No alteration in gene expression of components of the ubiquitin-proteasome proteolytic pathway in dystrophin-deficient muscles

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Abstract Increased expression of critical components of the ubiquitin-dependent proteolytic pathway occurs in any muscle wasting condition so far studied in rodents where proteolysis rises. We have recently reported similar adaptations in head trauma patients [Mansoor et al. (1996) Proc. Natl. Acad. Sci. USA 93, 2714–2718]. We demonstrate here that the increased muscle protein breakdown seen in mdx mice only correlated with enhanced expression of m-calpain, a Ca\textsuperscript{2+}-activated proteinase. By contrast, no change in mRNA levels for components of the ubiquitin-proteasome proteolytic process was seen in muscles from both mdx mice and Duchenne muscular dystrophy patients. Thus, gene expression of components of this pathway is not regulated in the chronic wasting that characterizes muscular dystrophy.

Key words: Skeletal muscle; Protein turnover; Ubiquitin; Proteasome; Calpain; Muscular dystrophy

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common sex-linked lethal disease in man, affecting approximately 1 in 3500 boys and resulting in a fatal evolution before the end of the third decade of life [1]. This disease is characterized by the lack of detectable dystrophin, a very large (400 kDa) cytoskeletal protein [2,3]. In DMD patients and in mdx mice, the animal model that reproduces DMD, there is a severe muscle wasting. This wasting results from enhanced protein breakdown in mdx mice [4,5].

Like all mammalian tissues, skeletal muscle contains multiple proteolytic systems. The best known system is the lysosomal pathway, which involves four major proteinases in muscle (cathepsins B, H, L and D). Increased specific activities of cathepsin B [6–8], H [8], L [7,8], and D [6] have been reported in dystrophic skeletal muscle. However, these increased activities of cathepsins were related to the infiltration of non-muscle cells in both dystrophic hamsters [7] and mdx mice [8], although some cathepsin L was co-localized in muscle cells as well as in invading macrophages [8]. Skeletal muscle also contains two major cytosolic proteolytic pathways, Ca\textsuperscript{2+}-dependent [9] and ATP-ubiquitin-dependent [10], respectively. Turner et al. [4] showed that the elevation of the concentration of intracellular Ca\textsuperscript{2+} in muscle fibers from young mdx mice resulted in an enhanced protein breakdown. Furthermore, MacLennan et al. [5] reported that the increased protein breakdown seen in mdx muscle was attributable to a non-lysosomal Ca\textsuperscript{2+}-activated proteolytic process. Recent data, however, demonstrated that both cathepsins and calpains (i.e., the Ca\textsuperscript{2+}-activated proteinases) do not contribute significantly to the overall muscle protein breakdown [11–15], and do not play an important role in the breakdown of actin and myosins [16–18]. By contrast, the ATP-ubiquitin-dependent proteolytic pathway which was previously believed to degrade short-lived and abnormal proteins [19,20] is responsible for the bulk of muscle protein breakdown [11–15,21–25] including the degradation of the long-lived contractile proteins [17,18].

Increased mRNA levels for critical components of the ubiquitin-proteasome-dependent proteolytic pathway have been systematically observed in various animal models of muscle wasting, such as fasting [11,25], denervation atrophy [25], cancer [13,21,22], acidosis [12], sepsis [14,18], simulated weightlessness [15], burn injury [26], and glucocorticoid administration [11,23]. We have also recently shown that similar adaptations prevailed in muscle biopsies from head trauma patients who exhibited negative nitrogen balance, increased whole body rate of protein breakdown, and enhanced urinary 3-methylhistidine excretion [24]. A concomitant stimulation of the ubiquitin-proteasome pathway with either the Ca\textsuperscript{2+}-dependent [13] or the lysosomal process [11,22] or both [14,15,17,24] seems to prevail in some of these catabolic states.

To our knowledge, the role of the ATP-ubiquitin-dependent proteolytic process has not been studied in muscular dystrophies, although an accumulation of ubiquitylated proteins was observed in muscle biopsies from patients with oculopharyngeal muscular dystrophy [27]. The present experiments were undertaken to determine (i) whether the ATP-ubiquitin-dependent proteolytic process is activated in muscular dystrophy, and (ii) whether a coordinate activation of the lysosomal and Ca\textsuperscript{2+}-activated proteinases prevails in such conditions. To address this question we measured mRNA levels for cathepsins D and L, m-calpain, ubiquitin, 14-kDa ubiquitin conjugating enzyme E2 (14-kDa E2), and subunits of the 20S proteasome in muscles from mdx mice and in muscle biopsies obtained from control and dystrophin-deficient patients.

2. Materials and methods

2.1. Animals

Mice of the mdx strain were kindly provided by Dr. J.-L. Guénét (Institut Pasteur, Paris, France). They were compared to C57Bl/6 control mice obtained from Ilfa Credo (L’Arbresle, France). The mice were maintained in a temperature-controlled room (22±1°C) on a 12-h light-dark cycle. They were fed ad libitum with a standard

Abbreviations: 14-kDa E2, 14-kDa ubiquitin conjugating enzyme E2; DMD, Duchenne muscular dystrophy

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2.2. Rates of protein turnover

Animals were killed by cervical dislocation at 5 weeks of age. Extensor digitorum longus muscles were carefully dissected and incubated at approximately resting length by pinning their tendons on plastic supports. All tissues were incubated at 37°C in Krebs-Henseleit buffer (NaCl 120 mM, KCl 4.8 mM, NaHCO₃ 25 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM and MgSO₄ 1.2 mM, pH 7.4) equilibrated with 95% O₂ and 5% CO₂, and containing 5 mM glucose, 0.1 U/ml insulin, 0.17 mM leucine, 0.10 mM isoleucine, and 0.20 mM valine to improve protein balance [28]. After 1 h of preincubation, muscles were transferred to a fresh medium of identical composition and further incubated for 2 h.

The rate of protein synthesis was determined by incubating muscles in a medium containing 0.5 mM [L-¹⁴C]phenylalanine (Amersham Corp., Amersham, UK; specific radioactivity in the medium 500 dpm/nmol), as previously described [13]. Tissues were homogenized in 10% trichloroacetic acid (TCA) and hydrolyzed in 1 N NaOH at 37°C. Tissue protein mass was determined using the biuret chemicinonic acid procedure [29]. Rates of phenylalanine incorporation were converted into tyrosine equivalents as described [28], and expressed in nmol tyrosine incorporated/mg protein/h.

Rates of protein breakdown were measured by following the rates of tyrosine release into the medium. Since muscle neither synthesizes nor degrades this amino acid, tyrosine release reflects the net breakdown of proteins. Thus, rates of total protein degradation were calculated by adding the rate of protein synthesis and the net rate of tyrosine release into the medium [28]. Tyrosine was assayed by the fluorometric method of Waalkes and Udenfriend [30].

2.3. Human muscle biopsies

Muscle biopsies were performed during orthopedic surgery, in the Department of Pediatric Surgery of the Hôpital Dieu Hospital in Clermont-Ferrand. The protocols were approved by an Ethical Committee (Consultative Committee for the Protection of Persons Undergoing Biomedical Research, Région d'Auvergne).

The characteristics of the patients and of the muscles sampled are given in Table 1. Five DMD patients presenting a lack of dystrophin were wheelchair bound, except the youngest one. All DMD patients were dystrophin (-). Control subjects were dystrophin (+) and not suffering from any neuromuscular pathology, and presented no abnormality in terms of muscle structure or fiber distribution. The age of the DMD patients studied ranged between 7 and 21 years. All DMD patients were wheelchair bound, except the youngest one.

2.4. Northern blot analysis

Tibialis anterior muscles from control and mdx mice and human muscle biopsies were rapidly excised, frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted as described by Chomczynski and Sacchi [31]. 20 μg of total RNA was electrophoresed in 1% agarose gel containing formaldehyde. RNA was electrophoretically transferred to a nylon membrane (GeneScreen, NEN Research Products, Boston, MA, USA) and covalently bound to the membrane following UV crosslinking. The membranes were hybridized with cDNA probes encoding chicken polyubiquitin [32], rat 14-kDa E2 [33], the rat [34] or human [35] C2 proteasome subunit, human cathepsin D [36], and human m-calpain [37]. The hybridizations were performed at 65°C with [³²P]cDNA fragments labelled by random priming. Following washes at the same temperature, the membranes were autoradiographed for 3-96 h at −80°C with intensifying screens on Hyperfilm-MP (Amersham, Little Chalfont, UK).

The characteristics of the patients and of the muscles sampled are given in Table 1. Five DMD patients presenting a lack of dystrophin were wheelchair bound, except the youngest one. All DMD patients were dystrophin (-). Control subjects were dystrophin (+) and not suffering from any neuromuscular pathology, and presented no abnormality in terms of muscle structure or fiber distribution. The age of the DMD patients studied ranged between 7 and 21 years. All DMD patients were wheelchair bound, except the youngest one.

Table 1

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>Muscle</th>
<th>Diagnosis</th>
<th>Dystrophin</th>
<th>Fiber necrosis and fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>M</td>
<td>deltoid</td>
<td>DMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>triceps surae</td>
<td>DMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>hip adductor</td>
<td>DMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>tensor fasciae latae</td>
<td>DMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>deltoid</td>
<td>DMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>deltoid</td>
<td>psychosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>triceps surae</td>
<td>ttopes walker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>piformis</td>
<td>sciatic compression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>hip adductor</td>
<td>cerebral palsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>hip adductor</td>
<td>femoral anteversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>paravertebral</td>
<td>kyphosis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M: male; F: female. The degree of fiber necrosis and fibrosis was evaluated by histoenzymatic analysis and classified as absent (−), moderate (+), intermediate (+++) or severe (+++). All DMD patients were dystrophin (−). Control subjects were dystrophin (+) and fiber necrosis and fibrosis (−).

2.5. Statistical analysis

All data are expressed as means±S.E.M. The unpaired Student’s t-test was used for statistical analyses. Significance was defined at the 0.05 level.

3. Results and discussion

Recent lines of evidence strongly suggest that the ATP-ubiquitin-dependent proteolytic pathway plays a major role in skeletal muscle. First, in several instances of muscle atrophy, there is no evidence for an activation of either the lysosomal [13,21] or the Ca²⁺-dependent [11,22] proteolytic process. Secondly, cathepsins and calpains only account for a minor part of overall protein breakdown in muscles from both control and cachectic animals where protein breakdown rises [11–15]. Thirdly, both lysosomal and Ca²⁺-activated proteinases do not degrade myofibrillar proteins [16–18]. By contrast, in any catabolic state so far studied in rodents [11–15,18,21,22,26], it has been reported that the bulk of increased protein breakdown is due to the activation of a non-lysosomal and Ca²⁺-independent process. This process is ATP-dependent [11–13,18,22,26], and increased expression of ubiquitin [11–15,18,21–26], 14-kDa E2 involved in the ubiquitylation of protein substrates [13–15,23,24,33], and subunits of the 20S proteasome [12–15,22–25], which is the proteolytic core of the 26S proteasome that degrades ubiquitin conjugates, systematically paralleled the changes in protein breakdown measured in incubated muscles. Furthermore, since the break-
Values are means ± S.E.M. for six animals. Protein turnover data are expressed in nmol tyrosine/mg protein/h (for more details see Section 2).

Table 2
Muscle/body weight ratios and protein turnover in the extensor digitorum longus (EDL) of control and mdx mice

<table>
<thead>
<tr>
<th>Muscle mass/body weight ratio</th>
<th>Protein synthesis</th>
<th>Total protein breakdown</th>
<th>Net protein breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibialis anterior</td>
<td>EDL</td>
<td>Control mice</td>
<td>1.551 ± 0.049</td>
</tr>
<tr>
<td>mdx mice</td>
<td>1.165 ± 0.169*</td>
<td>0.378 ± 0.022**</td>
<td>0.427 ± 0.057**</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.386</td>
<td>-0.091</td>
<td>+0.216</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for six animals. Protein turnover data are expressed in nmol tyrosine/mg protein/h (for more details see Section 2). *P < 0.05, **P < 0.005 vs. controls.
However, in human muscle biopsies, we observed no significant variation in mRNA levels for m-calpain (Fig. 2B) in accordance with recent observations by Sakamoto et al. [43]. The precise significance of changes in mRNA levels for Ca$^{2+}$-activated proteinases in different muscle wasting conditions is still unclear. Enhanced expression of m-calpain was associated with increased enzyme activity in the atrophying unweighted soleus muscle [15], suggesting a transcriptional regulation. By contrast, no change in μ- and m-calpain activity occurred in the muscles of fasted rabbits, although the mRNA levels for μ- and m-calpain were elevated [44]. Furthermore, a third calpain, p94, that is expressed abundantly only in skeletal muscle, has been characterized [45]. Mutations in p94 cause limb-girdle muscular dystrophy type 2A [46]. There is some evidence that p94 was expressed in proportion of dystrophin, whose absence causes DMD [43]. Interestingly, the expression of p94 was also down-regulated in the atrophying muscles from interleukin-6 transgenic mice [47]. Thus, muscle atrophy seems to be associated with decreased p94 mRNA levels.

Fig. 2. Quantification of mRNA levels for m-calpain in skeletal muscle from mdx mice (A) and DMD patients (B). Open bars, control muscles; black bars, dystrophic muscles. RNA was extracted, electrophoresed, transferred to a nylon membrane and hybridized with a $^{32}$P-labeled cDNA (m-calpain) or a $^{32}$P-labeled riboprobe (m-calpain), as described in Section 2. After stripping of the probes, blots were rehybridized with an 18S ribosomal oligonucleotide. Autoradiographic signals are expressed as a percentage of controls corrected for 18S rRNA abundance, to take into account slight variations in RNA loading. Data are means ± S.E.M. for n = 6 mice and 5-6 patients. Representative Northern blots are also shown.

Finally, MacLennan et al. [5] demonstrated that enhanced muscle proteolysis in mdx muscle was attenuated by inhibitors of thiol proteases (e.g. leupeptin and E-64 which inhibit both the calpains and cathepsins B, H, and L), but not by the weak base methylamine, which only affects lysosomal protein breakdown. The lack of significant variations in mRNA levels for both cathepsins D and L in mdx muscle supported these observations (Fig. 3). Alternatively, these data may suggest that the possible involvement of cathepsins in elevated protein breakdown during muscular dystrophy [6-8] did not reflect a transcriptional regulation.

In conclusion, we have shown that in contrast with all other muscle wasting conditions so far studied, there is no detectable activation of the ubiquitin-proteasome proteolytic pathway in dystrophic muscle from mdx mice and DMD patients. We suggest that the lack of change in mRNA levels for critical components of this pathway resulted from the very progressive changes in muscle mass that characterize muscular dystrophies. Alternatively, some mechanisms may prevent sustained increased expression of proteolytic genes in muscular dystrophy, to avoid excessive and rapid muscle wasting. In contrast, the increased mRNA levels for m-calpain in mdx muscle strongly support a selective activation of the Ca$^{2+}$-dependent proteinases. These proteinases may selectively degrade soluble or membrane proteins. For example, they could be responsible for the dramatic loss of all the components of the dystrophin-glycoprotein complex which is characteristic of dystrophin deficiency [48].

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