43. *Dynamic Expression of α B-Crystallin in Skeletal Muscle*

Effects of Unweighting, Passive Stretch and Denervation

By Yoriko ATOMI,^{*)} Shigeru YAMADA,^{*)} and Yeong-Man HONG^{**)}

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Abstract: Twenty-two kDa protein specifically decreased in slow soleus muscle atrophy after 2-week hindlimb suspension (HS) of rats. This protein is abundant in soleus muscle but less in fast plantaris muscle. We purified this protein, determined the partial amino acid sequence using its proteolytic fragment, and found that the 22-kDa protein was a α -crystallin B chain with a 95% homology to bovine α B-crystallin. Further we demonstrated that α B-crystallin did not decrease in passively stretched slow muscle even under HS, and did markedly increase in denervated and stretched fast muscle under HS. These results suggest that the expression of α B-crystallin is dynamically related to the muscle plasticity.

Key words: α B-crystallin; muscle atrophy; passive stretch; denervation.

Alpha-crystallin, the complex of two subunits of A chain and B chain, has been believed to be expressed only in the lens of eye for a long time. The most striking characteristics of α -crystallin is to form a large aggregate. α -Crystallin is chemically stable, sharing striking sequence similarity to small heat shock proteins of *Drosophila*.⁷⁾ In 1989, three researchers^{3), 5), 8)} reported that α -crystallin B chain was expressed in heart and skeletal muscle. However, no study to elucidate the cellular function of α B-crystallin in skeletal muscle has been reported until today.

Limb musculature in animals, especially slow muscle like the soleus muscle that functions to maintain posture and counteract gravitational force on earth, undergoes a significant reduction in mass during space flight or during unweighting condition, such as hindlimb suspension (HS).^{12), 16)} It is well known that passive stretch is one of the factors to attenuate muscle atrophy.⁵⁾ Myofibrillar proteins, especially slow muscle myosin, markedly decrease in the rat soleus muscle by HS.^{14), 15)} But the mechanism of slow muscle atrophy is poorly understood.

In this communication, we report that the 22-kDa protein, which specifically decreases during soleus muscle atrophy with unweighting, is α B-crystallin and that it does not decrease in the suspended soleus muscle with passive stretch and increase in the suspended plantaris muscle with passive stretch under sciatic nerve denervation.

In our preliminary study,^{1), 2)} we used a hindlimb suspension technique developed by Morey for rat¹¹⁾ as a model to induce muscle atrophy. We first analyzed the protein composition of the total muscle homogenate from control and atrophied soleus muscles after 7-week HS (70% lower muscle weight for

^{*)} Department of Sports Sciences, College of Arts and Sciences, University of Tokyo, 3-8-1, Komaba, Meguro-ku, Tokyo 153, Japan.

^{**)} Laboratory for Cell Technology, Ohtsuka Pharmaceutical Co., Ltd. Tokushima Prefecture, Japan.

Fig. 1. Appearance and disappearance of 22-kDa protein in rat soleus muscle under suspended, and both suspended and stretched condition. (A) One-dimensional SDS-PAGE of muscle soluble proteins. (B) Corresponding two-dimensional SDS-PAGE. C, control without any treatment; S, treated with suspension of hindlimb for 2 weeks; ST, stretched under suspended condition for 2 weeks. Coomassie Blue staining of soluble fractions ($30\ \mu\text{g}$ for one- and $60\ \mu\text{g}$ for two-dimensional SDS-PAGE) of total homogenate of rat soleus muscle. Arrow shows the 22-kDa protein. Molecular weight standards (Pharmacia) are given in kDa. Notice the decrease of 22-kDa protein in S and its slight increase in ST compared as in C.

Fig. 2. Immunological confirmation of 22-kDa protein by the antibody against 22-kDa protein. (A) Coomassie Blue staining of SDS-PAGE. (B) Corresponding immunostaining. 1 ($3\ \mu\text{g}$) and 2 ($1\ \mu\text{g}$), purified 22-kDa protein; 3, soluble and 4, total proteins of soleus muscle. Notice the specific immunostained reactions of this antibody with 22-kDa protein (A and B, 3 and 4).

the control), by SDS-PAGE,⁹⁾ and found 22-kDa protein which specifically decreased in the atrophied muscle.¹⁾ The 22-kDa protein was extracted in soluble fraction and decreased in suspended atrophied soleus muscle after 2-week HS, and expressed less in plantaris muscle.²⁾ No marked and specific change was observed in the total and soluble protein compositions of atrophied plantaris muscle.²⁾ In this communication on the basis of these preliminary results, we first recognized the specific change of the 22-kDa protein in atrophied muscle and further determined the effect of passive stretch of soleus muscle on the 22-kDa protein under the suspended condition, by means of both one-⁹⁾ and two-¹³⁾ dimensional PAGE (Fig. 1). As a result, we found that the 22-kDa protein reduced in its content under suspended condition (S in Fig. 1-A and B) and that it did not decrease in its content under suspended condition with passive stretch (ST in Fig. 1-A and B). The muscle weight also did not decrease in this condition. Further the protein map of two-dimensional PAGE showed that the 22-kDa protein was a single and neutral protein. From these results we considered the 22-kDa protein as a proper clue to analyze the molecular mechanism of muscle plasticity. Consequently we tried to purify the 22-kDa protein from rat red muscles which were mainly composed of slow muscles.

All procedures were performed at 4°C. Total red muscles of adult male rats were immediately frozen, and crushed under liquid nitrogen, solubilized into 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 2 mM dithiothreitol, and spun at 10,000 rpm for 20 min. Supernatant solution obtained after 2nd centrifugation was applied to Sephacryl-200 column equilibrated with the same buffer (2 mM EGTA in stead of 5 mM). The solution containing 22-kDa protein was eluted in the void volume with such a relatively low ionic strength solution in spite of a low molecular weight of SDS-PAGE. Since this fraction had a tendency easily to aggregate and precipitate in non-detergent buffer solution, this fraction was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EGTA, 2 mM dithiothreitol, 7 M urea and 0.1% triton X-100 (v/v). Pooled and dialyzed fraction was applied to DEAE-Sephacryl column chromatography equilibrated with the same solution. The eluted fraction mainly composed of 22-kDa protein was dialyzed against 0.1 M sodium acetate buffer, pH 4.5 containing 1 mM EGTA, 2 mM DTT, 7 M urea and 0.1% triton X-100, and applied to S-Sepharose column equilibrated with the same buffer. The fraction containing only 22-kDa protein was eluted at 0.3 M KCl as a small peak with 0-0.5 M KCl linear gradients. The 22-kDa protein fraction was dialyzed against 10 mM phosphate buffer (pH 7.0) saline (PBS) including with 7.0 M urea and 0.1% triton X-100 and concentrated by ultrafiltration. Thus, the 22-kDa protein was purified as a single band on SDS-PAGE (shown in Fig. 2-1, 2).

As the 22-kDa protein was purified, determination of its amino acid sequence was a next focusing point. Since the N-terminus of the protein was blocked, the protein was fragmented by lysylendopeptidase and each fragment was purified by the reverse phase HPLC as follows. Purified 22-kDa protein (50 μ g) dialyzed against 10 mM Tris-HCl buffer, pH 8.5, was incubated with lysylendopeptidase (S/E=50) for 6 hours at 30°C. The reaction mixture was diluted into the starting buffer of 0.1% trifluoroacetic acid (TFA) and applied to the reverse phase column (TSK gel DDS-120 T; Toyosoda Ltd. Japan) equilibrated with the same solution. Ten peaks were eluted with two-steps linear gradients of 0-15% and 15-60% of acetonitrile in 0.1% TFA. The amino acid sequence of one appropriate peak in them was firstly analyzed with HPLC

Table I. Amino acid composition of 22-kDa protein and bovine α B-crystallin

Amino acid	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Lys	His	Arg
Rat 22-kDa	77	43	73	118	80	28	32	0	47	11	49	81	15	63	61	108
Bovine α B-Cry.	78	34	76	121	86	31	35	0	49	9	51	87	17	64	60	108

Figures represent amino acid recovered (mol per 1000 mol). Tryptophan and phenylalanine were not determined. α B-Cry. means α B-crystallin.

(Amino Acid Sequencer, Applied Biosystem Co. Ltd. USA). Twenty-two amino acids from the fragment were determined: K Y R I P A D V D P L T I T S S L S S D G V. This sequence except for the 12th A in place of T was the same as that of α B-crystallin from bovine lens already reported.¹⁸⁾ Therefore, we compared the amino acid compositions of the 22-kDa protein from rat red slow muscle and those of bovine lens α B-crystallin separated¹⁹⁾ from its α -crystallin purchased (Table I). The amino acid compositions of the 22-kDa proteins were almost the same with those of bovine lens α B-crystallin, as shown in Table I. But there are some differences which could be explained as the replacement of several amino acids. We further confirmed that the 22-kDa protein is a α B-crystallin itself from complete sequence of the 22-kDa protein by cDNA cloning, cross reaction of the 22-kDa protein-antibody to eye's α B-crystallin and of eye's α B-crystallin antibody to the 22-kDa protein from slow muscle. The details of these results are now submitting to other journal.

We prepared a polyclonal antibody from the purified muscle 22-kDa protein using a rabbit as a host. The rabbit IgG was purified by ammonium sulfate salting out at 40% saturation and the Affi-Gel Blue (BIO-RAD) column chromatography. Transfer of proteins into nitrocellulose and immunoblotting were performed by the methods of Towbin *et al.*¹⁷⁾ with a slight modification. Anti-22k protein was generally used at 1:1000 to 1:2000 dilution. The antibody markedly reacted with a purified 22-kDa protein and the 22-kDa band of soluble and total muscle protein from rat soleus muscle (Fig. 2).

The 22-kDa α B-crystallin was expressed abundantly in slow muscle and reduced in the suspended muscle atrophy but not with passive stretch observed above. Continuous mechanical stretch or lengthening muscle is reported to increase muscle mass, while shortening of muscle length results in augmentation of muscle atrophy.^{6),10)} On the other hand, denervation to skeletal muscle is known to aggravate muscle atrophy⁶⁾ and cross innervation between slow and fast muscle to reverse physiological and biochemical characteristics dependent on fiber type.⁴⁾ Therefore, we further examined the effects of continuous passive stretch and denervation on both slow and fast muscles under HS on the expression of the 22-kDa protein in skeletal muscle. Innervated or denervated animal under HS was restrained in a fully dorsiflexed (stretched state) and plantarflexed (shortened state) position with tape, respectively. After 2 weeks of the experiment, soleus and plantaris muscles were removed from the suspended and the control groups without any treatment mentioned above. The soluble proteins separated from total homogenate of these muscles were analyzed by means of SDS-PAGE and immunoblotting described above (Fig. 3). In the suspended innervated soleus muscles the 22-kDa α B-crystallin slightly increased in the innervated and stretched state mentioned above (Fig. 1) compared with that in the control muscle, while decreased in shortened state, and its content was

Fig. 3. Effect of passive stretch (lengthening), shortening and denervation on protein compositions of rat suspended soleus and plantaris muscles. (A) Coomassie blue staining of muscle soluble proteins (30 μ g). (B) Corresponding immunostaining. C, control; S, suspended; and DS, denervated and suspended muscles. ST, stretched; and SH, shortened muscles. Arrow head shows 22-kDa α B-crystallin. Molecular weight standards (Pharmacia) are given in kDa. Notice clear increase of 22-kDa protein in denervated plantaris muscle by stretching under suspension, although 22-kDa protein was rather reduced slightly in denervated soleus muscle even by stretching under suspension.

the same as that of only suspended experiment as shown in Fig. 1. Next, in the suspended denervated soleus muscle, 22-kDa protein disappeared completely in the shortened state while it remained in the stretched state. In the suspended plantaris muscle in which 22-kDa protein was scarcely expressed in the control, the 22-kDa protein clearly appeared only in the denervated and stretched state. In the other conditions the 22-kDa protein was rarely expressed as well as the control. These results suggest that passive stretch increases the expression of the 22-kDa α B-crystallin in both slow and fast, especially denervated fast skeletal muscle even under suspension experiment. It seems that the innervation to slow muscle facilitates the expression of muscle α B-crystallin while the inner-

vation to fast muscle inhibits the expression of muscle α B-crystallin because the 22-kDa protein appeared only in the denervated state.

The present work suggests that muscle α B-crystallin may play a role during the process of slow muscle atrophy, regulated by innervation and passive stretch. The detailed results of this work will be reported elsewhere.

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