Coexistence of slow and fast isoforms of contractile and regulatory proteins in human skeletal muscle fibres induced by endurance training

P. G. SCHANTZ and G. K. DHOOT
Department of Physiology III, Karolinska Institute, Stockholm, Sweden, and Department of Immunology, University of Birmingham, UK.


The distribution of fast and slow isoforms of troponin C, I, and T components and myosin heavy chains was investigated in histochemically typed myofibrillar ATPase intermediate (IM) fibres, that is, fibres that stain after both acid and alkaline pre-incubation in stainings for myofibrillar ATPase. In addition to the previously described IM fibres of types IIc and 1B, fibres that displayed staining characteristics between types IIc and 1B were observed and termed type IIc–1B. The IM fibres constitute less than 1% of the fibres in normal human limb and abdominal muscles. The IM fibres studied here resulted from extensive endurance training of human triceps brachii muscle (n = 6) and were induced by conversion of a proportion (13%) of type II fibres. The immunohistochemical stains of serial sections with antibodies to slow isoforms of troponin I, T, C and myosin heavy chain showed no staining of type II fibres but intense staining of types I and 1B fibres, whereas type IIc fibres stained with intermediate intensity. The antibodies to fast isoforms of the troponin components and myosin heavy chain did not give rise to staining of type I fibres but dark staining of type II fibres. Type 1B fibres stained with intermediate intensity and type IIc was either as dark as type II or slightly lighter. Type IIc–1B fibres showed staining intensities intermediate between those observed for types 1B and IIc in the immunohistochemical stains. It is therefore concluded that training-induced myofibrillar ATPase intermediate human skeletal muscle fibres are characterized by the coexistence of slow and fast isoforms of contractile and regulatory proteins. Changes in the distribution of fast and slow isoforms of several of the myofibrillar proteins appeared to be induced in a co-ordinated manner.

Key words: fibre types, human skeletal muscle, myofibrillar, ATPase, myosin, physical training, troponin.

Human skeletal muscle is composed of fibres that can be distinguished by the histochemical myofibrillar ATPase staining reaction based on their differential susceptibility to acid and alkaline treatment. Fibre types I, IIA and IIB, the three main types, emanate from this classification system (Engel 1962, Brooke & Kaiser 1970b). Different isoforms of myosin heavy chains, troponin components and tropomyosin have been shown to be present in type I and type II fibres (Dhoot & Perry 1979, Billeter et al. 1981a, b, Baumann et al. 1984). Physiologically, they are distinguishable as slow- and fast-twitch fibres (Buchta & Schmalbruch 1970, Garnett et al. 1979). However, a small percentage of fibres,
usually less than 1% in human limb and abdominal muscles (Hedberg & Jansson 1976 as cited by Jansson & Kajser 1977, Hägmark & Thorstensson 1979, Eriksson 1982, Blomstrand & Ekbloom 1982), do not fall into either of these categories since they stain after both acid (pH 4.3 and 4.6) and alkaline (pH 10.3) pre-incubation. Depending on different staining characteristics of these fibres, two subgroups (types II C and I B) have been reported (Brooke & Kaisser 1970, Dubowitz & Brooke 1973, Eisen et al. 1975). When referring to these fibres as a group the term myofibrillar ATPase intermediate (IM) fibres will be used. Little is known about the nature of the isoforms of myofibrillar proteins in these fibres. Coexistence of slow and fast isoforms of whole myosin and myosin light chains has been demonstrated in training-induced IM fibres (Schantz et al. 1982). The aim of this study was to investigate further the composition of protein isoforms in the IM fibres, using antibodies directed against fast and slow isoforms of troponin I, C, T, and myosin heavy chains by the immunoperoxidase procedure. This was studied in biopsy specimens from human triceps brachii muscle with IM fibres produced by training (Schantz & Henriksson, 1983).

SUBJECTS AND METHODS

Biopsy specimens of the triceps brachii muscle from six subjects (two men, four women) were studied. The average age, weight and height were 26 years (range 20–29), 66 kg (50–98) and 1.70 m (1.60–1.98). The subjects were given information about the procedure and risks involved in the experiments before they volunteered to participate. The study was approved by the Committee on Ethics of the Karolinska Institute. The subjects had skied 800 km in the mountain terrain of northern Scandinavia. Teams of two skiers pulled a load of approximately 80 kg on one sledge. The distance was covered in 36 days of skiing with 5 days of rest evenly interspersed. Training-induced changes in muscle fibre type distribution in these subjects has been described elsewhere (Schantz & Henriksson 1983).

Muscle sampling. The biopsy specimens studied were obtained 5 days after training from the middle-lower portion of the medial head of the left or right triceps brachii muscle using the needle biopsy technique (Bergström 1962). The samples were mounted immediately in embedding medium (Tissue-Tek II, Lab-Tek Products, USA) and frozen in isopentane, cooled to its freezing point in liquid nitrogen. The samples were stored at −80°C until analysed.

Histochemical analysis. Serial transverse cross-sections (10 μm thick) were cut with a microtome at −20°C. The sections were stained for myofibrillar ATPase at pH 9.4 (Gomori 1941, Paty & Herman 1955) after pre-incubation at pH 4.3, 4.6 and 10.3. The fibres were classified into types I and II (Engel 1962) and into subgroups types IIA and IIB (Brooke & Kaisser 1970b) as well as type IIC (Brooke & Kaisser 1970a, Dubowitz & Brooke 1973) and type IB (Eisen et al. 1975). Fibres with slight to moderate inhibition of the myofibrillar ATPase reaction after pre-incubation at both pH 4.3 and 10.3 have been designated as type IIC-IB (see Results). The staining procedure, as well as fibre type classification, has been described in more detail elsewhere (Schantz et al. 1982). However, the staining procedure in the present study was slightly modified: glycine buffer 0.04 M CaCl₂ and acetate buffer 0.10 M NaAc were used, and the pre-incubation time at pH 4.3 was 5 min.

Production of antibodies and immunohistochemical procedures. The monoclonal antibodies raised against fast and slow isoforms of troponin components I, T, and C were from the same sera used in earlier studies. In brief, the slow and fast isoforms of troponin I, C, and T were purified from red and white muscle from the rabbit or chicken and injected into guinea-pigs for antibody production. The specificity of the antisera was tested by the standard Ouchterlonny immunodiffusion method against purified preparations of each of the polymorphic forms of the three components of the troponin complex, the α- and β-troponin and urea extracts from different muscles. Further confirmation was obtained by passing antisera through columns of antigen linked to either Sepharose or cellulose carbonate, as well as by immuno-histochemical staining procedures which showed that virtually no cells in normal adult skeletal muscle contained both fast and slow forms of the troponin components. It was concluded that the antibodies are specific for the polymorphic form of the protein which they are raised but not for the species from which the protein was derived. For a more detailed description of these matters, see Dhoot et al. (1978, 1979).

For the production of monoclonal antibodies to slow skeletal myosin heavy chain (MHCs) (clone 96J), mice were immunized with crude myosin prepared from mixed human skeletal muscle. For the production of monoclonal antibodies to fast skeletal myosin heavy chain (MHCf) (clone I M5), the mice were immunized with myosin purified from fast chicken skeletal muscle. Spleen cells from immunized mice were fused with a P3-NS1/Ag-1 plasmacytoma cell line as described by Lowe et al. (1981). The specificity of 96J, which-secretes antibody to human MHCs, has been described in detail elsewhere (Dhoot et al. 1986). In brief, this antibody bound only type I fibres in normal adult human triceps brachii muscle. Immunostaining (De Blas & Cherwinski 1983) of Western blots (Tobin et
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Table 1. Staining intensity of different fibre types observed by immunohistochemical stains of muscle biopsy samples from m. triceps brachii after endurance training

<table>
<thead>
<tr>
<th>Antibody</th>
<th>II</th>
<th>IIC</th>
<th>IIC-IB</th>
<th>IB</th>
<th>I</th>
</tr>
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<tbody>
<tr>
<td>Slow MHC</td>
<td>-</td>
<td>++</td>
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<tr>
<td>Slow TN-I</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Slow TN-T</td>
<td>-</td>
<td>++(+)</td>
<td>+</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Slow TN-C</td>
<td>-</td>
<td>+(+)</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fast MHC</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Fast TN-I</td>
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<td>Fast TN-T</td>
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<td>Fast TN-C</td>
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The degree of staining intensity is indicated by ‘+’. Lack of staining intensity is indicated by ‘-’; ‘≥’ designates that some unequal staining intensities were observed but that most displayed equal staining intensities.

Abbreviations: MHC = myosin heavy chains, TN = troponin.

of extracts of human or rabbit skeletal muscle using this antibody resulted in a positive reaction with the MHC in slow skeletal muscle or mixed muscles containing a significant number of type I fibres. Fast skeletal muscles containing no or a few type I fibres did not stain by this method. When Western blots of light meromyosin, heavy meromyosin and subfragment-1 prepared from slow skeletal muscle were tested for reaction with this antibody, only the light meromyosin band stained. Thus, the antibody produced by clone 9G7 reacted with the rod region of the myosin molecule (Dhoot et al. 1986). The immunostaining of Western blots of extracts of chicken skeletal muscle with antibody secreted by clone LM5 resulted in positive staining of MHC band present in fast skeletal muscle only. Light chains did not show any reaction with this antibody. Immunoperoxidase staining of frozen sections of normal human triceps brachii muscle showed that the reaction was restricted to type II cells. Thus, although this antibody was raised against chicken skeletal muscle, it cross-reacted with human skeletal muscle.

The antigens in the frozen serial sections (6 μm thick) were localized by the immunoperoxidase sandwich labelling technique as described by Dhoot et al. (1978). The antisera dilutions used for staining were 1/20 for polyclonal antibodies and 1/200 for monoclonal ascites fluid. The cell supernatants in the case of monoclonal antibodies were either used neat or diluted 10-fold.

Rating the intensity of the immunohistochemical stains

In a previous study (Schantz et al. 1982), subjective rating was found to be as discriminative as using a microphotometric method for assessing staining intensities. Since subjective rating was considerably less time-consuming, this method was used in the present study. Nine samples were semi-quantitated in this manner, and the number of IM fibres analysed in the different samples were in the ranges of 1–13 type IB, 13–30 type IIC-IB and 7–10 type IIC fibres. In some samples, the staining intensity varied within the section. In such cases the IM fibres were compared with neighbouring type I, type II, and other IM fibres.

RESULTS

Classification of the myofibrillar ATPase intermediate fibre types (IM fibres: types IIC, IB and IIC-IB). The staining patterns for types IIC and IB were originally described by Brooke & Kaiser (1970a) and Eisen et al. (1975), respectively. Type IIC was defined as fibres with a non-inhibited staining reaction after alkaline pre-incubation (pH 10.3) but with moderately inhibited staining reactions after acid pre-incubations (pHs 4.3 and 4.6), whereas type IB fibres were ascribed the opposite pattern of staining, that is, moderate inhibition and non-inhibition after alkaline and acid pre-incubations, respectively. In the present study there were fibres that stained in a similar manner as types IIC and IB, but applying strict criteria they could not be typed as IIC or IB. These fibres were characterized by slight to moderate inhibition after pre-incubation at both pH 4.3 and 10.3. In general, the degree of inhibition was greater at pH 4.3 than at pH 10.3. Only very rarely were fibres observed with the same degree of inhibition after both pre-incubation conditions. We have termed this group of fibres type IIC-IB. This distinction was considered to be of value not the least since the aim of this study
was to investigate the isoform composition of the different fibre types.

Staining for slow and fast isoforms of myosin heavy chains. With antibody directed against the slow isoform of myosin heavy chain (MHCs), the general staining pattern was that of equally intensely staining types I and 1B fibres, type II C staining intermediately to types I and II, and type II C–IB fibres intermediately to types 1B and II C (Table 1). In some cases, though, it was not possible to distinguish between the staining intensities of types I B and II C fibres or between types II C and II C–IB, whereas in all cases there was a difference between types IB and II C. Fibres barely stained at pH 4.3 usually stained for MHCs but to a lesser degree than greyer ones at pH 4.3. In general, type II fibres remained unstained. However, weak staining for MHCs was observed in some rare type II A fibres in four subjects. In contrast, in one subject a fibre that stained slightly grey after pre-incubation at pH 4.3 did not stain for MHCs.

In stains for the fast isoform of MHC (MHCf) the type I fibres remained unstained. Increasing staining intensity was noted in the order: types IB, II C–IB, II C and II. Different staining intensities were noted in types I B, and II C–IB, whereas slightly overlapping intensities were noted between types II C–IB, II C, and II, that is, some type II C–IB fibres displayed the same staining intensity as type II C fibres, and some type II C fibres stained as dark as type II fibres.

Stains for slow and fast isoforms of troponin T, I, and C. The staining pattern for slow isoforms of troponin I, T, and C was inverse to that obtained with antibodies to fast isoforms of these proteins (Table 1). With antibodies to fast isoforms of the troponin components, the type II C fibres stained either as dark as type II fibres or only slightly lighter. The type II C–IB fibres stained either as dark as type II C or somewhat lighter. Type IB fibres stained lighter than the type II C–IB fibres, whereas the type I fibres remained unstained. With antibodies to slow isoforms of the troponin I, T and C, type 1B fibres stained as dark as type I fibres. Type II C–IB fibres stained lighter than the type 1B fibres but generally darker than II C fibres. Type II fibres remained unstained. The rare type II A fibres and fibres barely stained at pH 4.3, which showed weak reactions with the antibody to MHCf, stained in the same manner with the antibody to the slow isoform of troponin I. Due to weaker staining, these fibres could not be distinguished from the other type II fibres with antibodies to slow isoforms of troponin T and C.

DISCUSSION

Unlike normal human triceps brachii skeletal muscle, which contain almost exclusively type I (slow-twitch) and type II (fast-twitch) fibres with slow and fast isoforms of several myofibrillar proteins compartmentalized within the respective fibre type, the studied muscles were characterized by a significant proportion of muscle fibres that contained variable amounts of fast and slow isoforms in the same cell. This group of fibres, corresponded to myofibrillar ATPase intermediate fibres (IM fibres: types II C, IB and II C–IB) as typed by the histochemical myofibrillar ATPase staining procedure.

It is of obvious interest to specify the origin of these fibres. Simultaneously with the increase in IM fibres by training, the proportion of type II fibres decreased by 13% (Schantz & Henriksson 1983). The experimental protocol used in the study cited above allowed the conclusion that the IM fibres studied here were induced by the training per se. Thus, an apparent explanation is that the type II fibres were converted into IM fibres. However, an alternative explanation is that type II fibres were lost through phagocytosis and the IM fibres formed through proliferation of satellite cells. This series of events has been demonstrated in conjunction with some patterns of chronic electrical stimulation (Gambke et al. 1985). In such a case, developmentally and subsequently adult forms of myosin would be expressed in the proliferating cells, with the coexistence of slow and fast isoforms of adult myosins being one of several possible developmental pathways (cf. Gambke et al. 1985, see Whalen 1985). Muscle fibres staining similarly to the IM fibres in the myofibrillar ATPase staining reactions also occur frequently in developing muscles (Brooke & Kaiser 1974, Colling-Saltin 1978). However, it is unlikely that this is the origin of the IM fibres since the present mode of activation was fundamentally
different from that of long-term electrical stimulation. Furthermore, there were no morphological signs of regression or de novo formation of muscle fibres, that is, no myotubes were observed and all fibres displayed normal shapes and sizes. Also, there were no signs of developmental proteins in the IM fibres: muscle with a dominance of fetal myosin stain, in contrast to the IM fibres, equally dark (black) in stains for myofibrillar ATPase after both acid and alkaline pre-incubation (cf. Riley 1973, Colling-Saltin 1978 and the staining pattern of the IM fibres as described in e.g. Schantz 1986), and none of the IM fibres contained neonatal myosin heavy chains (Thornell, Butler-Browne & Schantz as presented in Schantz 1986). It is therefore concluded that the IM fibres studied here are a result of conversion of type II fibres and do probably only contain adult forms of protein.

On the basis of myofibrillar ATPase stains, the fibres were divided into types II and I as well as IM fibres that could be further subdivided into types IIC, IIC–IB and IB. The IM fibres contained variable proportions of fast and slow isoforms of both the contractile and the regulatory proteins studied. Type IIC fibres had only a small proportion of slow isoforms and type IB fibres only a small amount of fast isoforms. Type IIC–IB fibres had intermediate levels of both fast and slow isoforms. On the other hand, type II and type I fibres showed the presence of only fast and slow isoforms, respectively. An exception to this was some rare type IIA fibres in four subjects in which small amounts of the slow isoforms of myosin heavy chains and troponin I were detected. Similar observations have been made with regard to rat muscle in conjunction with fibre type II to type I transformation (Müntener 1982). The results of the present study are compatible with previous results pertaining to training-induced IM fibres demonstrating the coexistence of slow and fast isoforms of whole myosin and myosin light chains (Schantz et al. 1982).

It should be pointed out that fibres in human masticatory muscles, which stain like the present IM fibres, have been shown to contain a mixture of neonatal and slow myosin (G.S. Butler-Browne, P.-O. Eriksson, G.J. Laurent & L.-E. Thornell, pers. comm.) and most likely also corresponding isoforms of myofibrillar ATPase (see Schantz 1986). But in the present IM fibres, slow and fast isoforms of myofibrillar ATPase probably coexist in a manner similar to that described above with regard to the contractile and regulatory proteins. The staining patterns of the IM fibres can easily be interpreted in conformity with that concept, and this is further supported by the suggested non-existence of developmental protein isoforms in the IM fibres (see above).

The levels of slow and fast isoforms in different cells indicate the conversion from a type II to type I cell via II (IIA) → IIC → IIC–IB → IB → I. Whether or not type II to type I fibre conversion can occur in response to endurance training in man has been a matter of controversy (Schantz 1986). The possibility of such a conversion has mainly been supported by animal studies (Müller 1974, Jaweed et al. 1977, Henckel 1983, Green et al. 1984, Luginbuhl et al. 1984). However, two studies on humans recently reported a significantly increased number of type I fibres (6%) after endurance training (Howald et al. 1985, Simonau et al. 1985). These studies support an interpretation of the role of IM fibres as a transitional stage in type II to type I conversion.

Normally cells that barely stained in the myofibrillar ATPase reactions after pre-incubation at pH 4.3 also contained small amounts of slow isoforms of myosin heavy chain and troponin I, but one cell was noted that was an exception to this rule. However, in general, there was a good correlation in the type II C, IIC–IB, and IB fibres between levels of fast and slow isoforms of different muscle proteins investigated in this study, suggesting some kind of coordinated control of the manner in which the switch in isofrom synthesis is induced. This agrees with the general view of changes with transformation induced by electrical stimulation or cross-innervation (Dhoot et al. 1981, Brown et al. 1983, Eldridge et al. 1984). Although this coordination of expression of fast or slow isoforms of troponin components and myosin heavy chain was observed in these muscles, this obviously cannot be expected to occur with all other myofibrillar proteins, as the number of isoforms and their patterns of distribution vary with different proteins. In some cases more than one isofrom of the same protein, for example, C-protein, can occur in cells of normal adult skeletal muscle (Dhoot et al. 1985). Another type
of co-ordination of the expression of different proteins also governs development as the number of developmental isofoms of different proteins varies.

The stimulus for initiation of a change in expression of slow and fast isofoms of contractile or regulatory proteins is far from clear, and it is beyond the scope of this article to discuss this matter in depth. However, it is of interest that the importance of motoneurone impulse frequency in governing muscle phenotype has previously been stressed, whereas several recent studies favour the importance of the total amount of nerve impulses rather than the impulse frequency (Pette & Vrbova 1985). Evidence also exists, however, for the potential importance of muscular activity per se. Kugelberg (1973, 1976) studied rat m. soleus undergoing type II to type I transformation during rapid growth and noted that motor units with IM fibres were moderately heterogeneous in the histochemical stainings. IM motor units mainly consisting of type IB fibres could also include some type I fibres and the same was valid for type IID and type IIB fibres. Kugelberg (1976) stated that these variations showed a distributional gradient indicating a more rapid rate of progression in the transformation process from type II to type I towards the deeper medial part of the muscle where the type I fibres are relatively most numerous. Support for the role of muscular activity per se is also found in the fact that the overloaded human heart responds with a similar shift in isomyosin composition as skeletal muscle despite the fact that the heart lacks motor innervation (Swynghedauw 1986). For a more detailed discussion of these matters, see Schantz (1986).

It is concluded that extensive endurance training can induce a conversion of type II fibres into myofibrillar ATPase intermediate (IM) fibres (types IID, IIIC-IB and IB), as determined by histochemical stains. Concomitantly, slow isofoms of myosin heavy chains and troponin C, I and T are synthesized resulting in a coexistence of fast and slow isoforms of these proteins in the IM fibres. An increasing content of slow isoforms is noted in the order of types IIIC, IIIC-IB and IB. The IM fibres are interpreted as a transitory link between types II and I during transformation processes.

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