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Connexin hemichannels explain the ionic imbalance and lead to atrophy in denervated skeletal muscles

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ABSTRACT

Denervated fast skeletal muscles undergo atrophy, which is associated with an increase in sarcolemma permeability and protein imbalance. However, the mechanisms responsible for these alterations remain largely unknown. Recently, a close association between *de novo* expression of hemichannels formed by connexins 43 and 45 and increase in sarcolemma permeability of denervated fast skeletal myofibers was demonstrated. However, it remains unknown whether these connexins cause the ionic imbalance of denervates fast myofibers. To elucidate the latter and the role of hemichannels formed by connexins (Cx HCs) in denervation-induced atrophy, skeletal myofibers deficient in Cx43 and Cx45 expression ($Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice) and control ($Cx43^{fl/fl}Cx45^{fl/fl}$ mice) were denervated and several muscle features were systematically analyzed at different post-denervation (PD) times (1, 3, 5, 7 and 14 days). The following sequence of events was found in denervated myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$ mice: 1) from day 3 PD, increase in sarcolemmal permeability, 2) from day 5 PD, increases of intracellular Ca^{2+} and Na^{+} signals as well as a significant increase in protein synthesis and degradation, yielding a negative protein balance and 3) from day 7 PD, a fall in myofibers cross-section area. All the above alterations were either absent or drastically reduced in denervated myofibers of $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice. Thus, the denervation-induced Cx HCs expression is an early event that precedes the electrochemical gradient dysregulation across the sarcolemma and critically contributes to the progression of skeletal muscle atrophy. Consequently, Cx HCs could be a therapeutic target to drastically prevent the denervation-induced atrophy of fast skeletal muscles.

1. Introduction

Denervated fast skeletal muscles undergo several metabolic, structural and functional alterations, which are characteristic of muscle waste [1–4]. During the first post-denervation (PD) week, myofibers show an increase in intracellular Na^{+} concentration and reduction in intracellular K^{+} concentration [5–10]. These alterations might be explained by a Na^{+}/K^{+} ATPase deficiency. However, denervated myofibers treated with ouabain, a Na^{+}/K^{+} ATPase inhibitor, show a further reduction in resting membrane potential (RMP) of approximately 10% [11], suggesting the presence of Na^{+}/K^{+} ATPase activity. Consequently, a reduction in Na^{+}/K^{+} ATPase activity does not fully explain the decrease in RMP of denervated myofibers. Notably, decreased RMP may also be facilitated by the down-regulation of ClC-1 chloride channels, which are crucial in membrane repolarization [12]. An alternative explanation could be the *de novo* expression of non-selective membrane channels induced by denervation. In this regard, at 7 days PD myofibers were shown to express connexin hemichannels (Cx HCs), TRPV2 channels and $P2X_7$ receptors, and to upregulate the pannexin1 channel levels [4]. Interestingly, the simultaneous deficiency in connexins (Cxs) 43 and 45 drastically diminishes the reduction in cross sectional area (CSA) of skeletal myofibers induced by denervation [4], suggesting strongly that these two Cx HCs play a relevant role in the mechanism that leads to muscle atrophy. However, it remains unknown if the expression of Cxs occurs simultaneous to or precedes the reduction in CSA of myofibers. It also remains unknown whether the expression of Cx HCs is involved in the increase in intracellular Na^{+} signal of denervated myofibers.

Under normal conditions, the muscle mass results from a balance between protein synthesis and protein degradation rates, both of which are enhanced in denervation-induced atrophy with predominance of protein degradation rate [13–15]. The increase in protein synthesis results from activation of the mTOR pathway [16,17]. On the other hand, at least four intracellular metabolic pathways involved in protein degradation are activated by Ca^{2+} in skeletal muscles: (1) calpains [18], (2) caspases [19], (3) cathepsins [20], and (4) ubiquitin

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proteasome [21]. The role of these metabolic pathways in protein degradation during muscle atrophy has been demonstrated in several reports [22–24]. During atrophy, the expression of both ubiquitin ligase atrogin-1 and MuRF-1 are activated [25]. The greatest increase in expression of these proteins occurs at about 3 days PD in rat medial gastrocnemius muscles [26]. Accordingly, overexpression of MuRF-1 in myotubes produces atrophy, whereas mice deficient in any of these ligases exhibit less atrophy PD [25,27,28]. The possible role of Cx HCs in activation of protein synthesis and degradation pathways induced by denervation remains unproven.

A common condition that activated the protein degradation pathways aforementioned is an increase in intracellular free Ca^{2+}. With regard to denervated myofibers, an increase in total intracellular calcium has been found [29], but to our knowledge the intracellular free Ca^{2+} signal has not been described. Since Cx43 HCs are permeable to Ca^{2+} [30], it is possible that the increase in total intracellular calcium found in denervated muscles is, at least in part, the consequence of enhanced Ca^{2+} influx through Cx43 HCs. However, and to our knowledge the intracellular free Ca^{2+} signal of denervated muscles and the possible role of Cx HCs as membrane pathways for Ca^{2+} influx have not been evaluated. In this work, a sequence of PD events was studied. Cx43 and Cx45 HCs were detected as early as 3 days PD, followed by simultaneous increases the intracellular Ca^{2+} and Na^{+} signals and evident protein imbalance at day 5 PD. Later on, a clear decrease in CSA of myofibers was evident from day 7 PD and on. All these alterations were not detected or greatly reduced in denervated muscles of $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice, strongly suggesting that Cx43 and 45 HCs play a critical role in denervation-induced atrophy of skeletal muscles, and their inhibition could drastically reduce the degenerative changes of denervated fast myofibers.

2. Methods

2.1. Reagents

Ehidium (Etd^{+}) bromide, *n*-benzyl *p*-toluenesulfonamide (BTS), suramin sodium salt, 1-Nitroso-2-naphthol, 1,2-dichloroethane and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluoromount-G with DAPI was purchased from Electron Microscopy Science (Hatfield, PA, USA). Dulbecco's modified Early medium (DMEM), fetal bovine serum (FBS) and collagenase type I were purchased from Invitrogen (Camarillo, CA, USA). FURA-2 AM and SBFI AM were purchased from Molecular Probes (Eugene, OR, USA). 4′, 6-diamidino-2-phenylindole (DAPI) and Halt Protease and Phosphatase Inhibitor Cocktail (100X) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Bio-Rad Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad (Hercules, CA, USA). Ketamine hydrochloride was purchased from Troy Laboratories Australia Pty Ltda. (Glendenning, Australia). Xylazine hydrochloride was purchased from Centrovet Ltda. (Santiago, Chile). L-(4,5-^{3}H(N)) leucine (^{3}H-leu) was purchased from PerkinElmer (Waltham, MA, USA). Ecoscint H was purchased from National Diagnostics (Atlanta, Georgia, USA). L-tyrosine was purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). Monoclonal anti-phospho-p70 S6 kinase (p-p70S6K) was purchased from Cell Signaling Cat: #2708 (Danvers, MA, USA). Polyclonal anti-atrogin-1 was purchased from ECM Biosciences Cat: #AP2041 (Versailles, KY, USA). Monoclonal anti-μ-calpain was purchased from Sigma-Aldrich Cat. #C5736 (St. Louis, MO, USA). Secondary antibody conjugated to Cy3 was purchased from Jackson Immuno Research Cat. #111-166-003 (Indianapolis, IN, USA).

2.2. Animals

All procedures were approved by the Institutional Bioethics Committee of the Pontificia Universidad Católica de Chile. Previously described muscle specific Cx43 and Cx45 deficient ($Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre) and control ($Cx43^{fl/fl}Cx45^{fl/fl}$) mice were used [4]. Wild type C57 BL/6 (C57) mice of the same age were also used for comparison with ($Cx43^{fl/fl}Cx45^{fl/fl}$) mice, and since these were found to present no significant differences in all parameters studied in this work, we used ($Cx43^{fl/fl}Cx45^{fl/fl}$) mice as control animals in order to reduce the total number of euthanized animals. All mice used in the experiments were 2-month-old males, which were maintained under light:dark 12:12 cycles with food and water *ad libitum*. In total, 132 mice were used.

2.3. Unilateral hind limb denervation

Under anesthesia with a mix 100 mg/kg ketamine and 10 mg/kg xylazine, a complete transection of the sciatic nerve in the left hind limb was done. The right hind limb was used as sham surgery. After 1, 3, 5, 7 and 14 PD days, the *flexor digitorum brevis* (FDB) and *tibialis anterior* (TA) muscles were carefully dissected from anaesthetized animals and different assays were performed. Then, animals were euthanized by cervical dislocation.

2.4. Isolation of myofibers from FDB muscles

Since myofibers of FDB muscles are easy to isolate and manipulate, freshly dissected FDB muscles were incubated for 30 min at 37°C in culture medium (DMEM/F12 supplemented with 10% FBS) containing 2% collagenase type I, 200 μM suramin, an inhibitor of P2 receptors that might result from ATP release to the extracellular milieu from damaged cells [4,31,32], and 10 μM BTS to reduce muscle damage caused by spontaneous contractions [33]. Then, the muscle tissue was transferred to a 15 mL tube (Falcon) containing 5 mL of culture medium, in which the tissue was gently triturated 10 times, using a Pasteur pipette with a wide tip to disperse single myofibers. Dissociated myofibers were centrifuged at 1,000 rpm for 15 s and washed twice with Krebs-HEPES solution (containing in mM: 145 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 10 HEPES-Na and pH 7.4) with 10 μM BTS (Krebs-HEPES-BTS). Finally, myofibers were resuspended in 5 mL of Krebs-HEPES-BTS solution, plated in plastic culture dishes or placed in 1.5 mL Eppendorf tubes, and kept at room temperature for time-lapse recording of Etd^{+} uptake or evaluation of intracellular Ca^{2+} and Na^{+} signals.

2.5. Time-lapse recording of Etd^{+} uptake

Cellular uptake of Etd^{+} was evaluated by time-lapse measurements as described previously [4,34]. In brief, freshly isolated myofibers plated in plastic culture dishes were washed twice with Krebs-HEPES-BTS solution. For time-lapse measurements, myofibers were incubated in Krebs-HEPES-BTS solution containing 5 μM Etd^{+}. The Etd^{+} fluorescence was recorded in regions of interest that corresponded to nuclei of myofibers by using a Nikon Eclipse Ti inverted microscope (Japan) with NIS-Elements software acquisition, while image processing was performed with ImageJ 1.46r software (National Institutes of Health).

2.6. Intracellular free-Ca^{2+} and Na^{+} signals

Myofibers isolated from FDB muscles were loaded with 5 μM FURA-2-AM (Ca^{2+}) or SBFI-AM (Na^{+}) in culture medium without serum for 45 min at 37 °C, and then washed three times with Krebs-HEPES-BTS solution. The experimental protocol for imaging involved data acquisition of light emission at 510 nm due to excitation at 340 nm and 380 nm. The ratio was obtained by dividing the emission fluorescence image value at 340-nm by the 380-nm excitation on a pixel-by-pixel base ($\Delta = F_{340\ nm}/F_{380\ nm}$).