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Fibre Composition and Tubulin Localization in Muscle of Thoroughbred Sprinters and Stayers

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ABSTRACT. Fibre type composition and tubulin distribution were studied in the gluteus medius muscle of Thoroughbreds. Needle biopsies were taken from 5 sprinters and 5 stayers after completion of their racing career. Histochemical analysis was performed on cryostat sections using a new inhibition-reactivation myofibrillar ATPase technique. No significant differences were found between sprinters and stayers in mean fibre type frequency, diameter and relative fibre type area. Immunocytochemical techniques with monoclonal antibodies TU-01 and TU-02 against the alpha-subunit of tubulin revealed its heterogeneous distribution in different fibre types. Type II fibres reacted strongly and type I fibres weakly with antibody TU-01, whilst the reverse staining pattern was found with antibody TU-02.

Key words. Horse muscle; histochemical analysis; tubulin

INTRODUCTION

Mammalian skeletal muscles are composed of a mixture of different fibre types. Inheritance of the fibre type composition and the influence of training on this have been extensively studied in locomotory muscles of laboratory animals and man.[1,16,21] The proportion of slow-twitch (type I) and fast-twitch (type II) fibres shows a high heritability value implying a marked genetic influence. In man, a high correlation has been found between fibre type proportion and athletic discipline. Long distance runners demonstrate increased frequency of type I fibres whilst a high proportion of type II fibres is characteristic of sprinters.[4] Similarly, various equine breeds differ in the proportion of type I fibres which reflects probably long-lasting selection for distinct muscle performance.[23]

Numerous studies have been published which describe muscular adaptations in response to increased physical activity. The oxidative capacity of muscle fibres and the whole fibre number are mainly increased, but the type I/type II fibre ratio can also be changed depending on training intensity and animal age.[1,14,21,22] Such studies suggest that not only hereditary factors are significant determinants but that certain environmental stimuli are also important in the establishment of the metabolic profile and fibre type composition of skeletal muscle.

Monoclonal antibodies against various contractile and regulatory myofibrillar proteins provide a precise tool for studying their isoform distribution in different fibre types of adult animals. Structural changes of myofibrillar proteins during ontogenetic development and training have also been revealed by immunocytochemical methods.[8,22] Differential localization of tubulin, one of the microtubular proteins, has been demonstrated in various animal tissues using monoclonal antibodies against alpha- or beta-tubulin subunits.[5,6] However, data about tubulin distribution in skeletal muscle and its relation to fibre types are unknown up to now.

In the present study, we have performed a histochemical analysis of gluteus medius muscle samples in Thoroughbred sprinters and stayers. The purpose of this investigation was to determine whether there is a relationship between fibre type composition and muscle performance as has been demonstrated in man. In addition, we have ascertained the distribution of alpha-tubulin in equine muscle using an immunocytochemical technique with two monoclonal antibodies against different tubulin epitopes. We have proved a correlation between muscle fibre types and heterogeneous tubulin distribution.

MATERIALS AND METHODS

Needle biopsies were taken from the gluteus medius muscle of the best 5 sprinters and 5 stayers after completion of their racing career (i.e. winners of listed or grade races over 1 200 m and 2 400 m, respectively). Biopsy samples were covered successively with Tissue Tek embedding medium (Miles, Naperville) and talcum powder and frozen in liquid nitrogen. Serial cross-sections were cut in a cryostat at −20°C, air dried for 15 min and stained immediately with histochemical or immunocytochemical techniques.

Histochemical analysis

Muscle fibres were classified into individual types according to the nomenclature of Brooke and Kaiser,[2] i.e. type I (slow-twitch), type II A and type II B (both fast-twitch). To demonstrate all three types in a single muscle section we used the inhibition-reactivation myofibrillar ATPase technique (IR technique) described recently for human muscles.[13] Briefly, the equine muscle sections (10 μm) were stained as follows:

1. Inhibition of myofibrillar ATPase (m-ATPase) by hydroxymercuribenzoate (HMB) solution (2.5 mM; 30 min; room temperature): 4-hydroxymercuribenzoic acid, Na-salt (Serva) was dissolved in 0.1 M Tris and pH was adjusted with 0.1 M HCl to 7.0–8.0.
2. Thorough washing in distilled water (twice for 1 min each).
3. Reactivation of m-ATPase activity by cysteine in incubation solution (35 min; 37°C). The solution contained 2.5 mM ATP-disodium salt (Serva), 50 mM potassium chloride, 27 mM calcium chloride and 10 mM L-cysteine · HCl (Reanal) in Michaelis' barbital buffer, pH adjusted to 9.4 with 1 M and 5 M KOH.
4. Visualization of m-ATPase, i.e. treatments in 1% calcium chloride (three times for 30 s each), 2% cobalt chloride (3 min), distilled water (four times for 30 s each), 0.1% ammonium sulfide (3 min) and tap water (5 min) were used. Other details including stability of the solutions and specificity of the enzymatic reaction are mentioned in the original article.[13]

Fibre type identification in the sections stained with the IR technique was performed by comparison of these sections with the serial ones processed by the m-ATPase methods of Guth and Samaha[10] using acid preincubation (pH 4.2; 10 min; room temperature) and of Tunell and Hart[24] with a formaldehyde-glycine pre-incubation at pH 7.25. The latter method allows simultaneous demonstration of all three fibre types in a single cryostat section of equine muscle[12] and gives a similar staining pattern to that of IR technique. The main advantage of the IR technique in comparison with others is the optimal color separation between the three fibre types over a broad pH range of HMB solution (pH 7.0–8.0 is utilizable). To estimate the oxidative capacity of individual fibre types, succinate dehydrogenase (SDH) activity was demonstrated according to Lojda's method.[19]

Microphotographs were taken from sections stained by the IR technique and the following histochemical parameters of I, II A and II B types were evaluated. In each sample, about 200 muscle fibres were counted for determination of fibre type frequencies and at least 30 fibres of each type were measured for estimation of fibre type diameters. The mean of two measurements at right an-

gles to one another was used for each fibre. Relative fibre area (RFA) occupied by the respective fibre type in the muscle section was determined from the mean size and percentage of each fibre type.[15] Differences in mean values of the studied parameters between Thoroughbred sprinters and stayers and within each horse group were tested statistically by analysis of variance.

Immunocytochemical analysis

A spectrum of mouse monoclonal antibodies against pig brain tubulin were prepared by the hybridoma technique. A detailed characterization of these antibodies including their cross-reactivity with alpha-tubulin of several species was carried out previously.[25] Monoclonal antibodies TU-01 and TU-02 against different epitopes of alpha-tubulin subunit were chosen to study tubulin distribution in equine muscle fibres. TU-01 belongs to the IgG1 and TU-02 to the IgM immunoglobulin class. Polyclonal rabbit antiserum to tubulin was used to decide whether any tubulin was present in the muscle fibres.

Cryostat sections (6 μm), serial to those used for histochemical analysis, were stained by modified indirect immunoperoxidase technique[20] which included the following steps:

1. Fixation in cold acetone-methanol mixture (1 : 1; 5 min).
2. Inhibition of endogenous peroxidase activity in a bath of 3% hydrogen peroxide (50 ml) and absolute methanol (200 ml) for 20 min.
3. Binding of monoclonal (diluted 1 : 20) or rabbit polyclonal antibodies (overnight; 4°C; a moist chamber).
4. Incubation with the peroxidase-conjugated swine antimouse (SwAM/Px) or swine anti-rabbit (SwAR/Px) immunoglobulins in the case of the polyclonal antibody was used in step 3 (both diluted 1 : 30; 1 h; a moist chamber).
5. Visualization of peroxidase activity: incubation solution was applied (75 mg of 3,3'-diaminobenzidine · 4 HCl (Sigma) was dissolved in 100 ml of 0.1 M Tris-HCl; pH 7.6) for 5 min; then 0.1 ml of 30% hydrogen peroxide was added and the incubation continued for 10 min.

Thorough washing in 0.92% phosphate buffered solution (PBS) (three times for 5 min each) was carried out between individual steps of the technique. All incubations (with the exception of step 3) were done at room temperature. PBS with 2% bovine serum albumin was used for dilution of monoclonal antibodies, SwAM/Px and SwAR/Px. The monoclonal and polyclonal antibodies were prepared at the Institute of Molecular Genetics (Prague), SwAM/Px and SwAR/Px were produced at the Institute for Sera and Vaccines Production, Prague.

The control sections were incubated as described above but primary antibodies (step 3) were replaced by PBS. No reaction was observed in these sections. Both histochemically and immunocytochemically stained sections were embedded in glycerin jelly.

RESULTS AND DISCUSSION

Three fibre types were demonstrated in the gluteus medius muscle of Thoroughbred sprinters and stayers by the IR technique. A comparison of the sections stained by the IR technique (Fig. 1 A) to serial sections processed for the m-ATPase activity[24] (Fig. 1 B) and after acid pre-incubation[10] (Fig. 1 C) revealed that light, intermediate and dark fibre classes correspond to the I, IIA and IIB types, respectively. As was found recently in human muscle sections treated with the IR technique, type IIB fibers were dark (as in equine muscle) but the staining intensities of I and IIA types were reversed, i.e. type I showed an intermediate and type IIA a light staining.[13] Thus, there is an apparent difference between human and equine skeletal muscles in their staining patterns using the IR technique and therefore the sensitivity of m-ATPase of the two species to HMB and cysteine must be distinct, too.

As far as the oxidative capacity of individual types, our result ascertained in Thor-

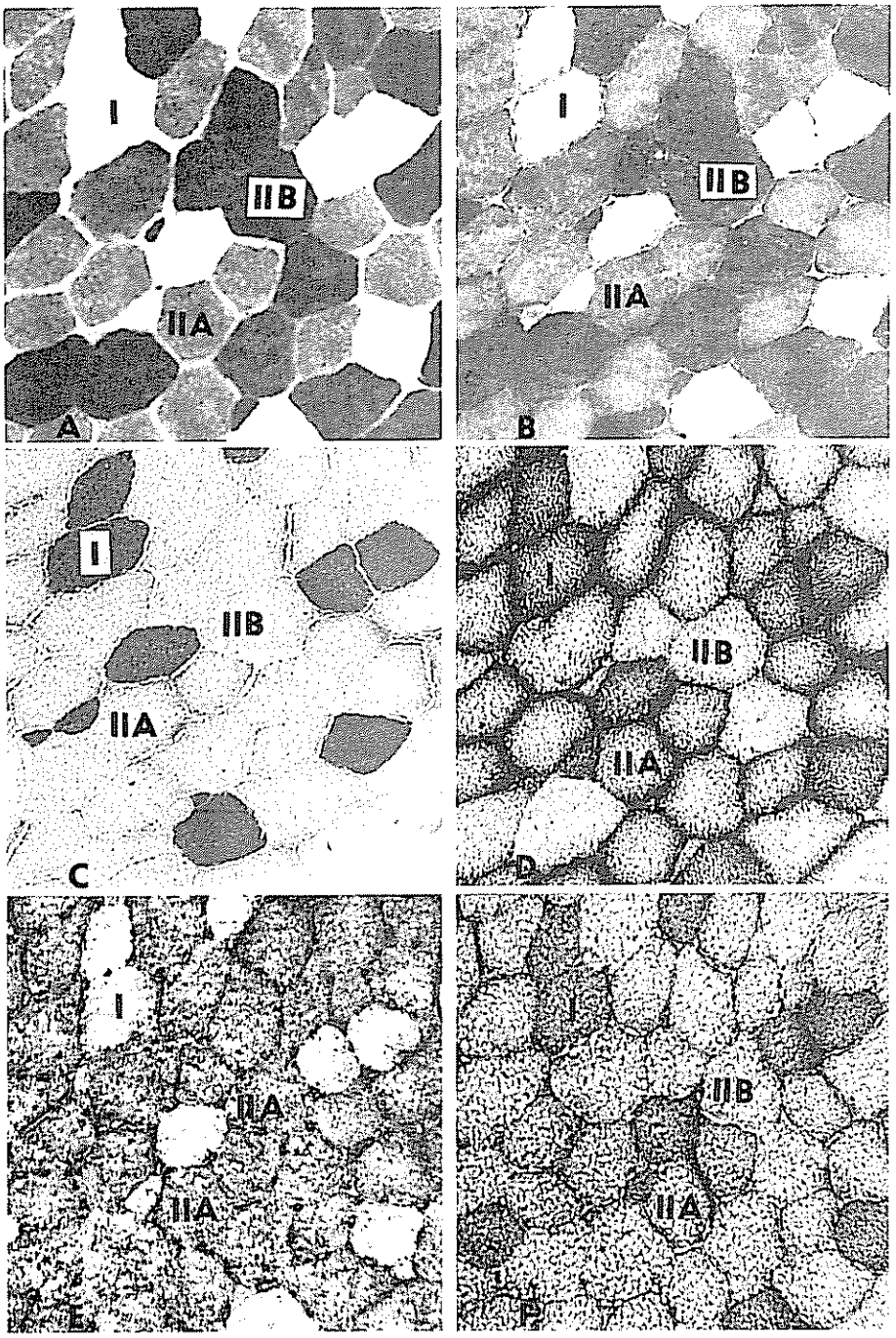

Fig. 1 Serial sections of equine gluteus medius muscle (×175). The IR technique (A) demonstrates three fibre categories which are classified as I, II A and II B types by comparison of this section with the following ones stained with technique of Tunell and Hart[24] (B) and Guth and Samaha (C) with preincubation at pH 4.2 [10] SDH activity (D) showed a continual spectrum of staining intensities. The heterogeneous tubulin distribution related to fast- and slow-twitch types and the reversal staining patterns were demonstrated with antibodies TU-01 (E) and TU-2 (F).

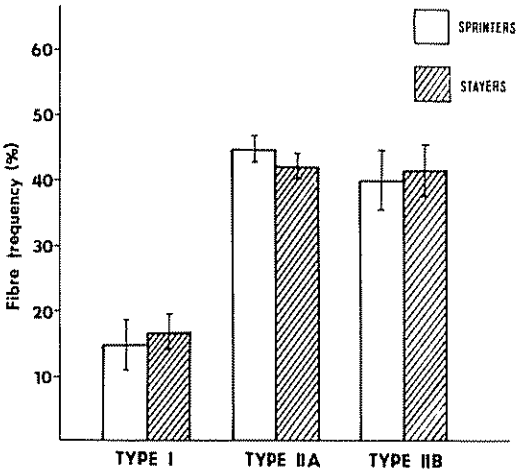

Fig. 2. The mean (±SD) fibre type frequencies in the equine gluteus medius muscle. Significant differences were ascertained between the type II B diameter on one side and the type I and type II A diameters on the other side within both Thoroughbred groups ($p<0.01$ with the exception of the type I versus type II B diameter in stayers where $p<0.05$).

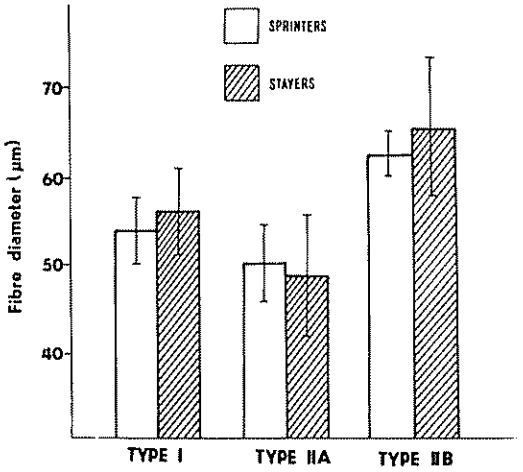

Fig. 3. The mean (±SD) fibre type diameters in the equine gluteus medius muscle. Significant difference ($p<0.01$) is seen only between slow- (type I) and fast-twitch (II A and II B types) frequencies within both Thoroughbred groups.

oughbreds is congruent with analysis of skeletal muscles of different equine breeds.[9,23] The SDH activity was low in type II B, intermediate to high in type II A and high in type I (Fig. 1 D). However, differentiation of fibres into three types only was rather artificial considering the fact that the SDH activity usually showed a continual spectrum from low to high level. This variable staining pattern can be related to the training response of Thoroughbreds since it is known that this will cause the oxidative capacity of muscle fibres to increase.[1,14,22] Thus, the SDH technique seems to be an unreliable method for fibre type classification especially in animals which are in the process of active training.

No statistically significant differences between Throughbred sprinters and stayers were found in the frequency, diameter and RFA of individual types. In both groups, the lowest frequency was ascertained in type I fibres, the frequencies of II A and II B types were almost the same (Fig. 2). Thus, in contrast to athletes,[4] there appeared to be no correlation between fibre type composition and sport discipline in the two groups of Thoroughbreds. Perhaps, genetic factors responsible for fibre type composition are more homogeneous in Thoroughbreds than in man. A further explanation may be the relatively similar length of track for which sprinters and stayers are trained (1 200 and 2 400 m, respectively). Consequently, no sufficient difference exists in the physical body load between the Thoroughbred groups which could cause a change in muscle fibre type composition.

A relationship was ascertained between diameter and fibre type. The lowest, intermediate and the highest mean values were demonstrated in the II A, I and II B types, respectively (Fig. 3). RFA of individual types reflects muscle performance more reliably than both the other parameters studied. However, again no significant differences were apparent in this characteristic between Thoroughbred sprinters and stayers (Fig. 4). RFA of type II B prevailed and RFA of type I was the lowest (about 15%) in both groups. A minor distinction was shown only in the proportion of RFA of both fast-twitch sub-

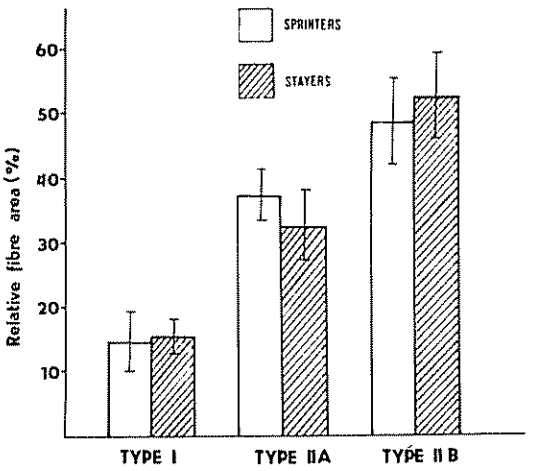

Fig 4. The mean (±SD) relative area of fibre types in the equine gluteus medius muscle. All differences between individual types within both Thoroughbred groups are statistically significant ($p<0.01$).

types. A higher RFA of type II A was characteristic of sprinters, whereas the opposite was true for RFA of type II B in stayers.

Fibre type composition of equine muscles is often studied with reference to the influence of training. Unfortunately, various breeds and skeletal muscles are used which differ in this characteristic so that only a limited comparison of our results with data in the literature is possible. As far as frequency of fibre type is concerned, our results are in agreement with findings in other Thoroughbreds.[11,23] Slight differences (2–5%) are explainable by the adaptive response of the gluteus medius muscle to various training demands. A change in the proportion of the two fast-twitch subtypes was ascertained (a significant increase in the type II A/II B fibre ratio) while the type I percentage was unaffected by training.[7,11,18] We found almost the same frequencies of II A and II B types. In this respect, Thoroughbreds in our study resemble inactive or late-age trained Standardbreds[7,18] and an untrained group of Thoroughbreds.[11] This is consistent with the fact that we analyzed the muscle samples which were taken from Thoroughbred sprinters and stayers after completion of their racing career.

Immunocytochemical studies using well characterized monoclonal antibodies against tubulin revealed subtle differences in its localization in various tissues.[5,6] Data on tubulin distribution in skeletal muscles are still missing. The present study outlines the tubulin heterogeneity in skeletal muscle. A comparison of immunocytochemically stained sections with serial ones treated for m-ATPase activity clearly demonstrated a correlation between the heterogeneous tubulin localization and fibre type distribution in the equine gluteus medius muscle. Monoclonal antibody TU-01 stained type II and type I fibres darkly and lightly, respectively (Fig. 1 E). The reversal staining pattern was seen in sections treated with monoclonal antibody TU-02 (Fig. 1 F). All fast-twitch (type II) fibres showed the same staining pattern and no difference was seen between the II A and II B subtypes.

Since the indirect immunoperoxidase technique with rabbit polyclonal antibody demonstrated homogeneous staining of whole muscle section (not shown) it is evident that all fibres contained the same level of immunoreactive tubulin. The heterogeneous tubulin distribution in fast- and slow-twitch fibre types can be explained by the presence of different isotubulins or with conformational modification of various epitopes on the tubulin molecules. Nothing is known about the genetic factors which determine alpha-tubulin in the horse. In vertebrates, alpha- and beta-tubulins are encoded by large multigene families[3] and several post-translation modifications have also been described.[17] We ascertained by immunoblotting that both TU-01 and TU-02 antibodies showed in the horse muscle homogenate only one band which correspond to alpha-tubulin. Thus, the specificity of the immunocytochemical reaction was proved and the conformational modification of tubulin is the probable explanation of its heterogeneous distribution in the equine muscle.

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