



Dexamethasone-induced muscular atrophy is mediated by functional expression of connexin-based hemichannels



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ARTICLE INFO

Article history:

Received 30 April 2016

Received in revised form 29 June 2016

Accepted 12 July 2016

Available online 18 July 2016

Keywords:

Connexons

Membrane leakage

Ethidium bromide

Purinergic receptors

Glucocorticoids

ABSTRACT

Long-term treatment with high glucocorticoid doses induces skeletal muscle atrophy. However, the molecular mechanism of such atrophy remains unclear. We evaluated the possible involvement of connexin-based hemichannels (Cx HCs) in muscle atrophy induced by dexamethasone (DEX), a synthetic glucocorticoid, on control (Cx43^{fl/fl}Cx45^{fl/fl}) and Cx43/Cx45 expression-deficient (Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre) skeletal myofibers. Myofibers of Cx43^{fl/fl}Cx45^{fl/fl} mice treated with DEX (5 h) expressed several proteins that form non-selective membrane channels (Cx39, Cx43, Cx45, Panx1, P2X₇ receptor and TRPV2). After 5 h DEX treatment *in vivo*, myofibers of Cx43^{fl/fl}Cx45^{fl/fl} mice showed Evans blue uptake, which was absent in myofibers of Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice. Similar results were obtained *in vitro* using ethidium as an HC permeability probe, and DEX-induced dye uptake in control myofibers was blocked by P2X₇ receptor inhibitors. DEX also induced a significant increase in basal intracellular Ca²⁺ signal and a reduction in resting membrane potential in Cx43^{fl/fl}Cx45^{fl/fl} myofibers, changes that were not elicited by myofibers deficient in Cx43/Cx45 expression. Moreover, treatment with DEX induced NF-κB activation and increased mRNA levels of TNF-α in control but not in Cx43/Cx45 expression-deficient myofibers. Finally, a prolonged DEX treatment (7 days) increased atrogen-1 and Murf-1 and reduced the cross sectional area of Cx43^{fl/fl}Cx45^{fl/fl} myofibers, but these parameters remained unaffected in Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre myofibers. Therefore, DEX-induced expression of Cx43 and Cx45 plays a critical role in early sarcolemma changes that lead to atrophy. Consequently, this side effect of chronic glucocorticoid treatment might be avoided by co-administration with a Cx HC blocker.

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1. Introduction

Glucocorticoids are frequently used as anti-inflammatory and immunosuppressive agents [1]. However, high doses and prolonged use induces undesired lateral effects such as reduction in tetanic stimuli-induced force [2] and muscular atrophy [3]. Nonetheless, the molecular mechanisms that explain the latter undesired effect are not completely understood.

Muscle wasting after glucocorticoid treatment is highly relevant and is called “steroid myopathy”. It is characterized by an insidious process

that causes weakness mainly in the proximal muscles of the upper and lower limbs and in the neck flexors [4,5]. An excess of either endogenous or exogenous corticosteroids can cause this condition. The excess of endogenous corticosteroid production can arise from adrenal tumors [6,7] and an excess of exogenous corticosteroids can result from steroid treatment for asthma, chronic obstructive pulmonary disease, and inflammatory processes, such as connective tissue disorders among others [8,9].

It has been suggested that glucocorticoids increase proteasome-dependent protein degradation [10] and inhibit protein synthesis [11]. Several noxious conditions that induce skeletal muscle atrophy (e.g. sepsis, cachexia and starvation) are also associated with an increase in circulating glucocorticoids levels [11], suggesting that these hormones constitute a common factor in skeletal muscle atrophy associated with these conditions. Accordingly, treatment with a glucocorticoid receptor antagonist (RU-38486) reduces muscle atrophy associated with sepsis [12]. Additionally, glucocorticoids have been shown to induce muscle

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atrophy in fast twitch fibers earlier than in slow twitch fibers [13,14]), which is probably related to higher glucocorticoid receptor expression in fast versus slow muscles [15].

The involvement of several pathways has been observed in glucocorticoid-induced muscle atrophy, including the activation of transcription factors FOXO and NF κ B [16,17]), of co-activators p300-C/EBP β [18] and of intracellular signaling pathways such as mTOR [19] and PI $_3$ K/Akt/GSK3 β [11,20]. In addition, it has been suggested that free radicals play a critical role in glucocorticoid-induced muscle atrophy [21]. The *de novo* expression of connexin hemichannels (Cx HCs) has been recently proposed to play a critical role in the mechanism underlying myofiber atrophy induced by denervation [22] and dystrophin mutations that cause muscular dystrophies [23]. Cx HCs are membrane channels formed by six connexin proteins, which communicate the intracellular space with the extracellular space, since they are permeable to ions and small molecules (e.g., ATP) [24].

Since glucocorticoids play a critical role in skeletal muscle atrophy in numerous conditions, and Cx HCs are relevant protagonists in some muscle pathological conditions, we evaluated the role of Cx HCs in glucocorticoid-induced muscle atrophy. After a few hours (5 h) of treatment with dexamethasone (DEX), myofibers of control (Cx43^{fl/fl}/Cx45^{fl/fl}) mice presented *de novo* expression of several non-selective membrane channels, as well as increases in membrane permeability, reductions in resting membrane potential and the activation of the transcription factor NF κ B. Moreover, longer DEX treatment (7 days) increased the levels of enzymes involved in protein degradation and reduced the cross-section area of myofibers. However, all the above changes were absent in myofibers deficient in Cx43/Cx45 expression (Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre). The possible role of Cx HCs in DEX-induced muscle atrophy is discussed further below.

2. Materials and methods

2.1. Reagents

Western Lightning chemiluminescence (ECL) detection reagents were purchased from Pierce (Pierce biotechnology, Rockford, IL, USA), and anti-rabbit or anti-mouse IgG antibodies-conjugated to Cy2 (green) or Cy3 (red) were purchased from Jackson ImmunoResearch laboratories (West Grove, PA, USA). Ethidium (Etd⁺) bromide was acquired from GIBCO/BRL (*Grand Island*, NY, USA), fluoromount-G from Electron Microscopy Science (Hatfield, PA, USA), brilliant blue G (BBG), N-benzyl-p-toluene sulphonamide (BTS) and collagenase type I from Sigma (St. Louis, USA), and A740003 from Tocris Bioscience (Bristol, UK). Phycoerythrin (PE) or FITC conjugated monoclonal anti-annexin V antibody were purchased from BD Biosciences (San Jose, CA, USA) and polyclonal anti-TRPV2 or anti-P2X $_7$ R antibodies from Abcam (Cambridge, MA, USA). Previously described polyclonal anti-Cx39, -43, -Cx45 and -Panx1 antibodies were used [22]. Rabbit polyclonal anti-phosphorylated-p65 NF κ B subunit antibodies were purchased from Cell Signaling (Boston, MA, USA). The atrogen-1 antibody was purchased from ECM Biosciences LLC (Versailles, KY, USA) and Murf-1 antibody was from Thermo Fisher Scientific (Waltham, MA, USA). Evans blue and LaCl $_3$ were acquired from Sigma (St. Louis, USA) and injectable dexamethasone (4 mg/ml) was purchased from Laboratorio Sanderson S.A (Santiago, Chile).

2.2. Animals

All protocols were approved by the Bioethics Committee of Pontificia Universidad Católica de Chile (protocol no. 176). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Two month old male Cx43^{fl/fl}/Cx45^{fl/fl}, Cx43^{fl/fl}:Myo-Cre, Cx45^{fl/fl}:Myo-Cre and Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre mice, previously described [22], were obtained from the animal facility of the Facultad de Ciencias

Biológicas, at Pontificia Universidad Católica de Chile. The Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre mice were skeletal muscle-deficient for Cx43 and Cx45 expression generated from breeding Cx43^{fl/fl} mice [45] and Cx45^{fl/fl} mice [46] with Myo-Cre mice, which express Cre recombinase under the control of the myogenin promoter and the MEF2C enhancer [47].

2.3. Ethidium uptake

Cellular uptake of ethidium (Etd⁺) was evaluated by time-lapse measurements as described previously [48]. In brief, freshly isolated myofibers from *Flexor digitorum brevis* (FDB) muscles, which have short myofibers that favor the isolation of healthy fibers, were plated onto plastic culture dishes and were washed twice with Krebs saline solution (in mM: 145 NaCl, 5 KCl, 1 CaCl $_2$, 1 MgCl $_2$, 5.6 glucose, 10 HEPES-Na, pH: 7.4). For time-lapse measurements, myofibers were incubated in recording medium containing 5 μ M Etd⁺. Etd⁺ fluorescence was recorded in regions of interest that corresponded to myofiber nuclei by using a water immersion Olympus 51W11 upright microscope (Japan). Images were captured with a Retiga 13001 fast cooled monochromatic digital camera (12-bit; QImaging).

2.4. Evans blue uptake

Cx43^{fl/fl}/Cx45^{fl/fl} and Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre mice treated or not with DEX were injected (i.p.) with Evans blue (80 mg/kg), and after 5 h mice were euthanized and *tibialis anterior* (TA) muscles were isolated and frozen. TA muscles were used because they are larger than FDB muscles, hence facilitating the elicitation of slices with conserved architecture. TA muscles also have a similar percentage of fast myofibers as FDB muscles. The frozen muscles were then cut in a cryostat (Leyca, CM1100, Buffalo Grove, IL, USA) in 10 μ m thick slices. These slices were mounted on microscope slides and analyzed in a fluorescence microscope Nikon eclipse Ti (Tokyo, Japan) at 590 nm (red) emission.

2.5. Ca²⁺ signal measurements

The basal intracellular free Ca²⁺ signal was measured in isolated myofibers using the Ca²⁺ indicator FURA-2AM and following the manufacturer's instructions. Briefly, isolated myofibers were incubated with FURA-2AM (5 μ M) in Krebs saline solution for 50 min at room temperature. Once the incubation period was over, the myofibers were washed with Krebs saline solution without dye, and placed on coverslips to measure the basal free Ca²⁺ signal under a fluorescence microscope Nikon eclipse Ti (Tokyo, Japan) as the 340 nm:380 nm fluorescence ratio.

2.6. Resting membrane potential (Vm)

Freshly isolated myofibers of *flexor digitorum brevis* muscles were used for evaluation of Vm, which was recorded in a whole cell current clamp configuration with conventional high resistance micropipettes (30 to 50 m Ω), 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 when crossing the membrane cell of healthy fibers, and which was stable for 5 s after access experiments were carried out with an Olympus IX 51 inverted microscope with Axopatch1-D amplifier.

2.7. Immunofluorescence

TA muscles were fast frozen with iso-methyl-butane cooled in liquid nitrogen. Then, cross-sections (20 μ m) were obtained using a cryostat (Leica, CM1100, Buffalo Grove, IL, USA) and fixed with 4% (wt/vol) formaldehyde for 10 min at room temperature. Sections were incubated for 3 h at room temperature in blocking solution (50 mM NH $_4$ Cl, 0.025%

Triton, 1% BSA in PBS 1X), incubated overnight with appropriate dilutions of primary antibody, washed 5 times with PBS followed by 1 h incubation with secondary antibodies conjugated to Cy2 or Cy3, and mounted in fluoromount G supplemented with DAPI. Immunoreactive binding sites were localized under a Nikon Eclipse *Ti* microscope equipped with epifluorescence illumination, and images were obtained using an ANDOR camera (model CLARA).

2.8. Cross sectional area (CSA)

The CSA of skeletal muscle fibers was measured as described previously [22]. In brief, cryosections of muscles were fixed with 4% formaldehyde, stained with hematoxylin and eosin, and the CSA of each myofiber was evaluated by using offline analyses with ImageJ software (National Institutes of Health).

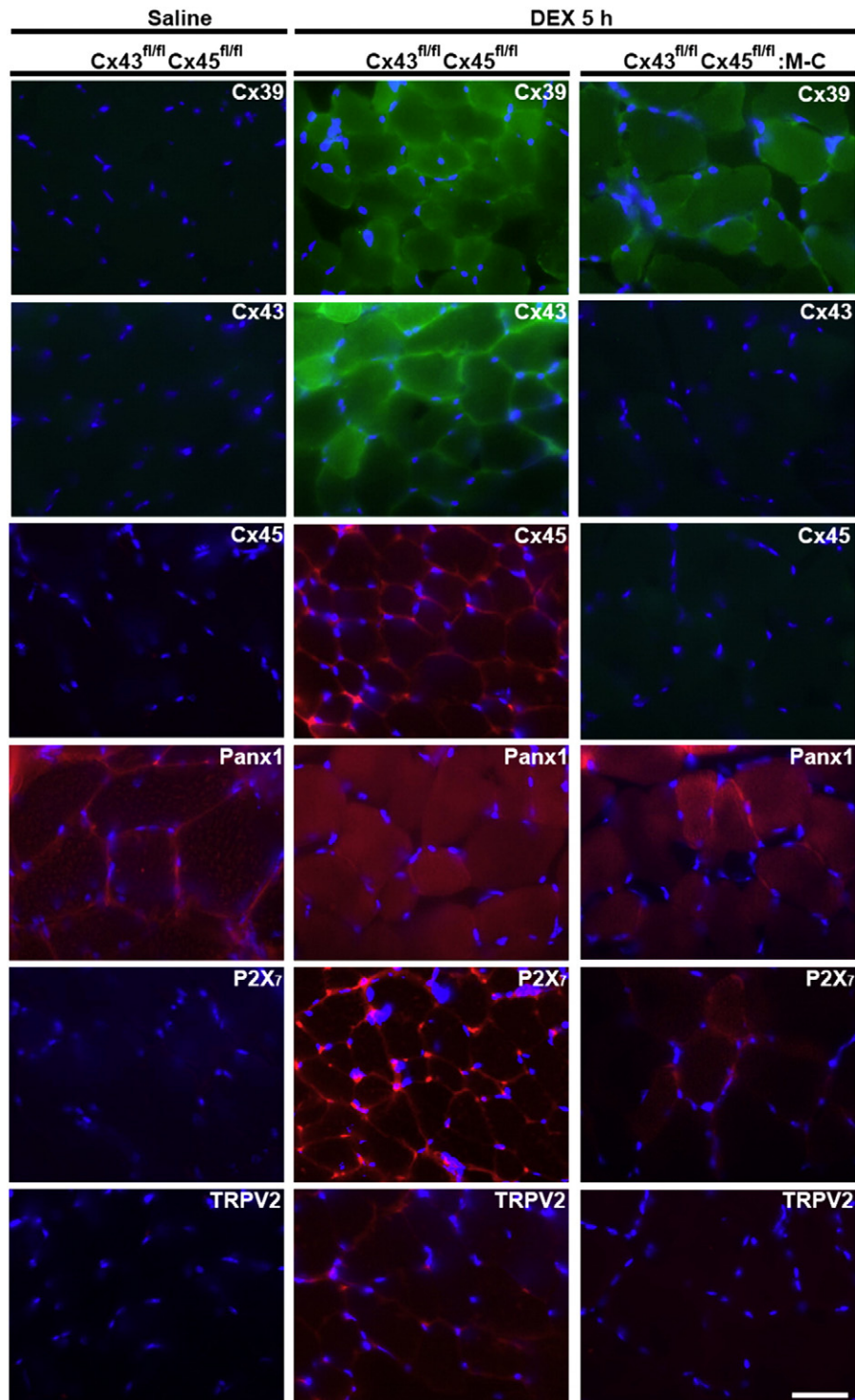


Fig. 1. Presence and distribution of Cxs 39, 43, 45, Panx1, P2X₇R and TRPV2 in dexamethasone-exposed skeletal muscles *in vivo*. By means of immunofluorescence assays, the presence and cellular distribution of Cx 39, 43, 45, Panx1, P2X₇R and TRPV2 was evaluated in cross-sections of TA muscle slices from Cx43^{fl/fl} Cx45^{fl/fl} (control), and Cx43^{fl/fl} Cx45^{fl/fl}:Myo-Cre (Cx43^{fl/fl} Cx45^{fl/fl}:M-C, myofibers deficient in Cx43 and Cx45 expression) mice treated for 5 h with dexamethasone (DEX) or Cx43^{fl/fl} Cx45^{fl/fl} mice treated with saline solution. Calibration bar: 50 μ m.

2.9. Western blots

Gastrocnemius muscles were used because they are larger than TA and FDB muscles, hence yielding more protein mass. They also have a similar percentage of fast myofibers. The muscles were isolated and washed with ice-cold saline containing protease inhibitors. Tendon-free muscles were minced in small pieces by using a razor blade and then homogenized (homogenizer; Brinkmann) and sonicated (Heat Systems Microson). Tissue homogenates were centrifuged for 15 min at $13,000 \times g$ and pellets were discarded. Then, samples were processed for Western blot analyses of proteins of interest as described previously [37]. Blots were incubated overnight with appropriate dilutions of primary antibodies diluted in 5% fat free milk–PBS solution. Then, blots were rinsed with 1% PBS solution–Tween 20 and incubated for 40 min at room temperature with HRP-conjugated goat anti-rabbit or anti-mouse IgGs (Santa Cruz Biotechnology). After five rinses, immunoreactive proteins were detected with ECL reagents according to the manufacturer's instructions (PerkinElmer).

2.10. Statistical analysis

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

3. Results

3.1. Connexins (39, 43 and 45), P2X₇R and TRPV2 are de novo expressed and mainly distributed on sarcolemma of skeletal myofibers exposed to dexamethasone

Since several non-selective membrane channels have been previously shown to be involved in muscular atrophy induced by denervation [22] and dystrophin dysfunction [23], we decided to investigate if the expression of Cx39, Cx43, Cx45, Panx1, P2X₇R and TRPV2 proteins is promoted by glucocorticoids. For this purpose, Cx43^{fl/fl}/Cx45^{fl/fl} mice (control mice) were treated with DEX, which is a potent synthetic glucocorticoid [25], and 5 h later the distribution of the above-mentioned proteins was analyzed by immunofluorescence in cryosections of TA muscles. In control muscles only Panx1 reactivity was detected (Fig. 1) whereas, myofibers from DEX-treated mice showed positive reactivity for Cx39, Cx43, Cx45, P2X₇R and TRPV2 as well as an upregulation of Panx1 reactivity (Fig. 1).

All proteins analyzed were mainly detected in the contour of the myofibers likely to correspond to the sarcolemma (Fig. 1). In addition, the same treatment in animals deficient in Cx43/Cx45 expression, specifically in myofibers, showed less P2X₇R and TRPV2 reactivity than the DEX-treated control myofibers. This suggests that Cx HCs are upstream of P2X₇R and TRPV2 expression.

3.2. Dexamethasone increases sarcolemma permeability through connexin-based hemichannels

Since several nonselective membrane channels were found in myofibers of DEX (5 h) treated mice and Cx HCs were previously shown to be the main protagonists of membrane permeability increase in denervated myofibers [22], we analyzed whether Cx HCs affect the sarcolemma permeability of myofibers from mice treated with DEX. For this purpose, Cx HC activity was evaluated using ethidium (Etd⁺) uptake assays in isolated myofibers of *flexor digitorum brevis* (FDB) muscles. After 5 h, DEX-treated myofibers of Cx43^{fl/fl}/Cx45^{fl/fl} mice showed a higher Etd⁺ uptake (~2.3 fold) than control myofibers (Fig. 2). The Etd⁺ uptake of myofibers from DEX-treated mice was completely blocked by La³⁺ (Fig. 2), which is a Cx HCs and P2X receptor inhibitor [26]. Since P2X₇R has been previously shown to be *de novo* co-expressed with Cx HCs and to contribute to changes in membrane permeability of denervated myofibers [22], we tested whether A740003 (a selective P2X₇R inhibitor [27]) affects the Etd⁺ uptake of myofibers from Cx43^{fl/fl}/Cx45^{fl/fl} mice treated with DEX. It was found that 10 μ M A740003 reduced Etd⁺ uptake to levels comparable to basal uptake of control myofibers (Fig. 2A), suggesting that in this system Cx HCs and P2X₇R work in an orchestrated fashion to increase membrane permeability of myofibers.

To evaluate the possibility that Cx HCs might mediate the sarcolemma permeabilization induced by DEX, transgenic Cx43^{fl/fl}:Myo-Cre, Cx45^{fl/fl}:Myo-Cre and Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre mice were used. It was found that myofibers derived from Cx43^{fl/fl}:Myo-Cre or Cx45^{fl/fl}:Myo-Cre mice treated with DEX showed ~50% increase in Etd⁺ uptake compared with DEX treated Cx43^{fl/fl}/Cx45^{fl/fl} mice (Fig. 2B), while in myofibers from DEX treated Cx43^{fl/fl}/Cx45^{fl/fl}:M-C mice the Etd⁺ uptake was slightly lower than that of control myofibers (Fig. 2). The above results indicate that both Cx43 and Cx45 contribute to the increase in membrane permeability and since Cx43^{fl/fl}:Myo-Cre and Cx45^{fl/fl}:Myo-Cre mice showed partial protection we decided to evaluate all other parameter only in Cx43^{fl/fl}/Cx45^{fl/fl}:M-C mice in order to reduce the number of euthanized animals.

Also, myofibers freshly isolated from control (Cx43^{fl/fl}/Cx45^{fl/fl}) mice treated with 300 nM DEX for 3 h showed an increased Etd⁺ uptake rate (Fig. 3), which was inhibited by 200 μ M La³⁺ and was absent

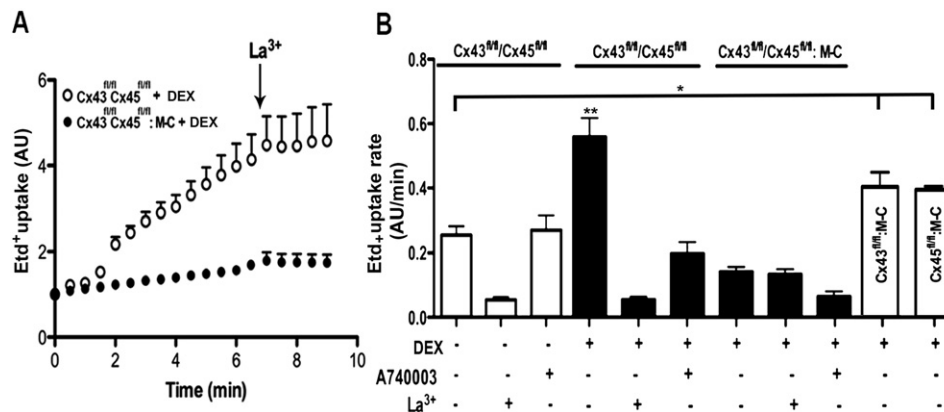


Fig. 2. Increased dexamethasone-induced membrane permeability of myofibers is mediated by connexin-based hemichannels. The activity of connexin-based hemichannels was measured with ethidium (Etd⁺) uptake assays in isolated skeletal myofibers from *flexor digitorum brevis* muscles of mice treated or not with dexamethasone (DEX, 8 mg/kg). Myofibers were disaggregated 5 h after DEX administration. A, representative curve of Etd⁺ uptake in myofibers from Cx43^{fl/fl}/Cx45^{fl/fl} mice treated or not with DEX, and acutely inhibited by La³⁺. B, Dye uptake rate of myofibers from Cx43^{fl/fl}/Cx45^{fl/fl}, Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre (Cx43^{fl/fl}/Cx45^{fl/fl}:M-C), Cx43^{fl/fl}:M-C and Cx45^{fl/fl}:M-C mice of 4 independent experiments as shown in A. * $P < 0.05$ respect to control condition without DEX treatment. ** $P < 0.01$ myofibers from DEX-treated Cx43^{fl/fl}/Cx45^{fl/fl} mice with regard to all other conditions.

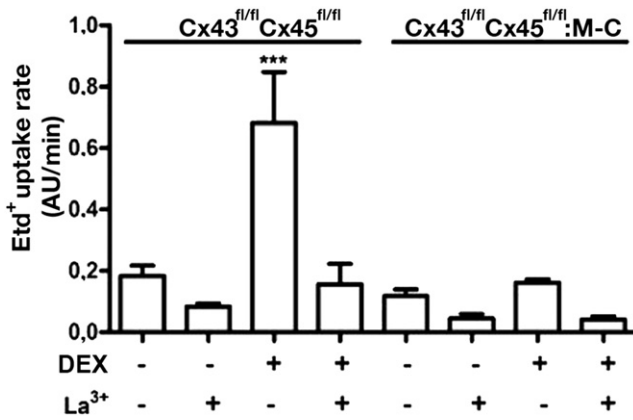


Fig. 3. *In vitro* dexamethasone stimulation of isolated skeletal myofibers induces increased membrane permeability mediated by connexin-based hemichannels. Isolated skeletal muscle fibers from *flexor digitorum brevis* muscle of Cx43^{fl/fl}Cx45^{fl/fl} (control) and Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre (Cx43^{fl/fl}Cx45^{fl/fl}:M-C; Cx43 and Cx45 expression deficient myofibers) were directly incubated for 3 h with 300 nM dexamethasone (DEX), after which the connexin hemichannels activity was evaluated through ethidium (Etd⁺) uptake assays in the absence or presence of 200 μM La³⁺. ***P < 0.001 DEX-treated myofibers with regard to all other conditions.

in myofibers deficient in Cx43/Cx45 muscle expression (Fig. 3). This indicates that the increase in Etd⁺ uptake through Cx HCs results from a direct interaction of DEX with the myofibers. In addition, Cx HC activity was measured *in vivo* through the previously described Evans blue uptake assay [22]. Evans blue was injected (i.p., 80 mg/kg) in mice previously treated with DEX (5 h), after which TA muscles were isolated and cryosections were obtained. It was observed that only myofibers of TA muscles from DEX-treated control mice (Cx43^{fl/fl}/Cx45^{fl/fl}) showed intracellular Evans blue staining (Fig. 4, middle panel). Notably, DEX-induced uptake was absent in myofibers deficient in Cx43/Cx45 expression (Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre) (Fig. 4, right panel) and was comparable to the results obtained in TA muscles from Cx43^{fl/fl}/Cx45^{fl/fl} mice treated with saline solution (Fig. 4, left panel). This confirms that Cx HCs are responsible for altered sarcolemma permeability in DEX-treated muscles.

3.3. Connexin-based hemichannels are responsible for elevated basal Ca²⁺ signal

Since Cx HCs are present in muscles exposed to DEX (Fig. 5), and considering that these channels have been described to be permeable to Ca²⁺ [28], it was analyzed if basal intracellular free Ca²⁺ levels of myofibers change when cells are treated with DEX for 5 h. To this end, freshly isolated myofibers of FDB muscles, from mice injected (i.p.) with DEX or DEX followed by carbenoxolone (Cbx, 80 mg/kg) injection

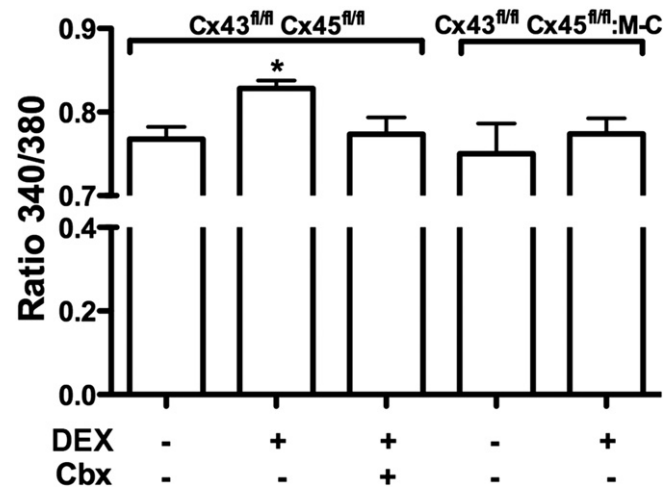


Fig. 5. Elevated basal Ca²⁺ signal induced by dexamethasone is mediated by connexin-based hemichannels in skeletal muscles. Isolated *flexor digitorum brevis* myofibers from Cx43^{fl/fl}Cx45^{fl/fl} (control) and Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre (Cx43^{fl/fl}Cx45^{fl/fl}:M-C) mice that were injected or not with 8 mg/kg dexamethasone (DEX), 80 mg/kg carbenoxolone (Cbx) or both, were used to evaluate basal Ca²⁺ signal, using the FURA-2AM assay analyzing the 340 nm/380 nm ratio. *P < 0.05 myofibers from DEX-treated Cx43^{fl/fl}Cx45^{fl/fl} mice with regards to all other conditions.

(30 min after DEX injection) were loaded with FURA-2AM probe, which was used to evaluate the intracellular Ca²⁺ signal. DEX induced a significant increase in basal Ca²⁺ signal, which was prevented by Cbx (a Cx HC and P2X₇R inhibitor) [29] (Fig. 5).

3.4. Connexin-based hemichannels mediate the drop in resting membrane potential induced by dexamethasone

It is already known that DEX treatment decreases the resting membrane potential (V_m) of myofibers [30]. Moreover, it is also known that Cx HCs are permeable to Na⁺ and K⁺ [31,32], and therefore V_m could be affected upon opening of Cx HCs. Thus, we analyzed if Cx HCs are responsible for the V_m drop induced by DEX. For this purpose, the V_m was measured in myofibers dissociated from FDB muscles of mice treated or not with DEX for 5 h. The V_m of myofibers from Cx43^{fl/fl}Cx45^{fl/fl} and Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice was similar (Fig. 6). Nonetheless, a significant reduction in V_m from -77.1 ± 2 mV to -64.3 ± 3 mV (Fig. 6) was found in myofibers of DEX-treated Cx43^{fl/fl}Cx45^{fl/fl} mice (Fig. 6). In contrast, myofibers deficient in Cx43 and Cx45 expression (Cx43^{fl/fl}/Cx45^{fl/fl}:Myo-Cre) showed only a slight reduction from -78 ± 4 mV to -71.6 ± 4 mV, which was not statistically significant, indicating that Cx HCs are responsible for the V_m drop in myofibers exposed to DEX.

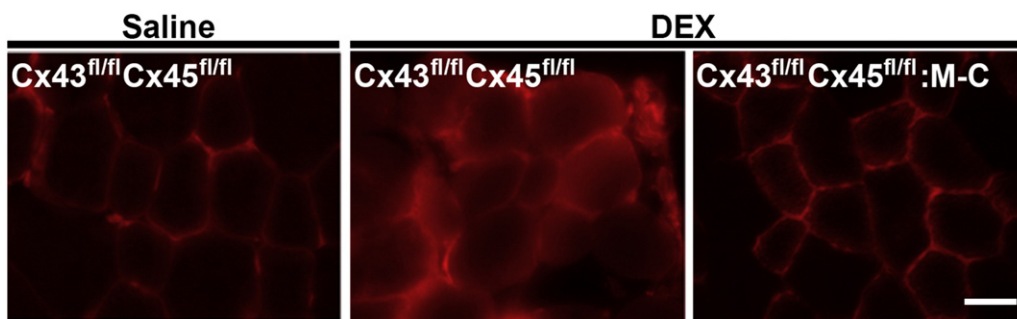


Fig. 4. Dexamethasone induced Evans blue uptake in myofibers *in vivo* through hemichannels. The Evans blue uptake assay was used to evaluate hemichannel activity *in vivo*. Cx43^{fl/fl}Cx45^{fl/fl} mice under control conditions and Cx43^{fl/fl}Cx45^{fl/fl} as well as Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice (Cx43^{fl/fl}Cx45^{fl/fl}:M-C; Cx43 and Cx45 expression deficient myofibers) treated for 5 h with dexamethasone (i.p., 8 mg/kg) were used. Mice were injected (i.p.) with Evans blue dye (80 mg/kg) 30 min after DEX injection, and after 5 h *tibialis anterior* muscles were dissected and processed to obtain cryosections. Then, the cryosections were inspected under a fluorescent microscope (λ emission 590 nm). Scale bar: 50 μm.

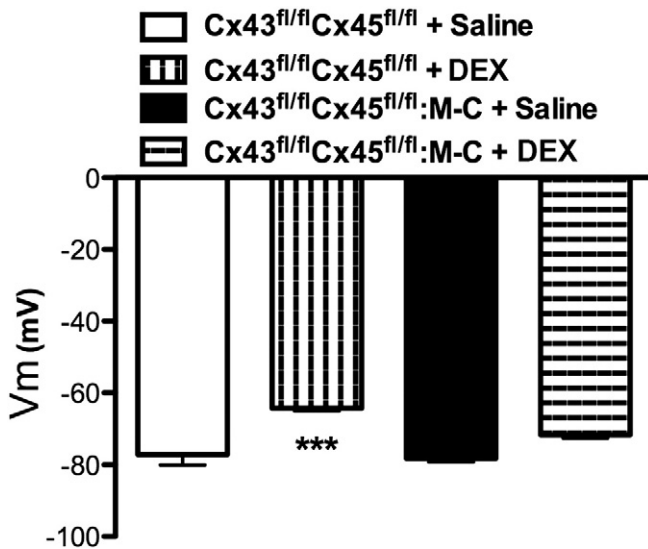


Fig. 6. Connexin hemichannels are responsible for the drop in resting membrane potential of myofibers exposed to dexamethasone. Mice were treated with dexamethasone (DEX) or saline solution, and 5 h later myofibers of flexor digitorum brevis muscles were isolated and the resting membrane potential (V_m) was evaluated using conventional microelectrodes. All measurements were performed within 1 h after plating freshly dissociated myofibers from Cx43^{fl/fl}Cx45^{fl/fl} and Cx43^{fl/fl}Cx45^{fl/fl}:M-C mice under control conditions (white bar) or after 5 h treatment with DEX (8 mg/kg of body weight, vertical lines bar) or mice deficient in Cx43 and Cx45 expression (Cx43^{fl/fl}Cx45^{fl/fl}:M-C) after 5 h treatment with saline solution (black bar) or DEX (horizontal line bar). *** $P < 0.001$ myofibers from DEX-treated Cx43^{fl/fl}Cx45^{fl/fl} mice with regards to all other conditions.

3.5. Dexamethasone induces a connexin hemichannel-dependent increase in mRNA of TNF- α and activation of NF κ B in skeletal muscles

Since NF κ B activation has been observed to be involved in muscular atrophy [33] and Cx HCs participate in the activation of NF κ B and in the synthesis of pro-inflammatory cytokines in denervated muscles [22], we decided to evaluate if Cx HCs play a similar role in skeletal muscles of mice treated with DEX. To accomplish this objective, TA muscles from

DEX-treated (5 h) Cx43^{fl/fl}Cx45^{fl/fl} or Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice were dissected and mRNA levels of the proinflammatory cytokines TNF- α , IL-6 and IL-1 β were evaluated by semiquantitative-PCR. Only TNF- α mRNA levels were elevated in muscles from DEX-treated Cx43^{fl/fl}Cx45^{fl/fl} mice, while levels of IL-6 and IL-1 β mRNA remained unaffected (Fig. 7A). In addition, activation of NF κ B was evaluated through the presence and distribution of phosphorylated p65 NF κ B subunits. Phosphorylated p65 subunits were detected mainly in the nuclei of TA muscles of DEX-treated Cx43^{fl/fl}Cx45^{fl/fl} mice, but not in muscles of control mice or Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice treated with DEX (Fig. 7B). This suggests that DEX-induced activation of NF κ B in muscles requires the expression of Cx43 and Cx45 HCs.

3.6. Dexamethasone-induced skeletal muscle atrophy does not occur in muscles devoid of Cx43 and Cx45 expression

Muscle atrophy was determined in the cross sectional area (CSA) of myofibers of TA muscles obtained from mice treated or not with DEX (700 μ g/kg per day, during 7 days). A significantly greater reduction of myofiber CSA (~50%) was detected in muscles of DEX-treated mice compared to control mice treated with saline (Fig. 8A, B). Moreover, the CSA of myofibers from Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice treated with DEX remained as that of myofibers from untreated mice (Fig. 8A), suggesting the beneficial consequences of the absence of Cx43/Cx45 expression. Additionally, two other molecules were evaluated, atrogen-1 and MurF-1, which are two E3 ligases from the ubiquitin-proteasome system that are elevated in glucocorticoid-induced muscle atrophy [15]. An increase in relative levels of these two proteins was observed. This increase was absent in muscles of DEX-treated Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice (Fig. 8C, D, E), suggesting that Cx HCs are upstream to the increase of atrogen-1 and MurF-1, and that the absence of Cx HCs prevents DEX-induced muscle atrophy.

4. Discussion

Our main results are that DEX-induced muscle atrophy is mediated by Cx HCs, which were expressed and activated after a few hours (5 h) of DEX treatment. These Cx HCs are responsible for increases in

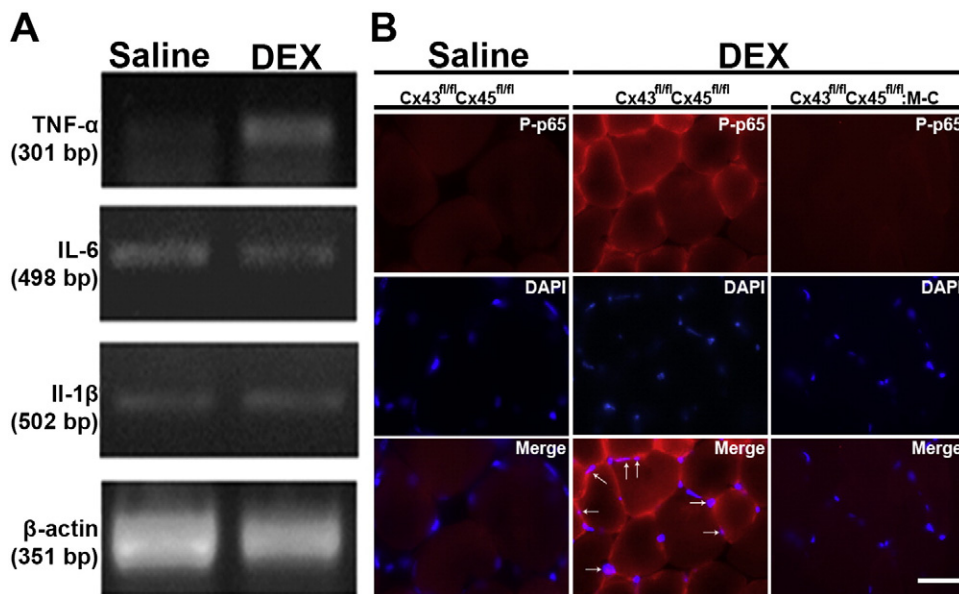


Fig. 7. Inflammatory signs of myofibers of skeletal muscles exposed to dexamethasone are attenuated by the lack of Cx43 and Cx45 expression. Mice Cx43^{fl/fl}Cx45^{fl/fl} (control) and Cx43^{fl/fl}Cx45^{fl/fl}:M-C (Cx43 and Cx45 muscle deficient) were treated with dexamethasone (DEX, 8 mg/kg), and 5 h later the tibialis anterior (TA) muscles were isolated and used for analysis of inflammatory signs. A, RT-PCR for TNF- α , IL-6 and IL-1 β mRNA. The predicted size (in bp, base pair) of the expected transcriptome is indicated in the left of each blot. B, the phosphorylated p65 subunit of NF κ B was detected in cross-sections of TA muscles by immunofluorescence in the nuclei of myofibers (white arrows). Nuclei were stained with DAPI. Calibration bar: 50 μ m.

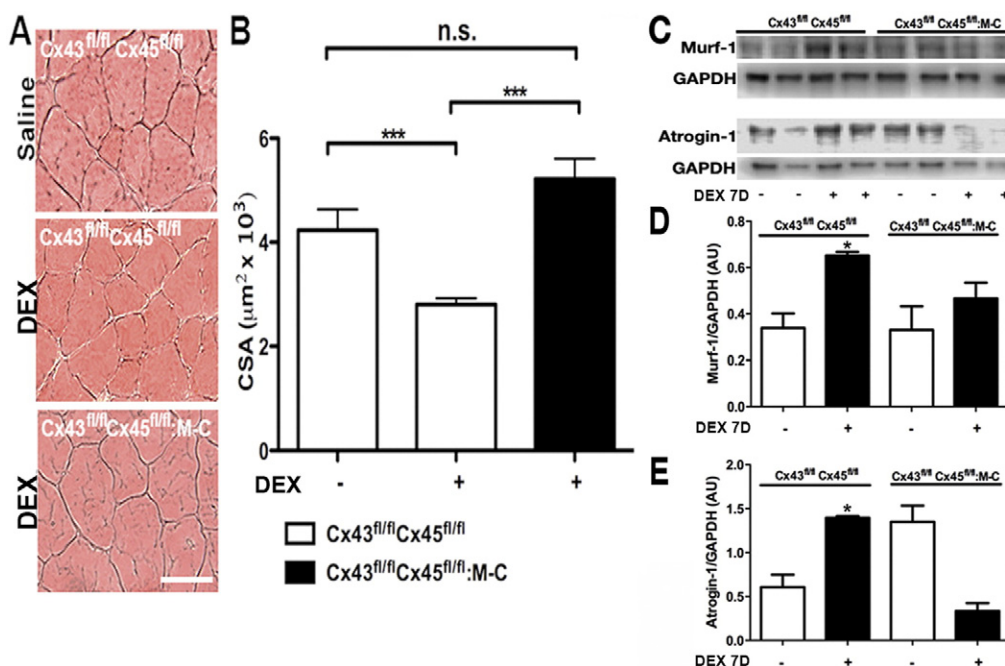


Fig. 8. Absence of Cx43 and Cx45 prevented dexamethasone-induced skeletal muscle atrophy. Cx43^{fl/fl} Cx45^{fl/fl} and Cx43^{fl/fl} Cx45^{fl/fl}: Myo-Cre (Cx43^{fl/fl} Cx45^{fl/fl}: M-C) mice were treated daily for 7 days with saline buffer or dexamethasone (DEX, 700 μg/kg), and then the cross sectional area (CSA) of myofibers of *tibialis anterior* muscles (TA) was measured by means of an off-line analysis of hematoxylin-eosin images. A, hematoxylin-eosin stained sections of TA; Calibration bar: 50 μm. B, quantification of CSA from images as shown in A. C, blots of the relative levels of Murf-1 and atrogin-1 of mice after 7 days of treatment with DEX. D and E, quantification of the blots by densitometry of Murf-1 and atrogin-1 reactive bands, as shown in C. *P < 0.05, with regards to DEX untreated Cx43^{fl/fl} Cx45^{fl/fl} muscles and DEX treated Cx43^{fl/fl} Cx45^{fl/fl}:M-C muscles, ***P < 0.001 CSA of myofibers from DEX-treated Cx43^{fl/fl} Cx45^{fl/fl}:M-C mice regarding myofibers from DEX-treated Cx43^{fl/fl} Cx45^{fl/fl} mice. n.s. = not significant difference between CSA of myofibers from untreated mice versus DEX-treated Cx43^{fl/fl} Cx45^{fl/fl}:M-C.

Ca²⁺ signal and reductions in resting membrane potential induced by DEX treatment. In addition, long-term treatment with DEX (7 days) induced atrophy, which was absent in myofibers deficient in Cx43/Cx45 expression.

Although the exact mechanism underlying steroid myopathy is unclear, it is considered to be multifactorial and related to decreased protein synthesis, increased protein degradation, alterations in carbohydrate metabolism, mitochondrial alterations, oxidative stress, and/or decreased sarcolemmal excitability [11,15]. By means of immunofluorescence, we found that DEX induces *de novo* expression of protein subunits (Cx39, 43 and 45, P2X₇R and TRPV2 channels) known to form non-selective membrane channels that can contribute to altering the transmembrane electrochemical gradient. Accordingly, we found that inhibition of either Cx HCs or P2X₇Rs recovered the permeability properties of the sarcolemma of DEX-treated myofibers. Moreover, we found that sarcolemma myofibers deficient in Cx43/Cx45 expression remained impermeable to Evans blue or Etd⁺ upon treatment with DEX. This indicates that Cx HCs are the main membrane pathway through which these dyes permeate the sarcolemma of myofibers exposed to DEX. Consistent with this finding, the known reduction in myofiber Vm induced by DEX [30] was not evident in myofibers deficient in Cx43/Cx45 expression. This suggests that in spite of the expression of several non-selective membrane channels found in myofibers exposed to DEX, Cx43/Cx45 HCs are the main causes of the electrochemical gradient disruption that explains the drop in Vm. Along the same lines of analysis, we found that control myofibers exposed to DEX presented higher levels of free intracellular Ca²⁺ signal and that lack of Cx43/Cx45 expression prevented this change induced by DEX. The increase in Ca²⁺ signal seems to be dependent on multiple pathways, between them, as a consequence of ATP released via Panx1 and Cx HCs that activate P2 receptors, leading to an increase in Ca²⁺ influx or its release from intracellular stores, depending on whether extracellular ATP acts on P2X or P2Y receptors, respectively [34,35]. The possible role of Ca²⁺ influx through P2X₇Rs is supported by our results

of elevated levels of P2X₇R reactivity in DEX-exposed myofibers. Also, it could be a consequence of the TRPV2 channels activity, which is expressed after DEX treatment. In addition, at least Cx43 and Cx45 hemichannels have been shown to be permeable to Ca²⁺ [28,36] and thus could contribute to Ca²⁺ influx. Thus, all these channels together might explain the observed Ca²⁺ increase, and it would be sufficient to block or abrogate the expression of Cx43/Cx45 HCs to prevent the increase of Ca²⁺ levels. These Cx HCs are upstream of the other channels. This interpretation is supported by the drastically reduced levels of P2X₇ and TRPV2 proteins observed in absence of Cx43/Cx45 expression (Fig. 1). Interestingly a similar change in Ca²⁺ signal has been reported to occur in myofibers of mdx mice and in myotubes of dysferlinopathies, which can also be explained by Ca²⁺ influx via Cx HCs [23,37]). The resulting increase in Ca²⁺ signal could lead to activation of the transcription factor NFκB, as demonstrated in other cell types [38], and consequently to the synthesis and release of pro-inflammatory cytokines [39]. Accordingly, we found increases in mRNA levels of TNF-α, but not in those of IL-6 or IL-1β, which would be in line with published data indicating that TNF-α is released before IL-1β and IL-6 in an inflammatory response [40].

These results are controversial with regard to commonly used criteria to design rational therapies, since glucocorticoids are well known anti-inflammatory drugs [41]. For example, glucocorticoids are the preference treatment for Duchenne muscular dystrophy, where skeletal muscles show infiltration of inflammatory cells [42]. However, a recent report demonstrated that myofibers of skeletal muscles of Duchenne patients and skeletal muscles of the animal model of this disease (mdx mice) express Cx HCs that promote NFκB activation and apoptosis [23]. Therefore, DEX treatment is likely to reduce the inflammatory response due to the infiltration of innate immune system cells, but promotes inflammasome activation and apoptosis of myofibers (inflammation of parenchymal cells). In this sense, it has been demonstrated that DEX induces upregulation of Cx43 at protein and mRNA level in hepatic stellate cells [44].

Hence, it is possible that the same occurs in other systems expressing glucocorticoids receptors like skeletal muscles [13]. Whether a similar mechanism explains the upregulation of Cx39 and Cx45 remains to be demonstrated.

The aforementioned mechanism suggests that the use of corticoids to treat Duchenne patients is controversial and perhaps inappropriate. Accordingly, it has been reported that corticoid treatment does not increase the life expectancy of Duchenne patients [43], probably because glucocorticoids induce the activation of NF κ B [16], which acts as a pro-inflammatory signal for muscular tissue as demonstrated in this report. It should be kept in mind that steroids have differential effects in different tissues (e.g., anti-inflammatory in the immune system and inflammatory in skeletal muscles). In particular, in non-immune systems tissues like skeletal muscles, glucocorticoids induce inflammation. It is highly relevant to describe that this pro-inflammatory response to steroids in muscle tissue is at least partially mediated by the activation of Cx HCs [22]. Since there are several drugs that have been proven to inhibit HCs and pannexins, it is desirable to test if the likely negative effects of steroids could be modulated by the inhibition of these hemichannels.

Since at least Cx43 HCs are permeable to Ca²⁺ [28], lack of atrophy in myofibers of Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice treated with DEX can be explained by the lack of Ca²⁺ influx, and hence the lack of activation of protein degradation pathways (as observed by the low atrogin-1 and Murf-1 levels), which has been previously shown to be involved in DEX-induced muscle atrophy [10]. The lack of activation of protein degradation pathways is probably related to the low intracellular Ca²⁺ signal observed in myofibers of Cx43^{fl/fl}Cx45^{fl/fl}:Myo-Cre mice, as was observed in this study. Moreover, DEX treatment for 7 days in normal mice induced muscle atrophy, which did not occur in the absence of Cx43 and Cx45 expression in muscles. This suggests that DEX-induced muscle atrophy is mediated by Cx HCs. Interestingly, the absence of Cx43Cx45 HCs completely prevented DEX-induced muscular atrophy. But in other conditions, like denervation, it just prevented ~75% of muscular atrophy [22], thus suggesting the involvement of additional mechanisms that lead to muscular atrophy.

In conclusion, DEX treatment is associated with early muscle atrophy and muscle inflammation. These effects can be explained by the expression and activation of Cx HCs. Inhibition of Cx HCs during early steroid treatment could prevent the activation of protein degradation pathways, Ca²⁺ influx, and unfolding of atrophy.

Ethical standards

The authors certify that the experiments comply with the current laws within Chile, where the experiments were performed. All protocols were approved by the Bioethics Committee of the Pontificia Universidad Católica de Chile (protocol no. 176) in accordance with the ethical standards stipulated in the 1964 Declaration of Helsinki and its later amendments. All efforts were made to minimize animal suffering as well as to reduce the number of animals used, and alternatives to *in vivo* techniques were applied, if available.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Transparency document

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

Acknowledgements

This work was partially supported by CONICYT/PAI Proyecto de Inserción en la Academia 79140023 (to LAC); Fondo Nacional de

Desarrollo Científico y Tecnológico (FONDECYT): grant 1141092 (to LAC, TR and JCS); 1150291 (to JCS); FONDECYT Postdoctorado grant 3160594 (EB); Iniciativa Científica Milenio-Economía P09-022-F (to JCS, BAC, CP and RS).

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