α7B Integrin changes in mdx mouse muscles after L-arginine administration

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Abstract **Muscle fibers attach to laminin in the basal lamina using two mechanisms, i.e., dystrophin with its associated proteins and α7β1 integrin. In humans, gene-mutation defects in one member of these complexes result in muscular dystrophies. This study revealed changes after L-arginine treatment of utrophin-associated proteins and the α7B integrin subunit in mdx mouse, a dystrophin-deficient animal model. In the two studied muscles (cardiac muscle and diaphragm), the α7B integrin subunit was increased in 5-week-old treated mice. Interestingly, the diaphragm histopathological appearance was significantly improved by L-arginine administration. These results highlight a possible way to compensate for dystrophin deficiency via α7β1 integrin.**

Keywords: α7β1 Integrin; Utrophin; mdx; Cardiac muscle; Diaphragm; L-Arginine

1. Introduction

The actin network located under the muscle membrane is linked to the extracellular laminin by two distinct mechanisms. The first one involves dystrophin at the inner cytoplasmic membrane. The linkage is mediated through dystrophin associations with transmembrane proteins, i.e., dystroglycan and sarcoglycan–sarcospan complexes [1–3]. There are also important associations with the subsarcolemmal complex, i.e., dystrobrevins, syntrophins and neuronal-type nitric oxide synthase (nNOS) [4]. The second mechanism involves muscle-specific α7β1 integrin. This transmembrane laminin receptor also links the extracellular matrix with the cell cytoskeleton of skeletal and cardiac muscles [5,6]. The dystrophin-associated protein complex (DAPC) and the α7β1 integrin both participate in the molecular continuity between the extracellular matrix and myofibers, which is essential for muscle membrane structural and functional integrity.

Defects in the dystrophin gene result in a lack of dystrophin, which weakens muscle fiber association with the surrounding basal lamina and underlies Duchenne and Becker muscular dystrophies [7]. The absence of dystrophin is followed by persistence of an homologous protein called utrophin [8]. Utrophin is present all around the muscle membrane during foetal development and then only maintained at the neuromuscular junction in normal muscle [9]. In dystrophin-deficient muscles, utrophin remains associated along the muscle membrane and links to different dystrophin-associated partners such as syntrophin and nNOS [10].

An absence of dystrophin partially disrupts membrane-bound nNOS [11] followed by defects in microvascular adaptation [12]. In contrast, a nNOS transgene improves the muscular dystrophy phenotype [13]. Contraction of dystrophin-deficient muscles therefore may not properly stimulate sufficient NO production to release vasoconstriction, resulting in local muscle ischemia [14].

The NO precursor is provided by L-arginine (L-Arg), which is involved in the urea synthesis cycle and is the nNOS substrate. If muscle fibers produce sufficient level of nNOS, elevated L-Arg concentrations could influence NO synthesis and perhaps improve muscle membrane integrity. This prompted us to treat a murine model of dystrophin deficiency, i.e., mdx mouse [15], with L-Arg. Utrophin, different members of the DAPC and α7B integrin were analyzed in cardiac muscle and diaphragm by Western blot and immunofluorescent detection. Here, we describe specific protein changes and potential associated improvements in muscle morphology induced by L-Arg treatment in dystrophin-deficient muscles.

2. Materials and methods

2.1. Antibodies

Polyclonal antibodies directed against C-terminal sequences of α-dystrobrevin (D124: GVSYVPYCRS), β-dystroglycan (LG5: PPPYVPPP), dystrophin (H4: SRGNIPGKPMREDTM); sarcospan (C525: CSLTASEGPQQKI); utrophin (K7: CPNVPSRPQAMC) and α7B-integrin (K6: DWHPELGPDGHPVPATA) were obtained by injecting the keyhole limpet hemocyanin-linked peptide as antigen according to a previously described protocol [16]. K6 specifically recognize the α7B isoform of the α7 subunit in the α7β1 integrin complex.

Commercial antibodies directed against caveolin-3 and nNOS were purchased from Santacruz Biotechnologies and BD Transduction Laboratory, respectively.

2.2. Laboratory animals and L-arginine administration protocol

Wild-type (C57BL/10) and dystrophin-deficient (mdx) mice were purchased from Jackson Laboratories. Intraperitoneal injections (250 μl vol.) were performed daily for 3 weeks. Control mdx and

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Abbreviations: NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; Ss, saline solution; SDS, sodium dodecyl sulfate

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C57BL/10 mice were injected with saline solution (L-Arg-diluent) while treated mdx animals were administered with L-Arg solution (250 mg/kg). Experiments were carried out on 5- and 13-week-old animals in duplicate. Thirty-six mice were used: Ss-treated C57BL/10 ($n = 2 \times 6$), Ss-treated mdx ($n = 2 \times 6$) and L-Arg-treated mdx ($n = 2 \times 6$). Animals were killed on the 22nd day post-treatment. Diaphragm and cardiac muscles were dissected, rapidly frozen in 2-methylbutane, cooled in liquid nitrogen and stored at −80 °C until use.

2.3. Preparation of tissue lysates

0.01 g from cardiac muscle or diaphragm were homogenized in 150 μl of SDS buffer (50 mM Tris/HCl, pH 8.0, 10% SDS, 5% β-mercaptoethanol, 10% glycerol, 10 mM EDTA). Samples were centrifuged at $13\,000 \times g$ for 10 min. Each supernatant was mixed with 50 μl of SDS buffer containing 0.01% bromophenol blue. Each tissue lysate was denatured for 3 min at 100 °C and submitted in duplicate to SDS–PAGE. One resulting gel was Coomassie blue stained and the other was transferred onto nitrocellulose.

2.4. Scanning densitometric measurement standardization

Using the NIH Image software package, the relative optical density of the myosin heavy chain (MHC) present in each tissue lysate was estimated from the Coomassie blue stained gel. Independently, a standard SDS–PAGE, corresponding to known myosin amounts, was stained with Coomassie blue. This was converted into a standard curve associating each scanning densitometric measurement of the MHC band with the corresponding amount of myosin. In reference to this curve, the protein concentration was equilibrated in each tissue lysate.

2.5. Western blot analysis

Tissue lysates were electroblotted onto nitrocellulose (0.2 μm). Each blot was blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 3% bovine serum albumin (w/v). All membranes were incubated with primary antibodies for 1 h at room temperature. After labeling, the membranes were washed in TBST and then incubated with the secondary antibody (phosphatase-conjugated goat anti-rabbit IgG, Chemicon International, 1/5000). Antibody-bound proteins were detected with *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate substrate, as previously reported [17].

2.6. Measurement of relative protein concentrations

Protein band intensities were assessed using the NIH Image software package. Means of arbitrary values obtained for the two assays were calculated. To test for statistically significant differences, a one-way analysis of variance (ANOVA) was used; in the case of significant differences, the Scheffe test was applied. Statistical significance was set at $P < 0.05$. Means of arbitrary values were translated into mean percentages by considering the C57BL/10 mean of the arbitrary value as 100%. Data were expressed as means ± SEM (in %).

2.7. Immunofluorescence studies

Unfixed cryostat sections (10 μl thick) of frozen diaphragm and cardiac muscles were incubated with anti-utrophin or anti-α7B integrin antibody for 1 h at room temperature on slides. After washing with phosphate-buffered saline (PBS), sections were incubated for 1 h at room temperature with the secondary antibody (Cy3-conjugated goat anti-rabbit IgG, Chemicon International, 1/4000). Finally, the sections were washed with PBS, the slides were then mounted with Mowiol (CalBiochem) and observed under a Nikon optiphot-2 microscope.

2.8. Histological analysis

Unfixed cryostat sections (10 μl thick) of frozen cardiac muscle and diaphragm from 5-week-old mice were incubated with haematoxylin (Sigma). After washing with distilled water, sections were incubated with eosin (Sigma). Finally, the sections were washed, the slides were then mounted with Mowiol and observed under a Nikon optiphot-2 microscope.

A subsequent image analysis was performed using the ImageTool IT3 software package. Muscle fiber cross-sections were analyzed in three steps: (a) determination of the muscle fiber size, (b) determination of the percentage of muscle fibers containing centralized nuclei and (c) assessment of the lymphocytic invasion. The geometrical parameter tested for the determination of the muscle fiber size was the minimal "Feret's diameter" (the minimum distance of parallel tangents at opposing borders of the muscle fiber) according to recent published data [18]. The variance coefficient (VC) of the "Feret's diameter" is defined as follows: VC = (standard deviation of the "Feret's diameter"/mean muscle fiber size) × 1000. The lymphocytic invasion was evaluated by measuring the inflammatory area as a percentage of the total area muscle cross-section.

3. Results

3.1. L-Arg treatment does not significantly alter the utrophin level in mdx mouse cardiac muscle and diaphragm

We first attempted to determine whether L-Arg treatment would modify protein levels in muscles. Tissue lysates from cardiac muscle and diaphragm were subjected to Western blot analysis with antibodies directed against utrophin, α7B-integrin and dystrophin-associated proteins (i.e., caveolin-3, α-dystrobrevin, β-dystroglycan, nNOS and sarcospan).

As expected, saline solution (L-Arg-diluent) did not modify the protein levels in Ss-treated C57BL/10 and Ss-treated mdx mice, as compared with protein levels in untreated mice (data not shown). In addition, L-Arg treatment had no significant effect on caveolin-3, α-dystrobrevin, β-dystroglycan, nNOS and sarcospan levels in both 5- and 13-week-old mdx mice (data not shown).

Fig. 1A shows the utrophin band obtained with the K7 polyclonal antibody and Fig. 2A shows the relative utrophin concentrations (percentage) in each treated animal-type. In each studied muscle, we observed clear overexpression of utrophin in Ss-treated mdx mice relative to Ss-treated C57BL/10 mice as previously showed [19]. Histograms revealed that L-Arg treatment had no effect on cardiac utrophin level in 5-week-old mdx mice. Surprisingly, L-Arg treatment reduced the cardiac utrophin level in 13-week-old mdx mice. Finally, diaphragm utrophin levels appeared increased in 5- and 13-week-old L-Arg-treated mdx mice but it was not significant according to our statistical tests (Fig. 2A).

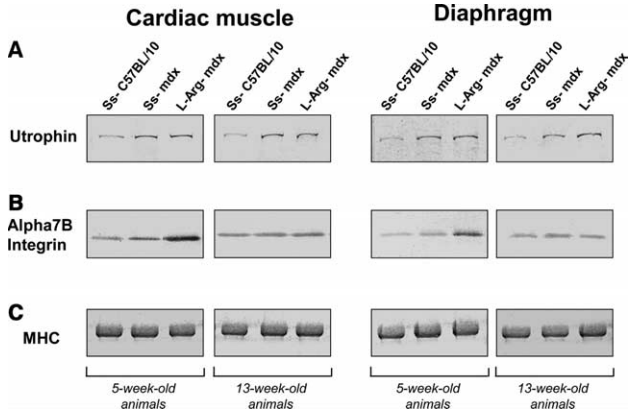

Fig. 1. Western blot detection of utrophin and α7B integrin in Ss-treated C57BL/10, Ss-treated mdx and L-Arg-treated mdx mice muscles. Western blots showing relative detection of (A) utrophin and (B) the α7B integrin subunit in tissue lysates from 5- and 13-week-old mice. The six left lanes contained samples from cardiac muscles, while the six right lanes contained samples from diaphragms. (C) Coomassie blue detection of MHC after SDS–PAGE of tissue lysates.