Increased caveolin-3 levels in mdx mouse muscles

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Abstract The density of skeletal muscle caveolae is increased in Duchenne muscular dystrophy and its genetic homologue, the mdx mouse. This structural change is significant as it may indicate muscle regeneration. We identified in mdx mouse tibialis anterior muscles significantly increased levels of caveolin-3, the chief protein in muscle caveolae, and reduced levels of neuronal nitric oxide synthase, an enzyme regulated by caveolin-3. Similar changes occurred in the corresponding mRNA levels. These data suggest that induction of caveolin-3 occurs and this may at least partly be responsible for increased number of caveolae, altered nNOS-caveolin cycle, and regeneration of dystrophic muscles.

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Key words: Muscular dystrophy; Duchenne; Mdx mouse; Caveolin; Nitric oxide

1. Introduction

Caveolins, 21–24 kDa proteins, are the chief structural components of caveolae, distinct cholesterol- and sphingolipid-rich vesicular invaginations of the plasma membranes [1]. Caveolins form homo-oligomeric complexes with each other. They also bind and regulate several different signaling molecules such as calcium-dependent nitric oxide synthases, G-proteins, Ha-Ras and Src family of tyrosine kinases [1,2].

Caveolin-3 is a newly identified member of the caveolin family of proteins. It is specifically expressed in skeletal, cardiac and smooth muscles [3,4]. In skeletal muscles, caveolin-3 binds and regulates neuronal nitric oxide synthase (nNOS) [5,6] and phosphofructokinase M, a key regulatory enzyme of glycolytic pathway [7]. Caveolin-3 is transiently associated with T-tubules during development [8]. Caveolin-3 is also associated with dystrophin [4], a protein that is mutated or absent in Duchenne muscular dystrophy (DMD). Defects in the expression of dystrophin-associated proteins cause the various forms of muscular dystrophies [9]. A recent study shows that mutated caveolin-3 is responsible for the development of a specific form of limb-girdle muscular dystrophy [10].

Morphological studies have identified a characteristic increase in the number of caveolae in DMD [11] and mdx mice [12]. We have studied the expressions of caveolin-3 and nNOS in mdx mice, animals that do not express dystrophin and are the genetic homologues of DMD [13]. While the expression of nNOS was reduced, caveolin-3 levels increased in dystrophic muscles that successfully compensate for the lack of dystrophin. These data suggest that caveolin-3 expression is induced and this, just like induction of utrophin [14], may aid muscle regeneration.

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2. Materials and methods

2.1. Laboratory animals

Control (C57BL/10SnJ) and *mdx* (C57BL/10ScSn Dmd^{mdx}) mice were purchased from Jackson Laboratories and were use at 1 year of age. Skeletal muscles were dissected, weighed, rapidly frozen and stored at −80°C until use.

2.2. NOS activity and Western blots

NOS activity was determined by the citrulline assay [15]. Caveolin-3 and nNOS proteins were detected by Western blot analysis as described before [15] using a variety of polyclonal (C38330, N31030, N53130) and monoclonal (C38320, N31020) antibodies from Transduction Laboratories, Lexington, KY. Quantitative analysis of Western blots was performed by Kodak BioMax 1D Image Analysis Software.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated according to Chomczynski and Sacchi [16]. Three μg total RNA was reverse transcribed with Superscript Preamplification system (Gibco BRL) according to the manufacturer's specifications. PCR amplifications were carried out using the following parameters: 5 min denaturation at 94°C, 2 min annealing at 62°C, followed by 35 cycles of 1 min at 72°C, 40 s at 94°C and 40 s at 62°C, with a final extension of 10 min at 72°C. For PCR amplification of a 271 bp nNOS and a 373 bp nNOSμ products the following primer pair was used: CAC CAG CAC CTT TGG CAA TGG AG (sense) and AAA GGC ACA GAA GTG GGG GTA (antisense). For PCR amplification of a 447 bp caveolin-3 product the primers were: ATG ATG ACC GAA GAG CAC ACG G (sense) and CCT TCG CAG CAC CAC CTT AAT G (antisense). A β-actin primer set for RT-PCR was purchased from Stratagene (Cat. #302110).

2.4. Northern blot analysis

Total RNA (30 μg) was separated on a 1% agarose gel, transferred to a nylon membrane, air dried, pre-hybridized for 20 min at 65°C in Rapid-hyb buffer (Amersham) and then incubated for 3 h at 65°C with [α-^{32}P]dCTP-labeled probes (mouse nNOS, mouse caveolin-3 and mouse β-actin PCR fragments). After extensive washing, autoradiography was performed at −80°C for 16 h.

3. Results

We have determined the expression of nNOS and caveolin-3 proteins by Western blot analysis in tibialis anterior (TA) muscles of mdx mice. In agreement with previous studies [17], our data indicate that the nNOS levels are greatly reduced in both the membrane (Fig. 1A) and the soluble fractions of mdx muscles (Fig. 1B). A second probe of the same blots with an anti-caveolin-3 monoclonal antibody revealed the presence of caveolin-3 protein in the membrane fractions of control TA muscles (Fig. 1A), and to a lesser extent in the soluble fractions of the same muscles (Fig. 1B). Identical results were obtained with an anti-caveolin-3 polyclonal antibody (not shown). It became also apparent from these studies that much more caveolin-3 protein is present in both the membrane and soluble fractions of mdx TA muscles (Fig. 1A,B). These studies suggested that the expression of nNOS and caveolin-3 may change reciprocally in mdx muscles. Ver-

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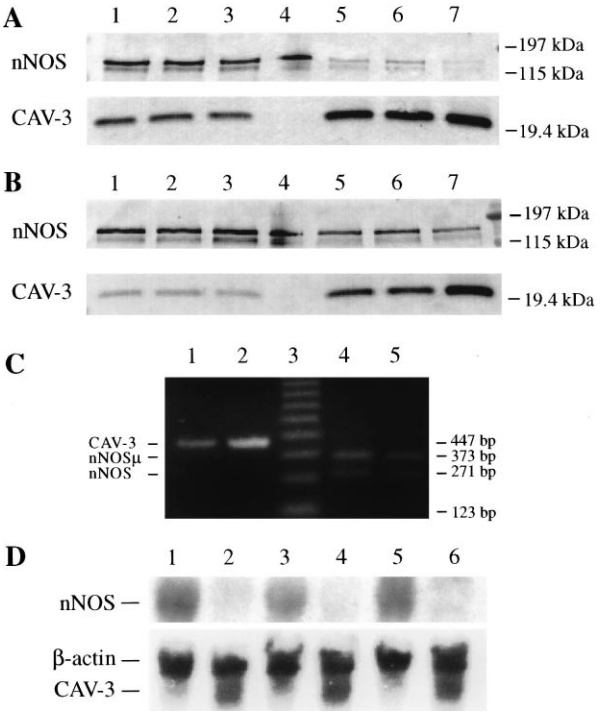

Fig. 1. nNOS and caveolin-3 protein and mRNA levels in mdx mouse muscles. A: Western blot analysis of nNOS and caveolin-3 protein levels in the membrane fraction of control and mdx mouse TA muscles. The nNOS proteins were detected with an anti-brain NOS monoclonal antibody (Transduction Laboratory, Lexington, KY) and caveolin-3 protein was detected with an anti-caveolin-3 monoclonal antibody (Transduction Laboratory, Lexington, KY). This antibody (clone 26) was originally produced and characterized by Song et al. [4]. Lanes 1–3, control TA membranes from three animals; lane 4, nNOS standard; lanes 5–7, mdx TA membranes from three animals. B: Western blot analysis of nNOS and caveolin-3 protein levels in the soluble fraction of control and mdx mouse TA muscles. Detections as described in A. Lanes 1–3, control TA; lane 4, nNOS standard; lanes 5–7, mdx TA. C: cDNA fragments of caveolin-3 and nNOS amplified by PCR after reverse transcription of TA mRNA. Lanes 1 and 2, 447 bp caveolin-3 cDNA fragment amplified from control (lane 1), and mdx (lane 2) muscles. Lane 3, 123 bp ladder. Lanes 4 and 5, 271 bp nNOS and 373 bp nNOSμ cDNA fragments amplified from control (lane 4) and mdx (lane 5) muscles. D: Northern blot analysis of nNOS and caveolin-3 mRNA levels in control (lanes 1, 3 and 5) and mdx (lanes 2, 4 and 6) mouse leg muscles.

ification of this finding required unequivocal identification and quantitation of these proteins.

We consistently identified in Western blots two protein bands (160 and 164 kDa) that specifically reacted with an anti-brain NOS monoclonal antibody (Fig. 1A,B) and two other anti-brain NOS polyclonal antibodies (data not shown). Previous studies of nNOS expression in mdx mice did not describe the simultaneous expression of two nNOS isoforms [17]. Nevertheless, the predominant expression of an alternatively spliced muscle specific nNOS isoform, nNOSμ that is approximately 4 kDa larger than nNOS, is well established [18]. Since the molecular masses of 160 and 164 kDa of the proteins that reacted with anti-brain NOS antibodies corresponded to the expected sizes of nNOS and nNOSμ, respectively, the possibility that these two nNOS isoforms are co-expressed was further investigated by RT-PCR.

The muscle specific nNOSμ is different from nNOS in that it contains a 102 bp insert between amino acid residues 839 and 840 [18]. This results in the expression of a nNOSμ protein that is 34 amino acid residues larger than nNOS. We used a pair of PCR primers to amplify the cDNA fragment that includes this alternatively spliced segment. This primer pair was expected to amplify a 271 bp cDNA fragment of nNOS and a 373 bp cDNA fragment of nNOSμ simultaneously if both mRNA are present together. Fig. 1C shows that this was, in fact, the case. This one set of primer pair amplified the expected two cDNA fragments in both control and mdx

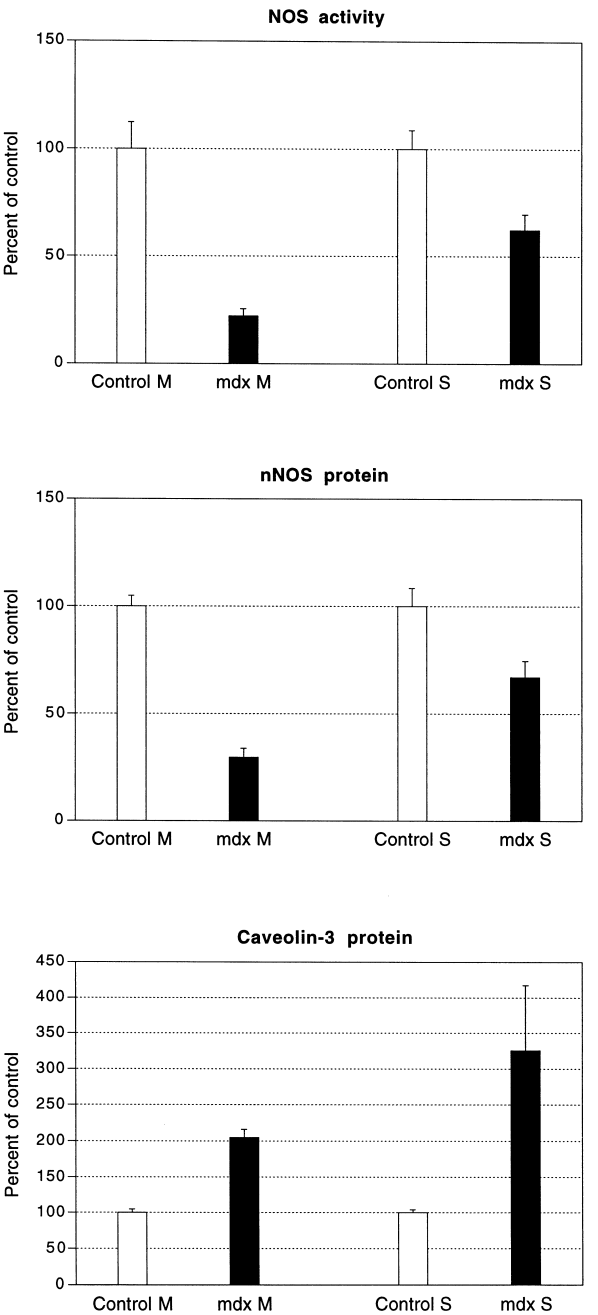

Fig. 2. Quantitative changes in NOS activity, nNOS protein and caveolin-3 protein levels in mdx mouse TA muscles. Top panel: NOS activity. Middle panel: nNOS proteins. The data represent the sum of two proteins, nNOS and nNOSμ. Lower panel: Caveolin-3 protein. All data were normalized to show percentages relative to controls (100%). Abbreviations: M, membrane fraction; S, soluble fraction. Statistics: mean values ± S.E.M., $n = 6$.

muscles, although the intensity of these bands was much stronger in controls than dystrophic muscles. Thus, nNOS and nNOSμ mRNAs are co-expressed in control muscles and the expressions of both of these messages are reduced in dystrophic muscles. We also investigated the expression of caveolin-3 mRNA in control and mdx muscles. RT-PCR amplification of a 447 bp caveolin-3 cDNA fragment revealed the presence of significantly increased caveolin-3 mRNA in mdx TA relative to control (Fig. 1C).

Northern blot analysis of mRNA levels in mixed leg muscles verified these findings. The mdx muscles that contained low nNOS mRNA also contained significantly increased caveolin-3 mRNA (Fig. 1D). The β-actin mRNA levels in Fig. 1D are shown to indicate equal loading.

To correlate Western blot data with the expression of functionally intact proteins, it was necessary to determine the NOS activity. TA homogenates were separated to membrane and soluble fractions by centrifugation at $105\,000 \times g$ for 1 h. The enzyme assays were performed at nearly saturating substrate concentrations (20 μM L-arginine). The mean NOS activities (pmol/mg/min) of six animals ± S.E.M. were as follows: control membranes 32.2 ± 4.03; control supernatants 35.4 ± 1.28; mdx membranes, 6.8 ± 1.20; mdx supernatants, 20.8 ± 2.98.

These data show that the NOS activity is significantly reduced in the membrane and the soluble fractions of mdx mouse TA muscles.

Since we assayed the same samples for NOS activity and for NOS protein levels, it was possible to determine if the changes in enzyme activity correlate with the changes in protein levels. Fig. 2 shows that the changes in mdx TA NOS activity correlate with the changes in NOS proteins. This suggests that the reduced enzyme activity in mdx muscles is due to the reduced enzyme levels. Fig. 2 also shows that nNOS and caveolin-3 protein levels change in the opposite way in the same mdx muscles.

4. Discussion

Our data showing increased caveolin-3 mRNA and protein levels in mdx mouse muscles are in agreement with the increased number of caveolae in dystrophin-deficient muscles [11,12]. We suggest that the induction of caveolin-3 could at least partly be responsible for this structural change.

Recent studies have determined that the activities of calcium-dependent NOS isoforms are regulated not only by calcium and calmodulin but also by caveolins. While binding of the calcium-calmodulin complex activates these enzymes, the binding of caveolins inhibits them [2,5,6,19]. This suggests the operation of a NOS-caveolin cycle that involves reversible binding and inhibition of NOS with caveolin depending on the cytosolic calcium concentrations [2,19]. The reciprocal change in nNOS and caveolin-3 may have an effect on the NOS-caveolin cycle in dystrophic muscles where cytosolic calcium is known to be increased.

Dystrophin deficiency results in the depletion of several associated proteins [9] and nNOS appears to be one of them [20]. In contrast to humans where regeneration is inefficient, the regeneration is extensive and efficient in mdx mice and this results in a mild phenotype [13]. The successful regeneration in the absence of dystrophin could be due to induction of utrophin expression in mdx mice [14,21–24]. The induction of caveolin-3 in mdx mouse muscles may also be an indication of differentiation and regeneration. This hypothesis is in agreement with data showing that caveolin-3 is substantially induced during differentiation of skeletal muscle myoblasts in culture [4] and that the density of caveolae is preferentially increased in regenerating muscles [12].

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