Published in final edited form as:

J Neurol Sci. 2010 July 15; 294(1-2): 43–50. doi:10.1016/j.jns.2010.04.007.

EXCESSIVE COLLAGEN ACCUMULATION IN DYSTROPHIC (MDX) RESPIRATORY MUSCULATURE IS INDEPENDENT OF ENHANCED ACTIVATION OF THE NF- κ B PATHWAY

KM Graham, R Singh, G Millman, G Malnassy, F Gatti, K Bruemmer, C Stefanski, H Curtis, J Sesti, and CG Carlson

Dept. of Physiology, Kirksville College of Osteopathic Medicine, AT Still University, 800 W. Jefferson St., Kirksville, MO 63501-1497

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- β 1) and fibrolytic (MMP-9) mediators is 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- β 1 with no corresponding increases in MMP-9 expression. Treatment with PDTC or UDCA did not influence collagen deposition or TGF- β 1 expression in the mdx respiratory musculature. These results indicate that dystrophic increases in collagen are the result of NF- κ B-independent signaling abnormalities, and that efforts to reduce excessive collagen accumulation will require treatments to more specifically reduce TGF- β 1 signaling or enhance the expression and/or activity of matrix metalloproteases.

Keywords

Duchenne Muscular Dystrophy; *Mdx* mouse; NF- κ B; Fibrosis; TGF- β 1; MMP-9; Collagen; Skeletal muscle; Triangularis Sterni; Diaphragm

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

Correspondence to: CG Carlson.

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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- β 1) and fibrolytic (MMP-9) mediators.

The potential role of the NF- κ B pathway in mediating excessive collagen deposition in the mdx respiratory musculature was examined by treating mdx mice *in vivo* with two NF- κ B inhibitors, pyrrolidine dithiocarbamate (PDTC) and ursodeoxycholic acid (UDCA). Each of these agents had previously been shown to be efficacious in inhibiting the NF- κ B pathway by enhancing cytosolic I κ B- 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- β 1) 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- κ B 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- κ B-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.

METHODS

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 CaCl₂, 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 (5N HCl) at 130° C for 12 hours at a concentration of 10 mg muscle wet weight/ml. Samples of the hydrolysate equivalent to 0.5 mg of muscle (50 μ l) were diluted with 2.25 ml of distilled water and neutralized with appropriate amounts of 0.1N 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 minutes with 2.0 ml of 0.2 M chloramine-T solution. In our initial experiments, the chloramine-T was dissolved in dH₂O. In later studies, a sodium-citrate buffer (25 gm/l citric acid monohydrate, 6 ml/l glacial acetic acid, 17 gm/l NaOH, 60 gm/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 gm). 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 minutes 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, 6.0 μg hydroxyproline) at 560 nm. 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).

Expression of TGF- β 1 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 $\mu\text{l/ml}$ protease inhibitor cocktail Sigma # 8340, 10 $\mu\text{l/ml}$ 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 sec). The supernatant cytosolic extract was immediately frozen (-80°C) for subsequent analyses of TGF- β 1 and MMP-9 expression. Total protein concentration was determined using the Bradford assay (Biorad 500-0006) in a standard 96 well plate.

TGF- β 1 expression in the cytosolic extracts was determined using an ELISA kit (R and D Systems, MB100B) following the manufacturer's instructions. TGF- β 1 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% non fat dehydrated milk (NFDM) in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 hr 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 hr at room temperature. The immunoblot detection was performed using ECL detection system (Amersham, UK) according to the instructions of manufacturer. After obtaining the immunoblot for MMP-9, the PVDF membrane was stripped for 30 minutes at 50°C and re-blocked with 5% NFDM in TBST for 1 hr 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.

Histology

Individual muscles were fixed overnight in 2% glutaraldehyde (Sigma G7526) in 0.1M cacodylate buffer and cut into appropriately sized blocks before being dehydrated, (ethanol: 30% 1 hour, 50% 1 hour, 75% 1 hour, 95% 2 one hour washes, 100% 2 one hour washes),

cleared in xylene (2 one hour washes), and embedded in paraffin (2 hours in 50% paraffin, 3 hours in 100% paraffin). The tissue was oriented to obtain 5 μ m 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 manufacturer's instructions (IMEB Inc., CAT#7228).

All images were viewed under an E. Leitz Wetzlar Microscope (NR. 502209) using either a 40 or 95X objective. Images of all fields on one section from each block were acquired using an Optronics DEI-750 camera (800 $\times$ 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), 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).

Drug Studies

In the PDTC studies, mdx mice (male and female) were given daily intraperitoneal (ip) injections of 50 mg/kg pyrrolidine 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.

Statistical Analysis

Sigmaplot 9.0 and Sigmastat v 3.0 were used. The means and standard errors were evaluated at the 0.05 level of significance using t-tests, Mann-Whitney Rank Sums tests, or Kruskal-Wallis one way ANOVA on Ranks (Sigma Stat 3.1).

RESULTS

Expression of hydroxyproline, TGF β 1, and MMP-9 in adult mdx and nondystrophic gastrocnemius and costal diaphragm

At 5 months of age, the mdx costal diaphragm exhibited a 3 fold higher mean hydroxyproline content than that in age-matched nondystrophic costal diaphragm (Fig. 1A; ***, $p < 0.001$). At this age, mdx mice also exhibited significantly higher hydroxyproline levels in the costal diaphragm than in the gastrocnemius muscle (Fig. 1A; $\tau\tau$, $p < 0.01$). Hydroxyproline content in the mdx gastrocnemius at 5 months was not significantly increased above the level seen in the nondystrophic gastrocnemius. Nondystrophic mice at 5 months of age did not exhibit a significantly higher hydroxyproline content in the costal diaphragm than in the corresponding gastrocnemius muscle (Fig. 1A).

At 14 months of age, the mdx costal diaphragm exhibited hydroxyproline levels that were approximately double those in the nondystrophic preparation (Fig. 1B; **, $p < 0.01$). Similar to the results at 5 months, the hydroxyproline content in the 14 month mdx gastrocnemius was not significantly higher than that in the nondystrophic preparation. By 14 months of age, however, both nondystrophic and mdx mice exhibited significantly higher hydroxyproline contents in the costal diaphragms than in the corresponding gastrocnemius muscles (Fig. 1B; $\tau\tau$, $p < 0.01$). Between 5 and 14 months, both nondystrophic and mdx mice exhibited age-dependent increases in hydroxyproline content in gastrocnemius and costal diaphragm muscles (Fig. 1B; $\mu, \mu\mu, \mu\mu\mu$, $p < 0.05, 0.01, \text{ and } 0.001$, respectively).

The expression of TGF- β 1 and MMP-9 were examined in cytosolic extracts from gastrocnemius and costal diaphragm muscles at 5 months of age. Extracts from both mdx gastrocnemius and costal diaphragms exhibited significantly higher expression of TGF- β 1 than the corresponding nondystrophic preparations (Fig. 2; *, $p < 0.05$). Both nondystrophic and mdx mice exhibited significantly elevated TGF- β 1 expression in the costal diaphragm than in the corresponding gastrocnemius muscles (Fig. 2; $\tau\tau$ and $\tau\tau\tau$, $p < 0.01$ and 0.001 , respectively).

In contrast to the results with TGF- β 1 (Fig. 2), the expression of MMP-9 was identical in cytosolic extracts from the costal diaphragms of nondystrophic and mdx mice, and was significantly elevated only in the mdx gastrocnemius muscle (Fig. 3; *, $p < 0.05$). Similar to the results obtained with TGF- β 1, both nondystrophic and mdx mice exhibited significantly higher expression of MMP-9 in the costal diaphragms than in the corresponding gastrocnemius muscles (Fig. 3; τ and $\tau\tau$, $p < 0.05$ and 0.01 , respectively). The ratio of TGF- β 1 to MMP-9 expression in 5 month old nondystrophic and mdx costal diaphragm and gastrocnemius muscles (Fig 4) paralleled the hydroxyproline content in these muscles particularly at 14 months of age (Fig 1). These results provide new evidence indicating that the accumulation of collagen (Fig. 1), the expression of TGF- β 1 (Fig. 2) and MMP-9 (Fig. 3), and the ratio of TGF- β 1 to MMP-9 expression (Fig. 4), are each dependent upon muscle origin in both nondystrophic and mdx mice.

Developmental aspects of collagen accumulation in the respiratory musculature of nondystrophic and mdx mice

The time course of collagen accumulation was examined in order to identify the earliest age at which excessive collagen accumulation is observed in the mdx mouse. Both nondystrophic and mdx mice exhibited muscle-specific collagen accumulation at 15 days of age. At this age, hydroxyproline content was significantly ($p < 0.05$) higher in the nondystrophic costal diaphragm than in either the crural diaphragm or the triangularis sterni (TS) muscles (Fig. 5A1). The identical sequence of hydroxyproline content (costal>crural>TS) was observed in the 30, 60, and 120 day old nondystrophic mice (Fig. 5A2, A3, A5).

Collagen expression in the developing mdx respiratory musculature depended upon muscle origin in a manner that roughly paralleled the results in nondystrophic mice (Fig 5B1-B5). At 15 days of age, the mdx costal diaphragm exhibited significantly higher hydroxyproline than either the mdx crural diaphragm or TS muscles (Fig. 5B1). The earliest excessive collagen accumulation was at 30 days of age, when the mdx TS muscle exhibited a significant increase in hydroxyproline content over corresponding nondystrophic levels (Fig. 5B2; σ , $p < 0.05$). By 60 days of age, hydroxyproline contents in all 3 mdx respiratory muscles were significantly larger than those in the corresponding nondystrophic muscles (Fig. 5B3; $\sigma\sigma\sigma$, $p < 0.001$). The largest proportional increase in hydroxyproline content was observed in the mdx TS muscle at 120 days of age (13.5 fold increase over nondystrophic TS; Fig. 5B5).

During the first 4 months, the costal and crural diaphragms of nondystrophic mice did not exhibit significant age-dependent changes in hydroxyproline, while the levels in the nondystrophic TS exhibited a significant (ANOVA, $p < 0.001$) peak at 90 days of age (Fig. 6A). In contrast, hydroxyproline levels in all 3 respiratory muscles of the mdx mouse steadily increased between 30 and 120 days of age (ANOVA, $p < 0.001$; Fig. 6B). These results indicate that collagen accumulation is highest in the costal diaphragms of both nondystrophic and mdx mice, and that the absence of dystrophin increases collagen levels in all 3 respiratory muscles, with the TS exhibiting the earliest (30 days, Fig. 5B2) and most profound (13.5 fold) increase (Fig. 5A5, B5).

Long term treatment with inhibitors of the NF- κ B pathway does not influence fibrosis in the mdx respiratory musculature

Previous studies in this laboratory indicated that a single *in vivo* dose of 50 mg/kg PDTC increased cytosolic levels of I κ B- α in the mdx costal diaphragm, and that daily treatment at this dose increased the resting potential and the survival of mdx TS striated muscle fibers [8] and the forward pulling tension exerted by the limb musculature of mdx mice [9]. To examine whether PDTC alters excessive collagen accumulation in dystrophic muscle, hydroxyproline contents were determined from our initial studies [8] in which mature mdx mice received at least 30 consecutive daily injections of 50 mg/kg PDTC. The results indicated that PDTC did not alter total collagen expression in either the costal or crural diaphragm. (Fig. 7A). Therefore, the potential action of PDTC was examined in a second study in which young adult (30 day old) mdx mice were treated for 30 consecutive days with either PDTC (50 mg/kg) or vehicle. The results again indicated that the treatment did not influence collagen accumulation in the costal diaphragm, crural diaphragm, or TS muscle (Fig. 7B). TGF β 1 expression in the costal diaphragm was also not significantly altered by the PDTC treatment (Vehicle treated: mean = 87.8 ± 32.8 (SEM) pg/mg protein; PDTC treated: mean = 103.3 ± 34.3 pg/mg protein).

The lack of influence of long term PDTC treatment on excessive collagen accumulation was further confirmed by morphometric analyses of trichrome sections of TS muscle from mature mdx mice treated either with vehicle or PDTC. Figure 7C summarizes the results of point-counting determinations of percent collagen in the caudal, middle, and cephalad regions of nondystrophic TS, and from TS muscles obtained from mature, vehicle- or PDTC-treated mdx mice. The results indicate that fibrosis was increased uniformly throughout the TS of vehicle-treated mdx mice (Fig. 7C2) and in the cephalad and middle TS regions of the PDTC treated mdx mice (Fig 7C3). The caudal TS region from the PDTC treated mdx mice exhibited a significant ($p < 0.05$) reduction in the proportion of collagen in comparison to the vehicle-treated preparations (Fig. 7C3). In this case, however, an independent morphometric examination indicated that the PDTC treatment significantly ($p < 0.05$) increased the density of fiber cross-sections in the caudal TS [15]. Thus, the observed reduction in the proportion of collagen in the caudal TS (Fig 7C3) was secondary to an increase in the density of muscle fibers and not to a reduction in collagen deposition.

To further determine whether inhibition of the NF- κ B pathway influences excessive collagen accumulation, mdx mice were treated with UDCA at a dose and treatment schedule that had previously been shown in this laboratory to significantly reduce nuclear p65 activation in the mdx costal diaphragm and significantly improve limb tension development [9]. Daily treatment of young adult mdx mice with UDCA had no effect on hydroxyproline content in the mdx costal diaphragm (Fig. 7D).

DISCUSSION

Excessive collagen accumulation in specific dystrophic muscles is produced by signaling abnormalities that favor the local expression of fibrogenic mediators

The results provide the first clear evidence that the deposition of collagen and the expression of fibrogenic and fibrolytic mediators are each dependent upon muscle location and/or function in both nondystrophic and mdx mice. In mature adult nondystrophic mice, collagen deposition was significantly higher in the costal diaphragm than in the gastrocnemius muscle (Fig. 1B). This can be explained by comparing the expression of fibrogenic (TGF β -1) and fibrolytic (MMP-9) mediators between the two muscles (Fig. 2–4). TGF β promotes fibrosis by increasing the number of fibroblasts in skeletal muscle [16], by reducing the expression of MMPs, and by increasing the expression of tissue inhibitors of matrix metalloproteases (TIMPs) and plasminogen activator inhibitors [17]. The mean TGF β -1/MMP-9 ratio in the nondystrophic

costal diaphragm was approximately 29 fold higher than that in the nondystrophic gastrocnemius (Fig. 4). These results demonstrate that increases in collagen deposition do not occur solely in dystrophic muscle, but are instead a normal consequence of local signaling mechanisms that depend upon muscle origin and/or function in nondystrophic muscle.

The conclusion that local signaling mechanisms modulate collagen expression in nondystrophic muscle has obvious implications regarding the excessive accumulation in specific dystrophic muscles (Fig. 1,5). Several previous reports have indicated that dystrophic skeletal muscle exhibits elevated TGF- β 1 expression [4–7], and it is generally considered that this depends upon cellular infiltration and growth [18], and the local differentiation of satellite cells into fibroblasts (cf [16]). The present results, however, demonstrate that the TGF β -1/MMP-9 ratio is inherently regulated by muscle origin and/or function in uninjured, non-inflamed, nondystrophic skeletal muscle (Fig. 4). It is therefore reasonable to propose that the elevated TGF- β 1 expression in dystrophic muscle (Fig. 2, 4) is secondary to disturbances in cell signaling that are independent of local injury or inflammation. This proposal is supported by our results showing that excessive collagen accumulation is associated with increases in the TGF β -1/MMP-9 ratio (Fig. 4) and is present in certain respiratory muscles prior to the development of substantial muscle injury, degeneration, or cellular infiltration (Fig. 5, 6).

A central conclusion of this study is that fibrosis, or excessive collagen deposition, in mdx muscle represents a quantitative, and not a qualitative, difference from nondystrophic muscle. This conclusion is consistent with observations indicating the presence of both perimysial and endomysial mast cells in nondystrophic muscle, and an increased number of fibrogenic mast cells that are located preferentially in the endomysium of dystrophic muscle [18]. Since mast cells are present in both nondystrophic and dystrophic muscle [18], the results of the present study showing muscle-specific differences in collagen deposition (Fig. 1, 5) suggest that local signaling mechanisms in normal and dystrophic muscle may control mast cell degranulation, mast cell migration, and/or mast cell growth.

The results of the present study suggest that the deposition of collagen in nondystrophic and mdx muscle depends upon the history of activation and the active or passive load that is present in the muscle during normal activity. Some differences in the expression of the disease between quadruped animal models and humans likely relate to differences in load-bearing during normal locomotion and activity. However, the results of this study showing that collagen expression in the mature nondystrophic costal diaphragm is significantly higher than that in nondystrophic gastrocnemius (Fig. 1B) suggest that the signaling environment normally present in the more persistently activated respiratory musculature may contribute to excessive collagen accumulation in the respiratory musculature of Duchenne patients. Future studies examining the expression of fibrogenic and fibrolytic mediators and the disposition of mast cells [18] in the respiratory and limb musculature may therefore be quite useful in developing treatments to specifically delay or prevent respiratory muscle fibrosis in Duchenne and related muscular dystrophies.

Inhibitors of the NF- κ B pathway do not influence collagen accumulation in dystrophic (mdx) muscle

Previous results from this laboratory indicated that PDTC stabilized cytosolic I κ B- α in the mdx costal diaphragm [8], increased the survival of striated muscle fibers [8], improved the resting membrane potential in mdx muscle [8], and increased active limb muscle tension in mdx mice [9]. The mechanism of action of PDTC in reducing nuclear NF- κ B activation involves cytosolic stabilization of the inhibitory protein I κ B- α [19] by inhibiting its ubiquitination [20]. This increases cytosolic levels of I κ B- α and reduces the proportion of NF- κ B in the nucleus [21]. Although PDTC effectively increased cytosolic I κ B- α in the mdx costal diaphragm [8], it was relatively ineffective at reducing nuclear p65 activation in the mdx costal diaphragm [9], which

we have shown exhibits a higher proportional increase in nuclear p65 activation than the mdx gastrocnemius muscle [14].

In contrast to PDTC, UDCA acts by inhibiting p65 transactivation in the nucleus without affecting cytosolic levels of I κ B- α [22]. Previous results from this laboratory indicated that the 30 day *in vivo* treatment with UDCA used in the present study significantly decreased nuclear p65 activation by 34% in the mdx costal diaphragm [9]. The improved efficacy of UDCA relative to PDTC is consistent with our results indicating that cellular p65 expression is substantially increased in dystrophic muscle and contributes significantly to elevated nuclear p65 activation in the mdx diaphragm [14]. These results suggested that agents to inhibit p65 transactivation or expression would be more effective than agents that increase cytosolic I κ B- α in dystrophic muscle [14]. In addition to decreasing nuclear p65 activation in the mdx costal diaphragm, UDCA, as administered in the present study, significantly improved the forward pulling tension of the limb musculature [9] and had positive morphological effects on the mdx TS muscle that were virtually identical to those produced by PDTC treatment [15].

Since neither PDTC nor UDCA, at concentrations that had independent positive effects on the dystrophic phenotype, negatively influenced hydroxyproline content or collagen accumulation in dystrophic muscle (Fig. 7), these results support the hypothesis that dystrophic increases in collagen accumulation are largely independent of elevated NF- κ B signaling [14,23]. From a practical perspective, this indicates that treatments to reduce excessive collagen accumulation in dystrophic muscle will require more direct intervention to either reduce TGF- β expression, reduce TGF β signaling, and/or enhance the expression and/or activity of matrix metalloproteases. Such treatments in combination with agents to reduce nuclear NF- κ B activation [9,14] would be expected to reduce excessive collagen accumulation and improve the morphology and function of dystrophic muscle fibers.

Summary

The results of this study lead to several new conclusions regarding collagen accumulation in nondystrophic and dystrophic (mdx) muscle. First, the results clearly indicate that different nondystrophic muscles exhibit distinct and characteristic levels of collagen accumulation (Fig. 1B; 5A) that are proportional to the relative expression of fibrogenic and fibrolytic mediators (Fig. 2–4). Second, the results show that the relative amounts of collagen accumulation in different muscles are quite similar in both nondystrophic and mdx mice, and that the absence of dystrophin leads to excessive collagen accumulation only in specific muscles, particularly those involved in respiration (Figs. 1, 5). Third, the results indicate that those dystrophic muscles exhibiting excessive collagen accumulation also exhibit increases in the relative expression of fibrogenic mediators (Figs. 2–4). Fourth, the dystrophic respiratory musculature exhibits excessive collagen accumulation prior to the onset of substantial muscle necrosis, degeneration, and cellular infiltration (Fig. 5, 6). Fifth, two distinct inhibitors of the NF- κ B pathway that negatively influence NF- κ B signaling in the mdx costal diaphragm and that have positive influences on the morphology and function of dystrophic muscle fibers [8,9] fail to reduce TGF- β 1 expression or collagen accumulation in the mdx costal diaphragm (Fig. 7). The results suggest that disturbances in cell signaling in dystrophic muscle fibers and satellite cells initiate excessive collagen accumulation by promoting increases in the relative expression of fibrogenic mediators (Fig 4) that are independent of excessive NF- κ B activation (Fig 7). The results therefore suggest that combination treatments to selectively reduce TGF- β 1 expression while independently reducing nuclear p65 activation may provide an improved strategy for reducing excessive collagen accumulation and improving muscle fiber integrity in Duchenne and related muscular dystrophies.

Acknowledgments

This research was funded by grants to CGC from the NIH (R15AR055360), the Association Française contre les Myopathies (AFM; 11832, 13980), Charley's Fund, the KCOM Biomedical Sciences Program, the Warner-Fermaturo (KCOM) fund, and a Strategic Research Grant (KCOM). The authors wish to thank Mrs. Bonnie King for her technical assistance in determining hydroxyproline levels, Dr. James Rhodes for his expertise and assistance with the histological studies, and Dr. Abbas Samadi for numerous helpful discussions.

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Figure 1.

Differences in hydroxyproline content (μg hydroxyproline/mg muscle) between gastrocnemius (medial and lateral heads with plantaris) and costal diaphragm muscles in both nondystrophic and mdx mice at 5 (A) and 14 months (B) of age. ** and *** indicate $p < 0.01$ and $p < 0.001$ comparing mdx and nondystrophic costal diaphragm preparations. ττ with brackets indicates $p < 0.01$ comparing corresponding (nondystrophic or mdx) costal diaphragm and gastrocnemius muscles. μ, μμ, and μμμ in (B) indicate $p < 0.05$, 0.01, and 0.001 comparing indicated values at 14 (B) and 5 months (A) of age. Shown are the means and standard errors. N is the number of preparations (mice). Black histobars – nondystrophic; white histobars – mdx.

Figure 2.

Differences in the expression of TGF β 1 (pg TGF β -1/mg cytosolic protein) between gastrocnemius and costal diaphragm muscles in both nondystrophic and mdx mice at 5 months of age. * indicates $p < 0.05$ comparing indicated mdx and nondystrophic preparations (gastrocnemius and costal diaphragm). $\tau\tau$ and $\tau\tau\tau$ with brackets indicates $p < 0.01$ and 0.001 , respectively, comparing corresponding (nondystrophic or mdx) costal diaphragm and gastrocnemius muscles. Shown are the means and standard errors. N is the number of preparations (mice). Black histobars – nondystrophic; white histobars – mdx.

Figure 3.

Differences in the expression of MMP-9 in cytosolic extracts from 5 month old nondystrophic and mdx gastrocnemius and costal diaphragm preparations. (A) Western blots of MMP-9 (top row) and GAPDH (bottom row) in mdx and nondystrophic gastrocnemius (left side), and in mdx and nondystrophic costal diaphragms (right side). (B) Densitometric results of the blots in (A) showing relative intensity of MMP-9 and GAPDH immunostaining. * indicates $p < 0.05$ comparing mdx and nondystrophic gastrocnemius preparations. τ and $\tau\tau$ indicate $p < 0.05$ and $p < 0.01$, respectively, comparing indicated costal diaphragm to gastrocnemius muscle preparations. Shown are the means and standard errors. N is the number of preparations (mice). Black histobars – nondystrophic; white histobars – mdx.

Figure 4.

Differences in the relative expression of TGFβ-1 and MMP-9 in cytosolic extracts from 5 month old nondystrophic and mdx gastrocnemius and costal diaphragm preparations. Relative expression (ordinate) is defined as the mean TGFβ-1 (pg/mg protein; Fig 2) value divided by the mean MMP-9/GAPDH (Fig 3) value for each condition. * and *** indicate $p < 0.05$ and $p < 0.001$ comparing mdx and nondystrophic preparations. τττ indicates $p < 0.001$ comparing indicated costal diaphragm to gastrocnemius muscle preparations. Shown are the means and standard errors (determined from the standard errors for the mean TGF-β1 and MMP-9 values). N is the smallest of the number of preparations (mice) used in each condition for the TGF-β1 (Fig. 2) and MMP-9 (Fig. 3) determinations. Black histobars – nondystrophic; white histobars – mdx.

Figure 5.

Hydroxyproline contents in the respiratory musculature of developing nondystrophic (A1 to A5) and mdx mice (B1 to B5). A1, B1 – Age is 15 days postnatal; A2, B2- 30 days; A3, B3- 60 days; A4, B4- 90 days, A5, B5- 120 days. A significant (ANOVA; $p < 0.05$) effect of muscle type on hydroxyproline content was observed in nondystrophic muscle at 15, 30, 60 and 120 days and in mdx muscle at 15, 60, 90, and 120 days. *, **, and *** with brackets indicate specific comparisons that showed significant (Kruskal-Wallis) differences at $p < 0.05$, 0.01, and 0.001 respectively. σ, σσ, and σσσ indicate significant elevations in hydroxyproline in mdx muscles at $p < 0.05$, 0.01, and 0.001, respectively. The N value for each condition represents the number of mice, except for the nondystrophic and *mdx* mouse TS muscle at 15 days (A1,

B1). At this age, the TS muscles from several individual mice were pooled in order to obtain a sufficient quantity of sample for the hydroxyproline assay and N refers to the number of samples assayed, the number of mice.

Figure 6.

Hydroxyproline contents increase with age in neonatal and young adult mdx (B), but not in nondystrophic (A) mice. Shown are the means and standard errors at 15, 30, 60, 90, and 120 days of age. *** indicates a significant effect ($p < 0.001$) of age on hydroxyproline content.

Figure 7.

Daily treatment of mdx mice with two distinct NF- κ B inhibitors, PDTC (A,B,C) and UDCA (D) does not alter collagen accumulation in the respiratory musculature. Shown are the means and standard errors. N represents the number of muscle preparations and mice (i.e., one muscle preparation per mouse). (A) Long term (3.5 months) daily treatment with PDTC in mature mdx mice (11.5 months at start of treatment) does not alter hydroxyproline content in either the costal (A1) or crural (A2) diaphragm. Mice were injected (ip) with either HEPES Ringer (vehicle) or 50mg/kg PDTC in HEPES Ringer. (B) One month daily treatment of 30 day old mdx mice with PDTC does not alter hydroxyproline content in the costal (B1) or crural (B2) diaphragm, or the TS (B3) muscle. Hydroxyproline content was determined at 2 months of age. (C) Point counting determinations of collagen accumulation in TS muscles (Masson's Trichrome stain) indicate substantial collagen accumulation in all three regions of mdx TS muscle. Preparations were obtained from mature nondystrophic and mdx mice treated with PDTC or vehicle as described in (A). (C1) Nondystrophic TS. (C2) TS from vehicle-injected mdx mice. (C3) TS from PDTC treated mdx mice. *** indicates a significant ($p < 0.001$) increase above levels seen in corresponding nondystrophic TS muscle regions. σ indicates a significant ($p < 0.005$) reduction in the fraction of collagen in the caudal region of the PDTC treated preparations. (D) One month daily treatment of 30 day old mdx mice with UDCA (40 mg/kg in isotonic saline vehicle, ip) does not alter hydroxyproline content in the mdx costal diaphragm.