Biochimica et Biophysica Acta 1862 (2016) 1891–1899

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

Luis A. Cea [a,*,1], Elisa Balboa [b,c], Carlos Puebla [b,d], Aníbal A. Vargas [b,d], Bruno A. Cisterna [b,d], Rosalba Escamilla [b,d], Tomás Regueira [c], Juan C. Sáez [b,d,**]

[a] Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[b] Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[c] Departamento Medicina Intensiva, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile
[d] Centro Interdisciplinario de Neurociencias de Valparaíso, Valparaíso, Chile

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, $P2X_7$ 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 $P2X_7$ receptor inhibitors. DEX also induced a significant increase in basal intracellular Ca^{2+} 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 NFκB 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 atrogin-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.

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

* Correspondence to: L. A. Cea, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile.
** Correspondence to: J. C. Sáez, Departamento de Fisiología, Pontificia Universidad Católica de Chile, Av. Libertador Bernardo O'Higgins 340, Santiago, Chile.
E-mail addresses: luiscea@med.uchile.cl (L.A. Cea), jsaez@bio.puc.cl (J.C. Sáez).
[1] Previous address Departamento de Fisiología, Pontificia Universidad Católica de Chile, Av. Libertador Bernardo O'Higgins 340, Santiago, Chile.

http://dx.doi.org/10.1016/j.bbadis.2016.07.003
0925-4439/

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 NFκB [16,17]), of co-activators p300-C/EBPβ [18] and of intracellular signaling pathways such as mTOR [19] and PI_3K/Akt/GSK3β [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 NFκB. 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). Phycoerithrin (PE) or FITC conjugated monoclonal anti-annexin V antibody were purchased from BD Biosciences (San Jose, CA, USA) and polyclonal anti-TRPV2 or anti-$P2X_7R$ antibodies from Abcam (Cambridge, MA, USA). Previously described polyclonal anti-Cx39, -43, -Cx45 and -Panx1 antibodies were used [22]. Rabbit polyclonal anti-phoshorylated-p65 NFκB subunit antibodies were purchased from Cell Signaling (Boston, MA, USA). The atrogin-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 μM Etd^+. Etd^+ fluorescence was recorded in regions of interest that corresponded to myofiber nuclei by using a water immersion Olympus 51W1I 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 μm 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. Ca^{2+} signal measurements

The basal intracellular free Ca^{2+} signal was measured in isolated myofibers using the Ca^{2+} indicator FURA-2AM and following the manufacturer's instructions. Briefly, isolated myofibers were incubated with FURA-2AM (5 μM) 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 Ca^{2+} 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 digitorium 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 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 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 μm) 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_4Cl, 0.025%