Journal of the Neurological Sciences 294 (2010) 43–50

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

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

Excessive collagen accumulation in dystrophic (mdx) respiratory musculature is independent of enhanced activation of the NF-κB pathway

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ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form 2 April 2010
Accepted 16 April 2010
Available online 13 May 2010

Keywords:
Duchenne muscular dystrophy
Mdx mouse
NF-κB
Fibrosis
TGF-β1
MMP-9
Collagen
Skeletal muscle
Triangularis sterni
Diaphragm

ABSTRACT

Skeletal muscle fibrosis is present in the diaphragm of the mdx mouse, a model for Duchenne dystrophy. In both the mouse and human, dystrophic muscle exhibits pronounced increases in NF-κ B signaling. Various inhibitors of this pathway, such as pyrrolidine dithiocarbamate (PDTC) and ursodeoxycholic acid (UDCA), have been shown to have beneficial effects on dystrophic (mdx) muscle. The present study characterizes the development of fibrosis in the mdx musculature, and determines the fibrolytic efficacy of PDTC and UDCA. The results indicate that collagen accumulation and the expression of fibrogenic (TGF-β1) and fibrolytic (MMP-9) mediators are dependent on muscle origin in both nondystrophic and mdx mice. Excessive collagen accumulation is observed in the mdx respiratory musculature prior to substantial muscle degeneration and cellular infiltration, and is associated with dystrophic increases in the expression of TGF-β1 with no corresponding increases in MMP-9 expression. Treatment with PDTC or UDCA did not influence collagen deposition or TGF-β1 expression in the mdx respiratory musculature. These results indicate that dystrophic increases in collagen are the result of NF-κB-independent signaling abnormalities, and that efforts to reduce excessive collagen accumulation will require treatments to more specifically reduce TGF-β1 signaling or enhance the expression and/or activity of matrix metalloproteases.

1. Introduction

In assessing the therapeutic utility of various signaling modulators in the treatment of Duchenne and Becker muscular dystrophy, it is necessary to view each of the primary symptoms of the disease as separate and possibly independent expressions of the dystrophic phenotype. A major phenotypic consequence of the lack of dystrophin is the development of muscle fibrosis [1–3], which is secondary to increases in the expression of TGF-β [4–7]. The present report directly compares total collagen expression between limb and respiratory muscles and provides new evidence that both nondystrophic and mdx muscles with different histories of activation accumulate different amounts of collagen and express different amounts of both fibrogenic (TGF-β1) and fibrolytic (MMP-9) mediators.

The potential role of the NF-κB pathway in mediating excessive collagen deposition in the mdx respiratory musculature was examined by treating mdx mice *in vivo* with two NF-κB inhibitors, pyrrolidine dithiocarbamate (PDTC) and ursodeoxycholic acid (UDCA). Each of these agents had previously been shown to be efficacious in inhibiting the NF-κB pathway by enhancing cytosolic IκB-α (PDTC; [8]) or reducing nuclear p65 activation (UDCA; [9]). Previous studies also indicated that these agents enhanced mdx limb tension development [9], and improved cell morphology and function in the mdx mouse respiratory musculature [8]. Neither PDTC nor UDCA reduced collagen accumulation in the mdx respiratory musculature.

The results indicate that collagen accumulation depends upon the relative expression of fibrogenic (TGF-β1) to fibrolytic (MMP-9) mediators in both nondystrophic and dystrophic (mdx) muscle, that excessive accumulation of collagen occurs in the mdx respiratory musculature prior to the appearance of substantial muscle degeneration and cellular infiltration, and that excessive collagen accumulation in dystrophic muscle is due to increases in the relative expression of fibrogenic mediators that are independent of enhanced activation of the NF-κB pathway. These results provide new evidence strongly suggesting that excessive collagen accumulation is not entirely dependent upon muscle degeneration and subsequent cellular infiltration, but is initiated by local, NF-κB-independent signaling abnormalities that increase the relative expression of fibrogenic to fibrolytic mediators in specific dystrophic muscles. Efforts to reduce excessive collagen accumulation in dystrophic muscle will therefore require more direct intervention in reducing TGF-β signaling or enhancing the local activity of matrix metalloproteases.

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doi:10.1016/j.jns.2010.04.007

2. Methods

2.1. Hydroxyproline assay

Mdx (C57Bl10-mdx) and nondystrophic (C57Bl10SnJ) mice were euthanized in accordance with established procedures (Institutional Animal Care and Use Committee; IACUC) and individual muscles were freshly isolated in HEPES Ringer (147.5 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 11 mM glucose, 5 mM HEPES, pH 7.35) using standard techniques [10]. The individual muscles were immediately flash-frozen and stored at −78° C until used in the hydroxyproline determinations. Hydroxyproline contents were determined using procedures adapted from Prockop and Udenfriend [11] and Switzer and Summer [12]. A complete description of the protocol used for these experiments is available at the Treat NMD website [13].

Individual muscles were weighed before being acid-hydrolyzed (5 N HCl) at 130 °C for 12 h at a concentration of 10 mg muscle wet weight/ml. Samples of the hydrolysate equivalent to 0.5 mg of muscle (50 µl) were diluted with 2.25 ml of distilled water and neutralized with appropriate amounts of 0.1 N KOH using phenolphthalein as a pH indicator. Sodium borate buffer (0.5 ml, pH 8.7) was then added, and the mixture oxidized for 25 min with 2.0 ml of 0.2 M chloramine-T solution. In our initial experiments, the chloramine-T was dissolved in dH_2O. In later studies, a sodium-citrate buffer (25 g/l citric acid monohydrate, 6 ml/l glacial acetic acid, 17 g/l NaOH, 60 g/l sodium acetate trihydrate) containing 30% 2 methoxyethanol (Sigma #284467) was used. Direct comparisons indicated that the methoxyethanol solution improved test reliability but did not result in different measurement outcomes.

The oxidation reaction was stopped by adding 1.2 ml of 3.6 M sodium thiosulfate. Since the pyrroline and pyrrole carboxylic oxidation products that are formed from hydroxyproline are not soluble in toluene, contaminating impurities were extracted by adding 2.5 ml toluene and saturating amounts of KCl (1.5 g). After appropriate mixing of the toluene and aqueous phases, the phases were separated by centrifugation. The toluene phase containing the extracted impurities was removed and discarded. The remaining aqueous layer containing the hydroxyproline products was removed and heated for 30 min in boiling water to convert the oxidation product of hydroxyproline, pyrrole-2-carboxylic acid, to pyrrole. The final pyrrole reaction product was removed in a second toluene extraction. In the final step, an aliquot of the toluene layer (1.5 or 2.0 ml) was mixed with a corresponding volume of Ehrlich's reagent (0.8 or 0.6 ml) for colorimetric assay against hydroxyproline standards (0.0, 0.5, 1.0, 2.0, 4.0, and 6.0 µg hydroxyproline) at 560 mµ. The total amount of hydroxyproline in the 2.5 ml of toluene extract was determined from the amount present in the 1.5 or 2.0 ml aliquot, and divided by the initial weight of muscle in the hydrolysate sample (0.5 mg) to obtain the hydroxyproline content (µg hydroxyproline/mg muscle wet weight).

2.2. Expression of TGF-β1 and MMP-9

Cytosolic extracts of gastrocnemius and costal diaphragm muscles were obtained using procedures described in Singh et al. [14]. The muscles were weighed after removing tendinous components, and frozen and homogenized by mortar and pestle in low salt lysis (LSL) buffer on ice (1 mg muscle wet weight/18 µl solution; in mM: 10 HEPES, 10 KCl, 1.5 $MgCl_2$, 0.1 EDTA, 0.1 EGTA, 1 dithiothreitol (DTT), 0.5 phenylmethylsulfonylfluoride (PMSF); 0.5 mg/ml benzamidine, 4.0 µl/ml protease inhibitor cocktail Sigma # 8340, 10 µl/ml phosphatase inhibitor Sigma #P2850, pH 7.9). To lyse the cells, the ground tissue was subjected to 2 freeze–thaw cycles (5 minute freeze on dry ice followed by thawing at room temperature), and was subsequently vortexed and centrifuged (13,000 rpm, 15 s). The supernatant cytosolic extract was immediately frozen (−80 °C) for subsequent analyses of TGF-β1 and MMP-9 expression. Total protein concentration was determined using the Bradford assay (Bio-rad 500-0006) in a standard 96 well plate.

TGF-β1 expression in the cytosolic extracts was determined using an ELISA kit (R and D Systems, MB100B) following the manufacturer's instructions. TGF-β1 levels are expressed in pg cytokine/mg protein. MMP-9 expression was determined using Western blots. Cytosolic extracts (20 µg total protein) were separated by 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% nonfat dehydrated milk (NFDM) in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 h at room temperature. Primary antibodies for MMP-9 were incubated with 5% BSA in TBST (1:1000, Cell Signaling Technology #2270) overnight at 4 °C. Secondary antibody horse reddish peroxidase (HRP) conjugated anti-rabbit IgG (1:1500, Jackson Laboratory) was incubated with 5% NFDM in TBST for 1 h at room temperature. The immunoblot detection was performed using an ECL detection system (Amersham, UK) according to the instructions of the manufacturer. After obtaining the immunoblot for MMP-9, the PVDF membrane was stripped for 30 min at 50 °C and re-blocked with 5% NFDM in TBST for 1 h at room temperature. Primary antibodies for the cytosolic marker glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:7000, Cell Signaling Technology #2178) were applied with 5% NFDM in TBST and incubated overnight at 4 °C. Densitometric analyses of the X-ray films were performed using Image J, Sigma Stat and Sigma Plot.

2.3. Histology

Individual muscles were fixed overnight in 2% glutaraldehyde (Sigma G7526) in 0.1 M cacodylate buffer and cut into appropriately sized blocks before being dehydrated (ethanol: 30% 1 h, 50% 1 h, 75% 1 h, 95% 2 one hour washes, and 100% 2 one hour washes), cleared in xylene (2 one hour washes), and embedded in paraffin (2 h in 50% paraffin and 3 h in 100% paraffin). The tissue was oriented to obtain 5 µm cross-sections of the TS fibers, which *in vivo* are attached to the sternum and project laterally towards tendinous insertions on the ribs at approximately 2 cm from their sternal origins. Preparations were stained with Masson's Trichrome in accordance with the manufacturer's instructions (IMEB Inc., CAT#7228).

All images were viewed under an E. Leitz Wetzlar Microscope (NR. 502209) using either a 40 or 95× objective. Images of all fields on one section from each block were acquired using an Optronics DEI-750 camera (800×600 pixels) and were viewed in Adobe Photoshop 5.5. A two dimensional grid was used to determine the percent collagen in each section. Each intersection on the grid was tallied according to what color it landed on; blue (collagen), red (muscle cytoplasm), and black (nuclei). The proportion of collagen was defined as the number of points falling on blue (collagen) divided by the total points falling either on blue or red (muscle cytoplasm).

2.4. Drug studies

In the PDTC studies, mdx mice (male and female) were given daily intraperitoneal (ip) injections of 50 mg/kg pyrollidine dithiocarbamate (PDTC; Sigma P8765) dissolved in a HEPES-buffered modified Ringers solution. Age-matched vehicle-treated mdx mice received daily injections of the HEPES Ringer solution. UDCA-treated mdx mice received 40 mg/kg UDCA in an isotonic saline (1.02% NaCl, pH 8.4). Saline-treated mdx mice served as controls. The procedures used in this study were approved by the IACUC.

2.5. Statistical analysis

Sigma Plot 9.0 and Sigma Stat v 3.0 were used. The means and standard errors were evaluated at the 0.05 level of significance using