The Effects of Age and Thyroid Hormone Status on the Expression of Type IIX Myosin Heavy Chain in Tibialis Anterior Muscle of the Mouse

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Abstract

The demonstration of a third "fast-twitch" fiber type (IIx) in muscles of rat and mouse raises the question of the relationship of this fiber type to the other two "fast-twitch" subtypes, IIa and IIb. The purpose of this study was to investigate the effects of age and thyroid status on myosin heavy chain (MyHC) expression in single fibers of the superficial portion of tibialis anterior (TAS) muscle in male C57BL mice. Hypothyroidism led to a decrease in the expression of IIB MyHC and an increase of IIX MyHC, while hyperthyroidism had no significant effect. In mouse TAS the proportion of pure IIx fibers and of hybrid fibers containing IIB and IIX MyHC increased with age over the range 2-8 months. These findings extend our knowledge of fiber type transitions in the mouse and suggest that IIb fibers can be converted to IIx fibers by an appropriate stimulus. It remains to be determined whether IIx fibers can be converted to IIa fibers.

Key words: myosin heavy chain, fiber type, IIx fiber, muscle transformation, thyroid hormone, ageing.

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Immunohistochemical analysis of adult rodent skeletal muscles has revealed the existence of four distinct sarcomeric myosin heavy chain (MyHC) isoforms, designated type I, IIA, IIX and IIB , which are expressed singly or in various combinations in single muscle fibers [31, 32, 39, 43]. It is well known that there is a certain degree of plasticity with regard to the phenotypic expression of the MyHC isoforms which is under the control of a number of factors, including age [23-26] and thyroid hormone [15, 17, 18, 36].

Fast-twitch muscles in rodents have been reported to exhibit slower contractile characteristics as the animal ages [9, 16]. That such a phenomenon may not be restricted to very old animals is suggested by the observation [23] of an increase, between 5 weeks and 8 months, in the proportion of motor units with prolonged times to peak tension. This was also shown to be accompanied by an increase in the proportion of fibers characterized with ATPase histochemistry as type I. In fast-twitch muscles, however, this relationship between altered physiological and histochemical properties has been questioned. For example, while some authors [19, 21, 34] detected a shift towards slower, or at least more oxidative, characteristics no such change was observed by Eddinger *et al.* [8] or Edstrom and Larsson [9]. The reason for this apparent disagreement is unclear, but may be due to differences among the various studies with regard to the range of ages of the animals used and to the inability of ATPase histochemistry to distinguish all fast fiber subtypes. In this latter regard, Gorza [13] and Parry and Zardini [31] have shown that the ATPase staining reaction of IIx fibers in mouse muscles is virtually indistinguishable from that of type IIb fibers after acid pre-incubation and from that of type IIa fibers after alkali pre-incubation.

Substantial evidence exists that thyroid hormone plays a role in MyHC isoform transitions during skeletal muscle development [5, 12, 36]. In the adult, altered thyroid hormone levels are followed by changes in MyHC protein composition or mRNA levels in a muscle specific manner [4, 17, 18, 20]. It may be generally stated that increased levels of thyroid hormone tend to push MyHC expression towards the "fast" end of the fiber type spectrum, manifested by a decrease in type I and an increase in type II fibers, with hypothyroid conditions producing the opposite effect.

It has been shown both in the rat [20, 25, 32] and mouse [43] that types IIB and IIX MyHC may co-exist within the same fiber, while types IIB and IIA MyHC are never found to be co-expressed without IIX MyHC being also present. This may suggest that any shift from type IIb towards a "slower" fiber type or conversely any shift towards the "faster" type IIb phenotype must involve the intermediate expression of IIX MyHC. Indeed, Kirschbaum *et al.* [20] showed that altered levels of activity as well as altered thyroid hormone levels yielded changes in MyHC expression in rat muscles that were consistent with this hypothesis. Similarly, Larsson *et al.* [24, 25] reported an age-related increase in the proportion of motor units containing type IIx fibers in the rat. The purpose of the present study was to determine the extent to which age and experimentally imposed hypo- and hyperthyroidism may modulate IIB and IIX MyHC expression in the superficial region of tibialis anterior muscle of the mouse which contains only these fiber types [31, 43]. A combined immunohistochemical and gel electrophoretic approach was used for the analysis of MyHC expression in both whole muscle and single muscle fibers since it is difficult with immunohistochemistry alone to demonstrate convincingly hybrid fibers containing IIX MyHC as one of the constituents due to the fact that the antibody employed to identify IIx fibers, BF-35 [39] does so by its *failure* to react with IIX MyHC.

Methods

Animals and muscles

The animals used in the age study were 2, 4 and 8 month old male C57BL mice. For the thyroid hormone study mice aged 3 months were used. All the animals were quartered in standard cages with food and water supplied *ad libitum*. The care and treatment of the animals were in accordance with the Guidelines of the Canadian Council on Animal Care. The animals were euthanized with an overdose of sodium pentobarbital (0.07 mg/g body weight; Somnotol, M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada). The right and left tibialis anterior (TA) muscles were removed from the mice in both the age and thyroid hormone study and weighed. For immunohistochemistry the right TA muscle was rapidly frozen in pre-cooled isopentane and stored at - 80°C until sectioned. Serial transverse sections 10 µm thick were cut on a cryostat microtome (Zeiss Microm, Microm Laborgerate Gmbh, Heidelberg) and mounted on gelatin-coated slides.

Immunohistochemistry

The sections were immunohistochemically processed using monoclonal antibodies (mAb's) against type I MyHC (BA-D5), type IIA MyHC (SC-71), type IIB MyHC (BF-F3) and all MyHC isoforms excluding type IIX (BF-35). The antibodies were generously provided by Dr. S. Schiaffino, Padua, Italy, and have previously been shown to react specifically with the above MyHC in muscles of mice. [13, 31, 43]. The secondary antibodies were peroxidase-labelled goat anti-mouse IgG (or IgM in the case of BF-F3) and diaminobenzidine was used as the chromogen. Stained serial cross sections of TAS muscle were photographed on a Zeiss microscope and all the fibers of the TAS (i.e. superficial to the aponeurosis) were counted. The percentages of total fiber number expressing IIX, IIB and both isoforms (IIX+IIB) were determined and expressed as a percentage of total fiber number.

Electrophoretic identification of MyHC

The procedure used for the gel electrophoretic analysis of the MyHC composition of single fibers was as previously described [31, 43]. In brief, the superficial portion of the left TA muscle (TAS) was separated from the deep portion, which was discarded. Strips of fibers tied to wooden sticks to maintain resting length, were immersed in skinning solution [37] for 24h with changes of solution at 1, 2, 4 and 8h. The muscle strips were stored at - 20°C in a mixture of 50% glycerol and 50% skinning solution until required. A small bundle of fibers was cut from the original strip of skinned muscle fibers under a dissecting microscope and single fibers were pulled out from one end of the bundle. The fibers were transferred to a capillary tube containing 20 µl of sample buffer and boiled for two minutes. Electrophoresis of the samples was carried out in 0.75 mm thick 6% polyacrylamide gels with 30% glycerol [6]. The gels were silver stained by the method of Morrissey [30].

Induction of hypothyroidism and hyperthyroidism

The animals for the thyroid hormone study were randomly divided into three experimental groups namely: 1) control 2) hyperthyroid 3) hypothyroid. Hyperthyroidism was induced by intraperitoneal injection of 3 µg 3,5,3-triiodothyronine/10 g body weight on alternate days for 5 weeks [5]. Hypothyroidism was induced by treatment with 1% $NaClO_4$ solution administered in drinking water for 5 weeks [42]. Animals in the control group received no treatment. Blood samples were collected by intracardiac puncture from deeply anaesthetized mice prior to euthanasia and removal of muscles. Following centrifugation, free thyroxine (T_4) and total triiodothyronine (T_3) serum concentrations were determined by radioimmunoassay.

Statistical analyses

The Statistical Analysis System (SAS) for Personal Computers software package (SAS Institute Inc., NC, USA) was used for all statistical computations. The means and S.E.M. were calculated for all the variables measured. A one-way analysis of variance (ANOVA) was used to test for the effect of either age or thyroid hormone on the proportion of type IIb, IIx+IIb and IIx fiber types. If the one-way ANOVA revealed a significant age or thyroid hormone treatment F ratio then post hoc t' test comparisons of least-squares means were performed to determine precise differences among age groups or thyroid treatment groups and the probability associated with these differences.

Results

Fig 1 shows an example of an electrophoretogram of TAS fibers and indicates that, as previously described [43], the IIX and IIB MyHC bands are clearly distinguishable. It is also clear from this figure that hybrid fibers exist in which IIX and IIB MyHC co-exist and that the ratio of these two MyHC differs among fibers. Since quantitation of MyHC expression was carried out primarily by gel electrophoretic analysis of single fibers, it was first necessary to demonstrate that this method yielded a random sampling of fibers within a given muscle. We, therefore, compared the data obtained from gel electrophoresis with those obtained from immunohistochemical analysis of the contralateral TAS of the same animals. Type IIb fibers were identified immunohistochemically by their intense staining with BF-F3 and BF-35 and a lack of staining with SC-71 and BA-D5, whereas type IIx fibers were negative with all of the antibodies used. Those fibers which exhibited an intermediate staining intensity with both BF-F3 and BF-35 were considered to be type IIx/IIb (i.e. hybrid fibers co-expressing IIB and IIX MyHC). These data are shown in Fig 2. There was a strong correlation ($r = 0.99$) between the proportion of fibers immunohistochemically identified in TAS muscle cross-sections as being type IIb, IIx/IIb and IIx and the proportion of single fibers identified on 6% polyacrylamide gels expressing IIB, IIX+IIB and IIX MyHC respectively in both the age and thyroid hormone study (Figure 1). This suggests that the results of our gel electrophoretic analysis were indeed representative of the entire TAS.

MyHC distribution in TAS: effect of age

Animal data and muscle weight for the age study are shown in Table 1. Both whole body weight and TA muscle weight increased significantly over the range of ages studied, though not proportionally, as reflected by a reduced muscle to body weight ratio with increasing age. The proportion of single TAS fibers expressing various MyHC isoforms identified by SDS-PAGE, at the three ages studied is shown in Figure 3. There was a significant decrease in the proportion of fibers expressing IIB MyHC with a parallel increase in fibers expressing IIX MyHC with increasing age. A small percentage of fibers that co-expressed IIB and IIX MyHC was present in each age group. These presumably represent hybrid, transforming fibers.

Table 1. Effect of age on body and TA muscle weight in normal mice.

Age (months)	n	Body weight (g)	TA wet weight (mg)	MW/BW (mg/g)
2	5	23.2 ± 0.4^{a}	44.0 ± 0.4^{b}	1.9 ± 0.02^{d}
4	5	31.2 ± 1.3^{a}	46.8 ± 1.4^{c}	1.5 ± 0.07^{d}
8	5	37.9 ± 1.4^{a}	51.6 ± 0.6^{bc}	1.3 ± 0.04^{d}

Abbreviation: MW = TA muscle weight, BW = body weight. Values are mean SEM.
$^{a\,b\,c\,d}$ Statistically different ($P < 0.05$) from the other with the same symbol.

MyHC distribution in TAS: effects of altered thyroid status

The efficacy of each of the treatments used to induce either hypothyroidism or hyperthyroidism was verified by determination of the thyroid hormone concentration in serum (Table 2). The T_3 and T_4 levels for both the hyperthyroid and hypothyroid group were significantly different from normal. The body mass of the treated animals was significantly less than control while the TA wet muscle weights (both absolute and normalised for body weight), though reduced, were not significantly different from control (Table 3).

Figure 4 shows the results of the analysis of the SDS-PAGE experiments on single fibers dissected from TAS muscles of hyperthyroid, hypothyroid and normal mice. A significant decrease in the proportion of fibers expressing IIB MyHC with a parallel increase in fibers expressing IIX MyHC was observed in the hypothyroid group compared with normal group. Hyperthyroidism did not induce any changes in MyHC expression. Hybrid fibers, in which IIB and IIX MyHC are co-expressed, were present in all three groups in similar proportions.

Discussion

In the present study we have demonstrated that a sample of single fibers taken for gel electrophoretic analysis of

Figure 1. Gel electrophoretogram of single fibers from mouse TAS muscle. Lane 1, extract of TA and SOL muscles showing (from slowest to fastest migrating) types IIA/IIX, IIB and I MyHC; Lanes 2-7, extracts of single TAS fibers. Lanes 2-4 are from hybrid fibers with different ratios of IIX:IIB MyHC, Lanes 5 and 7 are from pure IIB fibers and Lane 6 is from a pure IIX fiber.

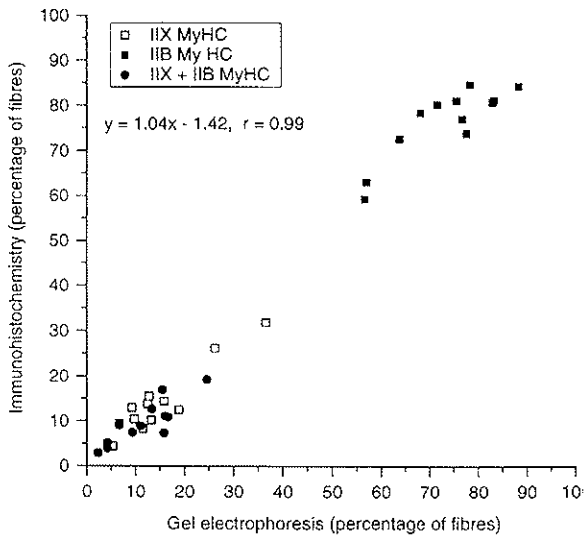

Figure 2. Reliability of immunohistochemical and electrophoretic determination of fiber type proportions in TAS muscle. Each point represents data from a single animal and relates the proportion of fibers expressing a given MyHC isoform by histochemical characterization to the proportion of single fibers sampled from the contralateral muscle expressing the same MyHC isoform as detected by gel electrophoresis. The two methods yielded virtually identical estimates of MyHC expression.

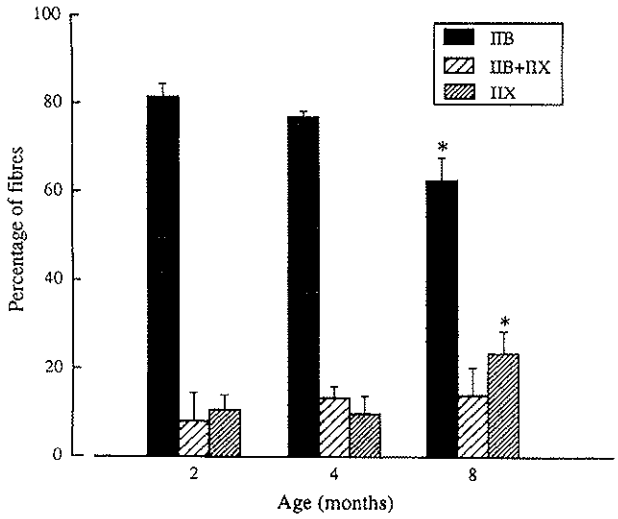

Figure 3. Proportion of type IIb, IIx/IIb and IIx fibers in TAS muscle of 2, 4 and 8 month old normal mice. Fiber types were determined by 6% SDS-PAGE of single fibers. 3 animals were used in each group. Number of single fibers used was: 140, 130 and 159 for 2, 4 and 8 month old mice, respectively.
** Significantly different from 2 and 4-month animals. P < 0.05, one-way ANOVA.*

Table 2. Serum T_3 and T_4 concentrations in hyperthyroid, hypothyroid and control mice.

Treatment group	n	Free T_3 (nmol/l)[a]	Total T_4 (pmol/L)[b]
Hyperthyroid	7	10.8 ± 3.8*	< 2*
Hypothyroid	7	0.4 ± 0.09*	< 2*
Control	5	0.8 ± 0.05	17.4 ± 1.1

[a] Limit of detection of assay = 0.2 nmol/l
[b] Limit of detection of assay = 2 pmol/l
Values are means ± SEM
* Significantly different from control, P < 0.05, one-way ANOVA.

Table 3. Body and TA muscle weight of hyperthyroid, hypothyroid and control mice.

Treatment group	n	Body weight (g)	TA muscle weight (mg)	MW/BW (mg/g)
Hyperthyroid	7	26.8 ± 0.6*	43 ± 1	1.6 ± 0.07
Hypothyroid	7	25.7 ± 0.9*	41.6 ± 2	1.6 ± 0.1
Control	5	31.2 ± 1.3	46.8 ± 1.4	1.5 ± 0.07

Abbreviation: MW = TA muscle weight, BW = body weight.
Values are mean ± SEM
* Significantly different from control, P < 0.05, one-way ANOVA.

MyHC composition is representative of the entire population of fibers within the muscle. Thus, the procedure complements the immunohistochemical approach and is particularly useful for identifying those fibers which co-express IIX MyHC together with the IIB MyHC. Furthermore, the procedure yields at least semi-quantitative data with regard to the precise admixture of the two myosin isoforms, information which is difficult to obtain from immunohistochemistry. We have, therefore, used the method of gel electrophoresis to assess the effects of ageing and altered thyroid hormone status on the conversion of type IIb to type IIx fibers in the superficial portion of the Tibialis Anterior muscle of mouse. Such a conversion would appear to be a natural extension of the hypothesis that there is a continuum of fiber types which express each of the four identified limb muscle-specific sarcomeric MyHC either exclusively or in orderly combinations.

The results of this study confirm that thyroid hormone exerts an effect upon MyHC isoform expression in mouse TAS muscle. Hypothyroidism led to a decrease in the proportion of fibers expressing IIB MyHC alone and an

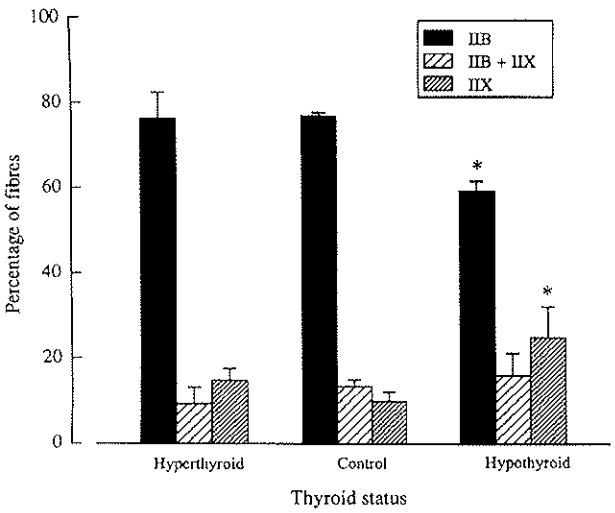

*Figure 4. Proportion of type IIb, IIx/IIb and IIx fibers in TAS muscle of hyperthyroid, hypothyroid and control mice. Fiber types were determined by 6% SDS-PAGE of single fibers. 3 animals were used in each group. Number of single fibers used was: 140, 152 and 130 for hyperthyroid, hypothyroid and control mice, respectively. * Significantly different from hyperthyroid and control. $P < 0.05$, one-way ANOVA.*

increase in the proportion of fibers expressing only IIX MyHC. The reduced IIB MyHC expression in hypothyroid mouse TAS is compensated for by the increase in IIX MyHC. This, together with the existence of single fibers co-expressing IIB+IIX MyHC in different proportions, strongly suggests that IIb and IIx fibers may be inter-convertible according to the following sequence:

IIB MyHC ↔ IIB+IIX MyHC ↔ IIX MyHC

although we should point out that in the present study we have not presented any evidence in favour of the conversion in the direction from IIX to IIB. The fact that the proportion of hybrid (IIx/IIb) fibers was not significantly different among the 3 animal groups (hypo-, hyper- and euthyroid) in our opinion merely suggests that the rates of accretion of IIX MyHC and degradation of IIB MyHC proceed at very similar rates and in no way detracts from the concept that the hybrid fibers represent a transitional population. Indeed, it is difficult to conceive by what other means a reduction of IIb fibers coupled with a quantitatively similar increase in IIx fibers could arise.

The lack of effect of elevated T_3 levels on MyHC expression is unlikely to be due to an insufficient time (5 weeks) for the treatment to have an effect in mouse since the half-life of skeletal muscle myosin is reported to be about 5 days [22]. Although it has been reported that elevated levels of thyroid hormone induce an increased expression of type IIA MyHC in rat soleus [4], there are several reports in the literature which indicate that this condition does not result in altered fiber type distribution in fast-twitch muscles of the rat. For example, Larsson *et al.* [27] observed that while chronic administration of T3 to rats over a 4-week period led to a reduction in the number of type I fibers and a concomitant increase in IIa and (in old rats) IIx fibres in the soleus, no shift in fibre type proportions was detected in EDL. Similarly, Fitzsimons *et al.* [11] reported the failure of hyperthyroidism to induce a change in myosin expression in the white gastrocnemius of the rat, although in the red portion of the same muscle they observed a decrease in the content of both slow and intermediate isomyosins together with an increase in fast isomyosins. The fiber type composition of white gastrocnemius is similar to that of TAS, being composed exclusively of IIb and IIx fibers. Kirschbaum *et al.* [20] also studied the effect of thyroid hormone on myosin expression in fast-twitch muscles of the rat both at the protein level and at the mRNA level. They also reported that hyperthyroidism did not significantly alter the percentage distribution of IIB MyHC and IIX MyHC at the protein level. However the IIB MyHC mRNA level was increased suggesting the possibility of post-transcriptional control.

The results of the present study also indicate that MyHC isoform expression in the fast-twitch TAS muscle of the mouse is age-dependent; in particular the percentage of fibers expressing IIB MyHC decreases from 2 to 8 months of age with a concomitant increase in the percentage of those expressing IIX MyHC. The presence of hybrid fibers, containing IIB and IIX MyHC, again suggests that there is a transformation of IIb fibers into IIx fibers. In support of this contention, Pettigrew and Noble [34] measured motor unit characteristics in rats ranging from 6 to 20 months and showed not only a reduction in the proportion of fast-fatiguable units (generally considered to comprise type IIb fibers) and an increase in slow (type I) units but also a significant increase in the proportion of "transitional-fast" units with characteristics intermediate between those of fast-fatiguable and so-called fast-intermediate motor units. Our demonstration of hybrid IIx/IIb fibers makes it tempting to speculate that these "transitional-fast" motor units were made up of fibers undergoing conversion from type IIb to IIx in these older rats. Indeed Larsson *et al.* [24, 25], using immuno-histochemical techniques to identify the MyHC composition in the fibers of the fast-twitch TA motor units in young and old rats, recently reported an age-related increase in the proportion of type IIx motor units as well as the presence of a population of motor units containing a mixture of type IIb and IIx fiber types in the old group that was not detected in their previous studies in which ATPase histochemistry was used to identify fiber types [1, 9, 26]. This finding has subsequently been confirmed by a gel electrophoretic approach [27] for rat muscles in which it is possible to separate the IIX and IIA MyHC isoforms. Such a result would be consistent with a progressive conversion of type IIb fibers to type IIx fibers with age in rat muscles as we have reported for the mouse.

The underlying cause of the age-related change in MyHC gene expression is not known. It may simply be a time-dependent phenomenon which occurs regardless of other disturbances to the system. Alternatively, it could be the result of an altered pattern of activity in the muscles of ageing animals, with a reduction in the level of phasic activity. Transformation of TAS muscle during growth and ageing may reflect such an adaptive process, although the physiological basis for an increased proportion of type IIx fibers is not clear from the present data. It is possible that these changes are associated with changes in function, although which comes first is unclear. For example, since IIx fibers have a higher oxidative capacity than IIb fibers [13, 31] an increase in IIx fibers in a muscle would confer a greater degree of fatigue resistance in that muscle, which could correlate with an increasing functional load due to an increasing body weight/muscle weight ratio.

Speed of contraction has been reported to decrease in aged rats [9, 16]. The mechanisms underlying this change are not yet well understood. However it has been reported that myosin ATPase activity decreases in both fast- and slow-twitch muscles with age [10, 16]. Furthermore, it has been shown that the intrinsic speed of shortening of muscle fibers is directly correlated to ATPase activity [2] and that this activity is determined by the type of MyHC expressed by a fiber [35, 40, 41]. Although the oldest animals used in the present study were only 8 months it should be noted that Kugelberg [23] reported that, over a range of ages similar to that which we have used, there is an adaptive transformation in the rat soleus such that type IIa fibers are progressively converted into type I fibers. This raises the question of whether the shift from IIB to IIX MyHC expression that we report here might be responsible for the observed slowing of contraction speed if this conversion continues as the animal ages further.

It has been suggested that the maximum velocity of shortening (Vo) of IIx fibers is less than that of IIb fibers. For example, Bottinelli et al. [3] measured Vo of single fibers from fast-twitch muscles of the rat and suggested that type IIB MyHC might be associated with higher Vo than type IIA and IIX MyHC, although the ranges of Vo of fast-twitch fibers showed some overlap. In addition, Gorza *et al.* [14] demonstrated that Vo of rat soleus following high frequency chronic stimulation was intermediate between that of normal soleus (composed almost exclusively of type I fibers) and that of EDL, which is composed primarily of type IIb fibers. Furthermore in these transformed muscles IIX MyHC was shown to be the major MyHC. Kirschbaum et al. [20] have also shown that chronic low frequency stimulation tends to have the same effect on MyHC expression in rat muscles as hypothyroidism, i.e. an increase in the level of IIX MyHC expression with a concomitant decrease in IIB MyHC. Thus, increased expression of IIX MyHC may partly account for the reduced Vo and ATPase activity in fast-twitch muscle with both ageing and hypothyroidism. That it may not be the sole factor is suggested by a recent report indicating that the sarcoplasmic reticulum from EDL, but not SOL, muscle of old (24 months) rats exhibits a reduced threshold for caffeine-induced calcium release [7].

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