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Pathological changes in levels of three small stress proteins, αB crystallin, HSP 27 and P20, in the hindlimb muscles of *dy* mouse

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Abstract

Using three different analyses, we investigated the levels of the three small stress proteins αB crystallin, HSP 27 and p20 in the slow-twitch soleus muscle and fast-twitch tibialis anterior muscle of normal and *dy* mice. All of these analyses (immunoassay, Western blot and immunohistochemistry) showed markedly increased levels of these stress proteins in fast-twitch type muscle (tibialis anterior muscle) of *dy* mouse. In contrast, the levels of αB crystallin, HSP 27 and p20 of *dy* mouse were reduced in slow-twitch type muscle (soleus muscle). Manipulation of this protective response may reduce injury and may have potential therapeutic application in congenital muscular dystrophy (CMD), which possesses a deficiency of laminin-α2 chain in muscle fiber basement similar to *dy* mouse. © 1998 Elsevier Science B.V.

Keywords: Muscular dystrophy; *dy* mouse; αB crystallin; HSP 27; p20

1. Introduction

The presence of stress- or heat-inducible proteins (HSP) has been recognized in a multitude of organisms. Among several families of HSP, the small or low molecular weight HSP are a diverse group of proteins with molecular masses ranging from 15 to 30 kDa. αB crystallin, a major structural protein of the vertebrate lens, is also expressed in various non-lenticular tissues [1,2] and is also a small HSP [3]. Both αB crystallin and HSP 27 share considerable sequence and structural similarity [4], associate in vivo [5,6], and are co-induced in response to heat and oxidative stress [7,8]. A 20-kDa protein (p20) was originally obtained as a by-product during the purification of human HSP 28 and αB crystallin, and this protein is comprised of amino acid sequences that are highly similar to those of αB crystallin and HSP 28 [9]. Although p20 was detected in various tissues, in particular in the slow-twitch soleus muscle, as were both αB crystallin [2] and HSP 27 [3], p20 is not inducible in response to various types of stress, and the function of this protein has not as yet been determined.

In the muscle of the patients of Duchenne muscular dystrophy (DMD), the up-regulation of HSP 90 and ubiquitin in regenerating fibers and ubiquitin in necrotic fibers in addition to increased levels of HSP

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70 in hypercontracted fibers were reported [10]. Although the locations of the mutated genes differ in *DMD* (Xp21) and *dy* (chromosome 10), the muscles of the *dy* mouse present abundant connective tissue infiltration and pronounced structural disorganization, ultimately leading to complete impairment of muscle function [11] similar to the muscles of DMD patients. In addition, the evidence points toward the loss of intracellular calcium homeostasis as the cause of the cellular degeneration in both DMD patients [12–14] and *dy* mice [15,16]. Although calcium alone is probably not involved in HSP induction, it may potentiate oxidative damage [17] and activate proteases [18].

Like HSP 27, αB crystallin has also been shown in vitro to prevent aggregation of denatured proteins in response to stress and to facilitate protein refolding upon removal of the stress, confirming its status as a molecular chaperone [19,20]. If small HSP act as a molecular chaperone under stressful condition in dystrophic muscles, it is probable that the changes of their levels parallel the demand of the muscles.

The purpose of the present study was to investigate whether changes in the levels of small stress proteins are observed in *dy* mouse in reaction to the metabolic stress characteristics of the disease. We report markedly increased levels of αB-crystallin, HSP 27 and p20 in fast-twitch type muscle (tibialis anterior) with reduction of these levels in slow-twitch type muscle (soleus).

2. Materials and methods

2.1. Animals and sample preparation

Mice of the strain C57BL/6J were used. *dy* mice were obtained by mating heterozygotes (*dy*/+: the autosomal gene *dy* is recessive). These mice first manifest the distinct symptom of dragging their hindlegs from about 2 weeks of age. For the immunoassay of each HSP, mice aged 3, 6, 9 and 12 weeks were studied. Adult mice (12 weeks old) were used for the Western blot and immunohistochemical analyses. The mice were killed, and the bilateral soleus and tibialis anterior muscles were sampled. All samples were kept frozen at −80°.

Frozen tissues were homogenized at 0°C in 10–50 volumes of 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM EDTA, and after being sonicated, each homogenate was centrifuged at 4°C at 125,000 × *g* for 20 min. The supernatants were kept frozen at −80°C for a few days until analyses for immunoassay and Western blot analysis.

2.2. Immunoassays of αB crystallin, HSP 27 and p20

Concentrations of αB-crystallin [2], HSP 27 [3] and p20 [9] in extracts of cells were determined by specific immunoassays as described previously. Because the assays were performed with the purified rat proteins as standards, the results are expressed as ng rat protein-equivalents.

2.3. Electrophoresis and western blot analysis

Equal amounts of muscle protein (5 μg) in sodium dodecylsulfate (SDS) sample buffer were electrophoresed on 10 or 12.5% SDS polyacrylamide gels according to the method of Laemmli [21]. Western blot analysis was performed as described previously [8] by using affinity-purified antibodies (0.05 μg/ml) raised in rabbits against the carboxy-terminal decapeptide of αB-crystallin [2], against the carboxy-terminal dodecapeptide of mouse HSP 27 [3] or against rat p20 [9], and peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. For detection of HSP 70, a mouse monoclonal antibody specific for the inducible form of HSP 70 (C92F3A-5; StressGen Biotechnologies, Victoria, British Columbia, Canada) and peroxidase-labeled antibodies raised in goat against mouse IgG (Medical and Biological Laboratories, Nagoya Japan) were used. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of a Western blot chemiluminescence reagent (Renaissance, Du Pont-NEN, Boston, MA, USA).

2.4. Immunohistochemistry

Serial 8-μm transverse cryostat sections were mounted on silanized slides (Dako Japan), fixed by cold acetone (4 min), and rinsed with cold (−20°C) phosphate-buffered saline (PBS) for 5 min. To inhibit endogeneous peroxidase, these slides were preincu-

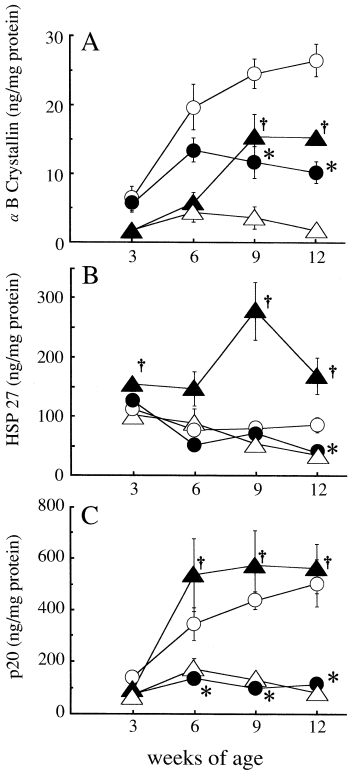

Fig. 1. Changes in the concentrations of αB crystallin, HSP 27 and p20 in muscles of normal and *dy* mice. Concentrations (ng/mg protein) of αB crystallin (A), HSP 27 (B) and p20 (C) in normal soleus muscle (open circles), *dy* soleus muscle (closed circles), normal tibialis anterior muscle (open triangles) and *dy* tibialis anterior muscle (closed triangles) were determined at 3, 6, 9 and 12 weeks of age. Each point shows the mean ± S.E.M. of results obtained in 5 mice. *: $p < 0.05$ compared with normal soleus muscle at the same age. †: $p < 0.05$ compared with normal tibialis anterior muscle at the same age.

bated with 0.3% hydrogen peroxide in absolute methanol for 20 min at room temperature (RT). Sections were blocked with 130 μl of 1.5% horse serum in PBS at RT. The primary antibody to the carboxy-terminal dodecapeptide of mouse HSP 27 [3], to the rat p20 [9] or to the inducible member of the HSP 70 (goat polyclonal anti-mouse HSP 70, Santa Cruz Biotechnology) was applied to the sections (1:50 dilution in PBS) for 60 min at RT. The sections were washed in PBS for 10 min, after which 130 μl of a 1:500 dilution (PBS) of biotinylated goat anti-rabbit (for HSP 27 and p20) and biotinylated rabbit anti-goat (for HSP 70) immunoglobulin G (Vector Laboratories) was applied for 30 min at RT. After they were rinsed in PBS (15 min), the sections were incubated for 30 min with peroxidase streptavidin conjugate (Vector Laboratories). The binding of primary antibodies was visualized with DAB (0.1 mg/ml; Aldrich, Milwaukee, USA) and 0.02% hydrogen peroxide.

2.5. Statistical analysis

All values are expressed as means ± S.E.M. Two-way analysis of variance (ANOVA) was used for determining the main statistical effects. A Scheffe's post-hoc test was conducted if the ANOVA indicated a significant difference. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Developmental profiles of αB crystallin, HSP 27 and P20 in the soleus and tibialis anterior muscles of normal and dy mice

The changes in the levels of the three stress proteins in the slow-twitch soleus muscle and the fast-

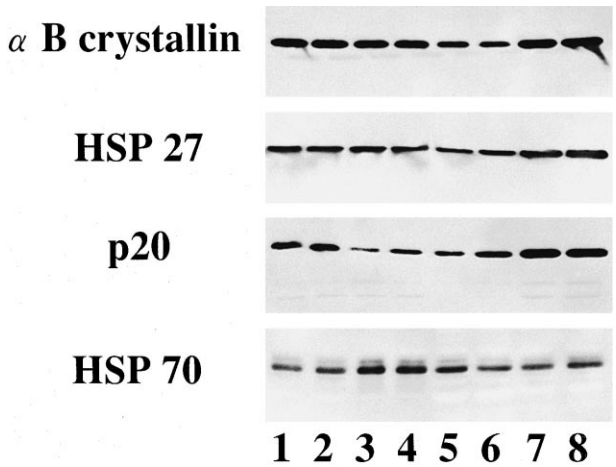

Fig. 2. Western blot analysis of αB crystallin, HSP 27, p20 and HSP 70 in extracts of muscles from normal and *dy* mice. Lanes 1 and 2, normal soleus muscle. Lanes 3 and 4, *dy* soleus muscle. Lanes 5 and 6, normal tibialis anterior muscle. Lanes 7 and 8, *dy* tibialis anterior muscle. Proteins transferred to the nitrocellulose sheet were stained with antibodies against αB crystallin, HSP 27, p20 and HSP 70 and visualized on X-ray film by use of a Western blot chemiluminescence reagent. Ten-microliter aliquots containing 5 μg protein were subjected to SDS-PAGE with subsequent Western blotting.

twitch tibialis anterior muscles were determined in normal and *dy* mice in the age range from 3 to 12 weeks. As shown in Fig. 1A, in the soleus muscle of normal mice, the levels of αB crystallin sharply increased from 3 to 6 weeks of age, and then gradually increased. In contrast, in the normal tibialis anterior muscle, the αB crystallin concentration did not change from 3 to 12 weeks of age. The results for both muscles almost are entirely consistent with the findings for the developmental profile of αB crystallin levels in the rat soleus and fast-twitch (extensor digitorum longus) muscles reported by Inaguma et al. [3].

In the soleus muscle of *dy* mice, the concentration of αB crystallin increased similarly to that in the control mice until postnatal week 6. However, the levels were unaltered thereafter. In the tibialis anterior muscle of *dy* mice, the concentrations of αB crystallin rapidly increased after 6 weeks of age.

The HSP 27 level of the soleus muscle in control mice did not change from 3 to 12 weeks of age, but that in the tibialis anterior muscle decreased gradually (Fig. 1B), as reported for the developmental profiles in rat hindlimb muscles [3]. Between 3 and 9 weeks of age, a similar change of HSP 27 level in the soleus muscle was also observed in the *dy* mice, and thereafter the level in *dy* muscle was significantly lower than that in normal muscle (Fig. 1B). In the tibialis anterior muscle, the HSP 27 level of *dy* mice increased rapidly after 6 weeks of age as did the concentration of αB crystallin.

Fig. 1C shows the developmental change in p20 levels in the normal and *dy* mice. In the normal mice, the p20 level of the soleus muscle sharply

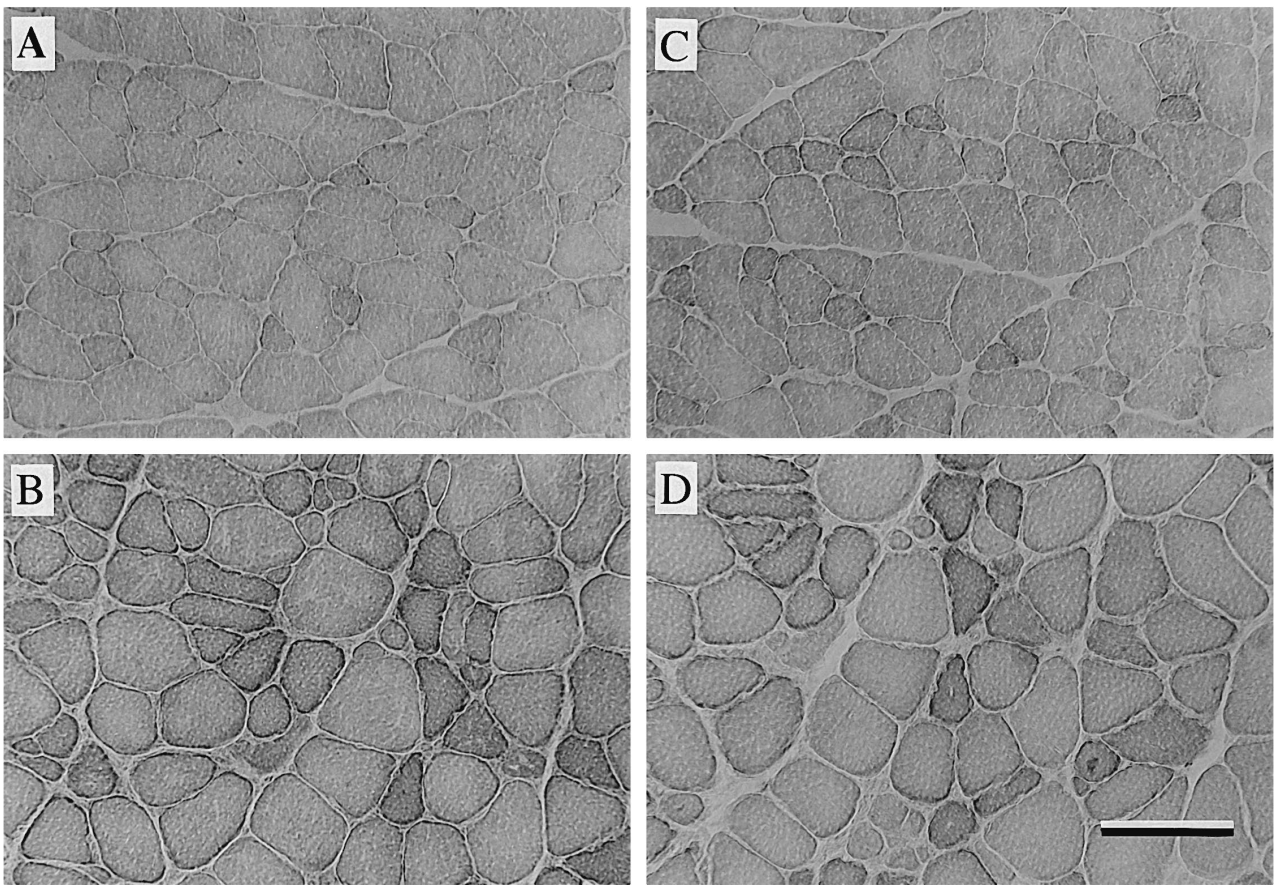

Fig. 3. Immunohistochemical staining of HSP 27 and p20 in sections of tibialis anterior muscle from normal (A and C) and *dy* (B and D) mice. Light photomicrographs showing cryosections stained immunohistochemically for HSP 27 (A and B) and for p20 (C and D). The specificity of staining patterns was confirmed by employing the primary antibodies that had been absorbed with the respective antigen (HSP 27 or p20). Primary antibodies were visualized by a standard avidin–biotin peroxidase method. Bar = 100 μm.

increased from 3 to 6 weeks of age, and then gradually increased. On the other hand, in the tibialis anterior muscle, the p20 level of normal mice did not change during the postnatal period. The developmental profile of p20 in both muscles was very similar to that of αB crystallin. In the soleus muscle of the *dy* mice, no significant changes during growth were found, but the p20 level in the tibialis anterior muscle sharply increased after 3 weeks of age in *dy* mice.

3.2. Western blot analysis of αB crystallin, HSP 27, P20 and HSP 70 in these muscles of normal and dy mice

Immunoblot analysis of whole muscle homogenates was performed to quantify the concentration of HSP 70 and to confirm the results of αB crystallin, HSP 27 and p20 in the obtained by immunoassays. In the tibialis anterior muscle, the levels of αB crystallin, HSP 27, and p20 in the *dy* mice were markedly enhanced compared to those in the normal mice. No difference between the groups was observed for αB crystallin or HSP 27 in the soleus muscle of both mice. (Fig. 2). These results are consistent with the data obtained by the immunoassays. However, the level of p20 in the *dy* soleus muscle was markedly lower than that in the normal muscle (Fig. 2). In contrast, the HSP 70 expression in the *dy* mice was not significantly different from that in the control mice in either the soleus or the tibialis anterior muscle (Fig. 2).

3.3. Immunohistochemical analyses of HSPs in tibialis anterior muscles of adult normal and dy mice

The constitutive expression of small HSP and HSP 70 was previously described in various cell and tissue types [1,22]. We investigated the pattern of HSP expression in normal and *dy* muscles. HSP 27 immunoreactivity was detected at low levels within a few fibers in sections from the normal tibialis anterior muscle (Fig. 3A). In contrast, pronounced immunoreactivity was observed in fibers with small diameter (probably oxidative fibers) in the dystrophic tibialis anterior muscle (Fig. 3B). Similarly to the HSP 27 immunoreactivity, the p20 antibody reactivity in *dy* muscle (Fig. 3D) was increased as compared with normal muscle (Fig. 3C). HSP 70 immunostaining revealed no clear difference in immunoreactivity between the control and *dy* tibialis anterior muscles (data not shown).

4. Discussion

Using three methods of analysis (immunoassay, Western blot and immunohistochemistry), we demonstrated significantly increased levels of αB crystallin, HSP 27 and p20 in the tibialis anterior muscle of *dy* mouse. The increase in the levels of these three HSP in *dy* mice is most likely a reaction to the metabolic stress characteristic of the disorder and may provide clues to the pathophysiology of this myopathy. Contrary to the response of the fast-twitch tibialis anterior muscle, the slow-twitch soleus muscle in *dy* mouse showed reduced levels of αB crystallin, HSP 27 and p20.

In the case of DMD patients, the up-regulation of HSP 90 and ubiquitin in regenerating fibers and of HSP 70 in some hypercontracted fibers was recently identified by immunohistochemical analysis by Bornman et al. [10]. Using three different analyses, we clearly demonstrated that the levels of αB crystallin, HSP 27 and p20 are significantly increased in the tibialis anterior muscle of *dy* mouse. Although the locations of the mutated genes differ in DMD (Xp21) and *dy* (chromosome 10), the muscles of the *dy* mouse present abundant connective tissue infiltration and pronounced structural disorganization [11] similar to the muscles of DMD patients.

In *dy* mouse, the loss of intracellular calcium homeostasis has been reported as the cause of cellular degeneration [15,16]. Although calcium induces other members of the stress protein families, i.e., the glucose regulated proteins [23], in several types of cells including macrophages [24], calcium alone is probably not involved in HSP induction. It may, however, potentiate oxidative damage [17] and activate proteases [18]. It has been suggested that the pathological damage in *dy* mouse may be the result of increased generation of reactive oxygen radicals caused by the absence of laminin-α2 chain [25,26] and subsequent alterations in calcium metabolism. Since co-induction of αB-crystallin and HSP 27 is known to occur in response to heat and oxidative stress [7,8], expression of the three small stress proteins might be

enhanced in fast-twitch tibialis anterior muscle by a common trigger(s) induced under the pathological condition. Furthermore, the small HSP present at increased levels may serve as molecular chaperones in the stressful condition of the muscle of *dy* mouse. Several studies have revealed that αB crystallin and HSP 27 prevent aggregation of denatured proteins induced by stress and facilitate protein refolding upon removal of the stress [19,20].

A second possibility for enhanced levels of these stress proteins would be continuous discharge of the fast-twitch muscle of *dy* mouse. The previous studies revealed that the repetitive high-frequency burst closely resembles the sustained multiple discharge typical of motor nerves of *dy* mice owing to focal amyelination [27,28]. Furthermore, the proposed increased activity of dystrophic muscle seems indirectly supported by the significantly greater glucose uptake of *dy* fast-twitch muscle than that of normal muscle [29]. In fact, continuous motor nerve activation is suggested to markedly increase the levels of αB crystallin and HSP 27 [30].

Contrary to the fast-twitch tibialis anterior muscle, the slow-twitch soleus muscle of *dy* mouse showed marked reduction of αB crystallin, HSP 27 and p20. The soleus muscle has been reported to contract almost continuously in (maintenance) of posture [31]. Additionally, since the amplitude and duration of the electromyographic activity does not change even during treadmill exercise [31], the soleus muscle seems to be maximally activated during posture maintenance. With the progression of disease, the *dy* mice gradually came to drag their hindlegs more and more, and therefore an antigravitational role of the soleus muscle seems to be unwarranted. Several investigations demonstrated that decreased activity induces reduction of the levels of small stress proteins in the soleus muscle [3,32,33]. For example, Atomi et al. [33] have reported an early and extreme decrease in the αB crystallin level in rat soleus muscle produced by hindlimb suspension, a model of unweighting. Furthermore, the HSP 27 concentration in the soleus muscle of the rat markedly decreased after denervation and tenotomy [3]. These results suggest that expression of small stress proteins in the slow-twitch muscle is maintained by both neural and mechanical factors. Indirectly, a study demonstrating diminishing glucose uptake in the soleus muscle of *dy* mouse [29] also supports the concept of decreased activity of this muscle.

Neither the soleus nor the tibialis anterior muscles in the *dy* mouse showed a significant change in the levels of HSP 70, as determined by means of Western blot analysis. Our results are in contrast with the finding of Bornman et al. [10], who recognized marked induction of HSP 70 in hypercontracted fibers in DMD patients, although the basis of the discrepancy with reported data is uncertain. Bornman et al. [10] failed to recognize significant induction of HSP 70, however, in fibers with normal, necrotic or regenerating characteristics. Therefore, it seems reasonable that a change of HSP 70 expression in some fibers would not be detected by Western blot analysis of whole muscle extracts as carried out in this study.

When skeletal muscle is damaged, mononuclear muscle precursors, the satellite cells, are activated and begin to proliferate and then fuse with each other, forming new multinucleated myotubes [34]. In different to DMD patients, the dystrophin-deficient *mdx* mouse presents muscle fiber necrosis but active muscle regeneration and slight or absent fibrosis. In the *mdx* mouse, satellite cells are reported to have a greater sensitivity to basic fibroblast growth factor (bFGF) compared to control satellite cells [35]. Therefore, satellite cells seem to be a potential target of gene therapy for muscular dystrophy, although it is unknown how small stress proteins modify the interaction among several growth factors, myogenic regulatory factors and satellite cells.

In conclusion, we propose the increased expression of the small HSP in the fast-twitch muscle, but the decreased expression of those in the slow-twitch muscle of *dy* mouse. Manipulation of this protective response may reduce injury and may have potential therapeutic application in congenital muscular dystrophy (CMD), which possesses a deficiency of laminin-α2 chain in muscle fiber basement similar to *dy* mouse.

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