Effects of thyroid hormone on fast- and slow-twitch skeletal muscles in young and old rats

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1. The effects of 4 weeks of thyroid hormone treatment on contractile, enzyme-histochemical and morphometric properties and on the myosin isoform composition were compared in the slow-twitch soleus and the fast-twitch extensor digitorum longus (EDL) muscle in young (3–6 months) and old (20–24 months) male rats.
2. In the soleus of untreated controls, contraction and half-relaxation times of the isometric twitch increased by 19–32% with age. The change in contractile properties was paralleled by an age-related increase in the proportions of type I fibres and type I myosin heavy chain (MHC) and slow myosin light chain (MLC) isoforms.
3. In the EDL of controls, contraction and half-relaxation times were significantly prolonged (21–38%) in the post-tetanus twitch in the old animals. No significant age-related changes were observed in enzyme-histochemical fibre-type proportions, although the number of fibres expressing both type IIA and IIB MHCs and of fibres expressing slow MLC isoforms was increased in the old animals.
4. Serum 3,5,3′,5′-tetraiodothyronine (T_4) levels were lower (34%) in the old animals, but the primary byproduct of T_4, 3,5,3′-triiodothyronine (T_3), did not differ between young and old animals.
5. The effects of 4 weeks of thyroid hormone treatment were highly muscle specific, and were more pronounced in soleus than in EDL, irrespective of animal age. In the soleus, this treatment shortened the contraction and half-relaxation times by 35–57% and decreased the number of type I fibres by 66–77% in both young and old animals. In EDL, thyroid hormone treatment significantly shortened the contraction time by 24%, but the change was restricted to the old animals.
6. In conclusion, the ability of skeletal muscle to respond to thyroid hormone treatment was not impaired in old age and the age-related changes in speed of contraction and enzyme-histochemical properties and myosin isoform compositions were diminished after thyroid hormone treatment in both the soleus and EDL.

Thyroid hormones are very important in the development of vertebrate skeletal muscle, and an intact thyroid gland is required both for the development of muscle mass and for the differentiation of biochemical and contractile characteristics of skeletal muscles (Finkelstein, Andrianakis, Luff & Walker, 1991). These developmental changes in skeletal muscles are well defined and are complete within a relatively short time period, and have received extensive scientific interest (Swynghedauw, 1986). After development and maturation, there is a slow on-going ageing process which continues to affect skeletal muscle progressively for the rest of life, resulting in profound alterations with a 'dedifferentiation' between slow- and fast-twitch muscles at an advanced age (Fitts, 1981). Ageing, on the other hand, has been given considerably less attention despite its importance. This is partly due to the complexity of the ageing process, which extends over a long period and involves a multitude of different factors; in addition, secondary influences related to disease, malnutrition and inactivity may obscure the 'primary' ageing process (Larsson, 1982). Thyroid hormones have been proposed to play a significant role also in the age-related changes in tissue function, and there is experimental evidence of lower circulating thyroid hormone levels in old rats (Sonntag, 1987). In previous studies, we have observed age-related changes in contractile, enzyme-histochemical and sarcoplasmic reticulum (SR) properties and in myosin heavy chain

(MHC) isoform composition mimicking changes observed in hypothyroid animals, such as slowing of the isometric twitch, an increased proportion of type I MHCs in slow muscle and decreased Ca^{2+} pump activity in the SR (Larsson & Edström, 1986; Edström & Larsson, 1987; Ansved & Larsson, 1989; Larsson & Salviati, 1989; Larsson, Ansved, Edström, Gorza & Schiaffino, 1991*a*; Larsson, Biral, Campione & Schiaffino, 1993).

Skeletal muscles are often characterized in terms of their maximum shortening velocity, which is primarily determined by the composition of the MHC isoform (for references see Larsson & Moss, 1993). The phenotypic expression of MHC isoforms is controlled by developmental, neuronal, hormonal and mechanical factors, thyroid hormones being among the most potent regulators of the MHC composition. The thyroid hormone state has been found to predominate over mechanical (Diffee, Haddad, Herrick & Baldwin, 1991) and neural (Nwoye, Mommaerts, Simpson, Seraydarian & Marusich, 1982) factors in the determination of MHC isoform expression, and an age-related change in the regulatory influence of thyroid hormones would accordingly be expected to have a strong impact on this expression and hence on the contractility.

The present study was undertaken to investigate the effects of long-term (4 week) treatment with thyroid hormone in young and old animals, with the aim of further elucidating the influence of ageing on skeletal muscle plasticity, and the possible influence of thyroid hormone on contractile properties and myosin isoform composition in fast- and slow-twitch skeletal muscles.

METHODS

The study was carried out on male albino rats (Wistar), fed *ad libitum* with standard laboratory food and tap water. The animals were anaesthetized with an intramuscular injection of fentanyl-fluanisone (0·2–0·3 ml kg^{-1}) followed by pentobarbitone (30 mg kg^{-1}) administered intraperitoneally. The anaesthesia was kept at a similar level between animals by noting the response to noxic stimuli and reflex responses. If necessary, additional intraperitoneal injections of pentobarbitone (20–30% of the initial dose) were given during the experiments. The anaesthetized rats were killed after the physiological measurements by removing the heart. To avoid unpredictable effects on the musculature in very old age, such as those of extreme obesity, disease and disuse, we chose rats that had not yet reached an advanced age for the old group. The rats were divided into young (3–6 months) and old (20–24 months) control and hyperthyroid groups. Hyperthyroidism was induced by subcutaneous injections of 3,5,3′-triiodothyronine (T_3, 300 μg (kg body weight)$^{-1}$) every second day for 4 weeks (Fitzimons, Herrick & Baldwin, 1990). The T_3 administration was terminated 2 days prior to the experiments. Several rats died during the T_3 hormone treatment (probably as a result of cardiac arrhythmias). These animals are presented in Fig. 1, but otherwise they were not included in the analyses. At the end of the experiments, fresh blood samples were collected for determination of serum thyroid hormone (T_3 and 3,5,3′,5′- tetraiodothyronine, T_4) concentrations in control and hyperthyroid animals, using commercial solid-phase time-resolved fluoroimmunoassay kits (DELFIA T_3 and T_4, Wallac Oy, Turku, Finland). All blood samples were taken between 11.00 and 18.00 h.

Physiological technique

The skin over the lower part of the left hindlimb was removed, and the tendon from the soleus or extensor digitorum longus (EDL) muscle was cut distally. For studies of the soleus, the tendons of the gastrocnemius and plantaris muscles were cut and the motor nerve to the soleus was cut 5–10 mm proximal to the motor point. When EDL was to be studied, the fascia overlying the muscle was removed, the tibial anterior tendon was cut and plantar flexor muscles were denervated by transecting the motor nerves.

The animal was placed in the prone position on a steel plate which was heated to maintain body temperature. The dissected limb was put into a bath through an elliptical hole in the wall of the bath. The hole was considerably larger than the thigh and the blood circulation was not impaired. The skin of the thigh was stretched over a flange around the hole to make the bath leak-proof. The limb was rigidly fixed in the bath by means of a steel rod drilled through the tibia close to the knee joint, and a clamp on the foot. Mineral oil was circulated through the bath. The bath temperature was thermostatically controlled at 36 °C (35·5–36·5 °C; Kugelberg & Lindegren, 1979).

The tendon of the soleus and EDL was oriented along the natural pull of the muscle and attached to a strain gauge (UC 2, Statham Instruments Inc., Oxnard, CA, USA; 300 Hz resonant frequency and a 120 μm displacement range of the sensing mechanism) and a load cell accessory (UL 4–10, Statham Instruments). The mechanical responses were amplified (AD 6, Medelec Ltd, Old Woking, Surrey, UK), displayed on an oscilloscope (M-scope, Medelec) and recorded on Kodak Linagraph direct print paper (Eastman Kodak, Rochester, NY, USA). Contractions were recorded with the muscle set at an optimum length as determined from the maximum isometric twitch force. The isometric twitch contraction time was measured from the beginning of contraction to peak force, and the half-relaxation time from the peak force to the time when the force had decreased by 50%. The muscles were stimulated with a short tetanus, 2 s in soleus and 0·5 s in EDL, with stimulation frequencies corresponding to 100 and 150 Hz in these two muscles, respectively. Prior to and within 10 s after each tetanus, the muscle was excited with a single supramaximal stimulus and the twitch properties were recorded. Tension–frequency responses were studied using stimulation trains with frequencies of 10, 20, 40, 50, 80, 100 and 200 Hz in the soleus and 10, 30, 50, 80, 100, 150, 200, 300 and 400 Hz in the EDL. The duration of each stimulation train was 2 s and the muscle was allowed to rest for 3 min between each train in order to avoid muscle fatigue (for details of experimental procedures and anaesthesia see Larsson & Edström, 1986; Ansved & Larsson, 1989).

Enzyme-histochemical techniques

After each experiment the soleus and EDL muscles were gently dissected away 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. It was

then cut at the motor point (soleus) or at its greatest girth (EDL) perpendicular to its longitudinal axis into 10 μm thick cross-sections with a cryotome at −20 °C.

The muscle fibres in the EDL cross-sections were stained for myofibrillar ATPase after alkaline and acid preincubations, and classified as types I, IIA and IIB (Edström & Larsson, 1987). 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, and the fibres were classified as types I, IC, IIC and IIA (Edström & Larsson, 1987; Ansved & Larsson, 1989). The total number of fibres of each type was counted on magnified photomicrographs of whole muscle cross-sections and the relative number of each type was calculated. Cross-sectional areas of the individual muscle fibres were measured by tracing their outlines by hand on magnified black and white photographic prints of myofibrillar ATPase-stained cross-sections with the aid of a digitizing unit connected to a microcomputer (Videoplan, Kontron GmbH, Munich, Germany). In the soleus, two hundred fibres of type I and fifty of each of types IC, IIC and IIA were measured and in the EDL, fifty each of types I, IIA and IIB. If the total number of fibres of the respective type was smaller than these numbers, then all fibres of that specific type were measured. The total muscle fibre area was calculated according to the formula: [(% type I × type I area) + (% type IIA × type IIA area) + (% type IIB (or type IC and IIC) × type IIB area (or type IC and IIC area))]/100 × total number of muscle fibres.

Chemical skinning and determination of myosin heavy and light chain isoform composition

The soleus and EDL were dissected away from surrounding tissue, tied to a wooden stick and stretched to 110–120% of their slack length. The muscle specimens were then chemically skinned for 24 h by exposure to a 'skinning' solution containing (mM): 5 K_2EGTA, 170 potassium propionate, 2·5 Na_2K_2ATP, 2·5 magnesium propionate and 10 imidazole buffer, pH 7·0, as described by Salviati, Sorenson & Eastwood (1982). The muscle specimens were then stored at −20 °C until analysed in the same skinning solution made up in 50% glycerol.

In each animal, ten chemically skinned muscle fibres from the soleus and EDL were incubated at room temperature overnight in a solubilization solution (2·3% (w/v) sodium dodecyl sulphate (SDS), 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol and 62 mM Tris–HCl, pH 6·8). The MHC composition was determined by SDS polyacrylamide-gel electrophoresis (PAGE) on 6% polyacrylamide gels, as described by Danieli Betto, Zerbato & Betto (1986). After electrophoresis the gel was stained with Coomassie Blue or silver (for details see Salviati *et al.* 1982; Salviati, Betto, Danieli Betto & Zeviani, 1983). The fibres were classified as types I, I/IIA, IIA/I, IIA, IIA/IIB, IIB/IIA and IIB, depending on the relative proportion of MHCs present in the respective fibre. In fibres co-expressing two MHCs, the dominating MHC is placed first.

The myosin light chain (MLC) composition and the different combinations of slow (MLC_{1s} and MLC_{2s}) and fast (MLC_{1f}, MLC_{2f} and MLC_{3f}) MLCs were determined on 10–20% SDS–PAGE linear gradient gels (Salviati *et al.* 1983).

Statistics

Means and standard deviations were calculated from individual values by standard procedures. Student's two-tailed independent t test was used for comparisons of two populations. When the two population variances were equal, i.e. when the F value was small, the pooled-variance t test was used; otherwise the separate-variance t test for means was used (see Norusis, 1988). One-way analysis of variance was performed for intergroup comparisons of different fibre types. Tukey's honestly significant difference test was subsequently used for comparing the groups. Differences were considered significant at $P < 0·05$.

RESULTS

In conformity with previous results of studies of this and other albino rat strains (e.g. ILAR, 1981; Larsson & Edström, 1986; Edström & Larsson, 1987), the body and muscle weights were increased in old compared with young animals (Table 1). T_3 treatment affected the body weight differently in young and old rats (Fig. 1). In the young controls, the body weight increased ($P < 0·001$) during the 4 weeks of treatment, while in the young

Table 1. Body and muscle weights together with thyroid (T_3, T_4) levels in young and old control and hyperthyroid animals

Group	Body weight (g)		Soleus weight (mg)	EDL weight (mg)	T_3 (ng ml^{-1})	T_4 (ng ml^{-1})
	Before	After				
Control						
Young (20)	367 ± 26	443 ± 60	222 ± 43	227 ± 34	2·1 ± 0·9	66·8 ± 11·3
Old (12)	580 ± 84	618 ± 100	276 ± 56	290 ± 54	2·1 ± 0·7	44·4 ± 7·6
P	<0·001	<0·001	<0·01	<0·001	n.s.	<0·001
Hyperthyroid						
Young (6)	379 ± 43	392 ± 46	202 ± 25	209 ± 30	1·6 ± 0·3	22·3 ± 7·7
Old (10)	552 ± 93	469 ± 78	236 ± 69	219 ± 39	2·6 ± 2·5	14·6 ± 9·0
P	<0·001	<0·05	n.s.	n.s.	n.s.	n.s.

Values are means ± S.D. The number of animals is given in parentheses in the first column. Here and in subsequent tables n.s. indicates not significant.

Table 2. Contractile properties of soleus muscle of young and old control and hyperthyroid animals

Group	Contraction time (ms)	Half-relaxation time (ms)	Twitch tension (g)	Tetanus tension (g)	Post-tetanus Contraction time (ms)	Post-tetanus Half-relaxation time (ms)	Post-tetanus Twitch tension (g)
Control							
Young (17)	37 ± 4	41 ± 8	39 ± 8	217 ± 47	33 ± 4	34 ± 7	36 ± 7
Old (8)	44 ± 11	53 ± 16	32 ± 12	193 ± 66	40 ± 10	45 ± 12	31 ± 12
P	<0·05	<0·05	n.s.	n.s.	<0·05	<0·01	n.s.
Hyperthyroid							
Young (6)	24 ± 2	26 ± 4	32 ± 10	183 ± 40	22 ± 2	23 ± 4	32 ± 10
Old (9)	22 ± 4	23 ± 4	27 ± 10	163 ± 63	22 ± 4	22 ± 3	32 ± 6
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Values are means ± S.D. The number of animals is given in parentheses in the first column.

T_3-treated animals it initially decreased. However, this initial decrease was followed by a gradual increase after the first week(s) of hormone treatment, and consequently there was no significant change in body weight after, as compared with before, T_3 exposure. Accordingly, the body weight tended to be lower ($P < 0·1$) in the young T_3-treated rats as compared with the age-matched controls, because of the pronounced body weight gain in the controls (Table 1). In the old animals, on the other hand, the body weight gradually decreased ($P < 0·001$) during the period of T_3 treatment, while no significant change was observed in the old controls, resulting in a significantly lower body weight in the treated rats than in the controls (Fig. 1, Table 1). Except for a lower EDL weight in old T_3-treated rats, there were no significant differences in muscle weights between hyperthyroid and control rats.

Thyroid hormones

The thyroid status was examined by measuring the serum concentrations of T_3 and T_4 (Table 1). In the control group, the T_4 level was lower in the old animals. The primary by-product of T_4, the active form of the thyroid hormone, T_3, did not differ between young and old animals (Table 1). As a consequence of the hormone treatment, the T_4 levels were highly depressed in the hyperthyroid rats. The wide scatter in T_3 concentrations after the treatment is probably related to a combined effect of endogenous and

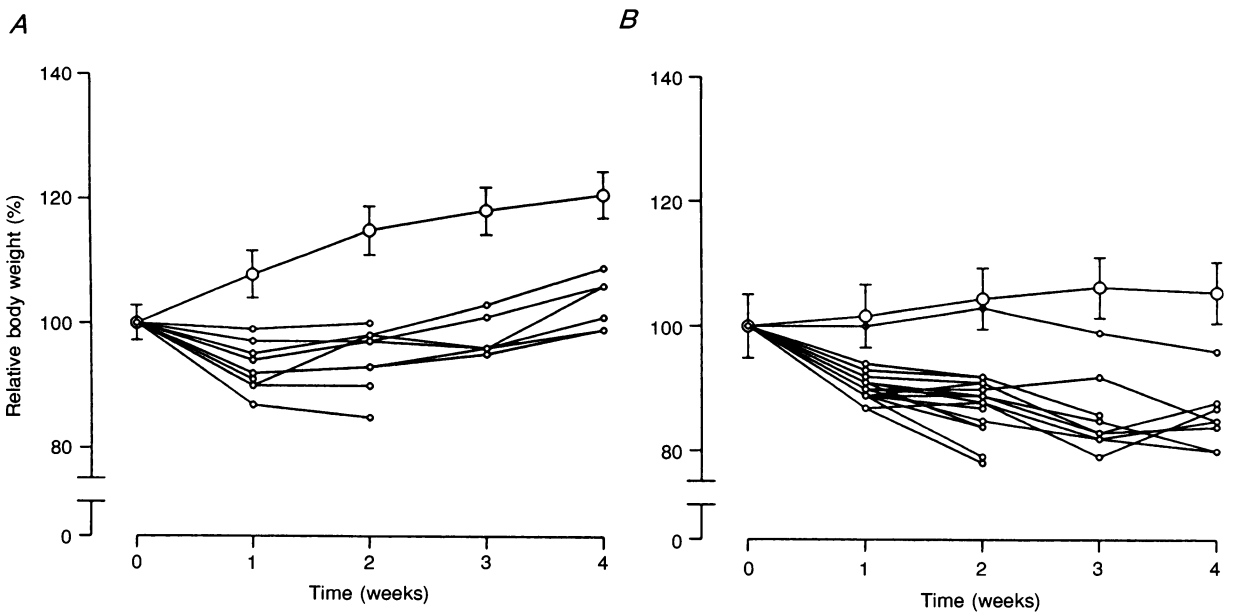

Figure 1. Body weight development
Body weight in young (*A*) and old (*B*) control and thyroid hormone-treated animals. Individual values are given for the animals treated with thyroid hormone (small circles) and means ± S.D. for the controls (large circles).

Table 3. Contractile properties of EDL muscle of young and old control and hyperthyroid animals

Group	Contraction time (ms)	Half-relaxation time (ms)	Twitch tension (g)	Tetanus tension (g)	Post-tetanus Contraction time (ms)	Post-tetanus Half-relaxation time (ms)	Post-tetanus Twitch tension (g)
Control							
Young (16)	14 ± 1	11 ± 2	68 ± 31	356 ± 134	14 ± 1	8 ± 2	79 ± 35
Old (5)	16 ± 3	13 ± 2	69 ± 25	321 ± 85	17 ± 3	11 ± 2	80 ± 29
P	(<0·1)	(<0·1)	n.s.	n.s.	<0·01	<0·05	n.s.
Hyperthyroid							
Young (6)	13 ± 1	11 ± 1	65 ± 12	362 ± 72	13 ± 1	9 ± 1	72 ± 11
Old (6)	13 ± 1	11 ± 5	59 ± 24	285 ± 86	13 ± 2	10 ± 5	65 ± 28
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Values are means ± S.D. The number of animals is given in parentheses in the first column.

exogenous T_3, despite the fact that T_3 treatment was terminated two days prior to blood sampling.

Contractile properties

In the control animals, an age-related increase was observed in the contraction and half-relaxation times of the isometric twitch in the soleus before and after a short tetanus (Table 2). A similar age-related increase was also noted in the EDL, though this was statistically significant only in the post-tetanus twitch (Table 3). The twitch and tetanus tensions were not significantly affected by age in either the soleus or EDL. The effect of a short tetanus on twitch tension was not affected by age. That is, the isometric twitch was depressed after a short tetanus in the soleus muscle of both young (pre-/post-tetanus tension: 0·92 ± 0·09) and old (0·91 ± 0·06) animals and was potentiated in the EDL of both young (1·17 ± 0·15) and old (1·15 ± 0·15) animals. Both the soleus and EDL tetanus tensions per muscle weight were slightly lower (15–29%; $P < 0·1$–$0·05$) in the old animals. However, there was no age-related difference in the specific tension, calculated as tetanus tension per total muscle fibre area measured at the motor point (soleus) or at the greatest girth (EDL) of the muscle, between young (2·46 ± 0·67 kg cm^{-2}) and old

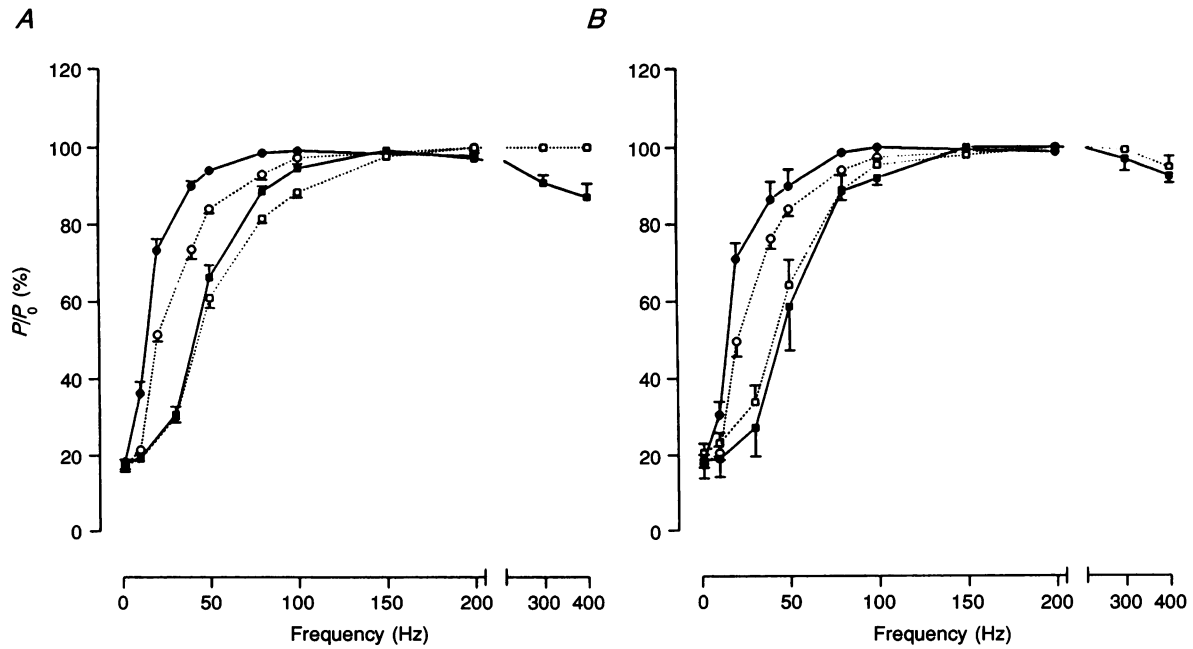

Figure 2. Tension–frequency relations
Tension–frequency curves for soleus (circles) and EDL (squares) muscles from young (*A*) and old (*B*) animals. *P*, tension; P_0, maximum tension. Curves obtained before (filled symbols) and after (open symbols) thyroid hormone treatment was given.

($2{\cdot}57 \pm 0{\cdot}52$ kg cm^{-2}) soleus or between young ($3{\cdot}78 \pm 1{\cdot}53$ kg cm^{-2}) and old ($3{\cdot}82 \pm 2{\cdot}04$ kg cm^{-2}) EDL muscles. In the soleus, all muscle fibres pass the motor point and accurate measurements of the total number of muscle fibres can accordingly be made from single cross-sectional cuts perpendicular to the long axis of the muscle at the motor point (see Gollnick, Timson, Moore & Riedy, 1981; Larsson & Edström, 1986) and specific tensions can be calculated with reasonable accuracy. In the EDL, on the other hand, total fibre number counts from single muscle cross-sections have to be considered with caution since the arrangement of muscle fibres is more complex than in the soleus. From direct counts of the total number of fibres in rat EDL, treated so as to permit isolation of each fibre, Gollnick *et al.* (1981) found that this number was almost 35% higher than counts reported from single cross-sections. Specific tensions based on total fibre counts from single cross-sections will accordingly be overestimated in the EDL.

In the hyperthyroid animals, the soleus contraction and half-relaxation times of the pre- and post-tetanus isometric twitch were shorter than in the controls, both in the young (35–37%, $P < 0{\cdot}001$) and old (50–57%, $P < 0{\cdot}001$) groups, resulting in higher fusion frequencies and rightward shifts of the tension–frequency curves (Fig. 2). Thus, 4 weeks of T_3 treatment made the soleus significantly faster in both young and old animals and the twitch properties were intermediate between those of the EDL and soleus muscles of the young controls. After T_3 treatment, the speed of isometric contraction was not affected by age, since T_3 effects were more pronounced in the old than in the young animals (Table 2). Tendencies ($P < 0{\cdot}1$–$0{\cdot}01$) towards a decrease in soleus twitch and tetanus tensions were observed in both young and old animals after treatment

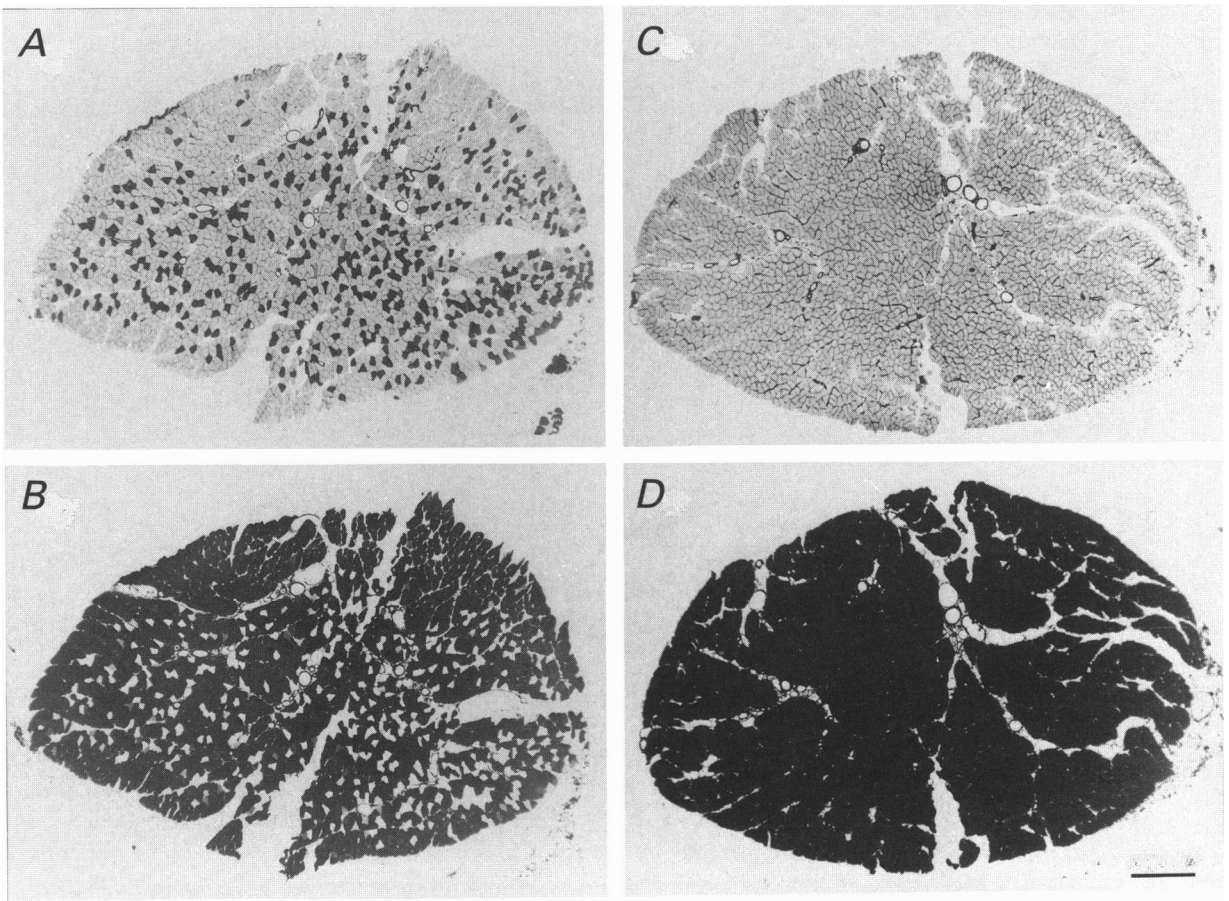

Figure 3. Enzyme-histochemical stainings of soleus cross-sections from controls
Transverse cryostat cross-sections of soleus muscle from young (*A* and *B*; 4 months) and old (*C* and *D*; 23 months) control rats. The sections were stained for myofibrillar ATPase after formaldehyde fixation (*A* and *C*) and after acid preincubation (*B* and *D*). Scale bar = 0·5 mm.

with T_3. The effect of a short tetanus on soleus twitch tension was significantly ($P < 0{\cdot}001$) altered by this treatment, i.e. the post-tetanic twitch depression was diminished in the young hyperthyroid rats and a slight potentiation (7%) of the post-tetanus twitch was observed in the old hyperthyroid animals. As was noted in the controls, tetanus tension per muscle weight tended to be lower in the soleus of the old animals as compared with that of the young ones, while the specific tension was not affected by age.

In the EDL, the effects of T_3 treatment were generally less pronounced than in the soleus (Tables 2 and 3). However, 4 weeks of T_3 treatment resulted in a 19–24% shortening ($P < 0{\cdot}1$–$0{\cdot}05$) of the pre- and post-tetanus contraction time in the old animals, while no significant change was seen in the young ones. The age-related difference in the speed of contraction in the EDL was, accordingly, lost after 4 weeks of T_3 treatment (Table 3), in the same way as in the soleus (Table 2). The potentiation of the twitch tension after a short tetanus was not affected by T_3 treatment in the EDL. Tetanus tension per EDL weight was lower ($P < 0{\cdot}05$) in the old T_3-treated animals, but the specific tension did not differ between young and old controls or T_3-treated animals.

Enzyme-histochemical properties

In the soleus of the control animals, the proportion of type I fibres was higher ($P < 0{\cdot}01$) and that of type IIA lower ($P < 0{\cdot}01$) in the old animals. The cross-sectional areas of the type II fibres (IIC and IIA) were smaller ($P < 0{\cdot}05$–$0{\cdot}01$) in the old than in the young controls (Figs 3 and 4, Table 4). In the EDL, the proportions of type I, IIA and IIB fibres did not differ between young and old animals, and the type II fibres (IIA and IIB) were

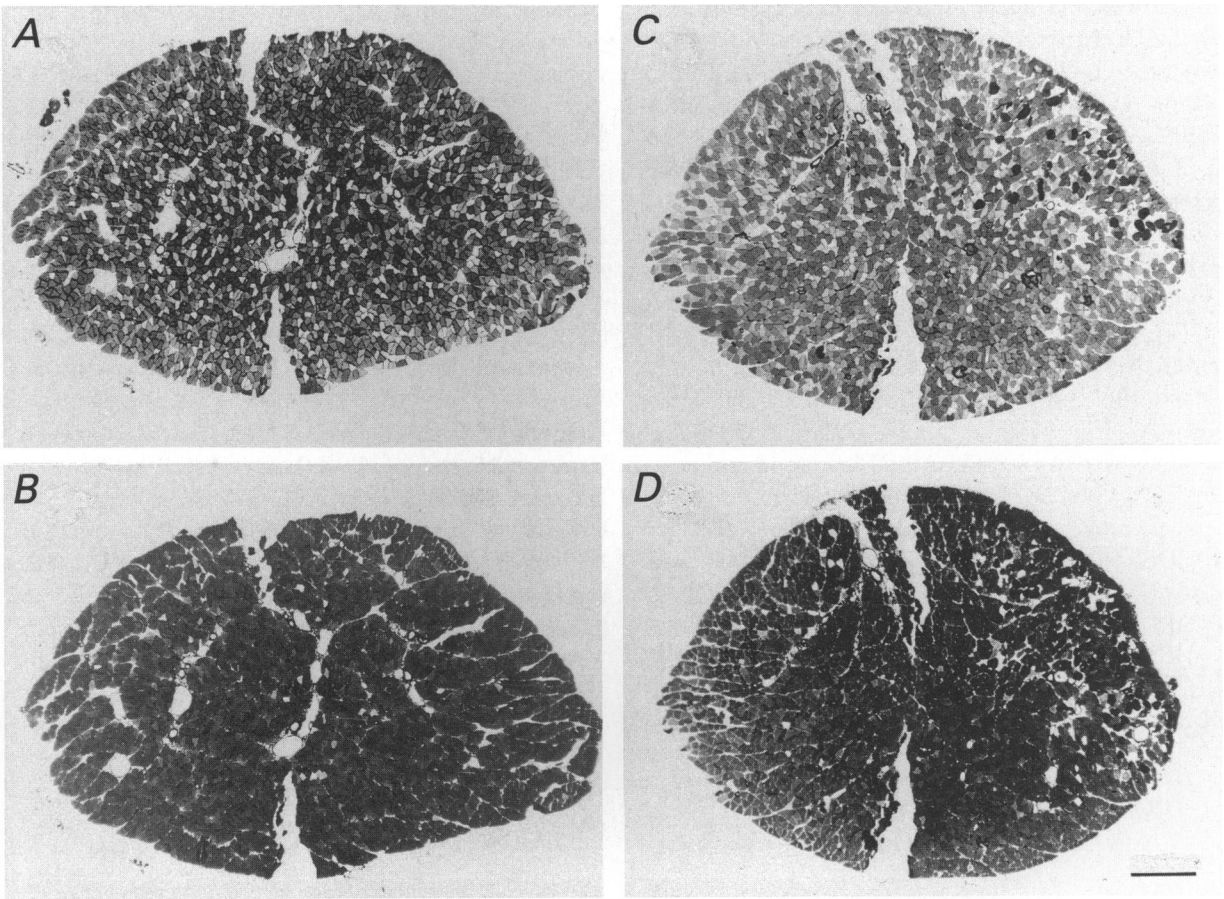

Figure 4. Enzyme-histochemical stainings of soleus cross-sections from thyroid hormone-treated animals

Transverse cryostat cross-sections of soleus muscles from young (*A* and *B*; 6 months) and old (*C* and *D*; 23 months) rats treated with thyroid hormone. The sections were stained for myofibrillar ATPase after formaldehyde fixation (*A* and *C*) and after acid preincubation (*B* and *D*). Scale bar = 0·5 mm.

Table 4. Enzyme-histochemical properties of the soleus in young and old control and hyperthyroid animals; total number of fibres and proportions and cross-sectional areas of different fibre types

Group	Number	Type I		Type IC		Type IIC		Type IIA	
		%	Area (μm^2)	%	Area (μm^2)	%	Area (μm^2)	%	Area (μm^2)
Control									
Young (17)	2560 ± 260	92·3 ± 6·3	2730 ± 510	1·6 ± 1·8	1960 ± 470	2·3 ± 2·8	2320 ± 480	3·9 ± 4·5	2670 ± 550
Old (6)	2680 ± 300	96·3 ± 5·7	2970 ± 510	0·5 ± 0·5	1970 ± 530	1·8 ± 3·1	1660 ± 550	1·2 ± 2·4	2080 ± 710
P	n.s.	<0·01	n.s.	n.s.	n.s.	n.s.	<0·01	<0·01	<0·05
Hyperthyroid									
Young (6)	2690 ± 260	26·5 ± 12·3	2010 ± 460	30·3 ± 4·9	1960 ± 450	39·5 ± 13·3	2230 ± 510	3·3 ± 4·8	2230 ± 1010
Old (10)	2730 ± 380	18·9 ± 14·0	1990 ± 320	39·4 ± 10·7	2280 ± 440	40·1 ± 15·9	2430 ± 650	1·6 ± 2·1	2400 ± 1010
P	n.s.	n.s.	(<0·1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Values are means ± S.D. The number of animals is given in parentheses in the first column.

larger ($P < 0·05$–$0·01$) in the old than in the young animals (Table 5).

In the soleus, 4 weeks of T_3 treatment had major effects on the enzyme-histochemical properties of both young and old animals. The proportion of type I fibres decreased ($P < 0·001$) and the proportions of types IC and IIC increased ($P < 0·001$), while no change was observed in the relative number of type IIA fibres in either young or old rats (Figs 3 and 4, Table 4). The average cross-sectional area of type I fibres decreased ($P < 0·01$–$0·001$) after T_3 treatment in both young and old animals. The type IIC fibres, on the other hand, were larger ($P < 0·01$) in old hormone-treated animals (Table 4). The enzyme-histochemical properties of the young and old hyperthyroid animals were intermediate between those of the control slow-twitch soleus and fast-twitch EDL muscles. In conformity with the contractile data, after T_3 treatment there were no significant differences in enzyme-histochemical or morphometrical characteristics of the soleus between young and old animals.

In the EDL, the effects of hyperthyroidism were less pronounced than in the soleus and differed slightly between young and old animals. In the young animals, the cross-sectional areas of all fibre types tended to increase ($P < 0·1$–$0·05$), while no significant change was observed in fibre-type proportions. In the old animals, on the other hand, the cross-sectional area of the type IIB fibres decreased ($P < 0·01$), while the proportion of type I fibres increased slightly ($P < 0·05$; Table 5).

Myosin isoform composition

Each myosin molecule consists of six subunits, which may be variably expressed in individual fibres: two heavy chains (MHCs) of about 200 kDa and four light chains (MLCs) with molecular mass between 16 and 25 kDa. Types I, IIA and IIB MHC isoforms were identified on 6% SDS–PAGE in eighty-five and seventy-seven single muscle fibres from control soleus and EDL muscles, respectively. Co-expression of two MHC isoforms is frequently observed in mammalian muscle fibres, and individual fibres were classified as types I, I/IIA, IIA/I, IIA, IIA/IIB, IIB/IIA and IIB, depending on the relative proportions of MHCs present. In the soleus, types I, I/IIA, IIA/I and IIA fibres conform with types I, IC, IIC and IIA fibres in the classification based on enzyme-histochemical staining (Staron & Pette, 1987; Ansved & Larsson, 1989). At the

Table 5. Enzyme-histochemical properties of the EDL in young and old control and hyperthyroid animals; total number of fibres and proportions and cross-sectional areas of different fibre types

Group	Number	Type I		Type IIA		Type IIB	
		%	Area (μm^2)	%	Area (μm^2)	%	Area (μm^2)
Control							
Young (7)	3610 ± 630	3·4 ± 1·1	900 ± 190	18·7 ± 4·7	970 ± 130	76·1 ± 4·4	2170 ± 250
Old (6)	3340 ± 410	3·3 ± 0·8	1090 ± 220	23·3 ± 6·4	1230 ± 190	72·0 ± 6·1	3030 ± 560
P	n.s.	n.s.	n.s.	n.s.	<0·05	n.s.	<0·01
Hyperthyroid							
Young (6)	3160 ± 330	3·3 ± 1·0	1170 ± 150	15·3 ± 7·8	1250 ± 300	80·5 ± 6·5	2890 ± 640
Old (10)	2910 ± 300	4·3 ± 1·1	1120 ± 210	24·3 ± 14·1	1070 ±140	69·8 ± 11·9	2180 ± 240
P	n.s.	(<0·1)	n.s.	n.s.	n.s.	(<0·1)	<0·01

Values are means ± S.D. The number of animals is given in parentheses in the first column.

single-fibre level in the EDL, types IIA, IIA/IIB, IIB/IIA and IIB were observed, while type II fibres were only separated into types IIA and IIB according to the myofibrillar ATPase staining of Brooke & Kaiser (1970). No type I fibres were identified at the single-fibre level in the EDL because of the small number of this fibre type.

In control fibres, age-related trends in the MHC patterns of the soleus and EDL fibres were similar to those found in the enzyme-histochemical analyses of the respective muscles (Fig. 5). It is not surprising that the results from the MHC analyses of single fibres were not completely identical with the data from the enzyme-histochemical analyses, since only a relatively small number of single fibres were characterized with respect to the MHC isoform composition (the total number of isolated fibres was less than 10% of the total number of fibres classified histochemically in a single muscle cross-section). However, additional information is provided by MHC analyses at the single-fibre level that cannot be obtained by the enzyme-histochemical techniques used in this study, especially in the EDL, where improved separation of type II fibres co-expressing types IIA and IIB MHCs is achieved. Interestingly, the number of muscle fibres expressing IIB MHCs was smaller in the EDL of the old than of the young animals, with a corresponding increase in fibres expressing types IIB/IIA and IIA MHCs in the old ones. In the T_3-treated animals, the MHC isoform composition was determined in a total of sixty-nine and sixty-two single muscle fibres from soleus and EDL muscles, respectively. In soleus fibres, T_3 treatment resulted in a significant decrease in type I MHCs in both young and old animals, with a concomitant increase in fibres co-expressing types I and IIA MHCs. The effect of T_3 treatment on the MHC composition was less marked in EDL than in soleus fibres, which agreed with observations from enzyme-histochemical and contractile measurements. It should be noted, however, that fibres co-expressing type IIA and type I MHCs (IIA/I) were only observed in the old thyroid hormone-treated rats (Fig. 5).

Myosin light chains (MLCs) were classified as essential (MLC_{1f} and MLC_{3f} in fast fibres and MLC_{1s} in slow) or regulatory (MLC_{2f} or MLC_{2s}), based on conditions for their dissociation from myosin. In agreement with the MHC composition, different combinations of slow and fast essential and regulatory light chains were seen at the single-fibre level (Table 6). In the control fibres, the proportion of fast MLCs was lower in the old soleus and EDL muscles than in the young ones (Table 6). After T_3 treatment, age-related and muscle-specific effects on the MLC composition were observed. In soleus fibres, the proportion of slow MLCs decreased in both young and old animals, but the relative amount of fast essential and regulatory MLCs was larger in the old as compared with the young animals. In EDL fibres, the amount of MLC_{3f} decreased in both young and old animals, while that of MLC_{1s} increased. Thus, the EDL fibres had acquired more slow MLC isoforms after T_3 treatment, particularly in the old animals (Table 6).

The fast isoform of the regulatory light chain (MLC_{2f}) was exclusively expressed in all EDL fibres irrespective of age and T_3 treatment. In the soleus, more than 80% of all control fibres in young and old animals expressed MLC_{2s},

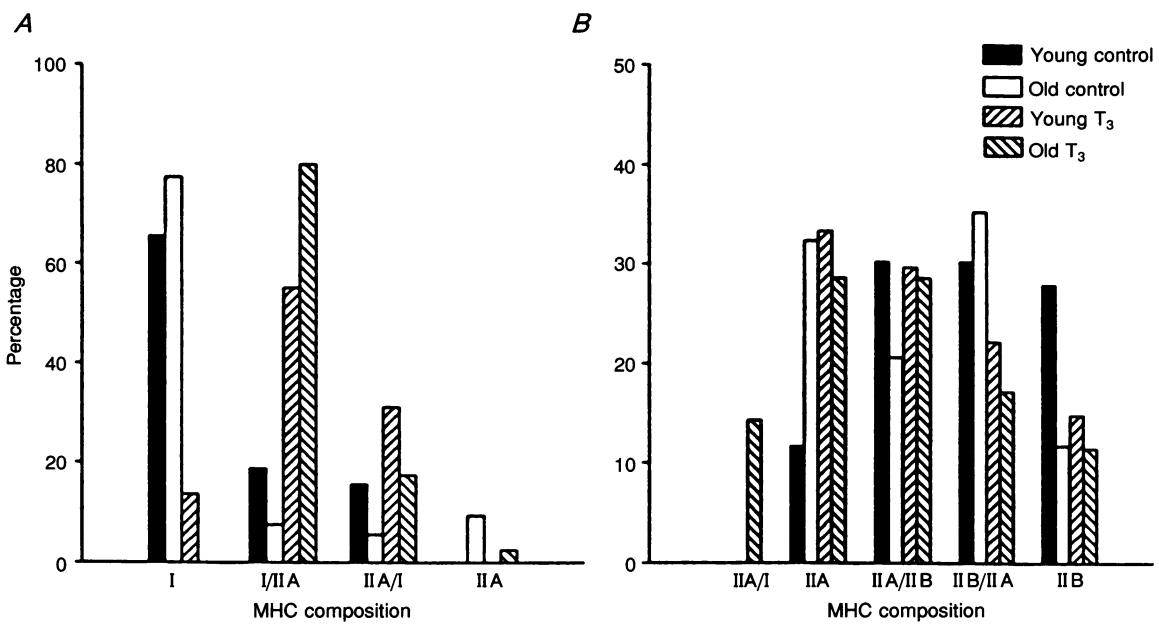

Figure 5. Myosin heavy chain (MHC) isoform composition
Composition of MHCs in chemically skinned single fibres from soleus (*A*) and EDL (*B*) muscles. The proportions of different fibre types are presented for young and old control and thyroid hormone-treated animals.

Table 6. Patterns of myosin light chain (MLC) isoform composition in single fibres from soleus and EDL muscles in young and old control and hyperthyroid animals

		Distribution (%)											
		MLC_{1s} MLC_{2s}	MLC_{1s} MLC_{1f} MLC_{2s}	MLC_{1s} MLC_{2s} MLC_{2f}	MLC_{1s} MLC_{2s} MLC_{3f}	MLC_{1s} MLC_{1f} MLC_{2s} MLC_{2f}	MLC_{1s} MLC_{1f} MLC_{2s} MLC_{3f}	MLC_{1s} MLC_{2s} MLC_{2f} MLC_{3f}	MLC_{1s} MLC_{1f} MLC_{2s} MLC_{2f} MLC_{3f}	MLC_{1s} MLC_{1f} MLC_{2f}	MLC_{1s} MLC_{1f} MLC_{2f} MLC_{3f}	MLC_{1s} MLC_{2f} MLC_{3f}	MLC_{1f} MLC_{2f} MLC_{3f}
Group	Muscle												
Control													
Young	Soleus (29)	48	—	—	17	—	21	—	10	3	—	—	—
	EDL (38)	—	—	—	—	—	—	—	—	—	—	—	100
Old	Soleus (53)	75	—	2	6	—	—	—	9	—	8	—	—
	EDL (37)	—	—	—	—	—	—	—	—	3	14	3	81
Hyperthyroid													
Young	Soleus (29)	10	—	—	—	7	—	31	48	—	3	—	—
	EDL (27)	—	—	—	—	—	—	—	—	—	—	30	70
Old	Soleus (40)	—	—	3	5	15	10	13	50	—	3	3	—
	EDL (40)	—	—	—	—	—	—	—	—	12	—	15	74

Mean values are given, with the total number of fibres in parentheses in the second column.

10–11% a combination of MLC_{2s} and MLC_{2f} and 3–8% MLC_{2f}. After T_3 treatment only 10–15% of all soleus fibres expressed the slow isoform of the regulatory light chain, while the majority of the fibres (81–86%) expressed a combination of MLC_{2s} and MLC_{2f} (Table 6). These findings are consistent with the loss of the post-tetanic isometric twitch depression in the soleus after administration of T_3. That is, twitch potentiation in rat muscle is regulated by MLC_{2f} and related to a phosphorylation-dependent increase in the rate constant for cross-bridge attachment to actin-binding sites, presumably as a result of a partial release of the myosin head from the thick filament backbone (Metzger, Greaser & Moss, 1989; Sweeney, Bowman & Stull, 1993).

DISCUSSION

The major observation in this study was the preserved ability of skeletal muscle to respond to the external influence of thyroid hormone with a switching of myosin isoforms and a change in contractile and enzyme-histochemical properties in old age. This is consistent with the results of a recent study on rat heart muscle, which showed a maintained initiation of cardiac hypertrophy in response to thyroid hormone in old age (Tomanek, Butters & Zimmerman, 1993). Tomanek *et al.* (1993) also observed that the old rats appeared to be more sensitive to thyroid hormones than the young ones, a finding similar to that in the soleus muscle in this study. They suggested that this was secondary to an increased sensitivity to thyroid hormones through an upregulation of receptor numbers (Tomanek *et al.* 1993). In skeletal muscle, it was recently found that T_3-receptor antibody-staining patterns were independent of age in both the rat and man (Schmidt *et al.* 1992). Thus, the exact mechanisms underlying the slightly more pronounced effect of T_3 on skeletal muscle in old animals remains uncertain. An alternative explanation to an upregulation of T_3 receptors in old age is an age-related difference in T_3 hormone dose per muscle mass. That is, the T_3 dose was given as a fraction of body weight (300 $\mu g\ kg^{-1}$), and the old rats were generally more obese than the young ones, resulting in a lower muscle to body weight ratio in old age.

The effects of ageing on the pulsative regulation of the concentration of circulating thyroid hormone are very complex, not fully understood and involve different hormones, which are at least partly interrelated (Sonntag, 1987). The present data conform with the observation in the majority of previous studies that a marked fall in the T_4 levels occurs in middle-aged and old rats while the circulating T_3 levels remained relatively unchanged, as a result of an increased T_4 to T_3 conversion rate. However, some authors have noted a decrease in both the T_4 and T_3 levels in ageing rats (Pekary *et al.* 1983; Sonntag, 1987). It is at present premature to speculate about the possible influence of thyroid hormones on the age-related changes in contractile, biochemical and enzyme-histochemical properties of skeletal muscle. However, the findings in this and previous studies of altered thyroid hormone levels during ageing together with the disappearance of age-related changes in skeletal muscle characteristics after thyroid hormone treatment, strongly indicate that this topic deserves further scientific attention.

Enzyme-histochemical properties and myosin isoforms

The observation that an age-related increase in the proportion of type I fibres occurs at the expense of a

decreasing number of type II A and atrophy of type II fibres in the soleus, as well as unaltered fibre-type proportions in the EDL, conforms with previous reports (for references see Larsson *et al.* 1991*a*). However, in fast-twitch rat hindlimb muscles the findings have been divergent regarding the effects of ageing on enzyme-histochemical properties. On the one hand, there have been reports of unaltered fibre-type proportions with age, as in this study, while others have observed an age-related change from a fast to a more slow fibre-type profile with an increasing number of muscle fibres of types I and II A at the expense of a decreasing number of type II B (for references see Larsson *et al.* 1991*a*, 1993). These age-related alterations in fibre-type proportions observed in rat fast-twitch muscles were mainly confined to an advanced age, indicating that there is a difference in the temporal sequence of such changes between fast- and slow-twitch muscles. However, MHC analyses at the single-fibre level revealed an increased number of fibres expressing type II A and II B/II A MHCs in the EDL of old animals, supporting the concept of an age-related transformation from type II B to II A MHCs, although this was not detectable with enzyme-histochemical techniques. These findings are in accordance with studies of the so-called II X MHC, which is expressed in a specific motor-unit type with contractile, morphological and biochemical properties intermediate between those of type II A and II B MHC motor units (Larsson, Edström, Lindegren, Gorza & Schiaffino, 1991*b*). These II X MHC units have been shown to undergo specific quantitative as well as qualitative changes during the ageing process, resulting in an increase in their number and in the total amount of II X myosin (Larsson *et al.* 1991*a*, 1993; Sugiura, Matoba, Miyata, Kawai & Murakami, 1992). These results indicate an age-related motor-unit transition from type II B to II X MHC, possibly preceding a transformation to type II A MHC and following the sequence II B→II XB→II X→II XA→II A (Gorza, 1990; Larsson *et al.* 1991*a*, 1993). This is supported by the differences in MLC isoform combinations between young and old control EDL muscles in the present study, the slow isoform of the essential light chains being observed only in old age.

It is well known that thyroid hormones are among the most potent regulators of muscle development and differentiation, controlling the transcription of one of the master genes orchestrating myogenesis (MyoD1) via T_3 nuclear receptors (Carnac *et al.* 1992). The regulation of the MHC multigene family is highly complex and all members of the MHC multigene family respond to T_3, but the mode is not intrinsic to any MHC gene and has been reported to be determined in a highly muscle type-specific manner and to be independent of growth hormone production and of innervation (Nwoye *et al.* 1982; Whalen, Toutant, Butler-Browne & Watkins, 1985). Expression of the type II A MHC gene is upregulated by T_3 in rat soleus, but downregulated by T_3 in the EDL muscle (Izumo, Nadal-Ginard & Mahdavi, 1986). The fast type II B MHC gene appears to be responsive in some rat muscles (soleus, diaphragm and masseter), but not in others (EDL; Izumo, *et al.* 1986). In agreement with previous reports from young animals (e.g. Nicol & Bruce, 1981; Fitzimons *et al.* 1990), the strong tissue-specific influence of T_3 hormone on fibre-type proportions and on the composition of heavy and light isoforms of myosin was confirmed in both young and old animals. That is, after T_3 treatment, marked alterations were observed in the slow-twitch soleus, while only slight or insignificant effects were noted in the fast-twitch EDL. In the soleus, the present findings at the protein level are consistent with an upregulation of the type II A MHC gene by T_3 (Izumo *et al.* 1986). After treatment with T_3, fibres co-expressing myosin of types I and II A increased and type I fibres decreased, as observed by enzyme-histochemical stainings and separations of MHCs. In the EDL, the type II A MHC gene is downregulated within days of T_3 treatment (Izumo *et al.* 1986) but the present results did not verify a significant change in the type II A myosin, at the protein level, in response to thyroid hormone treatment.

The essential myosin light chains, MLC_1 and MLC_3, are generated from a single genetic locus by transcription from two different promoters and alternate splicing of the pre-mRNAs. In both rat and human muscle, the muscle-specific regulation of the essential light chains depends on a strong enhancer element, located downstream from the MLC_1 gene promoter (see Wentworth, Donoghue, Engert, Berglund & Rosenthal, 1991). The effects of thyroid hormones on MLCs, however, are less well known than those on MHC mRNAs. To our knowledge, it is not known whether the T_3 effects on MLCs are exerted via gene transcription or via post-translational protein modifications. However, thyroid hormones have been reported to influence the MLC pattern in human muscle in two different ways, causing an increase in MLC_{3f} in type II fibres and a decrease in MLC_{1s} in type I fibres (Salviati, Zeviani, Betto, Nacamulli & Busnardo, 1986). The present results are consistent with this conclusion in both young and old animals, but the T_3-induced changes were more marked in the old than in the young soleus.

Impaired MHC switching in the EDL from old compared with young rats has been reported in response to cross-reinnervation by the soleus nerve (Clark & White, 1991). However, there were fewer neuronal contacts at the neuromuscular junctions after this intervention in the old rats. It was therefore suggested that the plasticity of individual muscle fibres is not necessarily impaired in old age, since the proportion of slow MHCs correlated with the number of innervated motor endplates in both young and old animals (Clark & White, 1991). The data from the present study strongly support the idea of maintained adaptability of skeletal muscle in old age, although age-related changes in motoneurones or motor endplates, or both, may impair the adaptability in old age in response,

for example, to physical training or chronic stimulation of the motor nerve.

Contractile properties

Various factors in the contractile apparatus play a role in the regulation of the maximum speed of shortening, but the intrinsic properties of the SR and the myosin isoform composition are the two key factors. It has been shown that the properties of SR are the strongest determinants of the speed of contraction of the isometric twitch, whereas the contractile proteins have a major influence on the maximum shortening velocity and the maximum rate of increase in tetanus force (see Larsson & Salviati, 1989). In the present study, our earlier observations on the effects of age on the isometric speed of contraction in slow- and fast-twitch muscles and single motor units have been confirmed (e.g. Larsson & Edström, 1986; Edström & Larsson, 1987; Ansved & Larsson, 1989; Larsson & Salviati, 1989; Larsson *et al.* 1991*a*). From our previous results we concluded that an age-related impairment of intrinsic SR function, i.e. the rate of Ca^{2+} uptake and the fractional rate of SR filling, and a decrease in SR volume were the most probable factors underlying the decreased speed of contraction in old fast-twitch motor units (Larsson & Salviati, 1989). In the slow-twitch units, on the other hand, no significant age-related changes were observed in the Ca^{2+} uptake activity of the SR. The fast to slow myosin isoform transition in ageing may account for part of the slowing in the soleus, although the dominating mechanism remains obscure (Larsson & Salviati, 1989). The inherent properties of the Ca^{2+} release channel are essential for the contraction time of the isometric twitch (Salviati & Volpe, 1988) and preliminary results show that another very important property of SR and determinant of intracellular Ca^{2+} level is affected by age, namely the Ca^{2+} release properties as indicated by age-related changes in the caffeine sensitivity in both the soleus and EDL (G. Salviati & L. Larsson, unpublished observations).

In conclusion, the strong influence of T_3 treatment on the MHC and MLC composition has been confirmed in this study, as well as the strong muscle tissue-specific effects of T_3. Further, it is demonstrated that the ability of skeletal muscle to adapt to this strong regulator of the skeletal muscle protein composition and function is not impaired during ageing.

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