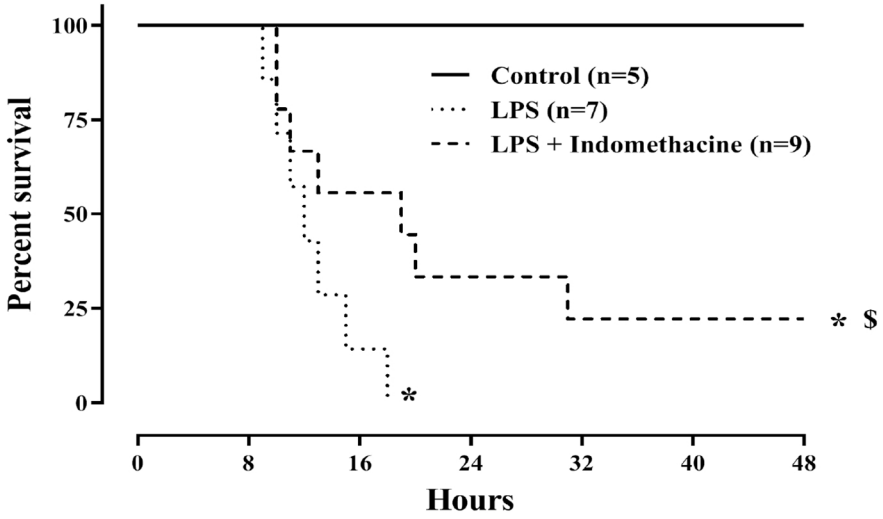

Fig. 4. Effects of indomethacin on survival 48 h after LPS administration. Solid and dotted lines represent controls and treated rats respectively. Results were considered significant when $P < 0.05$. $^{*}P < 0.05$ *vs* control and $^{\$}P < 0.05$ *vs* LPS after a Mantel-Cox test.

4. Discussion

While increased circulating plasma catecholamines are classically found in the early phase of septic shock [12], cardiac β-AR remodeling is poorly described in this disease. This study evaluated the protein expression of all three β-AR subtypes and functional remodeling in the hearts from rats at the early phase of the endotoxemic shock. An increased β-AR-induced contractility involving β_1-AR was demonstrated in papillary muscle from LPS rats without any modifications in the β_1-AR membrane protein level. Because endothelial dysfunction is largely expanded at the early phase of shock, we investigated its involvement in the β_1-AR-induced contractility. Finally, using inhibitors and antagonists of various endothelial signaling pathways we demonstrated an involvement of both COX1 and COX2 isoforms, in the endothelial dysfunction dependent increased β_1AR induced contractility.

4.1. Cardiac β-AR remodeling at the early phase of endotoxemic shock

Confirming our *in vivo* results, we observed alterations in papillary muscle contractility in basal conditions in LPS-treated rats. Our results using papillary muscles are consistent with another study using an isolated working heart model (data not shown), showing an increase in the response to isoproterenol on endotoxemic rats hearts independently of load conditions, which has been rarely evaluated in the literature at the early phase of endotoxemic shock. However, these results should be considered with caution. Indeed, the developed tension recorded must be standardized with the papillary muscle's diameter. The possibility for cardiac edema in the septic shock [26] could distort this parameter and the subsequent analyses. Thus, our results were expressed as the percentage of the peak tension measured in basal conditions to avoid this bias.

The global β-AR contractile response was increased in LPS-treated papillary muscles compared to control ones. This result is not in agreement with previous reports in the literature where β-AR contractility, evaluated using isoproterenol, was decreased [6,27–29]. However, in these studies the β-AR contractility was evaluated on isolated cardiomyocytes and/or after longer LPS challenges that could explain the reported differences.

To evaluate the specific involvement of β_1-AR, β_2-AR and β_3-AR subtypes in left ventricles, we measured total and membrane β-AR subtype proteins levels. Western blot analysis showed that total protein for all three subtypes were decreased, while by binding analysis, we saw that membrane protein levels for β_1- and β_2-AR were not modified indicating that AR membrane proteins are not different in both conditions. However, these binding experiments did not indicate possible functional effects of the specific β-AR. Previous experiments have demonstrated preserved β-AR expression at the cardiac membrane of rabbit heart 6 h after an LPS injection [6]. Nevertheless, in this study the specific expression of β_1-AR and β_2-AR were not investigated.

Functional remodeling was evaluated thanks to classical pharmacological experiments using isoproterenol associated with selective antagonists for each β-AR subtypes. We demonstrate that the isoproterenol increased cardiac contractility was dependent on β_1-AR.

4.2. Endothelial dysfunction involvement in the increased β_1-AR-induced contractility

As described by Brutsaert et al. in 2003, removal of the EE is associated with a switch to a faster relaxation time [30]. In our study, EE removal was confirmed by an accelerated relaxation in papillary muscles leading to a shortened contraction period (Fig. 2B), however this treatment could also have an impact on adrenoceptor binding capacities [31] and myocardial contractility [32]. When the EE was disrupted the β_1ARincreased response was completely abolished. Interestingly, Triton X-100, at concentrations lower than the one used in this study, is able to inhibit L-type voltage calcium channels [33] which could interfere with our results. However, the β_1-AR response was not modified in control papillary muscle following Triton X-100 treatment which is inconsistent with an action of this target in our model.

Mebazaa et al. demonstrated that the myocardium from endotoxemic rabbits is more sensitive to an endothelial control of contractile performance than myocardium from controls [8]. This control involves several endothelial signaling pathways such as NOS, endothelin receptors and COX pathways [8]. In our study, neither L-NMMA nor Bosentan induced any modification of the β_1-AR-induced contractility observed in papillary muscles from LPS-treated rats. However, when a non-specific COX inhibitor was used the increased β_1-AR-induced contractility was abolished. These data demonstrate for the first time the involvement of COX in the increased β_1-AR cardiac contractility at the early phase of endotoxemic shock. Regulation of β-AR function through COX has previously been demonstrated on isolated equine arteries where COX2 decreased the β_2-AR induced vasodilation [34].

However, in our study, the absence of blood components in the perfusion medium does not allow a perfect extrapolation of the mechanisms encountered *in vivo*. Indeed, leukocytes are known to regulate

EE-dependent decreased cardiac contractility during sepsis [9]. This decreased contractility is dependent on the production of ˙NO after the release of lysozymes by leucocytes.

When both COX isoforms were inhibited independently, the β_1-AR increased contractility was abolished suggesting the involvement of both COX1 and COX2. However, it seemed that isoproterenol potency was lower when using the COX2 inhibitor compared to the COX1 inhibitor. The lack of statistical difference may be due to the group sizes. This tendency is consistent with a major involvement of COX2 in this abolished β_1-AR-induced increased contractility. No increase in COX2 protein level expression in the left ventricle from LPS-treated rats at such an early stage was reported, yet it is in conflict with previous reports [35]. Modifications in the EE could easily be masked by other cells types.

COX isoforms are the rate limiting enzymes in the prostanoid biosynthesis pathways [36]. Prostanoids are recognized at the membrane of cardiomyocytes by seven-transmembrane receptors coupled to G proteins [37,38]. Interestingly, a previous report demonstrated that Treprostinil, a prostacyclin analogue, improved isoproterenol-induced cardiac contractility using both isolated cardiomyocytes and Langendorff experiments in healthy rats [39].

Whether the endothelial COX dependent increased β_1-AR-induced contractility involves only the activation of the PKA pathway or is also activated by the Epac/CaMKII pathway remains to be determined. While the activation of Epac pathways through prostanoid receptors has never been demonstrated in cardiomyocytes, several studies have demonstrated activation of Epac pathways through prostanoids receptors in rat microglia [40], in human mesenchymal stem cells [41] or fibroblasts [42]. The possible deleterious effects of the β_1-AR stimulation were not evaluated in this study but should not be excluded and could explain the positive effects of indomethacin on the survival of LPS-treated rats over 48 h. These effects of indomethacin confirm the results from numerous previous preclinical reports [43]. However, clinical studies using COX inhibitors a few hours after sepsis diagnosis, did not demonstrate any beneficial effects during the septic shock [44–46].

The endotoxemic shock model is probably not the most relevant model for studying septic shock. Nevertheless, it reproduces an acute endothelial dysfunction such as can be observed in septic shock but also in other situations such as after cardiopulmonary bypass or myocardial infarction. Some of these circulatory failure situations are potentially linked to endotoxin translocation during ischemia-reperfusion damage to the gastrointestinal mucosa [47].

5. Conclusion

Our work demonstrates an increased β_1-AR-induced cardiac contractility at the early phase of endotoxemic shock. The removal of the EE abolished this increased β_1-AR response, suggesting involvement of endothelial dysfunction in the regulation of this phenomenon. We demonstrate the involvement of both COX1 and COX2 isoforms in this increased β_1-AR contractility, and show an improved survival in LPS-treated rats treated with indomethacin 1 h after LPS administration. These results suggest a potentially beneficial effect of COX inhibitors in the management of the cardiac dysfunction observed at the initial phase of septic shock.

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Authors' contributions

DR and BR performed most of the experimental work; ME and DG performed papillary muscle experiments; LA, AG, AE, NM, BL, assisted experiments; IM participated in the study conception; BR, BL, CG participated in study conception and design as well as data analysis and interpretation; DR, BR, MF, CG, BL drafted the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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