Effects of thyroid hormone receptor gene disruption on myosin isoform expression in mouse skeletal muscles

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Effects of thyroid hormone receptor gene disruption on myosin isoform expression in mouse skeletal muscles. Am J Physiol Regulatory Integrative Comp Physiol. 278: R1545–R1554, 2000.—Skeletal muscle is known to be a target for the active metabolite of thyroid hormone, i.e., 3,5,3'-triiodothyronine (T3). T3 acts by repressing or activating genes coding for different myosin heavy chain (MHC) isoforms via T3 receptors (TRs). The diverse function of T3 is presumed to be mediated by TR-α1 and TR-β, but the function of specific TRs in regulating MHC isoform expression has remained undefined. In this study, TR-deficient mice were used to expand our knowledge of the mechanisms by which T3 regulates the expression of specific MHC isoforms via distinct TRs. In fast-twitch extensor digitorum longus (EDL) muscle, TR-α1-, TR-β-, or TR-αβ-deficient mice showed a small but statistically significant decrease (P < 0.05) of type IIB MHC content and an increased number of type I fibers. In the slow-twitch soleus, the β/slow MHC (type I) isoform was significantly (P < 0.001) upregulated in the TR-deficient mice, but this effect was highly dependent on the type of receptor deleted. The lack of TR-β had no significant effect on the expression of MHC isoforms. An increase (P < 0.05) of type I MHC was observed in the TR-α1-deficient muscle. A dramatic overexpression (P < 0.001) of the slow type I MHC and a corresponding downregulation of the fast type IIA MHC (P < 0.001) was observed in TR-αβ-deficient mice. The muscle- and fiber-specific differences in MHC isoform expression in the TR-αβ-deficient mice resembled the MHC isoform transitions reported in hypothyroid animals, i.e., a mild MHC transition in the EDL, a dramatic but not complete upregulation of the β/slow MHC isoform in the soleus, and a variable response to TR deficiency in different soleus muscle fibers. Thus the consequences on muscle are similar in the absence of thyroid hormone or absence of thyroid hormone receptors, indicating that TR-α1 and TR-β together mediate the known actions of T3. However, it remains unknown how thyroid hormone exerts muscle- and muscle fiber-specific effects in its action. Finally, although developmental MHC transitions were not studied specifically in this study, the absence of embryonic and fetal MHC isoforms in the TR-deficient mice indicates that ultimately the transition to the adult MHC isoforms is not solely mediated by TRs.

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including MHC genes via nuclear T3 receptors (TR) (4, 6, 15, 17, 35). TRs exert their effect through binding to specific DNA segments named thyroid-responsive elements (TRE), which are located upstream of the promoter of responsive genes (18). In vivo, in the absence of T3, TRs may still bind to TRE to repress basal transcription, suggesting that TRs possess a dual capacity to regulate transcription by both T3-dependent and T3-independent mechanisms (5, 14). TRs are encoded by two distinct genes, c-erb-Aα and c-erb-Aβ, located on chromosomes 3 and 17, respectively. The c-erb-Aα gene is alternatively spliced into TR-α1 and TR-α2 isoforms, and the c-erb-Aβ gene is spliced into TR-β1 and TR-β2, respectively. The TR-α1, TR-β1, and TR-β2 isoforms can activate transcription, whereas TR-α2 is unable to bind to T3. The exact physiological role of TR-α2 has remained unknown (see Refs. 4 and 13).

The extensive literature to date on hormonal regulation of myogenesis highlights that thyroid hormones are major determinants of the muscle phenotype, but the molecular mechanism of thyroid hormone action on skeletal muscle and its MHC isoform composition via specific TR isoforms are unclear. Mice deficient for one or several TRs are useful tools to elucidate the roles played by individual TRs. We have therefore used homologous recombination to delete TR-α1, TR-β gene, or both genes to study the influence of TRs in regulating the expression of MHC isoforms in fast- and slow-twitch skeletal muscles. These results have been reported in short form elsewhere (37).

MATERIALS AND METHODS

TR-deficient mice and assay of thyroid hormone levels in serum. The study was carried out on genetically altered male mice (2-6 mo) lacking the α2, β2, or both α1 and β-thyroid hormone receptors (TR-α1–/–, TR-β–/–, or TR-α1–/–β2–/–). TR-deficient mice were obtained by disrupting the TR-α1 (34) or TR-β gene (10). Mice were genotyped as described and documented in detail previously by Southern blot analysis and PCR, using primers specific for TR-α1 and TR-β (10, 34).

In addition, careful Northern blot analysis was performed to determine the consequences on gene expression. Mice deficient for TR-α1 still express the nonligand binding protein TR-α2, and no perturbation of the expression of the adjacent rev-erb-Aα gene has been observed in the TR-deficient mice (10, 34; Vennstrom, unpublished observations). Mice deficient for the expression of TR-β are the result of deletion of part of the DNA binding domain of TR-β, which furthermore results in a frameshift in the coding sequence. Consequently, no TR-β1 or TR-β2 protein is detected in these mice, as shown in detail by Forrest et al. (10). Tissues from these mice were further examined by determining the specific nuclear T3 binding capacity and by gel-shift analysis for the presence of specific TR proteins that could bind a T3 response element (14). Mice deficient for all T3 binding receptors (TR-α1 and TR-β) were produced by crossing the TR-α1–/– and TR-β–/– strains, as recently described (14). The genetic background differs between the different mouse strains. TR-α1–/– mice were derived from E14 ES cells of strain 129/Ola, and the chimeric offspring was mated to BALB/c mice. TR-β–/– mice derive from 129/5v ES cells, and the progeny was crossed with C57BL/6j mice. The TR-α1–/–β2–/– compound knockout mice thus have a genetic background stemming from all four mouse strains. For all experiments, wild-type (WT)-control mice were produced from double heterozygote crosses with the respective receptor-deficient strains. The control strains were bred in parallel form with the receptor-deficient strains for three to four generations, at which point new heterozygotes produced inbreeding, followed by renewed heterozygote breeding for homozygote WT and knockout strains. Mice were housed under 12:12-h light-dark cycles. The measurement of serum thyroid hormone levels were determined using “Amerlex-MAB” kit, according to the method described previously (34). Animals were killed either by spinal dislocation or by lethal injection of pentobarbital sodium. Blood was collected from the abdominal aorta, allowed to clot, and centrifuged; the serum was collected and stored at −20°C until assayed. The study was approved by the ethical committee at the Karolinska Institute.

Enzyme histochemical staining and fiber cross-sectional area measurement. The soleus and extensor digitorum longus (EDL) muscles were dissected free from surrounding tissue and clamped at approximately the in situ length. The muscle was subsequently weighed, frozen in freon, chilled with liquid nitrogen, and stored at −80°C until processed further. The muscle was cut at the motor point (soleus) or at its greatest girth perpendicular to its longitudinal axis (EDL) into 10-μm-thick serial sections with a cryostat (Frigocut E, Reichert-Jung, Heidelberg, Germany) at −20°C.

In the soleus, cross sections were stained for myofibrillar ATPase at pH 9.4, after 55 min of formaldehyde fixation at 4°C and after acid preincubation at pH 4.35 (Fig. 1, for details, see Refs. 21 and 22). The soleus muscle is fusiform with a discrete tendon, the muscle fibers are oriented in parallel form with an angle of −2–5° to the long axis, and all fibers pass through the motor point. An accurate estimation of the total number of fibers can accordingly be made from a single transverse cross section at the motor point. The number of soleus fibers of each type was counted on magnified photomicrographs of whole muscle cross sections.

In the EDL, cross sections were stained for myofibrillar ATPase after an acid preincubation at pH 4.55 and classified as type I (black), type IIB (light gray), and intermediate (dark gray) fibers (Fig. 2). The intermediate fiber type includes type IIX, type IIXA, and type IIXB (see Ref. 16). In the EDL, not all fibers pass through the eyes of the muscle, and accurate measurements of total muscle fiber number cannot accordingly be made from a single muscle cross section (21).

Cross-sectional areas of individual muscle fibers of different types were measured by tracing their outlines on magnified images of myofibrillar ATPase-stained cross sections with the aid of a digitizing unit connected to a microcomputer (Vidas, Kontron, Munich, Germany; or Optimas 61, Optimas). Cross-sectional areas were measured from a total of 50 fibers of types I and IIA in the soleus and of types IIB and intermediate in the EDL. The number of intermediate fibers in soleus and type I fibers in EDL muscle was very small (<50), and these fiber types have therefore not been included in the statistical analyses.

Determination of MHC isoform composition. The MHC composition was determined by SDS-PAGE (20). The total bis-acrylamide concentrations were 4% (wt/vol) in the stacking gel and 7% in the running gel, and the gel matrix included 30% glycerol as described previously (23). The ammonium persulfate concentrations were 0.04 and 0.029% in the stacking and separation gels, respectively, and the gel solutions were degassed (1 Torr) for 15 min at 18°C. Polymerization was subsequently activated by adding N,N,N’,N’-tetramethylethylenediamine to the stacking (0.1%) and separation gels (0.07%). Single 10-μm soleus and EDL muscle cross sections were placed in sample buffer in a plastic microfuge
tube and stored at −80°C until analyzed. Sample loads were kept small to improve the resolution of the MHC bands. The gels were placed in the electrophoresis apparatus (SE 600 vertical slab gel unit, Hoefer Scientific Instruments) connected to a power supply and a cooling unit. Electrophoresis was performed at 120 V for 22–24 h with a Tris-glycine electrode buffer (pH 8.3) at 15°C (20, 23). Separating gels were silver stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics, Sunnyvale, CA) with a high spatial resolution (50-µm pixel spacing) and 4,096 optical density levels to determine the relative contents of the MHC isoforms. The volume integration function was used to quantify the amount of protein, and background activity was subtracted from all pixel values (ImageQuant Software v3.3, Molecular Dynamics). Immunoblotting experiments have been performed in our laboratory to determine the migration order of the four MHC isoforms separated by the type of gels used in this study. The migration order from slowest- to fastest-migrating MHC isoform is as follows: IIA-IIIX-IIIB-I (23).

Fig. 1. Enzyme histochemical staining of mATPase at pH 4.35 in soleus cross sections. A: 3,5,3'-triiodothyronine (T3) receptor (TR)-α1 wild-type (WT); B: TR-β WT; C: TR-α1/β WT; and D: magnified picture of C. a: TR-α1/−; b: TR-β/−; c: TR-α1/−β/− mice; and d: magnified picture of c. △, arrow, and * denote type I, type intermediate, and type IIA fibers, respectively. Bar = 200 µm.

Statistics. Means ± SE were calculated from individual values by standard procedures. A two-way ANOVA was applied to test the effect of TR deficiency, mouse strain, and interactions among TR-α1/−, TR-β/−, and TR-α1/−β/− groups. Differences were considered significant at P < 0.05. If any significant interaction was found, Tukey's test (honestly significant difference) was applied.

RESULTS

Animals and thyroid hormone levels. Body and muscle weights were compared between the TR-deficient mice and the corresponding age- and gender-matched WT mice (Table 1). Significant differences were observed in these properties between TR-deficient and WT mice.
Serum total 1-thyroxine levels in TR-α1−/−β−/− deficient mice (129.4 ± 13.1 pmol/l) were significantly higher (P < 0.001) than those in WT mice (13.8 ± 0.7 pmol/l).

Muscle fiber number, cross-sectional areas, and fiber type proportions. In the soleus, total fiber numbers and cross-sectional fiber areas were lower (P < 0.05) in TR-deficient mice than in the age-, gender-, and strain-matched WT mice (Tables 2 and 3). Significant differences (P < 0.001) were observed in muscle fiber type proportions between the TR-deficient and WT mice (Fig. 1). However, the two-way ANOVA demonstrated significant interactions (P < 0.001) among the three mouse background strains: no effect in the TR-β−/−, a moderate effect in the TR-α1−/−, and a strong effect in the TR-α1−/−β−/− mice (Table 3). The proportion of the type I fibers was 51% higher (P < 0.001) in the TR-α1−/−β−/− mice than in the WT mice, and a 21% difference (P < 0.001) was observed in the TR-α1−/− mice. A correspondingly lower (P < 0.001) proportion of the fast-twitch type IIA fibers was observed in both TR-α1−/−β−/− and TR-α1−/− mice (Table 3). Despite the dramatic shift in fiber type proportions in the TR-α1−/−β−/− mice, some muscle fibers retained their type IIA enzyme histochemical staining pattern (Fig. 1).

In the EDL, total fiber number was not calculated because accurate measurements cannot be made from single EDL muscle cross sections. A significant (P < 0.001) effect of TR deficiency on a cross-sectional area was only observed in the intermediate fiber type (Table 2). The two-way ANOVA demonstrated a strong interac-
among 3 mouse strains. Significantly different from its wild-type (WT): *

Proportion (significant interactions (P < 0.001) in type I fiber proportion was restricted to the between the TR-deficient mice, and the increase (P < 0.001) was observed in both soleus and EDL, i.e., reductions in

Body and muscle weights in WT and TR-deficient mice (Fig. 3). The four MHC isoforms SDS-PAGE from single 10-µm-thick soleus and EDL muscle cross sections (Fig. 3). The four MHC isoforms identified in mouse skeletal muscle were referred to as IIA, IIX, IIB, and I in the order of increasing electrophoretic mobility; the type I (β/slow) MHC being the fastest-migrating myosin isoform. However, the distance between the IIA and IIX bands is shorter in the mouse compared with that in the rat (Fig. 3). We did not detect embryonic or fetal MHC in either soleus or EDL muscle.

In the slow-twitch soleus, a type IIA-to-type I MHC isoform shift (P < 0.001) was observed in the TR-deficient mice. The two-way factor analysis demonstrated significant differences in the extent of fast-to-slow myosin isoform transitions among the three TR-deficient groups; the type I MHC content did not differ significantly between the TR-β-/ and TR-β+/ mice, a moderate increase of type I MHC (P < 0.05) was observed in the TR-α1-/β-/- group (63.9 ± 5.1 vs. 50.7 ± 5.0% type I, Fig. 4), and a dramatic increase (P < 0.001) was observed in the TR-α1-/β-/- group (86.9 ± 2.7 vs. 41.4 ± 3.3% type I, Fig. 4). Despite the 45% difference in type I MHC content between the TR-α1-/β-/- and WT groups, the MHC transition was not complete, and 13% type II MHCs were expressed in the TR-deficient mice (Fig. 4). There was no TR-deficient effect on the expression of type IIX or IIB MHC isoforms in the soleus. However, significant mouse strain differences (P < 0.01–0.001) were observed in the expression of IIX and IIB MHCs among three different mouse background strains; i.e., the relative content of the MHC IIX was higher in the TR-β-/ and TR-β+/ mouse strain. In fact, type IIX MHC was not detected in either TR-α1-/β-/- or TR-α1+/β+/ mice, and type IIB MHC was only detected among the TR-β-/ and TR-β+/ mice (Figs. 3 and 4).

In general, there was a close correspondence between fiber type proportions (calculated from the enzyme histochemical stainings) and the MHC isoform expression (determined from electrophoretic protein separations). However, significant differences were also observed between the two methods. In the soleus, the differences in the TR-α1-/β- and TR-α1-/β-/- mouse strains can be explained by the coexpression of type I and IIA MHC isoforms in the type IIC fibers. In the TR-β-/ mouse strain, on the other hand, the percentage of type IIB fibers determined by enzyme

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Body Wt, g</th>
<th>Soleus Wt, mg</th>
<th>EDL Wt, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR-α1</td>
<td>5 / 6</td>
<td>29.7 ± 1.2 / 30.5 ± 1.1</td>
<td>9.6 ± 1.2 / 8.0 ± 0.9</td>
<td>124.0 ± 7.0 / 11.5 ± 0.8</td>
</tr>
<tr>
<td>TR-β</td>
<td>5 / 6</td>
<td>28.8 ± 1.3 / 37.0 ± 5.3</td>
<td>9.4 ± 1.3 / 9.2 ± 1.9</td>
<td>134.0 ± 11.0 / 12.2 ± 2.2</td>
</tr>
<tr>
<td>TR-α1/β</td>
<td>5 / 11</td>
<td>30.8 ± 2.8 / 19.8 ± 5.5</td>
<td>8.4 ± 1.8 / 4.9 ± 1.3</td>
<td>100.0 ± 21.0 / 6.7 ± 1.8</td>
</tr>
</tbody>
</table>

TR-deficient effect

| Difference among 3 mouse strains | NS | P < 0.01 | P < 0.05 |

| Interactions | NS | P < 0.01 | P < 0.001 |

Values are expressed as means ± SE. A 2-way ANOVA was applied to test for effects of TR-deficiency and differences among 3 mouse strains. Significantly different from its wild-type (WT) group, i.e., a significant decrease (P < 0.001) in fiber size was only observed in TR-α1-/β-/- mice. A higher proportion (P < 0.01) of type I fibers was observed in the TR-deficient mice compared with WT mice, but significant interactions (P < 0.05) were observed between the TR-deficient mice, and the increase (P < 0.001) in type I fiber proportion was restricted to the TR-α1-/β-/- mice (Table 4).

Thus the same general TR-deficient effects were observed in both soleus and EDL, i.e., reductions in muscle weights, total fiber number (only measured in the soleus), and cross-sectional fiber areas were more pronounced in the TR-α1-/β-/- deficient group compared with the TR-α1-/β-/- and TR-β-/ mice.

MHC isoform composition. A total of four distinct MHC isoforms were separated on the silver-stained 7% SDS-PAGE from single 10-µm-thick soleus and EDL muscle cross sections (Fig. 3). The four MHC isoforms identified in mouse skeletal muscle were referred to as IIA, IIX, IIB, and I in the order of increasing electrophoretic mobility; the type I (β/slow) MHC being the fastest-migrating myosin isoform. However, the distance between the IIA and IIX bands is shorter in the mouse compared with that in the rat (Fig. 3). We did not detect embryonic or fetal MHC in either soleus or EDL muscle.

In the slow-twitch soleus, a type IIA-to-type I MHC isoform shift (P < 0.001) was observed in the TR-deficient mice. The two-way factor analysis demonstrated significant differences in the extent of fast-to-slow myosin isoform transitions among the three TR-deficient groups: the type I MHC content did not differ significantly between the TR-β-/ and TR-β+/ mice, a moderate increase of type I MHC (P < 0.05) was observed in the TR-α1-/β-/- group (63.9 ± 5.1 vs. 50.7 ± 5.0% type I, Fig. 4), and a dramatic increase (P < 0.001) was observed in the TR-α1-/β-/- group (86.9 ± 2.7 vs. 41.4 ± 3.3% type I, Fig. 4). Despite the 45% difference in type I MHC content between the TR-α1-/β-/- and WT groups, the MHC transition was not complete, and 13% type II MHCs were expressed in the TR-deficient mice (Fig. 4). There was no TR-deficient effect on the expression of type IIX or IIB MHC isoforms in the soleus. However, significant mouse strain differences (P < 0.01–0.001) were observed in the expression of IIX and IIB MHCs among three different mouse background strains; i.e., the relative content of the MHC IIX was higher in the TR-β-/ and TR-β+/ mouse strain. In fact, type IIX MHC was not detected in either TR-α1-/β-/- or TR-α1+/β+/ mice, and type IIB MHC was only detected among the TR-β-/ and TR-β+/ mice (Figs. 3 and 4).

In general, there was a close correspondence between fiber type proportions (calculated from the enzyme histochemical stainings) and the MHC isoform expression (determined from electrophoretic protein separations). However, significant differences were also observed between the two methods. In the soleus, the differences in the TR-α1-/β- and TR-α1-/β-/- mouse strains can be explained by the coexpression of type I and IIA MHC isoforms in the type IIC fibers. In the TR-β-/ mouse strain, on the other hand, the percentage of type IIB fibers determined by enzyme

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Soleus</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
<td>Type IIA</td>
<td>Type IIB</td>
</tr>
<tr>
<td>TR-α1</td>
<td>5 / 6</td>
<td>724 ± 22 / 703 ± 74</td>
<td>649 ± 30 / 658 ± 59</td>
</tr>
<tr>
<td>TR-β</td>
<td>5 / 6</td>
<td>783 ± 56 / 748 ± 65</td>
<td>757 ± 35 / 749 ± 45</td>
</tr>
<tr>
<td>TR-α1/β</td>
<td>5 / 11</td>
<td>815 ± 30 / 704 ± 37</td>
<td>637 ± 23 / 549 ± 34</td>
</tr>
</tbody>
</table>

TR-deficient effect

| Difference among 3 mouse strains | NS | P < 0.05 | P < 0.05 |

| Interactions | NS | P < 0.01 | P < 0.001 |

Values are expressed as means ± SE in µm². A 2-way ANOVA was applied to test for effects of TR-deficiency and differences among 3 mouse strains. Significantly different from WT: *P < 0.001; significantly different from TR-α1/β-deficient mice: †P < 0.005.
Table 3. Total fiber number and enzyme histochemically classified fiber type proportions of soleus muscles in the three WT and TR-deficient (−/−) mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Total Fiber Number</th>
<th>Type I, %</th>
<th>Type I+IIC, %</th>
<th>Type IIA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR-α1</td>
<td>5</td>
<td>587 ± 46</td>
<td>38.9 ± 2.0</td>
<td>59.7 ± 3.8*</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>511 ± 25</td>
<td>39.2 ± 2.5</td>
<td>57.8 ± 3.7*</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>TR-β</td>
<td>5</td>
<td>705 ± 51</td>
<td>34.9 ± 0.4</td>
<td>31.9 ± 1.6†</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>581 ± 58</td>
<td>41.6 ± 1.8</td>
<td>39.0 ± 1.5‡</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>TR-α1/β</td>
<td>5</td>
<td>537 ± 18</td>
<td>39.7 ± 1.3</td>
<td>90.4 ± 1.5‡</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>416 ± 10.8</td>
<td>69.2 ± 6.0</td>
<td>59.0 ± 1.4</td>
<td>8.1 ± 1.5*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A 2-way ANOVA was applied to test for TR deficiency and differences among 3 mouse strains. Significantly different from its WT: *P < 0.01; significantly different from TR-α1-deficient mice: †P < 0.05; significantly different from TR-β WT or TR-β-deficient mice: §P < 0.05; significantly different from TR-α1/β WT or TR-α1/β group: ¶P < 0.05.

The histochemistry method was 28 and 15% higher compared with the type IIA MHC contents determined by SDS-PAGE in TR-β−/− and TR-β+/+ mice, respectively. This cannot be explained by coexpression of type IIA MHC in the IIC and IIC fibers constituting only 2% of the total fiber population. Furthermore, the IIX and IIB MHCs combined contents amounted to ~25 and 12% in TR-β−/− and TR-β+/+ mice, respectively. However, we were unable to identify these changes in IIX and IIB MHC of the soleus sections from TR-β−/− and TR-β+/+ mouse strains, indicating that the IIX and IIB MHCs were coexpressed in fibers where the IIA MHC isoform was dominating and the fibers stained as type IIA fibers in the mATPase stained sections.

In the fast-twitch EDL, the type IIB MHC content was lower (P < 0.05) in the TR-deficient than in the WT mice, irrespective of mouse background strain (Fig. 5). TR deficiency had, on the other hand, no significant effect on the expression of types I, IIA, and IIX MHC isoforms. However, it is interesting to note that 3 of 11 TR-α1−/−β−/− mice expressed type I MHC, ranging between 9 and 12%, and 4 of 11 expressed IIA MHCs, ranging between 10 and 22% (Fig. 3), whereas types I and IIA MHC were not expressed in any other TR-deficient or WT mice, except for one TR-α1-deficient mouse expressing 12% type IIA MHC. Finally, mouse strain-specific differences (P < 0.001) were observed in the expression of IIX and IIB MHC isoforms; i.e., lower IIX and higher IIB MHC contents were observed in the TR-β−/− and TR-β+/+ mice compared with the other two groups.

Table 4. Enzyme histochemically classified fiber type proportions of EDL muscle in the three WT and TR-deficient (−/−) mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Animals</th>
<th>Type I, %</th>
<th>Type Intermediate, %</th>
<th>Type IIB, %</th>
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</thead>
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<tr>
<td></td>
<td>WT −/−</td>
<td>WT −/−</td>
<td>WT −/−</td>
<td>WT −/−</td>
</tr>
<tr>
<td>TR-α1</td>
<td>5</td>
<td>3.7 ± 1.3</td>
<td>30.4 ± 4.4</td>
<td>69 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.7 ± 1.3</td>
<td>30.8 ± 1.8</td>
<td>65.8 ± 1.8</td>
</tr>
<tr>
<td>TR-β</td>
<td>5</td>
<td>0.2 ± 0.1</td>
<td>29.8 ± 3.9</td>
<td>63.8 ± 2.6</td>
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<tr>
<td></td>
<td>6</td>
<td>0.2 ± 0.1</td>
<td>30.5 ± 2.6</td>
<td>69.3 ± 2.6</td>
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<tr>
<td>TR-α1/β</td>
<td>5</td>
<td>6.7 ± 1.4</td>
<td>28.8 ± 3.4</td>
<td>70.9 ± 3.3</td>
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<td></td>
<td>11</td>
<td>32.1 ± 3.0</td>
<td>31.2 ± 3.0</td>
<td>61.3 ± 2.3</td>
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</table>

Values are expressed as means ± SE. A 2-way ANOVA was applied to test for TR deficiency and differences among 3 mouse strains. Significantly different from its WT (†P < 0.001); significantly different from TR-α1-deficient mice (*P < 0.05); significantly different from TR-α1/β-deficient mice (‡P < 0.05).
content was only detected in fibers occupying >2% of total EDL cross-sectional area, i.e., above the critical level (3%) of detection by the silver-stained SDS-PAGE used in this study (23).

**DISCUSSION**

Our data allow several conclusions to be drawn. First, thyroid hormone is one factor that influences normal growth, i.e., significantly lower body weights were observed in TR-α1-/-β-/- mice but not in TR-α1-/- and TR-β-/- mice. The lower body weight was accompanied by a correspondingly lower muscle weight, which was caused by smaller and fewer muscle fibers. Second, a major fast to slow MHC isoform switching was observed in the slow-twitch soleus, and a minor transition was noted in the fast-twitch EDL in TR-deficient mice. However, the transition was not complete in either the soleus or the EDL, and all MHC isoforms expressed in the WT mice were also expressed in the TR-deficient mice, albeit in different proportions. Third, a muscle-type, receptor type-, and muscle fiber-specific effect of TR deficiency on MHC isoform composition was observed; i.e., 1) a dramatic fast-to-slow MHC isoform transition was observed in the slow-twitch soleus, but the effects in the fast-twitch EDL were restricted to a moderate decrease in the type IIB MHC content and a slight increase in type I fiber number, 2) in the slow-twitch soleus, the TR-deficiency effect on the expression of MHC isoforms differed among the TR-α1, TR-β, and TR-α1/β mouse strains, i.e., there was no effect in the TR-β strain, whereas there were significant effects in the TR-α1 (P < 0.05) and in the TR-α1/β (P < 0.001) strains. In the fast-twitch EDL, on the other hand, the relative change in MHC content was similar in all three TR-deficient groups, and 3) the fast to slow MHC isoform transition was not complete in the soleus of the TR-α1/β-deficient mice, and some muscle fibers retained type IIA enzyme histochemical characteristics. Fourth, all presently known adult myosin isoforms expressed in extrafusal muscle fibers from slow- and fast-twitch hindlimb mouse muscles were detected in the TR-deficient mice, and embryonic or fetal MHC isoforms were not expressed in either soleus or EDL from mice lacking TR-α1, TR-β, or both receptors. Although this study did not specifically address developmental myosin isoform transitions, the results from this study infer that ultimately the transition to an adult MHC isoform expression is not solely mediated by TRs.

**Effects of TR deficiency on body and muscle weights.** Thyroid hormone has been considered to be necessary for normal growth, and the anabolic effect of T3 is well established (33). The comparison between TR-deficient mice and the corresponding age- and gender-matched WT controls showed 36% lower body weights, which were accompanied by a parallel 34% reduction in both soleus and EDL muscle weights in the TR-α1-/-β-/-.
mice, whereas no decrease in body and muscle weights were observed in TR-α<sub>1</sub>-/- or TR-β<sub>2</sub>-/- mice. The present results are in conformity with previous observations of 30% lower body weight in TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice (14) and no decrease in body weight in either TR-α<sub>2</sub>- or TR-β<sub>2</sub>-deficient mice (10, 34). The significantly lower body weight in TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice is tightly coupled to thyroid hormone effects on normal muscle growth, i.e., the size and number of muscle fibers. Previously, it has been shown that serum levels of insulin-like growth factor I are significantly reduced in TR-α<sub>1</sub>-/- or TR-β<sub>2</sub>-/- mice by 21%. Furthermore, in the pituitary, growth hormone (GH) mRNA levels were fivefold lower, and the content of GH protein was 2.7-fold lower (P < 0.01) in TR-α<sub>1</sub>-/β<sub>1</sub>-/- than in WT mice. In contrast, GH mRNA levels were not significantly altered in TR-α<sub>1</sub>-/- or TR-β<sub>2</sub>-/- mice (14). Thus the consequences on muscle size and number of muscle fibers. Previously, it has been shown that serum levels of insulin-like growth factor I are significantly reduced in TR-α<sub>1</sub>-/- or TR-β<sub>2</sub>-/- mice by 21%. Furthermore, in the pituitary, growth hormone (GH) mRNA levels were fivefold lower, and the content of GH protein was 2.7-fold lower (P < 0.01) in TR-α<sub>1</sub>-/β<sub>1</sub>-/- than in WT mice. In contrast, GH mRNA levels were not significantly altered in TR-α<sub>1</sub>-/- or TR-β<sub>2</sub>-/- mice (14). These results suggest a primary requirement for the interaction between TR-α<sub>1</sub> and TR-β in the control of GH gene transcription, consistent with the identification of T<sub>3</sub> response elements in the rat GH gene (12, 29) and that GH deficiency is the most probable reason for the lower body and muscle weights in TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice.

Possible mechanisms underlying MHC transitions in TR-deficient mice. The present results showed that the expression of MHC isoforms in the soleus is unaltered in TR-β<sub>2</sub>-/- mice, moderately altered in TR-α<sub>1</sub>-/- mice, and dramatically changed in TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice. Thus individual TR-α<sub>1</sub> and TR-β are only playing a limited role in regulating MHC isoform expression. The results of TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice exhibited dramatic changes in growth and MHC isoform expression and established the existence of additional T<sub>3</sub> response pathways in which TR-α<sub>1</sub> and TR-β cooperate with, or substitute for, each other. Thus the use of such pathways significantly extends the spectrum of T<sub>3</sub> actions of TR-α<sub>1</sub> or TR-β alone.

The muscle-specific difference in the response to hypothyroidism in rodent fast- and slow-twitch muscles (see Ref. 4) was mimicked in the TR-α<sub>1</sub>-/β<sub>1</sub>-/- mice. That is, a dramatic, but not complete, upregulation of the β/slow MHC isoform was observed in the slow-twitch soleus, whereas only a slight, but significant, upregulation of the β/slow MHC isoform was observed in the fast-twitch EDL. Thus the consequences on muscle are similar in the absence of thyroid hormone or absence of thyroid hormone receptors, indicating that TR-α<sub>1</sub> and TR-β together mediate the known actions of T<sub>3</sub>.

The mechanisms underlying the muscle-specific difference in response to circulating thyroid hormone levels remain unknown. Recently, Haddad et al. (15) demonstrated that, despite a divergent pattern of TR mRNA expression in different muscle types, it is unlikely that these muscle-specific differences in TR expression pattern account for qualitative and quantitative changes in MHC isoform expression under altered thyroid hormone state. In addition to muscle-specific differences, phenotypically identical muscle cells in the same muscle respond differently to hypo and hyperthyroidism (2, 3, 7, 8, 21–24, 26, 33, 36). A similar result was observed in the TR-deficient animals, i.e., a complete MYHC isoform was restricted to a subset of the muscle fibers in the TR-deficient mice. The diversity of muscle cell types is determined not only by multiple hormonal and mechanical factors and innervation, but also from the developmental history of the muscle cell (30). That is, a significant heterogeneity of muscle cell precursors has been demonstrated at different developmental stages, and primary and secondary generation muscle fibers have been observed to differ with respect to the pattern of MHC isoform expression (30). The adaptive range for MHC isoform transitions may accordingly vary between different muscle cells, being dependent on the muscle cell developmental history (1). Alternatively, the expression of nuclear co-repressor proteins or muscle receptors, which primarily act as transcriptional repressors, may vary between different muscle cells that appear phenotypically identical, affecting the adaptive range of MHC transitions in specific muscle cells. This context, it is of interest to note that the rev-erb-A<sub>α</sub> orphan nuclear-receptor gene partially overlaps the TR<sub>α</sub> gene. The rev-erb-A<sub>α</sub> receptor belongs to the same superfamily of transcription factors as TRs, and it has been suggested that it could interfere with the TR<sub>α</sub> expression (25). The rev-erb-A<sub>α</sub> receptor has no known ligand, and it acts as a negative transcriptional regulator. Thus the expression of the rev-erb-A<sub>α</sub> receptor could have major consequences for the cellular thyroid hormone responsiveness. This is supported by recent observations in our group of a significant upregulation of the β/slow MHC isoform in the slow-twitch soleus, but not in the EDL, in rev-erb-A<sub>α</sub> receptor-deficient mice (P. Pircher, P. Chomez, B. Vennström, L. Larsson, unpublished observations). In the EDL, the rev-erb-A<sub>α</sub> receptor was expressed in a muscle fiber-type specific pattern, whereas no such pattern was observed in the slow-twitch soleus. The variable expression of the rev-erb-A<sub>α</sub> receptor observed in muscle cells expressing an identical set of MHC isoforms may be one factor underlying the muscle-fiber specific differences in response to thyroid hormone in the soleus muscle.

In small rodents, thyroid hormone is barely detectable in embryonic and neonatal stages, but it increases a few days after birth. Parallel with this increase, embryonic and fetal MHC isoforms are repressed and adult isoforms are expressed. This thyroid hormone-induced myosin isoform switching appears to be general in all mammalian limb muscles investigated to date, including both mouse and human (6). Furthermore, the developmental myosin isoform transition has been shown to be inhibited by hypothyroidism and accelerated by hyperthyroidism, being independent of the action of both motoneuron and GH activation (1–3, 11, 33). The exact mechanisms by which T<sub>3</sub> induces the switching from an embryonic/fetal to an adult MHC isoform expression remain unknown. The absence of embryonic/fetal MHCs in any of the TR-deficient mice suggests that T<sub>3</sub> receptors are not required for this transformation. Alternatively, T<sub>3</sub> is not an obligate developmental regulator of MHC isoform transitions, and the absence of TRs will primarily cause a develop-
mental delay in the adult MHC isoform expression. This is supported by the reduced cochlear hair cell potassium ion conductance during early development and the normal ion conductance at later stages of development in TR-β−/− mice (28) as well as by the slower upregulation of adult myosin isoforms in hypo-
MHC expression and the interaction between
hormone regulation of myofibrillar protein expression. Future studies to improve our understanding of thyroid
MHC isoform expression is the focus of ongoing and
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perspective
perspective
promoting growth and differentiation of MHC isoforms
via nuclear thyroid hormone receptors. The TR-α1 and TR-β appear to be able to, at least in part, functionally
substitute for or cooperate with each other. A compara-
tive study of TR-α1−/−, TR-β−/−, and TR-α1−/β−/− mice
revealed only limited roles that could be ascribed
individually to TR-α1 or TR-β. The results from this
study emphasize the complex interplay between TRs
and other cell- and muscle-type specific factors, which
play a very important role during development and
differentiation.

Perspectives

Myosin is a major structural component of skeletal
muscle, and it is considered to be the molecular motor
that converts free energy derived from its hydrolysis of
ATP into mechanical work. The extensive literature
to date on hormonal regulation of myogenesis highlights
that thyroid hormones are major determinants of the
muscle phenotype, but the molecular mechanism of
thyroid hormone action (via specific nuclear TRs) on
skeletal muscle MHC isoform composition is unclear.
Mice deficient for one or several TRs are therefore
useful tools to elucidate the roles played by individual
TRs in regulating the expression of myosin motor
proteins and for our understanding of the motor handi-
cap associated with the hypothyroid myopathy. The
influence of the rev-erb-α orphan nuclear receptor on
MHC expression and the interaction between rev-
erb-α and TRs in the transcriptional regulation of
MHC isoform expression is the focus of ongoing and
future studies to improve our understanding of thyroid
hormone regulation of myofibrillar protein expression.

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