Fiber-type-specific αB-crystallin distribution and its shifts with T3 and PTU treatments in rat hindlimb muscles

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Atomi, Yoriko, Kyoko Toro, Tsuyoshi Masuda, and Hideo Hatta. Fiber-type-specific αB-crystallin distribution and its shifts with T3 and PTU treatments in rat hindlimb muscles. J Appl Physiol 88: 1355–1364, 2000.—Changes in αB-crystallin content in adult rat soleus and extensor digitorum longus (EDL) were examined after 8 wk of 3,5,3'-triiodothyronine (T3) and propylthiouracil (PTU) treatments. Cellular distributions of αB-crystallin expression related to fiber type, and distribution shifts with these treatments were also examined in detail from the gray level of reactivity to specific anti-αB-crystallin antibody. αB-crystallin content in both soleus and EDL muscles was significantly decreased after T3, and that in EDL was significantly increased over twofold after PTU treatment. In both control soleus and EDL muscles, the gray level of type I fibers was higher than that of type II fibers. αB-crystallin expression among type II subtypes was muscle specific; the order was type I > IIα > IIx > IIb in control EDL muscle and type I > IIα > IIa in soleus muscle. The relation was basically unchanged in both muscles after T3 treatment and was, in particular, well maintained in EDL muscle. Under hypothyroidism conditions with PTU, the mean αB-crystallin levels of type IIα and IIx fibers were significantly lower than levels under control conditions. Thus the relation between fiber type and the expression manner of stress protein αB-crystallin is muscle specific and also is well regulated under thyroid hormone, especially in fast EDL muscle.

stress protein; immunohistochemistry; thyroid hormone; propylthiouracil; 3,5,3'-triiodothyronine

THERE ARE MANY TYPES of heat shock proteins (HSPs) that are constitutively expressed in both prokaryotic and eukaryotic cells. HSPs play fundamental roles, such as transcriptional regulation, nascent protein folding, and protein transport to endoplasmic reticulum and/or mitochondria (28). αB-crystallin is one of the small HSPs (sHSPs) and can function as a molecular chaperone (20). αB-crystallin not only is a major lens protein but also is expressed in other tissues such as heart, skeletal muscle, and kidney (5, 7, 13, 15, 23, 25). In skeletal muscle, some reports indicated that HSP70 (31) and αB-crystallin are constitutively expressed at levels higher in the slow-twitch muscles than in the fast-twitch muscles (4, 25). This higher expression of HSPs in the slow-twitch muscles might be related to their higher metabolic and protein turnover rates compared with fast-twitch muscles (34). Locke et al. (29) reported that the shift in the type I fiber composition to type II composition in rat hindlimb muscle after 3,5,3'-triiodothyronine (T3) treatment or incapitation of synergistic muscles was accompanied by changes in HSP72 content, which suggests a significant relationship between HSP72 content and the composition of the type I muscle fiber myosin heavy chain (MHC). However, there is no study showing a precise relationship between the fiber type and the expression of stress proteins in the skeletal muscle.

In skeletal muscle, αB-crystallin is expressed at a higher level in slow-twitch soleus muscles and heart compared with fast-twitch plantaris and extensor digitorum longus (EDL) muscles (4, 5, 22). We have found that αB-crystallin specifically decreases in atrophied soleus muscle, but the expression of αB-crystallin can be sustained if the muscle is passively stretched (3, 4). Recently, it was reported that the expression of αB-crystallin is related to maintaining the stability of the cytoskeleton (2, 23, 33). αB-crystallin localizes at Z bands in skeletal muscle (5) where many cytoskeleton-relating proteins assemble and mechanical stress is intensively transferred. If we consider the function of stress protein as a chaperone, possible fiber-type-dependent expression of αB-crystallin might indicate differences in its structure and function related to cytoskeletons, depending on the differences in the actomyosin contracting system in the skeletal muscle.

In this study, the relationship between αB-crystallin expression and the muscle fiber type was precisely investigated by means of an immunostaining technique using anti-myosin monoclonal antibodies and an anti-αB-crystallin antibody in rat skeletal muscle. We also examined the relationship between αB-crystallin and muscle fiber type after fiber transformation with T3 and propylthiouracil (PTU) treatments, which induce shifts in the expression of MHC isoforms (17, 26). Here, we show that the expression of αB-crystallin significantly decreased after T3 treatment in both soleus and EDL muscles and significantly increased after PTU treatment in EDL muscle. The intensity of αB-crystallin for each fiber in EDL muscle is systematically related to fiber type, and the relationship is better maintained under the influence of thyroid hormone.
METHODS

Animals. The same experiments were performed twice for biochemical (experiment 1) and immunohistochemical (experiment 2) analyses of αB-crystallin expression. Adult male Wistar rats weighing 201–229 g (n = 18 × 2) were randomly assigned to one of three groups designated control, hyperthyroid (T3), and hypothyroid (PTU). All animals were provided with standard rat Chow ad libitum and were housed at room temperature with a 12:12-h light-dark cycle. The rats assigned to the T3 group were injected subcutaneously with 300 µg/kg body wt of T3 (sodium salt) every other day. The rats in the PTU group were supplemented with 0.1% PTU in drinking water throughout the 8-wk experimental period. After 8 wk, the rats were anesthetized with diethyl ether, and then the EDL and soleus muscles were dissected. The conditions of these two experiments were the same, except that the animals in experiment 1 were older than those in experiment 2 by 1 wk.

Purification of αB-crystallin. For measuring the protein content of αB-crystallin in muscle homogenate, αB-crystallin was purified from bovine lens, which is detailed in a previous study (1).

Preparation of antibodies. Antibody C1 was raised against COOH-terminal (SH)KPAVPAAAQK peptides of rat αB-crystallin (synthesized and purified by Dr. S. Aimoto, Research Center for Protein Engineering, Institute for Protein Research, Osaka University, Osaka, Japan) conjugated to BSA with m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce Chemical, Rockford, IL) (35). IgG, precipitated by 50% NH4SO4 and dialyzed against PBS, was purified by using COOH-terminal peptide-conjugated affinity chromatography using MBS linker and EAH Sepharose 4B (Pharmacia Research, Osaka, Japan).

Preparation of muscle samples for SDS-PAGE and immunoblot analyses. The procedure followed Atomi et al. (4, 5) with a slight modification. Muscles were dissected out, immediately frozen in liquid nitrogen, and stored at −70°C until use. Muscle samples were crushed in liquid nitrogen and were directly solubilized in low-salt buffer containing 20 mM KCl, 2 mM sodium phosphate, pH 6.8, 2 mM EGTA, and 0.1 mM diisopropyl fluorophosphate. Protein concentration was determined with bicinchoninic acid protein assay kit using BSA as standard. Muscle homogenates in control, T3, and PTU groups (experiment 1) were applied to each lane. The band concerned was clearly separated in the gradient gel and identified to be αB-crystallin with Western blotting against anti-αB-crystallin antibody.

Immunohistochemistry. For immunohistochemical analysis, small pieces from the midsection of the muscles were frozen in melting isopentane and stored at −70°C. Cross sections were cut at 10 µm thickness on a cryostat microtome at −25°C. Muscle sections were sequentially incubated in PBS for 10 min, 1.5% goat serum in PBS for 30 min, anti-αB-crystallin Ig for 1 h, PBS for 10 min, FITC-labeled goat anti-rabbit IgG (TAGO, Burlingame, CA) at room temperature for 1 h, and PBS for 10 min. The sections were mounted in glycerol-para-phenylenediamine and were examined with epifluorescent illumination with a Zeiss Axiosplan photomicroscope. Controls using preimmune rabbit serum did not show any significant staining. For analysis of immunohistochemical staining, sections were visualized with a video camera attached to a microscope, and images were processed with a microcomputer-based image analysis system (IBAS, Carl Zeiss, Oberkochen, Germany). More than 250 muscle fibers were digitized per muscle using a computer that calculated the average gray level of pixels in a circle drawn within each fiber. The gray level of each fiber was measured by pixel at 0–256 graduation. This graduation of gray level was determined relative to the sections with the highest and the lowest pixilation. Type I fibers demonstrated the highest level, and the lowest level was observed mostly in type IIb fibers.

Skeletal muscle fiber-type classification. To compare the staining pattern of αB-crystallin in a muscle composed of different fiber types, 10-µm sections were consecutively cut and mounted on slides. These consecutive sections were then incubated in the five different antibodies. The specific activities of the five antibodies [BF-G6 (39), BF-35, SC-71 (40), BA-F8, and BF-13 (8)] are shown in Table 1. Four fiber types were designated as type I, type IIa, type IIx, and type IIb. These monoclonal antibodies were a generous gift from Dr. S. Schiaffino, Department of Biomedical Science, University of Padova (Padova, Italy). Bound antibodies were revealed by FITC-labeled goat anti-mouse IgG.

Statistical analysis. Data are given as means ± SD of number of fibers (n). Statistical analysis of data was performed according to ANOVA, Student’s unpaired t-test, or its modified method [by Welch and Aspin (see Ref. 41)]. Differences at P < 0.05 were regarded as significantly different.

RESULTS

Antibody characteristics. The polyclonal antibody against COOH-terminal peptide of αB-crystallin purified by peptide affinity chromatography specifically

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type IIx</th>
<th>Type IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF-F8</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BF-13</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>SC-71</td>
<td>+/-</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BF-G6</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BF-35</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 1. Staining intensities in rat muscle reacted to antibodies against MHC isoforms

Muscle homogenate were applied to each lane. The band concerned was clearly separated in the gradient gel and identified to be αB-crystallin with Western blotting against anti-αB-crystallin antibody.
reacted to a 22-kDa α-B-crystallin in the homogenates of rat soleus and EDL muscles (Fig. 1). Criteria to determine fiber type with monoclonal antibodies against MHCs produced by Dr. Schiaffino were well ascertained in this study for the skeletal muscles of Wistar rats (Table 1).

Quantification of α-B-crystallin in soleus and EDL muscles. In experiment 1, the α-B-crystallin content in soleus and EDL muscles was determined by the intensity stained with CBB and calculated using NIH Image software. α-B-crystallin content of EDL muscles was 0.8% of that of soleus muscles and roughly agreed with the ratio by immunoassay (22).

Physical characteristics of rats after EDL and soleus muscles were treated with T<sub>3</sub> and PTU. Body weights after the 8-wk experiment were significantly lower in T<sub>3</sub>- and PTU-treated animals than in the control rats (Table 2). The soleus muscle weight significantly decreased in PTU-treated animals, whereas the EDL muscle weight significantly decreased in both T<sub>3</sub> and PTU groups. This finding was consistent with previous studies of thyroid hormone-treated rats (17).

Specific changes of α-B-crystallin after T<sub>3</sub> and PTU treatment in soleus and EDL muscles. The α-B-crystallin protein content in the total muscle homogenate in the experimental groups was calculated from the areas in SDS-PAGE stained with CBB (Fig. 2, Aa and Ab). The approximation with a quadratic equation used for this calculation was obtained from the areas for five different contents of purified α-B-crystallin. The relation between them became linear after logarithm exchange. Typical examples of α-B-crystallin in total muscle homogenate of soleus and EDL muscles in control, T<sub>3</sub>, and PTU groups subjected to SDS-PAGE and Western blotting are shown in Fig. 2B. The α-B-crystallin content in total muscle homogenate, identified by Western blotting and estimated using the standard calibration curve for α-B-crystallin with Western blotting, was similar to the mean values obtained by CBB staining. The mean α-B-crystallin protein content was significantly lower in both T<sub>3</sub>-treated soleus (17%) and EDL (55%) muscles than in those of the control group (Fig. 2C). Although mean α-B-crystallin protein content after PTU treatment did not significantly change in soleus muscle, it significantly increased over twofold in EDL muscle (150%).

Fiber-type frequencies of the EDL and soleus muscles in control and in T<sub>3</sub>- and PTU-treated rats. About 10% of 250 fibers examined in the experimental groups reacted to antibodies against more than two MHC isoforms. These fibers were not included in the fiber-type distribution analysis in this study. Fiber-type frequencies of EDL and soleus muscles in control, T<sub>3</sub>, and PTU groups are shown in Table 3. After T<sub>3</sub> treatment, fiber frequencies of type I and type IIx significantly decreased in EDL muscle. In soleus muscle, fiber frequencies of type I significantly decreased to a level that was similar to that found in EDL muscle, whereas type IIa levels increased after hyperthyroidism was induced. On the other hand, significant increases in type I and IIa and significant decreases in type IIx and IIb were observed in hypothyroid EDL muscle. Significantly decreased type IIa fiber frequencies were observed in PTU-treated soleus muscles. These muscle-type-specific changes of fiber type after T<sub>3</sub> and PTU treatments are consistent with the results obtained in thyroid hormone-treated rats (17).

Serial relation of α-B-crystallin expression to fiber type in control soleus and EDL muscles. The relationship between the expression of α-B-crystallin and MHC isoforms was examined in control soleus and EDL muscles (Fig. 3). Because the staining intensity against anti-α-B-crystallin antibody was different between fibers, the relationships between the reactivity to anti-α-B-crystallin antibody and the reactivity to anti-MHC

![Fig. 1. Characteristics of anti-αB-crystallin COOH-terminal peptide antibody. Rat total proteins of soleus and extensor digitorum longus (EDL) muscle homogenates (lanes 1 and 2) were subjected to SDS-PAGE and followed by staining with Coomassie brilliant blue (CBB) (A, lanes 1 and 2; 7.5 µg per each lane) or immunoblotted with anti-αB-crystallin COOH-terminal peptide antibody (B, lanes 3 and 4; 1 µg per each lane). Molecular mass markers (lane M) are phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactoalbumin (144 kDa).](http://jap.physiology.org/DownloadedFrom/http://jap.physiology.org/content/69/6/1357/F1)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T&lt;sub&gt;3&lt;/sub&gt;</th>
<th>PTU</th>
</tr>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>556±22</td>
<td>465±30†</td>
<td>324±18†</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>184±29</td>
<td>201±15</td>
<td>140±20*</td>
</tr>
<tr>
<td>EDL, mg</td>
<td>268±27</td>
<td>198±57†</td>
<td>163±11†</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>427±17</td>
<td>354±31†</td>
<td>264±32†</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>140±14</td>
<td>131±10</td>
<td>118±12*</td>
</tr>
<tr>
<td>EDL, mg</td>
<td>207±16</td>
<td>178±21*</td>
<td>135±22†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6–7 animals/group. BW, body weight; EDL, extensor digitorum longus; T<sub>3</sub>, 3,5,3'-triiodothyronine; PTU, propylthiouracil. *P < 0.05, †P < 0.01, ‡P < 0.001, statistical significances of experimental groups vs. control groups.

![Table 2. Mean body weight and muscle mass of rats in control, T<sub>3</sub>, and PTU groups for biochemical (experiment 1) and immunohistochemical (experiment 2) analyses](http://jap.physiology.org/DownloadedFrom/http://jap.physiology.org/content/69/6/1357/Table2)
antibodies were examined. Type I fibers showed a stronger reactivity to anti-αB-crystallin IgG than did type II fibers in both soleus and EDL muscles. The differences in the reactivity to anti-αB-crystallin IgG among the fast subtypes were faint in soleus muscle and considerable in EDL muscle. In soleus muscle, the staining intensities of type IIa and IIx fibers were almost identical but that of type IIx was slightly higher.
Table 3. Fiber-type frequencies after thyroid T₃ and PTU treatments

<table>
<thead>
<tr>
<th>Muscle Group</th>
<th>Soleus</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>T₃</td>
</tr>
<tr>
<td>Type I fibers, %</td>
<td>82.7 ± 5.4</td>
<td>72.8 ± 5.2†</td>
</tr>
<tr>
<td>Type IIa fibers, %</td>
<td>16.6 ± 5.0</td>
<td>26.3 ± 4.9†</td>
</tr>
<tr>
<td>Type IIx fibers, %</td>
<td>0.5 ± 0.8</td>
<td>0.4 ± 0.9</td>
</tr>
<tr>
<td>Type IIb fibers, %</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Uncharacterized fibers, %</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 animals/group. *P < 0.05, †P < 0.01, statistical significance of experimental groups vs. control group.

than that of type IIa (Fig. 3A, top). In EDL muscle, type IIa, IIx, and IIb fibers showed moderate, weak, and faint immunoreactivity, respectively (Fig. 3A, bottom). The distributions of the gray levels of αB-crystallin per fiber type in EDL muscle (Fig. 3A, bottom) reinforced the above results. From these observations, it was suggested that the expression of αB-crystallin in rat skeletal muscles varies systematically with fiber type; however, this variation in expression might be muscle specific.

Maintained fiber-type-specific αB-crystallin expression in hyperthyroidism. Typical sections of soleus and EDL muscles from control and experimental groups stained with anti-αB-crystallin antibody are shown in Fig. 4A. Most of the fibers of soleus muscles both in control and T₃ groups (except for an unknown few in control soleus muscles) were classified into only two groups of type I and type IIa. The gray levels of type I fibers in soleus muscle were consistently higher than those of type IIa fibers in other areas in the muscle sections of the T₃ group (data not shown). Few fibers expressed type IIx, although the number of fibers expressing both type IIa and type IIx increased after T₃ treatment. Although the difference in the intensities of the gray level between type IIa and IIx was very small, any intensity of the gray level of five type IIx fibers observed in the areas where type IIx and IIa fibers were closely located was slightly higher than that of type IIa. Because the differences among the gray levels of type I, IIa, and IIx fibers and the numbers of type IIx were too small, especially in the soleus muscle treated with T₃ or PTU, statistical analysis was not performed for the data. From these observations for both control and T₃ groups, the intensities of anti-αB-crystallin antibody in soleus muscles were greater in the order of type I > type IIx ≳ type IIa. The relative percentages of the mean gray values of these five type IIx fibers and four type IIa fibers found near areas of T₃ soleus were ~75% and 62% respectively, compared with the mean gray values for nine type I fibers (100%).

The mean gray levels, showing the intensities of αB-crystallin expression, of the different fiber-type groups for EDL muscles in the all control, T₃, and PTU groups were significant (P < 0.001, Table 4). This means that the averages of the gray levels among three type II fiber groups were statistically different from each other. After T₃ treatment, the mean gray level of αB-crystallin was significantly less in the type IIa
group and was significantly larger in the type IIb group compared with results shown in controls. The relation between the αB-crystallin expression and fiber type observed in control EDL muscles was consistently maintained in T₃ EDL muscles. Distributions of the gray levels after T₃ treatment were almost the same as those in the control group for EDL (Fig. 4, A-C, right). The orders of intensities of the gray levels in control muscles showed the same pattern as in controls.
Type II groups are significantly different with fiber of each group. The activity of each fiber.

The mean gray level of the reactivity to anti-α-B-crystallin antibody in control, T3, and PTU groups in EDL muscles.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>T3</th>
<th>PTU</th>
</tr>
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<tbody>
<tr>
<td>Type I</td>
<td>n</td>
<td>256.0 ± 0.0 (100%)</td>
<td>237.3 ± 16.4 (100%)</td>
</tr>
<tr>
<td>Type IIa</td>
<td>n</td>
<td>188.1 ± 30.6 (73.5%)</td>
<td>175.5 ± 25.9* (74.0%)</td>
</tr>
<tr>
<td>Type IIx</td>
<td>n</td>
<td>70.9 ± 31.1 (27.7%)</td>
<td>82.2 ± 28.7 (34.6%)</td>
</tr>
<tr>
<td>Type IIb</td>
<td>n</td>
<td>37.5 ± 18.1 (14.6%)</td>
<td>44.7 ± 9.2† (18.8%)</td>
</tr>
</tbody>
</table>

Values are means ± SD. Values in parentheses are percentages for type I in each group; n shows counted numbers included into each group. Various gray levels have been normalized using the highest value seen in type I fiber (all set to 100%) and the lowest value observed in type II fiber of each group. *P < 0.05, †P < 0.01, ‡P < 0.001, statistical significance of experimental groups vs. control group. All mean gray level of type II groups are significantly different with P < 0.001 in each experimental group.

groups with reference to fiber type did not change in both soleus and EDL muscles.

Hypothyroidism condition depressed α-B-crystallin expression levels in type IIa and IIx in EDL muscle. Twenty percent of fibers were uncharacterized in PTU-treated soleus muscle. Most of the fibers in soleus muscles were included and classified into two groups of type I and type IIa as shown in Table 3. The gray levels of type I fibers in PTU-treated soleus muscles were consistently higher than those of type IIa fibers in these groups. More precise examination was not performed for soleus muscle. In EDL muscle, the mean α-B-crystallin levels of type IIa and IIx were significantly less (45% and 57% for control) in the PTU than in the control group (Table 4, Fig. 4). This means that fiber-type-specific expression of α-B-crystallin is maintained only under the influence of thyroid hormone in both soleus and EDL muscles.

DISCUSSION

Immunohistochemical fiber typing. Both immunohistochemical and electrophoretical analyses have revealed that most of the fibers express only a single type of MHC and can therefore be classified as type I, IIa, IIb, and IIx fibers. Only a few contain detectable levels of two MHC isoforms (11, 16, 38, 42). Monoclonal antibodies used in this study could not detect the coexpression of type IIa with type IIx or type IIx with type IIb MHC because none of them did specifically stain type IIx MHC. Type IIx MHC was identified by negative staining with the other antibodies used. Some fibers identified as type IIa or type IIb might contain various amounts of type IIx MHC isoform coexisting with a predominant one. It is not known whether anti-MHC antibodies used in this experiment react to embryonic and neonatal MHC isoforms. Therefore, a possible induction of these nonadult myosin isoforms under hypothyroidism (a reduction in circulating T3 level) (26) could not be detected in this experiment.

Evaluation of α-B-crystallin expression by immunoreactivity of each fiber. Although qualitative classification with immunocytochemical and histochemical techniques is available for fiber typing in skeletal muscle, it is difficult to quantitatively evaluate protein content per fiber with reference to fiber type by MHC expression. Possible reasons for this are as follows. 1) The thickness of muscle sections is not perfectly constant. 2) The reactivity of the protein in the muscle sections, therefore, is not necessarily constant. 3) Even if they are constant, there is some technical difficulty in staining the fibers equally. 4) Distribution of muscle fibers in a muscle section is usually mosaic and variable, especially under various physiological conditions. All these could induce variations in the reaction to antibody IgG, even if a given protein content per muscle fiber is reacted to a given IgG content. For these reasons, in the present study, we selected muscle fibers in the areas where there was relatively constant staining, to quantify α-B-crystallin from the gray level of each muscle section, and we classified them with respect to fiber type in each experimental muscle.

The analysis using BF-35 antibody, which detects type IIx by negative reactivity to type IIx, was available for EDL muscle but not for soleus muscle. The reason for the difference in the reactivity between soleus and EDL muscles is unknown at present. Therefore, the classification of type IIx in soleus muscle was performed by the comparison of reactivity to the other four anti-myosin antibodies. From the difficulty to quantify the relation in soleus muscle, the above-mentioned results of the relation observed for soleus muscles were described only in the text.

Effects of thyroid state on MHC gene expression. It is useful to examine the changes of protein expression relating to muscle fiber type under the various thyroid hormone levels, which directly and/or indirectly control expressions of muscle-specific genes. Hypothyroidism induces alterations in MHC phenotypes consisting of an increased expression of the slow type I MHC isoform, coupled with a decreased expression of the fast type IIa and IIx MHC. These alterations vary depending on muscle type (26). In this study, the changes of fiber type were evaluated by anti-myosin antibodies with T3 and PTU treatments for 8 wk. In slow soleus muscle, PTU-induced hypothyroidism caused a slight increase in the expression of type I MHC and a decrease in the expression of type IIa/IIx MHCs. In fast-twitch EDL muscle, hypothyroidism caused increases in type I and IIa MHC expression coupled with a decrease in type IIx and IIb MHC expression.
These results were consistent with previous results in rat soleus muscle (26) and in the fast-twitch plantaris muscle (17). In contrast, hyperthyroidism of hind-limb skeletal muscles induces the upregulation of type IIa/IIx MHC expression of the fibers in the soleus muscle and induces the downregulation of both type I and IIa/IIx MHC isoforms as well as a concomitant increase in the expression of type IIB MHC isoform in plantaris muscles (17). These findings about changes after thyroid hormone treatment agreed with the previous results (17, 26). The results in the present study also roughly agreed with the assumption that the expression of αB-crystallin in skeletal muscle might be dependent on the similar mechanism to the changes of fiber-type, from the response to T3/PTU treatment.

The thyroid hormone can alter the transcriptional rate of a gene by direct interaction with a thyroid hormone receptor-thyroid hormone responsive element (TR-TRE) of the target gene promoter. TR-TRE has been found in the promoter regulatory regions of several genes expressed in skeletal muscle including those genes for type I MHC (14), α-actin (10), sarcoplasmatic reticulum Ca2+-ATPase pump (19), and myogenic factors of MyoD and myogenin (12). MyoD mRNA and myogenin mRNA preferentially accumulate in fast- and slow-twitch muscles, respectively (21).

Effects of thyroid hormone on the expression of αB-crystallin in soleus and EDL muscles. Biochemical analysis in the present study showed that 8-wk T3 treatment significantly decreased the expression of αB-crystallin in both soleus and EDL muscles. Further PTU treatment increased it over twofold in EDL muscle, although significant change was not observed in soleus muscle. The difference of reactivity in muscles may be related to other factors, such as Ca2+ sensitivity (9), myogenic factor (21), and possibly the mechanical environment, which may regulate myosin expression.

T3-regulated fiber-type transformation and αB-crystallin expression. Interestingly, a complete serial order in the quantity of αB-crystallin expression in EDL muscle, that is, I > IIa > IIx > IIB, matches the sequence of fiber-type transition inferred from studies of MHC expression in normal and electrically stimulated muscles (16, 38). Furthermore, this relationship received some modifications after the withdrawal of thyroid hormone in PTU treatment but almost unchanged under T3. This means that the relation between αB-crystallin and fiber type is well maintained under physiological influence of thyroid hormone. There is at least one E box in MRE, the muscle-specific regulatory region, in the upstream of αB-crystallin gene (15), but no TRE was found there. Therefore, possible indirect regulation for αB-crystallin expression by thyroid hormone might be related.

Possible physiological significances of strict relation between SHSP, αB-crystallin, and fiber type. Previously, we have found that a 22-kDa protein that specifically decreases in slow-twitch muscle atrophy is αB-crystallin. αB-crystallin does not decrease by hindlimb suspension with passive stretch (4). αB-crystallin localizes at Z disks of glycerinated myofibrils (5). Although the function of αB-crystallin in skeletal muscle is still unknown, a recent study (20) showed the role of αB-crystallin as a molecular chaperone. One possible function of αB-crystallin in slow-twitch muscle is a chaperone for proteins that are easily denatured. Because slow-twitch muscles have higher metabolic and protein turnover rates compared with fast-twitch muscles (34), it seems that slow-twitch muscles may have an essential need for a chaperone system.

It is found in our recent study that αB-crystallin completely associates with tubulin/microtubule and intermediate filament in L6 myoblast cells (unpublished data) and that αB-crystallin binds temperature dependently to the tubulin molecule by immunoprecipitation with anti-αB-crystallin antibody and can suppress tubulin aggregation by complex formation (1). It is well known that tubulin is a labile protein that is easily denatured even in physiological condition. Together, the strict correlation between the expression of αB-crystallin and fiber type in the present study suggests that the function of αB-crystallin keeps dynamic stability and/or metabolic activity in skeletal muscle, particularly in slow-twitch muscle.

The amino acid sequence of αB-crystallin has a striking similarity to the sequence of shSPs, and increasing evidence indicates that αB-crystallin has a function similar to shSPs. The expression of αB-crystallin as well as shSPs is induced by various stresses (37). Recently it was found that overexpression of shSPs, including αB-crystallin, can prevent cells from dying by reactive oxygen species (ROS) induced by tumor necrosis factor-α and stimuli of ROS-inducing reagents (32). From these observations and from the fact that slow-twitch soleus muscle is continuously subjected to much more stress, including high temperature, oxidant injury, higher rate of protein turnover, and continuous contraction with passive stretching compared with the other skeletal muscles, it seems reasonable to assume that the constitutive expressions of αB-crystallin/shSP and other HSPs (described below) are required in slow-twitch muscle.

Other stress proteins in muscle. Although many stressors can induce HSP synthesis in various cultured cells from bacteria to humans, only a few studies have demonstrated the function of mammalian HSPs in vivo. Studies on diseases causing the abnormal expression of HSPs have been carried out in recent years (6). Increased expression of HSPs has been demonstrated in vivo by heat shock in anesthetized animals (18, 36). Physiological stress during treadmill running to exhaustion induces synthesis of HSP72 and HSP90 in rat peripheral lymphocytes, spleen cells, and soleus muscles (29). In addition, HSP70 is constitutively expressed in soleus muscle (30) and shows type I MHC-dependent expression (31). Such expression patterns and the localization and their changes of stress protein, including HSP70 and αB-crystallin in the previous (2, 4), and the present results suggest the existence of unknown characteristics that could explain different phenotypes of skeletal muscle fibers.
In summary, stress protein b-crystallin expression was muscle specific and fiber-type specific in both soleus and EDL muscles from the histochemical analysis using specific antibodies for myosins and b-crystallin and was significantly lower under T3 treatment in both muscles. This relation was well maintained in physiological conditions under thyroid hormone influence.

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