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De novo expression of functional connexins 43 and 45 hemichannels increases sarcolemmal permeability of skeletal myofibers during endotoxemia

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ABSTRACT

Endotoxemia caused by bacterial lipopolysaccharides (LPSs) leads to severe skeletal muscular deterioration, starting with higher membrane permeability and decline in resting membrane potential (RMP). However, the molecular mechanism of such changes remains unclear. Here, we evaluated the possible involvement of connexin43- and connexin45-based hemichannels (Cx43 and Cx45 HCs, respectively) as putative mediators of sarcolemmal dysfunctions induced by LPS in control ($Cx43^{fl/fl}Cx45^{fl/fl}$) and Cx43/Cx45 expression-deficient ($Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre) skeletal mice myofibers. At 5 h of endotoxemia, control myofibers presented Cx43 and Cx45 proteins forming functional HCs. Additionally, myofibers from endotoxic control mice showed dye uptake *in vivo*, which was inhibited by carbenoxolone, a Cx HC blocker. A similar increase in membrane permeability was observed in myofibers freshly isolated from skeletal muscle of mice treated for 5 h with LPS, which was blocked by the Cx HC blocker and was absent in myofibers from mice simultaneously treated with LPS and boldine, which is a Cx HC blocker. The increase in sarcolemmal permeability was mimicked by isolated myofibers treated with pro-inflammatory cytokines (TNF-α and IL-1β) and occurred at 5 h after treatment. Endotoxemia also induced a significant increase in basal intracellular Ca^{2+} signal and a drop in RMP in control myofibers. These two changes were not elicited by myofibers deficient in Cx43/Cx45 expression. Therefore, sarcolemmal dysfunction characterizing endotoxemia is largely explained by the expression of functional Cx43 and Cx45 HCs. Hence, current therapy options for individuals suffering from endotoxic shock could be greatly improved with selective Cx HC inhibitors avoiding the underlying skeletal muscle dysfunction.

1. Introduction

Systemic infection with bacteria produces a severe condition clinically known as sepsis associated-intensive care unit acquired weakness (ICUAW). One prominent characteristic of this condition is sarcolemmal injury in both respiratory and peripheral skeletal muscles [1]. At the cellular level, significant depolarization (reduction in resting membrane potential: RMP) and permeabilization of the sarcolemma to small fluorescent dyes have been described [2], but the molecular explanation of these changes remains largely unknown. Recently, it was demonstrated that sepsis induces the expression of proteins that form non-selective channels in skeletal muscle sarcolemma, and it was proposed that they might form functional channels, explaining the previously described channelopathy of the septic condition [3]. However, functional evidence has not been reported to date.

Several changes observed under the septic condition also occur in endotoxemia induced with lipopolysaccharides (LPSs) from Gram negative bacteria [4]. LPSs located in the outer membrane of these

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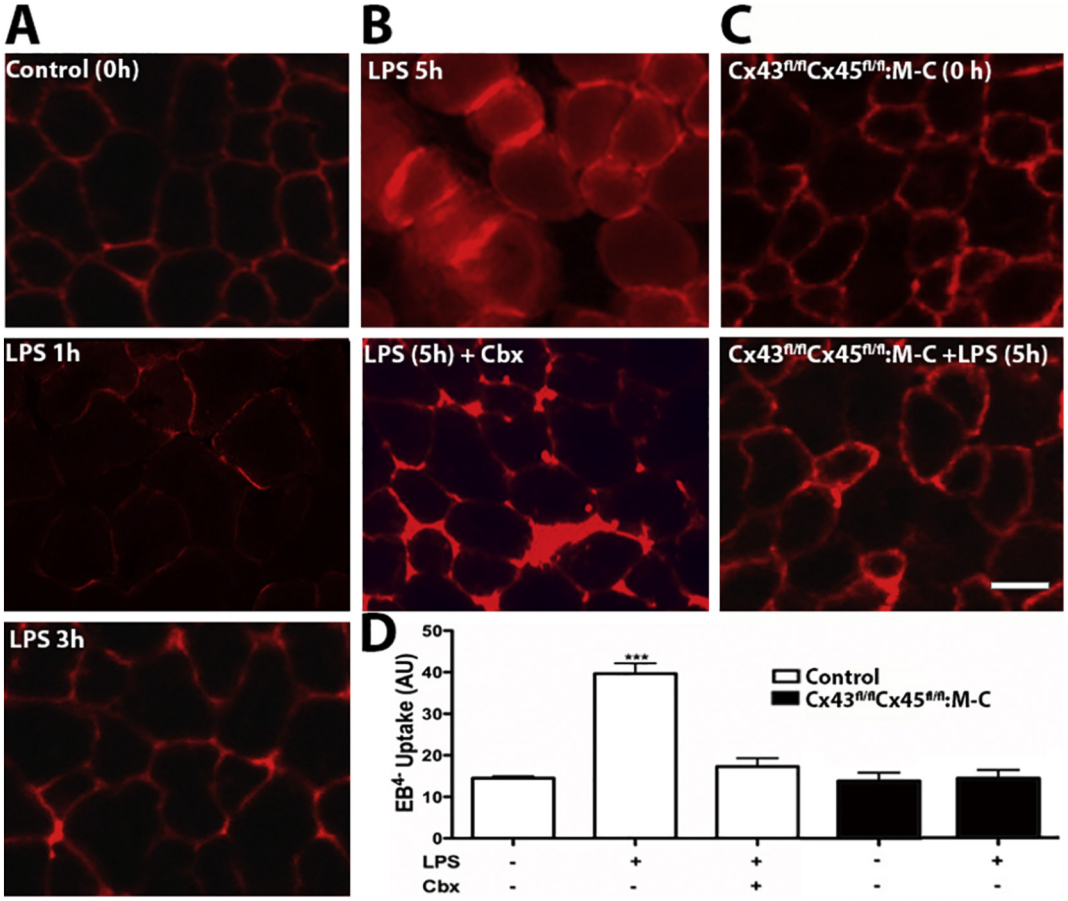

Fig. 1. The sarcolemma of skeletal myofibers in septic mice is permeable to Evans blue. A. The uptake of Evans blue by myofibers of TA muscles from control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) was evaluated *in vivo* after 1, 3 and 5 h of i.p. LPS injection (3 mg/kg), and compared to that of mice injected only with Evans blue dye. B. The possible involvement of Cx HCs was evaluated in myofibers from mice treated with LPS or LPS plus carbenoxolone (i.p. Cbx injection, 80 mg/kg), a Cx HC blocker. C. The Evans blue uptake was also evaluated in $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice (which cannot express Cx43 or Cx45 in skeletal muscles after myogenin expression) injected or not with LPS. D. The graph shows the intracellular fluorescence intensity detected under different conditions. Scale bar: 50 μm. $n = 4$ independent experiments and each plotted value corresponds to the mean ± SE of 80 myofibers recorded $^{***}p < 0.001$.

bacteria are recognized by immune system cells, among others. Consequently, endotoxemia causes an acute systemic inflammatory condition, inducing fast and severe loss of whole body proteins, especially from skeletal muscles [5]. Significant reductions in resting membrane potential (RMP) in skeletal myofibers have been observed as early outcomes of endotoxemia [6–8]. Such response appears to be mediated by macrophages that release pro-inflammatory cytokines, including TNF-α, which induces sarcolemmal depolarization in skeletal myofibers [9]. An observation possibly associated to RMP reduction is that diaphragm myofibers from rats treated with LPS show hyperpermeability to a low molecular weight tracer dye [2]. To our knowledge, it remains unknown whether a similar increase in sarcolemmal permeability also occurs in non-respiratory skeletal myofibers, as well as its underlying molecular mechanism.

Several studies have shown that muscular atrophy induced by LPS or sepsis results from an increase in protein degradation through an ubiquitin/proteasome-dependent pathway [10]. Accordingly, the amounts of Atrogin-1 or Murf-1 increase in muscles of animals subjected to cecal ligation/puncture-induced sepsis [11] or after LPS administration [5]. LPS-induced endotoxemia is also characterized by early increases in amounts of pro-inflammatory cytokines (*e.g.*, TNF-α, IL-1β and IL-6) [12].

Most intra venous (i.v.)-injected LPS is cleared by the liver (~40%), but a great amount is also distributed in the spleen, lung, kidney, and adrenal glands [13]. However, since skeletal muscles represent a significant portion of the whole body mass, a considerable amount of LPS is also accumulated in this tissue [13]. Skeletal muscles show a fast metabolic response to intraperitoneally (i.p.)-injected LPS. In dog muscles, LPS increases the amount of glucose-6-phosphate, phosphocreatine, and ATP within 5 min [14]. LPS also upregulates muscle amounts of TNF-α and IL-1 β mRNAs within 30 min [15]. Greater expression of proinflammatory cytokines has been associated with the presence of connexin-based hemichannels (Cx HCs) in conditions that affect skeletal muscles, such as denervation [16] and Duchenne muscular dystrophy [17]. Whether a similar association occurs in response to LPS remains unknown. Along the same line of thought, it has been reported that LPS treatment on C6 or HeLa cells transfected with Cx43 potentiates ATP release through Cx43 HCs induced by a divalent cation-free solution, which increases the open probability of Cx HCs [18]. This potentiation was not observed in cells transfected with truncated Cx43 [19], which does not form active Cx HCs [20], or upon treatment with Gap27 peptide, which is a Cx HC inhibitor [19].

Another critical response promoted by LPS is the increase in glucocorticoids (GCs) [21,22], which exacerbates some LPS-induced signaling [23]. In addition, GCs-induced skeletal muscle atrophy has been directly related to the *de novo* expression of functional Cx43 and Cx45 HCs. Dexamethasone, a synthetic GC, induces the expression of Cxs in fast skeletal myofibers from mice just 5 h after administration, followed by an increase in Cx HC activity and a reduction in myofiber RMP in the *flexor digitorum muscle*, a non-respiratory muscle [24].

In the present study, we explored whether the expression of functional Cx43 and Cx45 HCs in skeletal muscles might explain the

channelopathy that characterizes endotoxemia. We found *de novo* expression of poorly selective Cx HCs and their absence was enough to prevent the increase in sarcolemmal permeability, as well as RMP reductions and greater intracellular Ca^{2+} signal. Therefore, the expression of functional Cx HCs was likely to play a relevant role in the channelopathy observed in entotoxemia.

2. Results

2.1. During endotoxemia, the sarcolemma of myofibers become permeable to Evans blue via Cx HC

Since the EB^{4-} can cross the cell membrane *via* Cx HCs [16,17,24], we decided to test whether the sarcolemma of myofibers from endotoxic mice was permeabilized *in vivo*. This possibility was tested in tibialis anterior (TA) muscles at different times (1, 3 and 5 h) after LPS administration to control ($Cx43^{fl/fl}Cx45^{fl/fl}$) and Cx43/Cx45 skeletal muscle deficient mice ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C). EB^{4-} was detected predominantly in the extracellular space surrounding myofibers in TA muscles from mice not treated with LPS (0 h), as well as in muscles from control mice after 1 or 3 h treatment with LPS (Fig. 1). However, EB^{4-} staining was clearly visualized in the intracellular compartment of myofibres at 5 h after LPS treatment in control mice (Fig. 1A, B). In contrast, the dye remained preferentially in the interstitial space of muscles from mice treated with LPS (5 h) plus carbenoxolone (Cbx) (Fig. 1B, D, LPS + Cbx and graph) or from $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice (which cannot express Cx43 or Cx45 in skeletal muscles after myogenin expression) treated with LPS (5 h), similar to that observed in control mice (time = zero) or 1 and 3 h after treatment with LPS only.

2.2. Endotoxemia induces de novo expression of functional Cx HCs in skeletal myofibers, which is prevented and blocked by boldine

Endotoxemia was induced in control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) with i.p.-injected LPS (3 mg/kg). It was evaluated the presence of Cx43 and Cx45 proteins in TA muscles slices from $Cx43^{fl/fl}Cx45^{fl/fl}$ and $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice treated or not with LPS (5 h). It was found that only myofibers from $Cx43^{fl/fl}Cx45^{fl/fl}$ mice treated with LPS showed positive reactivity (red signal, Supplementary Fig. 1). Also, freshly isolated skeletal myofibers were dissociated from *flexor digitorum brevis* (FDB) muscles, and Cx43 and Cx45 immunoreactivity as well as Cx HC activity were evaluated.

Myofibers from LPS-treated control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) showed strong Cx43 and Cx45 immunoreactivity (Fig. 2A) at the border of myofibers, suggesting the presence of these proteins in the sarcolemma. These two proteins were neither detected in myofibers from control mice not treated with LPS nor in myofibers from mice treated with LPS but do not express Cx43 and Cx45 in skeletal muscles after myogenin expression ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C); the immunoreactivity was mainly located intracellularly and not in the sarcolemma of cells from mice that were simultaneously treated with LPS and 50 mg/kg boldine (Supplementary Fig. 1). Boldine is an alkaloid derived from the endemic Chilean tree called *Peumus boldus*, and has antioxidant and anti-inflammatory properties [25,26]. Boldine has been shown to block Cx HCs and drastically reduce pathological conditions in which Cx HCs play a relevant role in the inflammatory response [27,28]. Thus, we evaluated the acute effect of boldine on myofibers from LPS-treated mice as well as in myofibers from mice treated with 50 mg/kg boldine 30 min before the LPS administration (Fig. 2C). Since LPS induced the *de novo* expression of Cx43 and Cx45 (see above), it likely followed that myofibers presented functional Cx HCs. This possibility was investigated in myofibers freshly isolated from control ($Cx43^{fl/fl}Cx45^{fl/fl}$) mice treated with LPS (5 h) in which we observed that these myofibers showed ~3-fold increases in ethidium (Etd^+) uptake, whereas myofibers of Cx43/Cx45 expression-deficient mice ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C) showed very low Etd^+ uptake (Fig. 2B and C). In fact, the Etd^+ uptake rate of Cx43/Cx45 expression deficient myofibers was about the same as that of control myofibers from mice not treated with LPS (Fig. 2B and C). The Etd^+ uptake rate of myofibers from control mice treated with LPS was acutely inhibited by La^{3+}, Cbx and boldine (Fig. 2C, last white bar and last black bar) applied during the dye uptake assay (Fig. 2B and C), these three agents are Cx HC blockers [27,29]. It is worth noting that the low Etd^+ uptake rate detected in myofibers of $Cx43^{fl/fl}$ $Cx45^{fl/fl}$: M-C mice was not significantly affected by LPS or the HC blockers tested (Fig. 2C).

Consistent with the lack of Cx43 and Cx45 immunoreactivity in the sarcolemma of myofibers isolated from control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) treated *in vivo* with LPS plus boldine (Fig. 2A) showed an Etd^+ uptake rate comparable to that of myofibers from control mice not treated with LPS or of myofibers deficient in the expression of Cx43/Cx45 (Fig. 2B and C). Interestingly, myofibers from control mice treated with LPS + boldine presented intracellular immunoreactive puncta (Supplementary Fig. 2), with spot like appearance and might represent vesicles traffic to and/or from the sarcolemma. The area under the curve (AUC) of fluorescence intensity *vs* distance graph for myofibers from control mice treated with LPS was 9.821 and 7.480 for Cx43 and Cx45, respectively. However, these values of myofibers from control mice treated with LPS + boldine were 3.673 and 3.303, for Cx43 and Cx45, respectively, suggesting that boldine induced HC inhibition might be followed by down regulation and degradation of Cx HCs.

2.3. Endotoxemia increases intracellular Ca^{2+} signal in skeletal myofibers via open Cx HCs

Since elevated intracellular free Ca^{2+} activates proteolysis involved in muscle atrophy [30], and the expression of functional Cx HCs is associated with an increase in intracellular Ca^{2+} signal in skeletal myofibers under pathological conditions [17,24,31], we analyzed whether LPS affects intracellular Ca^{2+} signal. With this purpose, myofibers freshly isolated from FDB muscles of mice treated during 5 h with LPS, were loaded with FURA-2 to evaluate intracellular Ca^{2+} signal. We found that myofibers obtained from LPS-treated control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) showed an increase of ~25% in Ca^{2+} signal with respect to that of control myofibers under basal conditions (Fig. 3). In addition, the intracellular Ca^{2+} signal of myofibers $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C was not affected by LPS (Fig. 3, black bar), and values were comparable to those observed in myofibers from control mice simultaneously treated with LPS and Cbx (80 mg/kg) or LPS and boldine (50 mg/kg) (Fig. 3). To explore if this Ca^{2+} signal came from extracellular space, myofibers from LPS treated mice were exposed *in vitro* to boldine, a Cx HC blocker [27,28]. We found that 45 min incubation with 50 μM boldine reverted the Ca^{2+} signal to values comparable to those of control myofibers not treated with LPS (Fig. 4A). Additionally, the same protocol was applied to $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice and found that myofibers did not show variations in intracellular Ca^{2+} signal after *in vivo* treatment with LPS (Fig. 3) or *in vitro* treatment with LPS followed by *in vitro* treatment for 45 min with boldine (Fig. 4B).

2.4. The LPS-induced decrease in RMP of myofibers is due to the expression of Cx43 and Cx45 HCs

For the past century it has been known that LPS induces a reduction in skeletal myofiber RMP [6–8], but the underlying mechanism has remained unknown. Since Cx43 HCs are permeable to Na^+, K^+ and Ca^{2+} [32–34], and open Cx HCs significantly affect cells RMP [24,35], we studied whether the expression of Cxs in myofibers of endotoxic mice could explain the decline in RMP. To this end, RMP was measured in myofibers freshly dissociated from FDB muscles 5 h after mice were treated with LPS. Under control conditions, myofibers from $Cx43^{fl/fl}Cx45^{fl/fl}$ and $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice presented almost identical RMP and in the normal range (Fig. 5). Nonetheless, a significant reduction in RMP from −70.1 ± 2 mV to −60.3 ± 3 mV (5) was found

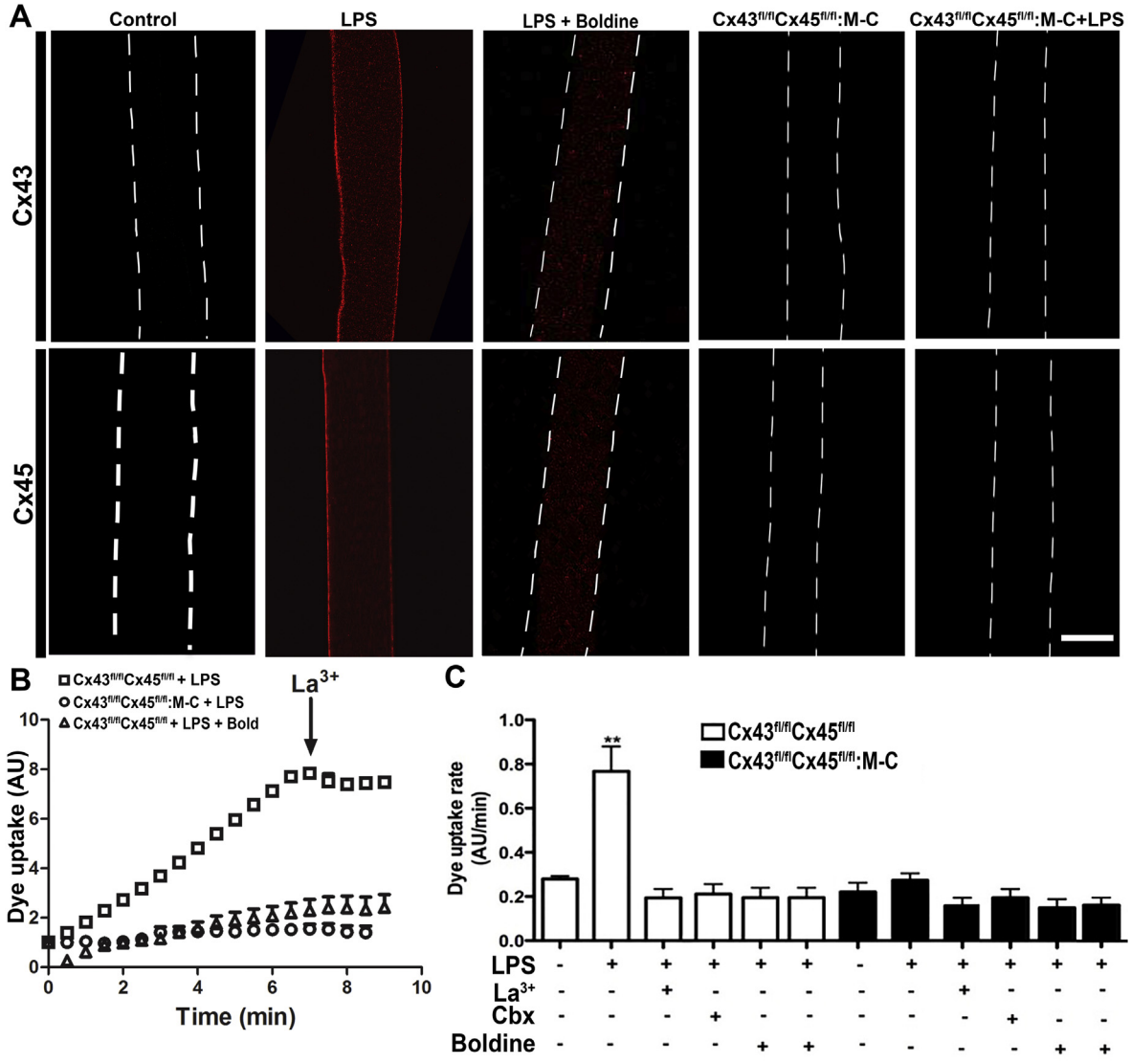

Fig. 2. LPS induces the *de novo* expression of functional Cx43 and Cx45 HCs, which is prevented and are blocked by boldine. Freshly isolated skeletal myofibers from control ($Cx43^{fl/fl}Cx45^{fl/fl}$) and Cx43/Cx45 muscle expression deficient ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C) mice treated or not with LPS were used to evaluate the immune reactivity of Cxs 43 and 45 and the presence of functional Cx HCs. A) Reactivity of Cxs 43 and 45 in FDB myofibers freshly isolated from control mice ($Cx43^{fl/fl}Cx45^{fl/fl}$) under basal conditions, 5 h after treatment with LPS (3 mg/kg, i.p. injected) (LPS) or 5 h after treatment with LPS and previous 20 min boldine injection (50 mg/kg) (LPS + boldine) and myofibers from $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice treated or not with LPS by 5 h mice were detected using specific anti-Cx primary antibodies followed by incubation with a secondary antibody conjugated to Cy3 (red fluorophore). B) Graph showing the fluorescence intensity of Etd^+ uptake in a time lapse experiment using myofibers from control ($Cx43^{fl/fl}Cx45^{fl/fl}$ + LPS) and Cx43/Cx45 expression deficient mice ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C + LPS) after 5 h treatment with LPS or myofibers from control mice after treatment with LPS and boldine ($Cx43^{fl/fl}Cx45^{fl/fl}$ + LPS + bold). Each plotted value represents the mean ± SE of the fluorescent intensity recorded in 20 cells every 30 s during recording. After 5 min recording, La^{3+} (200 μM) was applied in order to block Cx HCs. C). Graph showing Etd^+ uptake rate of myofibers from untreated control mice as well as myofibers from control $Cx43^{fl/fl}Cx45^{fl/fl}$ mice and $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice after 5 h treatment with LPS or LPS + boldine as in B. Also during recording, these myofibers were treated with 200 μM La^{3+}, 100 μM carbenoxolone (Cbx) or 100 μM boldine (from left to the right, last white bar and last black bar) 5 min before Etd^+ uptake evaluation (acute inhibition). Each bar represents the mean ± SE of $n = 4$. In each experiment a minimum of 80 cells were recorded. $^{**}p < 0.01$.

in myofibers of LPS-treated control ($Cx43^{fl/fl}Cx45^{fl/fl}$) mice (Fig. 5). In contrast, myofibers deficient in Cx43/Cx45 expression ($Cx43^{fl/fl}Cx45^{fl/fl}$:M-C) showed no statistically significant change in RMP upon treatment with LPS (−71.6 ± 4 mV untreated and −70 ± 5 mV LPS-treated mice), indicating that Cx HCs play a critical role in RMP decline in myofibers from LPS-treated mice.

2.5. Pro-inflammatory cytokines increase sarcolemmal permeability of isolated myofibers in a Cx43 and Cx45 expression-dependent manner

To investigate the possible mechanism by which LPS induces functional Cx-HC expression, we analyzed the direct effect of LPS on freshly isolated myofibers from control mice. To this end, myofibers were incubated during 1, 3 or 5 h with LPS, and the Cx-HC activity was analyzed using the Etd^+ uptake assays. We observed that LPS did affect the Cx-HC activity of myofibers (Supplementary Fig. 3), suggesting that its mechanism of action is indirect and possibly mediated by another molecule(s). We hypothesized that the mechanism includes the involvement of pro-inflammatory cytokines since macrophages are activated by LPS and release pro-inflammatory cytokines, such as TNF-α and IL-1β. In this way, it was likely that these cytokines affect the expression of connexins in myofibers. This possibility was studied in freshly isolated myofibers from FDB muscles of $Cx43^{fl/fl}Cx45^{fl/fl}$ and $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C mice. We found that after 5 h treatment with

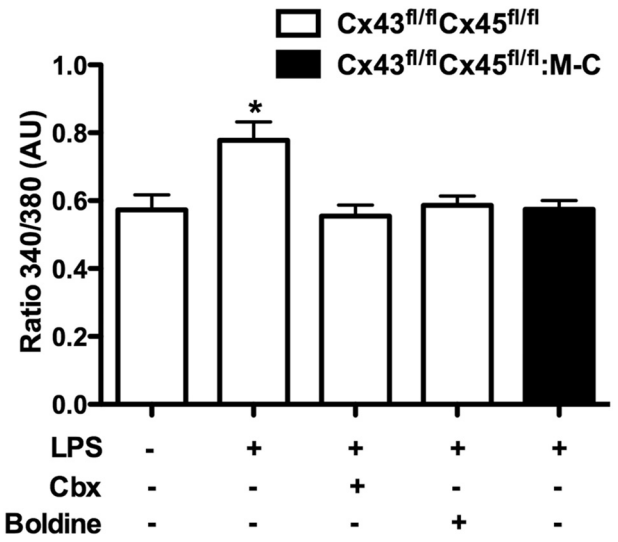

Fig. 3. The intracellular Ca^{2+} signal of skeletal myofibers from endotoxic mice is elevated due to Ca^{2+} influx through Cx HCs. Freshly isolated myofibers of FDB muscles from LPS injected control ($Cx43^{fl/fl}$ $Cx45^{fl/fl}$) or Cx43/Cx45 expression deficient ($Cx43f^{l/fl}$ $Cx45^{fl/fl}$:M-C) mice were used to evaluate Ca^{2+} signal using FURA-2 under basal conditions or after 5 h treatment with LPS. The Ca^{2+} signal increase did not occur in myofibers of mice treated with LPS pretreated 20 min before with boldine (Bold, 50 mg/kg) or carbenoxolone (Cbx). Boldine and Cbx are two Cx HC blockers. $n = 4$ independent experiments with 20 fibers per experiment. $^{*}p < 0.05$.

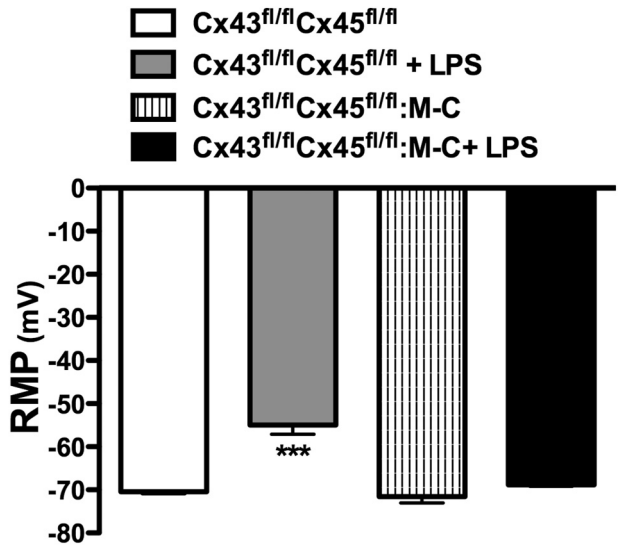

Fig. 5. Lack of Cx43/Cx45 expression prevents LPS-induced reductions in RMP of skeletal myofibers. After 5 h of LPS or saline administration to animals, freshly isolated myofibers of FDB muscles from control ($Cx43^{fl/fl}$ $Cx45^{fl/fl}$) and Cx43/Cx45 expression deficient ($Cx43^{fl/fl}$ $Cx45^{fl/fl}$:M-C) mice, were used to evaluate resting membrane potential (RMP). Each value corresponds to the mean ± SE ($n = 4$ independent experiments with 20 fibers per experiment). $^{**}p < 0.01$.

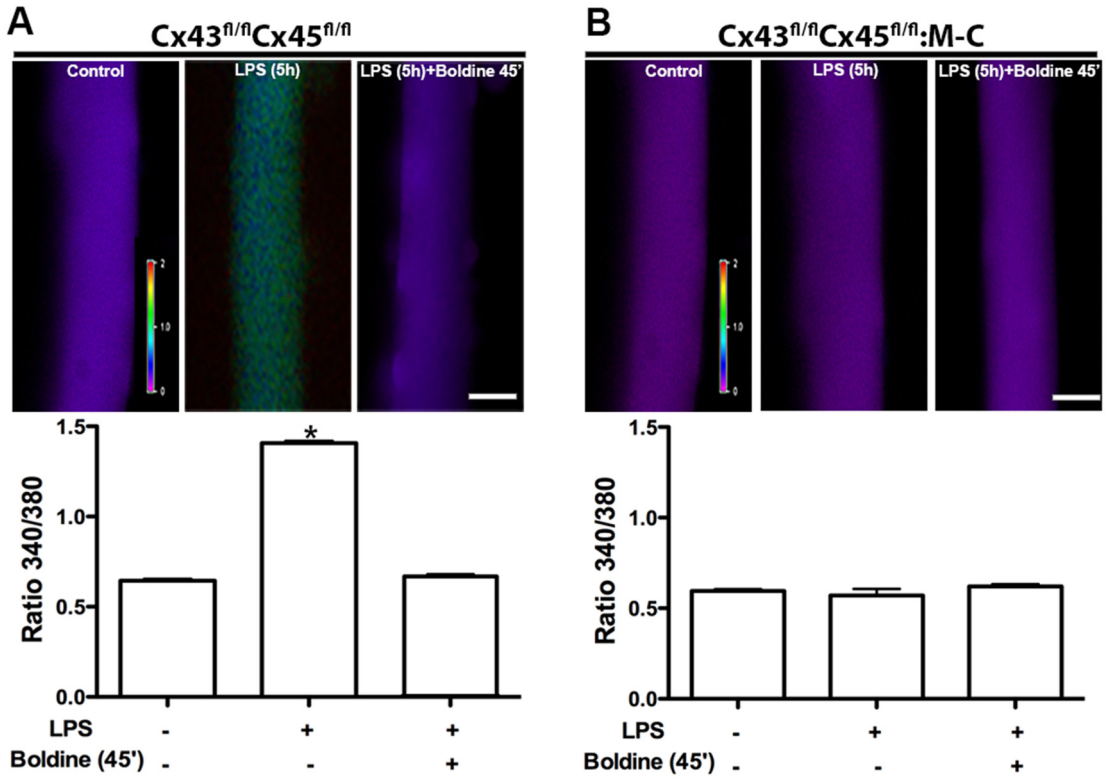

Fig. 4. Boldine restores the intracellular Ca^{2+} signal induced by LPS. After 5 h of LPS or saline administration to animals, freshly isolated myofibers of FDB muscles from control ($Cx43^{fl/fl}$ $Cx45^{fl/fl}$) and Cx43/Cx45 expression deficient mice ($Cx43^{fl/fl}$ $Cx45^{fl/fl}$:M-C) were used to evaluate if boldine restore the increased Ca^{2+} signal induced by LPS. A. Top panels, representative images of myofibers from control mice treated with LPS (5 h, middle and right panel) or not (left panel) loaded with FURA-2 and directly incubated with boldine (50 μM) (right panel). The quantification of these conditions is shown in the bottom graph. B. Shows the same conditions described in A but in myofibers $Cx43^{fl/fl}$ $Cx45^{fl/fl}$:M-C mice. $^{*}p < 0.05$ respect to control and boldine conditions. $n = 3$ animals with 20 myofibers analyzed per condition.

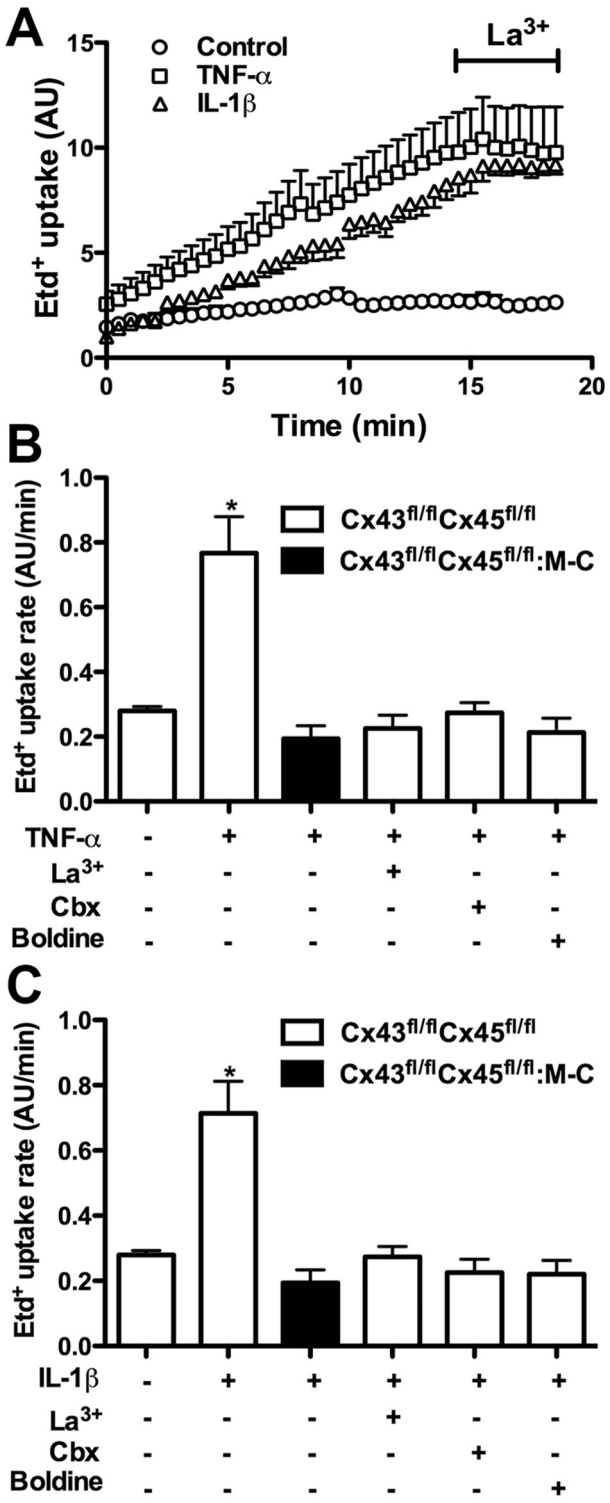

Fig. 6. Pro-inflammatory cytokines increase sarcolemmal permeability *via* Cx HCs in freshly isolated skeletal myofibers. Isolated myofibers from control mice were incubated with TNFα (10 ng/mL) or IL-1β (10 ng/mL) for 5 h. The Etd^+ uptake was evaluated in control and cytokine-treated myofibers during the first 10 min and then La^{3+} (200 μM), a Cx HC blocker, was applied. $n = 4$, a total of 80 myofibers were recorded.

10 ng/mL TNF-α or 10 ng/mL IL-1β, significantly increase the Etd^+ uptake as compared to basal conditions and was completely blocked by 200 μM La^{3+}, 100 μM carbenoxolone or 50 μM boldine acutely applied during dye uptake recording. Whereas in $Cx43^{fl/fl}Cx45^{fl/fl}$:M-C myofibers the cytokines did not change the basal Etd^+ uptake (Fig. 6B and C, black bar). Additionally, we evaluated the Ca^{2+} signal induced by direct application of TNF-α and IL-1β to freshly isolated myofibers from control mice, and found an increase in Ca^{2+} signal that after 45 min treatment with boldine (50 μM) returned to basal values, suggesting that this increase in Ca^{2+} is due to influx from the extracellular space (Supplementary Fig. 4).

2.6. Glucocorticoids are not involved in endotoxemia-induced Cx HC expression

It is known that LPS increases the blood concentration of glucocorticoids (GCs) and is part of the mechanism that induces cachexia in mice [21,22]. Moreover, treatment with GCs is known to induce the expression of Cx HCs [24]. Thus, we tested whether the LPS-induced expression of functional Cx43 and Cx45 HCs is mediated by endogenous GCs characteristic of endotoxemia. With this purpose, we assayed whether the inhibition of glucocorticoid receptors (GCRs) with mifepristone (MIFE), a well-known GCR inhibitor [36], prevents the GC-induced increase in sarcolemmal permeability. MIFE (20 mg/kg) was injected 20 min before LPS administration, and myofibers were isolated 5 h after LPS administration to evaluate sarcolemmal permeability mediated by Cx HCs. We found that MIFE did not prevent the increase in Etd^+ uptake of myofibers from mice treated with LPS (Supplementary Fig. 5), suggesting that GCs are not responsible for the LPS-induced expression of Cx43 and Cx45 HCs. To control the effectiveness of MIFE, we increased sarcolemmal permeability to dyes by inducing the expression of Cx HCs in myofibers of control mice treated with dexamethasone (8 mg/kg i.p.), a synthetic GC, as described previously [24]. We found that MIFE completely prevented the dexamethasone-induced increase in Etd^+ uptake (Supplementary Fig. 5B), indicating that the dose of MIFE and the evaluation for Cx expression were appropriate.

3. Materials and methods

3.1. Reagents

Anti-rabbit and anti-mouse IgG antibodies-conjugated to Cy3 (red) were purchased from Jackson Immunoresearch laboratories (West Grove, PA, USA). Evans blue (EB^{4-}), carbenoxolone (Cbx) N-benzyl-p-toluene sulphonamide (BTS), suramin sodium salt, collagenase type I, TNF-α, IL-1β and LPS from *E. coli* were obtained from Sigma-Aldrich (St. Louis, USA). Ethidium (Etd^+) bromide was from GIBCO/BRL (Grand Island, NY, USA), fluoromount-G DAPI conjugated was from Electron Microscopy Science (Hatfield, PA, USA). Monoclonal Anti-Cx43 antibody was purchased from BD Biosciences (San Jose, CA, USA) and polyclonal anti-Cx45 antibody was purchased from Invitrogen (Carlsbad, CA, USA). Boldine hydrochloride was prepared by Härting company (Santiago, Chile) and was obtained from boldo bark (*Peumus boldus* Molina, Monimiaceae), and the purity of the alkaloid was checked by means of HPLC (high-performance liquid chromatography) analysis (99%) as described previously [27].

3.2. Animals

Male 2–3 months-old mice were used for this study. $Cx43^{fl/fl}$ $Cx45^{fl/fl}$ mice were used as control mice and $Cx43^{fl/fl}$ $Cx45^{fl/fl}$:Myo-Cre mice, which cannot express Cxs 43 and 45 in skeletal myofibers after myogenin expression, were generated by mating $Cx43^{fl/fl}$ and $Cx45^{fl/fl}$ mice with Myo-Cre mice, which express Cre recombinase under the control of myogenin promoter (MYF4) and MEF2C enhancer [37].

3.3. LPS treatment

Two–three month-old mice were injected intraperitoneally (i.p.)

with LPS from *E. coli* (3 mg/kg, Sigma-Aldrich Cat #L2630) suspended in sterile PBS. After 1, 3 or 5 h of LPS administration, mice were euthanized in deep anesthesia with isoflurane followed by cervical dislocation. All procedures were approved by the Institutional Bioethics Committee of the Pontificia Universidad Católica de Chile.

3.4. Evans blue uptake in vivo

This is a modified protocol according to Cea and collaborators [16]. In brief, animals were i.p. injected 5 min before LPS injection with Evans blue (EB^{4-}, 80 mg/kg) dissolved in a sterile saline solution. To inhibit the *in vivo* EB^{4-} uptake of myofibers, Cbx (80 mg/kg), a Cx HC blocker, was administered (i.p.) 20 min before the simultaneous administration of EB^{4-} and LPS injections. Then, 1, 3 or 5 h later the animals were euthanized, muscles dissected and fast-frozen in isopentane precooled in liquid nitrogen, and EB^{4-} fluorescence intensity was quantified in cross-sections in intracellular regions by using a conventional Nikon Eclipse *Ti* fluorescent microscope (λ excitation, 545 nm; λ emission, 595 nm).

3.5. Isolation of mouse skeletal myofibers

A protocol previously described by Cea and collaborators was used [16]. Briefly, myofibers were dissociated from flexor digitorum brevis (FDB) muscles (fast muscles). Plantaris tendons and connective tissue were removed from anesthetized mice. Then, FDB muscles were carefully dissected and immersed in culture medium (DMEM/F12 supplemented with 10% FBS) containing 0.2% collagenase type I, incubated for 3 h at 37 °C, and transferred to a 15-mL test tube (Falcon) containing 5 mL of culture medium. Then, muscle tissue was gently triturated 10 times by using a Pasteur pipette with a wide tip to disperse single myofibers. Dissociated myofibers were centrifuged at 1,000 rpm for 15 s (model 8700 centrifuge; Kubota) and washed twice by sedimentation, first in PBS solution and then in Krebs buffer (in mM: 145 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 10 Hepes-Na, pH 7.4) containing 10 μM BTS (contraction inhibitor) to reduce myofiber damage during the isolation procedure. Finally, fibers were suspended in 5 mL of Krebs Hepes buffer containing 10 μM BTS, plated in plastic culture dishes or placed in 1.5-mL Eppendorf tubes, and kept at room temperature.

3.6. Time-lapse recording of Etd^+ uptake

Cellular uptake of Etd^+ was evaluated in time-lapse measurements as previously described [16,38]. Briefly, freshly isolated myofibers plated onto plastic culture dishes were washed twice with Krebs buffer solution. For time-lapse measurements, myofibers were incubated in recording medium containing 5 μM Etd^+. Etd^+ fluorescence intensity was recorded in regions of interest that correspond to myofiber nuclei by using a water immersion Olympus 51W1I upright microscope (Japan). Images were captured with a Retiga 13,001 fast cooled monochromatic digital camera (12-bit; QImaging) every 30 s, and image processing was performed offline with ImageJ software (National Institutes of Health).

3.7. Immunofluorescence

The freshly isolated myofibers and *tibialis anterior* muscles slices were obtained and processed as described previously [16,17,24]. Briefly, samples were incubated at 4 °C for 12 h with diluted primary anti-anti-Cx43 (1:300) or anti-Cx45 (1:300) antibodies followed by four washes with HSS and then, incubated with an appropriate dilution of Cy3-conjugated goat anti-rabbit IgG antibodies. Samples were rinsed with HSS, mounted with fluoromount G on glass slides and representative images were acquired in a confocal microscope, Olympus fluoview 1000 (Tokyo, Japan) for myofibers. To muscle slices, DAPI was incorporated to label nuclei and a Nikon *ti* eclipse epifluorescence microscope was used to perform observation and take pictures.

3.8. Intracellular Ca^{2+} signal

Basal intracellular Ca^{2+} signal was evaluated in freshly isolated myofibers by using the ratiometric probe, FURA 2-AM. Myofibers were incubated in Krebs-Ringer buffer (in mM: 145 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 10 HEPES-Na, pH 7.4) containing FURA2-AM (2 μM) for 55 min at room temperature. Then, the Ca^{2+} signal was evaluated using a Nikon Eclipse *Ti* microscope equipped with epifluorescence illumination, and images were obtained with a Clara camera (Andor), at 2 wavelengths (λ) 340 and 380 nm, calculating the ratio 340 : 380. Additionally, freshly isolated myofibers from mice treated with LPS or treated with TNF-α or IL-1β were incubated with boldine (50 μM) by 45 min to revert the increase in intracellular Ca^{2+} signal.

3.9. Evaluation of resting membrane potential (RMP)

Freshly isolated myofibers of FDB muscles were used to measure RMP. The latter was recorded in a whole cell current clamp configuration using conventional high resistance micropipettes (30 to 50 mΩ), which contained 3 M KCl. The bath medium was Krebs saline solution at pH 7.4 at room temperature. The recorded RMP corresponded to the potential measured after impelling the sarcolemma of healthy myofibers, which was stable for at least 5 s after access experiments were carried out with an Olympus IX 51 inverted microscope with an Axopatch1-D amplifier.

3.10. Statistical analysis

Results are presented as mean ± standard error (SE). Two populations were compared using the logarithm of ratio and posterior Student's *t*-test. For multiple comparisons with a single control, a non-parametric one-way ANOVA followed by the Tukey's multiple comparison test was used. Analyses were carried out using GRAPHPAD software. $p < 0.05$ was considered statistically significant.

4. Discussion

In this study, we demonstrated that endotoxemia induced the *de novo* expression of functional Cx HCs in skeletal myofibers within a few hours (5 h), through a mechanism likely to be associated to the expression of pro-inflammatory cytokines. Cx HCs explain to a great extent, if not completely, the increase in sarcolemmal permeability, the increase in intracellular Ca^{2+} signal and the reduction in RMP. Hence, Cx HCs are proposed as novel molecular targets to reduce skeletal muscle channelopathy associated to LPS-induced endotoxemia.

A previous study demonstrated that during endotoxemia small fluorescent molecules permeate the sarcolemma of myofibers from the diaphragm [2]. Here, we extended this observation to other skeletal muscles both *in vivo* and *in vitro*, and demonstrated that the increase in permeability to fluorescent molecules (EB^{4-} and Etd^+) is due to the *de novo* expression of Cx HCs. This interpretation is supported by several findings described herein, since myofibers of endotoxic mice showed: 1) *in vivo* uptake of EB^{4-} that was blocked by Cbx, 2) positive Cx43 and Cx45 immunoreactivity in the sarcolemma, 3) elevated Etd^+ uptake mediated by membrane pathways inhibited by Cx HC blockers, and 4) the absence of Etd^+ uptake in myofibers deficient in Cx43 and Cx45 expression. In other pathological conditions including sepsis myofibers also express Cx39 [3,16,17,24,31], and Cx39 HCs are not permeable to Ca^{2+} [35], but might contribute to reduce the RMP if present in endotoxemia, as it does in HeLa cells transfected with Cx39 [35]. Therefore, if Cx39 HCs were expressed in endotoxemia the complete prevention of the LPS-induced reductions in RMP and intracellular Ca^{2+} signal increases in Cx43/Cx45 expression deficient myofibers might, in

part, results from compensatory mechanism such as Na^+/K^+ ATPase and Ca^{2+}/Na^+ exchangers. In addition, as observed in sepsis 3, it remains to studied whether other non-selective channels such as $P2X_7$ receptors, TRPV2 channels and Panx1 channels co-expressed with Cxs, are also expressed in endotoxemia.

In agreement with previous work [2], we found a reduction in RMP of myofibers in response to *E. coli* LPS-induced endotoxemia. This response was due to the expression of functional Cx43/Cx45 HCs, since it did not occur in Cx43/Cx45 expression deficient myofibers and was prevented by Cbx and boldine, which are two Cx HC blockers [27–29]. The latter was also observed in freshly dissociated myofibers from LPS treated control mice, in which the acute application of boldine inhibited Etd^+ uptake. On the other hand, the reduction in Cx HC activity induced by boldine in myofibers of control mice treated with LPS was associated with a drastic reduction of Cx HCs immunoreactivity located in the sarcolemma and significant accumulation of intracellular Cx immunoreactive spots that could correspond to down regulation of Cx HCs, which are internalized and traffic from the sarcolemma to a degradation pathway. A similar mechanism has been described for pannexin 1 channels upon high extracellular ATP concentration that first block the channels and after about 15 min causes down regulation of the channel [39]. Alternatively or simultaneously, the long term inhibition of Cx HC by boldine over the LPS induced expression of Cx43 and/or Cx45 HCs might prevent the activation of an intracellular signaling pathway that promotes the expression of these Cxs. In support to this interpretation a similar response has been previously reported in LLC-PK1 cells transfected with Cx43 and treated with Cd^{2+} that induces opening of Cx43 HCs and activation of Cx43 expression *via* a JNK-dependent pathway [40]. Future studies are required to demonstrate the precise mechanism of action of boldine treatment over the cell signaling mediated by Cx HCs.

It is noteworthy that RMP reduction in myofibers of rabbits occurs before deep hypotension, suggesting that RMP reduction is produced before septic shock and not as a consequence of it [6]. It also suggests that this change is an early predictor of successive sepsis-associated changes. Possible mediators of RMP reduction are pro-inflammatory cytokines released by macrophages and/or as outcomes of the activated local inflammasome [41]. Accordingly, TNF-α is known to reduce RMP of rat myofibers in a concentration-dependent manner within the first two hour post-treatment *ex vivo* and *in vivo* [9]. Moreover, we found that freshly isolated myofibers treated with TNF-α or IL-1β for just 5 h presented high sarcolemmal permeability to Etd^+ and the intracellular Ca^{2+} signal likely to be mediated by Cx HC since they were blocked by La^{3+}, a Cx HC blocker [29]. In addition, the expression of Cx HCs has been described to reduce skeletal myofibers RMP [24] and that of HeLa cells transfected with Cxs [35]. RMP reduction is likely due to HC permeability to Na^+ [32], K^+ [33] and Ca^{2+} [34]. Thus, the elevated Cx HC activity found in myofibers of septic mice most likely plays a relevant role in reducing the electrochemical gradient across the sarcolemma. Low excitability of myofibers during sepsis might result from long lasting membrane depolarization, which increases the number of inactivated Na^+ channels. On the other hand, a consequence of stronger intracellular Ca^{2+} signals could be the activation of Ca^{2+}-dependent calpains, which in turn activates the ubiquitin-proteasome system leading to muscular atrophy that characterizes severe sepsis [42].

LPS is known to elevate the serum concentration of several intermediary molecules, including GCs and pro-inflammatory cytokines, which are dramatically up-regulated by LPS. Both of them could be possible candidates mediating skeletal muscle responses. For instance, GCs like dexamethasone induce the expression of functional Cx HCs in skeletal muscles [24]. However, these molecules were discarded in the present work, since the inhibition of GCRs with MIFE did not prevent the expression of Cx HCs. Moreover, skeletal muscles of endotoxic animals have been shown to present abundant infiltration of inflammatory cells [43], and activated innate immune cells are known synthesize and release proinflammatory cytokines in response to LPS [44]. Thus, as mentioned in the previous paragraph, the effect of LPS in the muscle is likely to be mediated by TNFα and/or IL-1β. Consistent with this possibility, it has been suggested that LPS activates the inflammasome signaling pathways in macrophages [45]. Additionally, Cbx, which is a Cx HC and Panx1 channel blocker [29], decreases the release of pro-inflammatory molecules in an animal model of polymicrobial sepsis and significantly increases animal survival rates [46].

All alterations observed in myofibers of mice treated with LPS could explain the channelopathy of skeletal myofibers in endotoxic or septic subjects. In this sense, it was reported that sepsis induces the expression of non-selective channels in the sarcolemma, and it was proposed that channelopathy in skeletal muscles could result from a reduction in the electrochemical gradient across the cell membrane [3]. In support of this hypothesis, we showed in the present work that LPS-induced endotoxemia is associated with a reduction in RMP and an increase in the intracellular Ca^{2+} signal of skeletal myofibers. These two changes where due to Cx HC expression, because their absence or blockade prevented skeletal muscle changes, indicating that these channels could be the main cause of channelopathy in sepsis. Therefore, Cx HCs could be a novel target to efficiently reduce or prevent channelopathy in skeletal muscles, and perhaps the inflammation of the biggest organ (skeletal muscles) that is affected very early during multi organ failure. Skeletal muscle protection could increase life expectancy of septic subjects.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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