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The effect of ageing and immobilization on structure and function of human skeletal muscle fibres

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Biopsy samples were taken from vastus lateralis muscle of seven young (YO, age 30.2 ± 2.2 years), and seven elderly (EL, age 72.7 ± 2.3 years) subjects and two elderly subjects whose right leg had been immobilized for 3.5 months (EL-IMM, ages 70 and 75). The following main parameters were studied: (1) myosin heavy chain (MHC) isoform distribution of the samples, determined by SDS-PAGE; (2) cross-sectional area (CSA), specific force (P_o/CSA) and maximum shortening velocity (V_o) of a large population (n = 593) of single skinned muscle fibres, classified on the basis of MHC isoform composition determined by SDS-PAGE; (3) actin sliding velocity (V_f) on pure myosin isoforms determined by *in vitro* motility assays; (4) myosin concentration in single fibres determined by quantitative SDS-PAGE. MHC isoform distribution was shifted towards fast isoforms in EL and to a larger extent in EL-IMM. In EL and, more consistently, in EL-IMM we observed a higher percentage of hybrid fibres than in YO, and noted the presence of MHC-neonatal and of unusual hybrid fibres containing more than two MHC isoforms. P_o/CSA significantly decreased in type 1 and 2A fibres in the order YO → EL → EL-IMM. V_o of type 1 and 2A fibres was significantly lower in EL and higher in EL-IMM than in YO, i.e. immobilization more than counteracted the age-dependent decrease in V_o. The latter phenomenon was not observed for V_f. V_f on myosin 1 was lower in both EL and EL-IMM than in YO. V_f on myosin 2X was lower in EL than in YO, and a similar trend was observed for myosin 2A . Myosin concentration decreased in type 1 and 2A fibres in the order YO → EL → EL-IMM and was linearly related to the P_o/CSA values of corresponding fibre types from the same subjects. The experiments suggest that (1) myosin concentration is a major determinant of the lower P_o/CSA of single fibres in ageing and especially following immobilization and (2) ageing is associated with lower V_o of single fibres due to changes in the properties of myosin itself, whereas immobilization is associated with higher V_o in the absence of a change in myosin function.

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Ageing and disuse are the two main conditions leading to skeletal muscle atrophy in humans. In both conditions, muscle force decreases (Bruce *et al.* 1989; Narici *et al.* 1997); in ageing, slowing of movement occurs (Larsson *et al.* 1979). Although a likely explanation for such impairment of muscle performance is loss of muscle mass (Grimby & Saltin, 1983; Narici *et al.* 1997), recent evidence suggests that a significant contribution might come from changes in the properties of muscle fibres. Maximum shortening velocity (V_o; Larsson *et al.* 1997) and specific force (P_o/CSA; Larsson *et al.* 1997; Frontera *et al.* 2000) of type 1 and 2A muscle fibres from vastus lateralis muscle were found to be lower in elderly subjects than in young controls (Larsson *et al.* 1997), although lower V_o was not invariably observed in both fibre types (Krivickas *et al.* 2001). Higher V_o values have been observed following disuse (bed rest or spaceflight) in both slow and fast fibres (Widrick *et al.* 1997, 1999, 2001), although some contradictory results have been reported (Larsson *et al.* 1996). In the same studies, data concerning variations in P_o/CSA are less consistent. Lower P_o/CSA values have been reported in some (Larsson *et al.* 1996; Widrick *et al.* 1999), but not all (Widrick *et al.* 2001) of the studies, and a large degree of variability was observed in subjects exposed to the same unloading model and the same extent of disuse (Widrick *et al.* 1999).

The age- and disuse-related changes in V_o and P_o/CSA, occurring in fibres that contain the same MHC isoform, cannot be explained on the basis of the myosin isoform-based regulation of skeletal muscle fibre properties (Schiaffino & Reggiani, 1996; Bottinelli & Reggiani, 2000; Bottinelli, 2001). The mechanisms underlying such changes have been the subject of several very recent

reports. It has been suggested that the decrease in the P_o/CSA of single intact mice fibres as a result of ageing could be due to alterations in excitation–contraction coupling (Renganathan *et al.* 1997). However, such a phenomenon is unlikely to account for the whole decrease in P_o/CSA observed in single skinned muscle fibres that lacked excitation–contraction coupling and were maximally activated by exposure to calcium concentrations above maximum (Larsson *et al.* 1997; Frontera *et al.* 2000). The lower V_o of slow, type 1 fibres, in aged muscle has been attributed to changes in the intrinsic properties of MHC-1 determined by *in vitro* motility assays (IVMA) (Hook *et al.* 2001). In contrast, the higher V_o observed following unloading in humans is thought to depend on alterations in the structure of the sarcomere, i.e. on a lower thin filament density and on a higher thin–thick filament distance (Widrick *et al.* 1999). No consistent explanations have been given for the decrease in P_o/CSA following unloading, although a hypothesis has been put forward (Riley *et al.* 2002).

The controversial findings on the age-related modifications of skeletal muscle fibres may be explained by several determinants, among which disuse and partial denervation of muscle fibres (Campbell *et al.* 1973) are the most important and all of which can have a variable role in different populations of subjects. On the other hand, the lack of a clear picture on disuse-related modifications of single muscle fibres might be due to the fact that most models of disuse have used a rather short time scale (~2 weeks) and did not completely prevent neuromuscular activity (i.e. spaceflight with countermeasures).

The present work focuses on (1) the mechanisms underlying the alterations in force and velocity of shortening at the single fibre level that take place during ageing and following a very pronounced decrease in neuromuscular activity, namely immobilization, and (2) the role of immobilization in determining the age-dependent modifications of skeletal muscle fibres. The study was performed on biopsy samples from young (YO), elderly (EL) and elderly immobilized (EL-IMM) subjects whose right leg had to be immobilized for 3.5 months before total knee arthroplasty. The parameters determined were P_o, CSA and V_o of single skeletal muscle fibres, actin sliding velocity (V_f) on pure myosin isoforms (using IVMA), myosin concentration in single fibres and MHC isoform distribution.

In this study, the immobilization protocol was prolonged and very strict, ensuring pronounced fibre atrophy and enabling clear-cut information to be collected. For the first time, the structure and function of single muscle fibres and the function of isolated myosin following immobilization in the elderly have been studied and a comparison performed with mobile elderly subjects.

Preliminary reports of these results have been presented in abstract form (D'Antona *et al.* 2000; Reggiani *et al.* 2001).

METHODS

Subjects and muscle biopsy

Sixteen male subjects were enrolled in the study and divided in three groups: (1) healthy controls (YO, $n = 7$) with no previous record of muscular diseases or traumatic lesions, aged 30.2 ± 2.2 years, and who were not involved in any regular training or exercise activity; (2) elderly subjects (EL, $n = 7$) with no previous record of muscular disease or traumatic lesions, aged 72.7 ± 2.3 years, and who were not involved in any exercise activity (average walking activity outside home less than 1 h a day); (3) elderly subjects (EL-IMM, $n = 2$), aged 70 and 72, whose right leg had been completely immobilized for 3.5 months in an extended position for substitution of a total knee arthroplasty.

Muscle samples were taken by needle biopsy under local anaesthesia from the right vastus lateralis muscle of four YO and four EL subjects and by surgical biopsy from the two EL-IMM subjects, and from three YO and three EL subjects. The study was approved by the ethical committee of the University of Pavia and conformed to the standards set by the Declaration of Helsinki (last modified in 2000). After subjects had been fully informed of the goal of the experiments and of the risks involved in the bioptic procedure, written informed consent was obtained.

Experimental approach

Samples of vastus lateralis muscle were quickly immersed in ice-cold skinning solution (see below) and divided in several small bundles (about 100 fibres each). Most of the bundles were stored for up to 3 weeks before being used for single fibre dissection and functional analysis. Analysis of MHC isoform distribution of vastus lateralis muscles was performed on a pool of all the bundles from the samples that remained after single fibre dissection. On the day of the biopsy, some bundles from three YO, three EL and the two EL-IMM subjects were used for myosin extraction from single fibres and subsequent analysis of actin sliding velocity in *in vitro* motility assays. Some bundles from the same eight subjects (3 YO, 3 EL and 2 EL-IMM) were used for single fibre dissection and subsequent quantification of myosin content.

Single fibre dissection, solutions and experimental set-up

The methods used for single fibre dissection, the solutions and the experimental set-up have been previously used and described in detail (Bottinelli *et al.* 1996; Pellegrino *et al.* 2003). Briefly, segments of muscle fibres were chemically skinned and attached to the beams of the force transducer (AE 801 SensoNor, Horten, Norway) and the isotonic lever (model 101 vibrator, Ling Dynamic System, Royston, UK) in the experimental set-up.

Skinning (5 mM EGTA, pCa 9.0), relaxing (5 mM EGTA, pCa 9.0), pre-activating (EGTA 0.5 mM, pCa 9.0) and activating (EGTA 5 mM pCa 4.5) solutions were prepared as previously described (Bottinelli *et al.* 1996). The isotonic lever could either keep the length of the fibre segment constant to elicit isometric contractions, or impose on the specimen quick releases of preset amplitude completed in 2 ms. A stereomicroscope was fitted over the apparatus to view the fibre at × 20–60 magnification during the mounting procedure and during the experiment. The set-up was placed on the stage of an inverted microscope (Axiovert 10, Zeiss, Germany), which allowed us to view the fibre at × 320 magnification.

Single fibre analysis

Single fibre analysis was performed as previously described (Bottinelli *et al.* 1996; Pellegrino *et al.* 2003). In all experiments the temperature was set at 12 °C and the sarcomere length at 2.5 μm, i.e. optimal sarcomere length for force development. The cross-sectional area (CSA) of the specimen was determined, assuming a circular shape, from the mean of the three diameters measured at × 320 magnification, without correction for swelling, i.e. for the increase in the CSA of muscle fibres of ~20 % that is known to occur upon membrane removal (Godt & Maughan, 1977). To determine V_o, slack-test manoeuvres were employed (Bottinelli *et al.* 1994). V_o was expressed as fibre lengths per second (L s^{-1}) and P_o/CSA was expressed as kN m^{-2}. At the end of the mechanical experiment, fibres were put in 20 μl of standard buffer (Laemmli, 1970) and stored at –20 °C for subsequent analysis of MHC isoform content.

Fibre typing and myosin isoform identification

The MHC isoform composition of the single muscle fibre segments used in the mechanical experiments was determined by polyacrylamide-gel electrophoresis on 6 % polyacrylamide slab gels after denaturation in SDS (SDS-PAGE) using a procedure previously described (Bottinelli *et al.* 1996; Pellegrino *et al.* 2003). In the MHC region, three bands corresponding to the three adult MHC isoforms (MHC-1, MHC-2A and MHC-2X) could be separated in YO. One or two bands in the MHC region, fibres were classified in three pure fibre types (1, 2A, 2X) and two hybrid fibre types (1–2A and 2AX). Examples of MHC separation are reported in Fig. 1. The same electrophoretic protocol followed by densitometric analysis of MHC bands was used to determine the MHC isoform composition of whole muscle samples as previously described (Harridge *et al.* 1996) (Fig. 1*A*). In single fibres and muscle samples from two EL and the two EL-IMM subjects, a fourth band was observed in the area of migration of MHCs with a velocity greater than MHC-2A (Fig. 1). The fourth band was identified as MHC-neonatal using a combination of Western blot and immunohistochemical analysis. Western blot was performed after transferring proteins from unstained gels to a nitrocellulose sheet. Transfer was obtained by electrophoresis and the semidry transfer procedure (Towbin & Gordon, 1984). A generic anti-myosin antibody (BF-49) (Schiaffino *et al.* 1989) was used to ensure that the extra band was a myosin band (Fig. 2*A*). The neonatal nature of the myosin isoform, suggested by the migration velocity of embryonic and neonatal isoforms in the rat (Schiaffino & Reggiani, 1996), was demonstrated by using commercially available anti-MHC-neonatal (NCL-MHCn, Novocastra, Newcastle, UK). The antibody, which does not stain denaturated myosin and therefore does not work in Western blots, has been used on cross-cryosections of muscle bundles (for method see Bottinelli *et al.* 1991) from the two EL-IMM subjects to assess whether some fibres contained MHC-neonatal (Fig. 2*B*). As both EL-IMM samples showed the presence of an extra myosin isoform in the gels and some fibres were stained by the anti-neonatal-MHC antibody in cross-sections, it was concluded that the extra band in the gels was MHC-neonatal.

Myosin light chain (MLC) separation was performed as previously described (Bottinelli *et al.* 1994) on 10–20 % linear polyacrylamide gradient slab gels. Two regulatory MLC isoforms, the slow isoform MLC2s and the fast isoform MLC2f, and three alkali MLC isoforms, the slow MLC1s and the two fast isoforms MLC1f and MLC3f, were separated.

Myosin extraction and *in vitro* motility assay

For analysis of actin sliding velocity (V_f) on pure myosin isoforms (types 1, 2A and 2X) from YO, EL and EL-IMM, myosin was extracted from single fibres and loaded in an *in vitro* motility assay (IVMA) using a technique previously developed (Canepari *et al.* 1999; Pellegrino *et al.* 2003). Actin filaments were prepared from acetone powder obtained from rabbit skeletal muscles, labelled with rhodamine falloidine and visualized by epifluorescence. The sliding of actin filaments was studied at 25 °C in an assay buffer of the following composition: 25 mM Mops (pH 7.2), 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 200 μg ml^{-1} glucose oxidase, 36 μg ml^{-1} catalase, 5 mg glucose and 2 mM ATP. Velocity of sliding of actin (V_f) was expressed in μm s^{-1}.

As skeletal muscles generally express all or most myosin isoforms, they invariably provide a mixture of myosin isoforms that does not enable a precise comparison of the different myosin types. On the other hand, as single muscle fibres generally contain only one myosin isoform, they represent a very useful source of pure myosin isoforms for use in IVMA (Canepari *et al.* 1999). Long fibres (length ~10 mm) are required to provide, upon extraction, a sufficient quantity of myosin for IVMA (Canepari *et al.* 1999). Needle biopsy samples do not provide sufficiently long fibres for this purpose. Thus single muscle fibres were dissected only from the surgical biopsies of the 3 YO, 3 EL and 2 EL-IMM subjects. The experimental approach uses a very short portion of the fibre (0.2–0.3 mm) for myosin identification by SDS-PAGE (see above) and the rest of the fibre length (~10 mm) for myosin extraction (Canepari *et al.* 1999). The latter approach enabled us to load into the *in vitro* motility assay only pure myosin isoforms and discard samples containing more than one MHC isoform (hybrid fibres). It is in fact well known that the actin sliding velocity on myosin

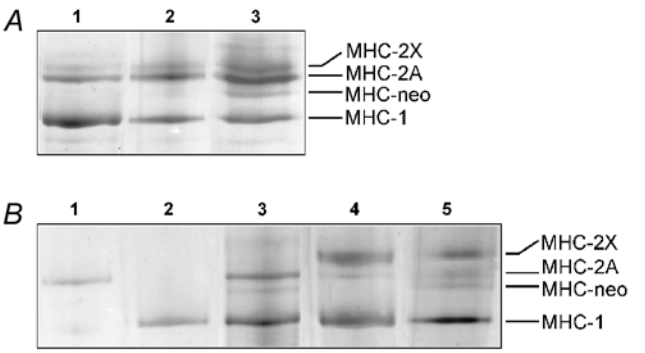

Figure 1. Electrophoretic (SDS-PAGE) separation of myosin heavy chain (MHC) isoforms in fibre bundles and in single muscle fibres of young (YO), elderly (EL) and elderly immobilized (EL-IMM) subjects

A, SDS-PAGE analysis of MHC isoforms in muscle bundles. Vastus lateralis samples of a YO, an EL and an EL-IMM subject were loaded in lanes 1, 2, and 3 respectively. Four MHC isoforms were separated and their area of migration is indicated by the labels on the right. In lanes 1 and 2 the samples from YO and EL subjects show the three adult MHC isoforms. In lane 3 the sample from the EL-IMM subject shows, besides the three adult MHC isoforms, a fourth band. This band was identified as MHC-neonatal (MHC-neo)(see Methods). *B*, SDS-PAGE analysis of single muscle fibres. The areas of migration of the four MHC isoforms are indicated by the labels on the right. Lane 1: a fibre from EL containing MHC-2A (type 2A); lane 2: a fibre from EL containing MHC-1 (type 1); lane 3: a fibre from EL-IMM containing MHC-1, MHC-2A and MHC-neonatal (MHC-neo)(type 1–2-neo); lane 4: a fibre from EL-IMM containing all three adult MHC isoforms (type 1–2AX); lane 5: a fibre from EL-IMM containing all four MHC isoforms (type 1–2-neo).

samples containing a mixture of slow and fast myosins is disproportionately affected by the slowest myosin, which acts as an internal load applied to the fastest myosins (Harris *et al.* 1994). The method of MHC identification used can detect ~1 % of a contaminating MHC (Bottinelli *et al.* 1994), an amount that is unlikely to significantly affect the functional properties of single fibres or of myosin in IVMA. The procedure for V_f determination was less successful with myosins from EL-IMM subjects than with myosins from YO and EL, possibly due to the lower concentration of myosin in EL-IMM (see Results). This, together with the fact that myosin extraction was done only on fresh muscle samples on the day of the biopsy, is responsible for the somewhat lower number of IVMA assay experiments performed on myosin from EL-IMM subjects in comparison with YO and EL.

Myosin quantification in single fibres

Myosin content was quantified in single muscle fibres from YO, EL and EL-IMM subjects using gel electrophoresis by determining the brightness–area product (BAP) of the MHC band of each fibre and calculating myosin content from BAP using a standard curve obtained in each gel by loading known amounts of a myosin standard.

Single muscle fibres, 5–10 mm long, were dissected in skinning solution under a dissecting microscope. The length of the fibre segment was determined at × 20–40 magnification. The specimen was then transferred in a skinning solution-filled chamber to the stage of an inverted microscope and mounted using aluminium clips between two hooks. By turning the aluminium clips, both the width and depth of the segments could be measured at × 320 magnification every 0.5–1 mm along the length of the segment. On average, 10 measurements for depth and 10 for width were made. The volume of the specimen was calculated from the length and the mean values of width and depth. The segment was then placed in 20 μl of standard buffer (Laemmli, 1970) and stored at room temperature for 4 h and at 4 °C for 14 h. Subsequently, aliquots of the 20 μl standard buffer in which the segment was dissolved were loaded on 10–20 % linear polyacrylamide gradient gels (Bottinelli *et al.* 1994), run at 100 V overnight at room temperature and stained with Coomassie brilliant blue R-250. This protocol is generally used to separate low molecular weight proteins such as myosin light chains and has been employed here to visualize MHC isoforms in a single band and make myosin quantification easier and more precise. In the same gels, known amounts of a myosin standard were loaded to enable determination of a standard curve. Two myosin standards were tested. One is commercially available (SIGMA M3889) and the concentration was verified with the Bradford method. The other was prepared from rat muscle using a previously described procedure (Canepari *et al.* 1999) with two further purification steps obtained by myosin precipitation and re-suspension in a high ionic strength buffer. The high purity of the myosin sample was indicated by the ratio of the absorbance at 280 nm and 260 nm (1.6; Tikunov *et al.* 2001) and by SDS-PAGE analysis. The myosin concentration in the sample was determined by measuring the absorbance at 280 nm. Calibration curves of both standards were determined in the same gel in order to test the correspondence of the myosin concentration of the two standards. The standard curves agreed within 5 %. The same myosin standard obtained from rat muscle was used in all determinations of myosin concentration in single fibres reported in the present work. Gels were imaged using a high-resolution scanner (Arcus II, Agfa). The BAP of the myosin bands was determined using the software Adobe Photoshop 5.5 (Adobe) (Geiger *et al.* 2000). BAP corresponds to the number of pixels of the whole MHC band times the mean intensity level of the pixels. The BAPs of the myosin standards were plotted *versus* the known amount of myosin loaded and a standard curve was built (Fig. 3*B*). From such curve the amount of myosin in the single fibre segments was determined. From the amount of myosin in the MHC band, the volume of standard buffer containing the segment

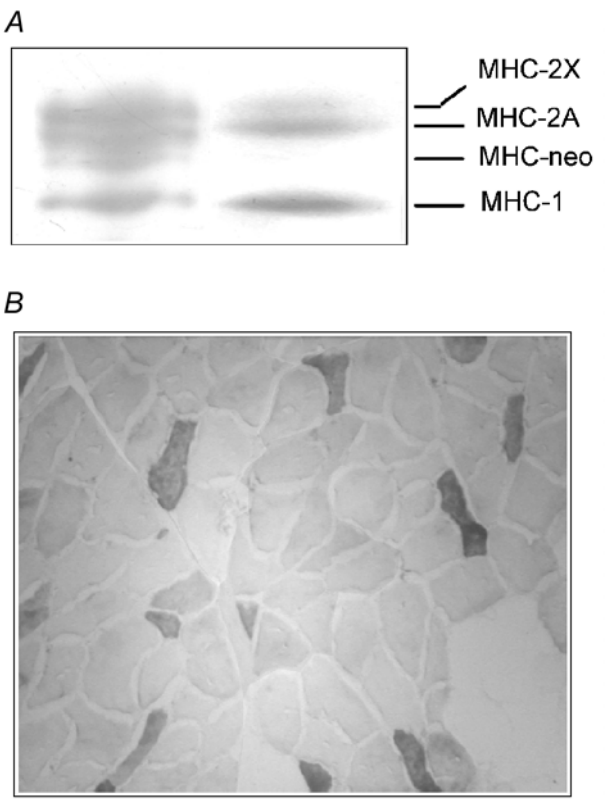

Figure 2. Identification of the extra protein band in the area of migration of MHC isoforms

A, Western blot analysis of a sample from one EL-IMM subject (lane 1) and of a sample from one YO subject (lane 2). The antibody specific for all myosin isoforms (BF-49) was used. Four bands are stained in the EL-IMM sample, whereas the usual three adult MHC isoforms are stained in the sample from YO. *B*, a cross-cryosection of a muscle sample from one EL-IMM subject stained by the antibody for MHC-neo (NCL-MHCn). Some fibres are stained and therefore contain MHC-neonatal.

and the volume of the fibre, myosin concentration was calculated and expressed in μM.

The reproducibility of fibre myosin quantification was tested using several procedures. Aliquots of each fibre buffer were often loaded either in the same gel or in different gels. This approach enabled us to use only the MHC bands whose BAP fell in between the lowest and the largest standard (i.e. between 0.5 and 4.0 μg of myosin). To verify the repeatability of myosin extraction by the standard buffer, some 6 mm fibres were cut into two segments either of equal length (3 mm) or of different lengths (2 and 4 mm), which were separately put in standard buffer and separately loaded in gels after the usual 18 h (4 h at room temperature and 14 h at 4°C). Determinations were very repeatable, as shown in Fig. 3*A*. No attempt was made to ensure that the standard buffer actually extracted 100 % of the myosin from a fibre segment. As the values of myosin concentration obtained (184–215 μM) are very consistent with previous estimates (He *et al.* 1997) and determinations (Tikunov *et al.* 2001), it is likely that myosin extraction was close to 100 %. However, the goal of the analysis of myosin concentration was mainly to compare single fibres from YO, EL and EL-IMM subjects. The procedures used to ensure repeatability of the measurements indicate that the data can be safely used for comparative purposes.

Statistical analysis

Data were expressed as means ± S.D., unless otherwise stated. Statistical significance of the differences between means was assessed by ANOVA followed by the Student-Newman-Keuls

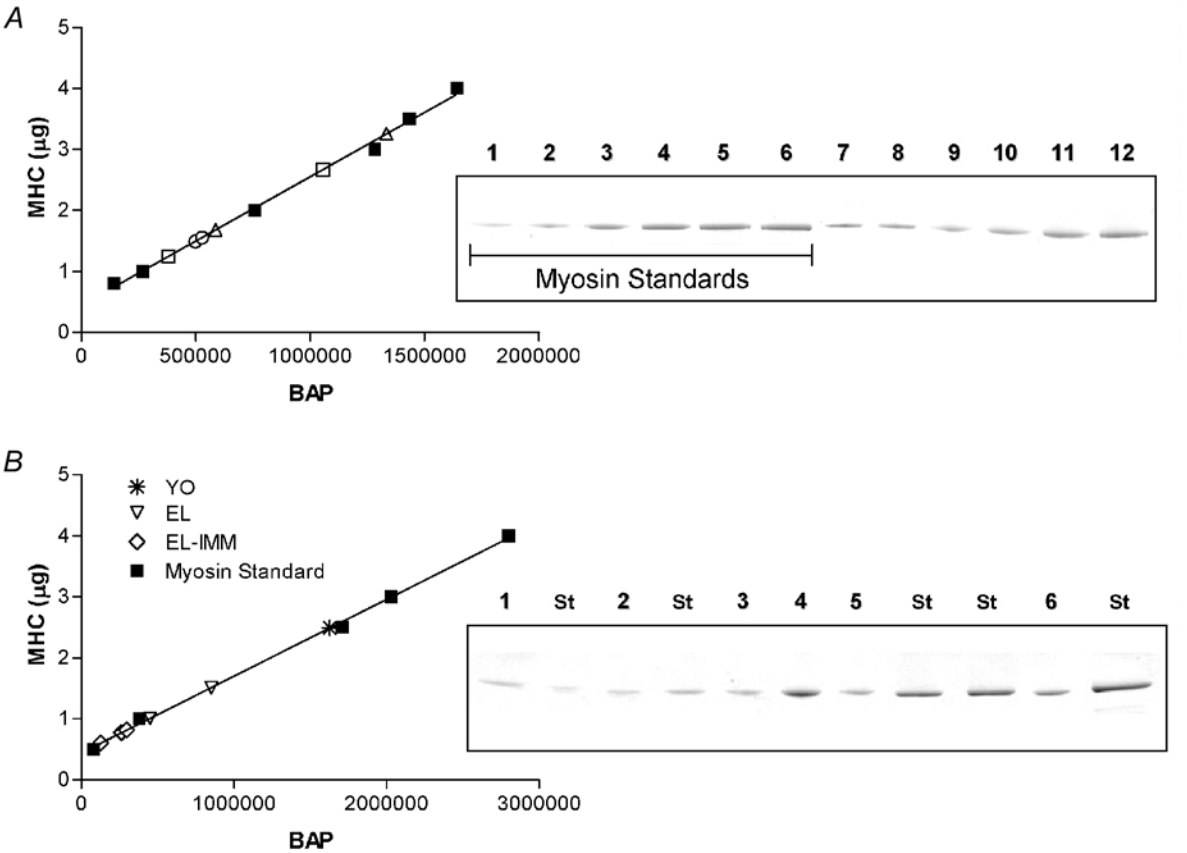

Figure 3. Quantification of myosin concentration in single muscle fibres

A, an example of the procedure used to ensure precise determination of myosin content in single fibres. The graph shows the relation between the brightness–area product (BAP) and the MHC content, expressed in μg, of the bands of the gel in the inset on the right. Six samples of 0.5, 1.0, 2.0, 3.0, 3.5, and 4.0 μg of a myosin standard were loaded in lanes 1–6, respectively. The BAPs of such bands were plotted in the graph (■) and were used to build a standard curve (continuous line). The slope of the standard curve is very significantly different from zero ($P < 0.0001$, $r^2 = 0.996$). Two segments of the same fibre of equal length were loaded in lanes 7 and 8 and their BAPs were plotted in the graph with open circles. Two segments of the same fibre of different length (2 and 4 mm) were loaded in lanes 9 and 11 and their BAPs were plotted in the graph with open squares. Two segments of the same fibre of different lengths (3 and 6 mm) were loaded in lanes 10 and 12 and their BAPs were plotted in the graph with open triangles. *B,* an example of the procedure used to determine myosin content in single fibres. Five samples of 0.5, 1.0, 2.5, 3.0, and 4.0 μg of the myosin standard were loaded from left to right in the gel in the inset (lanes indicated with St) and used to build a standard curve (■) in the graph. The standard curve had a slope very significantly different from zero ($P < 0.0001$, $r^2 = 0.998$). Segments of single fibres from YO (✳), EL (▽), EL-IMM (◇) were loaded in lanes 1–6 and their BAPs plotted in the graph to determine myosin content using the standard curve.

test. A probability of less than 5% was considered significant. Statistical analysis and linear regression analysis for slack-test determination were performed using Prism Graphpad 4.0 (Graphpad, CA, USA) software. Slopes were considered significantly different from zero if the $P < 0.05$. Comparison of the slopes of two linear regressions was also performed by Graphpad 4.0 using a method discussed in detail by Zar (1998).

RESULTS

MHC isoform and fibre type distribution

Figure 4*A* shows the MHC isoform distribution determined by SDS-PAGE (Fig. 1*A*) and densitometry of myosin isoform bands. MHC-2A content was found to significantly decrease and MHC-2X content was found to significantly increase in the order YO → EL → EL-IMM. MHC-1 was significantly lower in EL-IMM than in YO and in EL, whereas no difference was observed between the MHC-1 content of YO and EL. Collectively, the results indicate a shift towards a faster phenotype in EL and, to a greater extent, EL-IMM. In SDS-PAGE gels of the two EL-IMM subjects and of two of the seven EL subjects an extra band was found in the area of migration of MHC isoforms. This extra band, which could be identified as the MHC-neonatal isoform using an approach described in the Methods (Fig. 2), represented 4.5 ± 5.6% and 7.97 ± 2.38% of all MHC isoforms in EL and EL-IMM subjects, respectively. Interestingly, variability between the two EL-IMM subjects was low and both subjects showed a clear shift towards fast MHC isoforms. MHC isoform distribution in the two subjects was: MHC-1, 14.4 ± 3.2 (S.D. of three repeated determinations) and 26.8 ± 3.9%; MHC-2A, 29.0 ± 1.1 and 24.3 ± 5.2%; MHC-2X, 46.3 ± 5.3 and 42.7 ± 5.2%; MHC-neonatal, 9.8 ± 0.9 and 6.1 ± 1.9%.

The analysis of the distribution of fibre types in a large population of single fibres ($n = 730$, of which 593 were used for functional analysis) (Fig. 4*B*) suggests a shift towards a faster phenotype, consistent with the analysis of MHC isoform distribution by SDS-PAGE (Fig. 4*A*). Analysis of the MHC isoform content of single fibres enabled identification of hybrid fibre types. Interestingly, hybrid type 1–2A fibres were significantly more frequent in EL (16%) than in YO (6%) and hybrid type 2AX were significantly more frequent in EL-IMM (30%) than in YO (6%) and EL (6%). In EL-IMM, 5% ($n = 6$) of the fibres contained all three adult MHC isoforms, and 3% of the fibres ($n = 3$) contained MHC-neonatal. MHC-neonatal was always in association with both MHC-1 and MHC-2A or with MHC-1, 2A and 2X (Fig. 1). The fibres containing

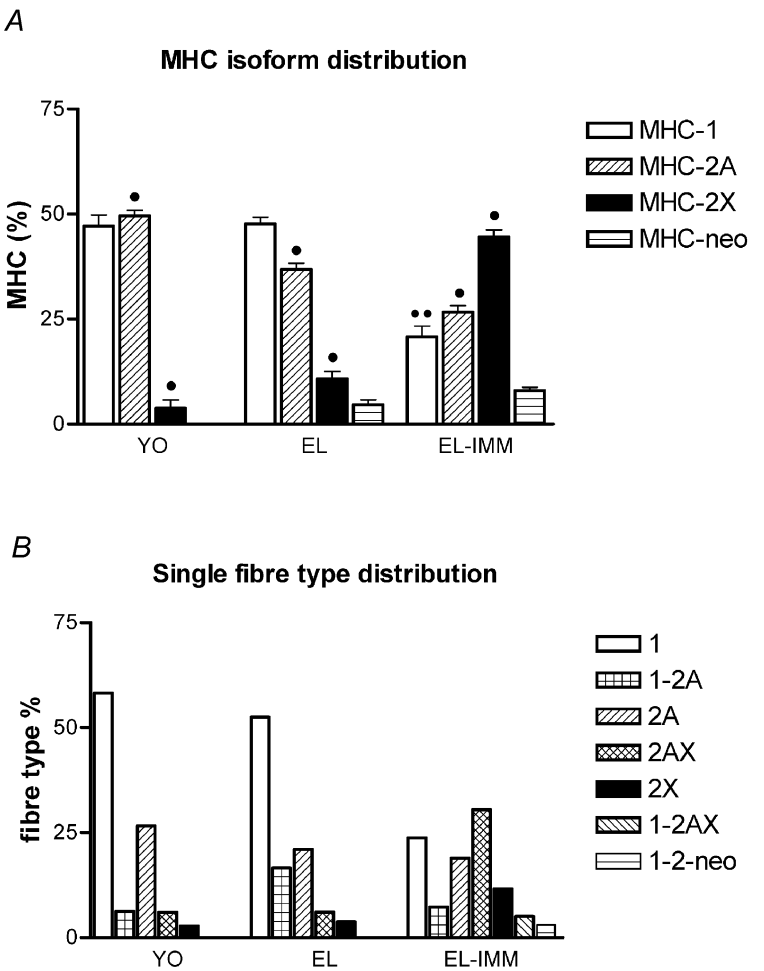

Figure 4. Myosin heavy chain (MHC) isoform distribution and fibre type distribution in young (YO), elderly (EL) and elderly immobilized (EL-IMM) subjects

A, MHC isoform distribution determined from biopsy samples of the three groups of subjects by SDS-PAGE separation and subsequent densitometric analysis of MHC bands. The height of each vertical bar represents the mean values (± S.E.M.). *B*, fibre type distribution in a population of single muscle fibres ($n = 730$) identified on the basis of MHC isoform composition determined by SDS-PAGE. Hybrid fibre types containing more than two MHCs were found (1–2AX, 1–2–neo). In the group 1–2–neo the fibres containing MHC-neo together with two or three adult MHCs were grouped. • Significantly different from all the other subject groups, •• significantly different from EL, $P < 0.05$.

MHC-neo were, therefore, grouped in the type 1–2–neo. None of fibres from YO was found to contain more than two MHC isoforms; hybrid fibres contained either 1 and 2A or 2A and 2X.

Fibre size and force of single muscle fibres

Figure 5*A* shows the mean values of CSA of the different fibre types determined in the population of fibres used for functional analysis. CSA of type 1 fibres was found to significantly decrease in the order YO → EL → EL-IMM. Type 1–2A, 2A and 2X fibres from EL-IMM were significantly smaller than corresponding fibre types from YO. Fibre atrophy following immobilization appeared more pronounced for type 1 fibres (−51 %) than for type 2A (−26 %) and type 2X (−24 %). Fibre atrophy following ageing was also somewhat more pronounced for type 1 fibres (−22 %) than for type 2A (−12 %), whereas no significant decrease in fibre CSA was observed in type 2AX and 2X fibres.

Fibres containing all three adult MHC isoforms (type 1–2AX) and two or more adult MHCs plus MHC-neonatal (type 1–2–neo) had somewhat lower CSA values than the other fibre types from EL-IMM: 4057 ± 1285 μm^2 ($n = 6$) for type 1–2AX, and 3182 ± 527 μm^2 ($n = 3$) for type 1–2–neo. Immunohistochemical analysis of muscle bundles from EL-IMM subjects using an anti-MHC-neonatal antibody (Fig. 2*B*) showed that the majority of MHC-neonatal-containing fibres had a small size. The small size probably prevented them from being dissected. It is, therefore, not surprising that none of the fibres used for mechanical analysis was found to contain only MHC-neonatal, and that the few MHC-neonatal-containing fibres studied were hybrids.

Figure 5*B* shows the mean values of specific force (P_o/CSA) of the same fibres reported in Fig. 5*A*. When type 1, 2A and 2X fibres were compared within each subject group, in agreement with previous observations (Bottinelli & Reggiani, 2000), P_o/CSA was significantly lower in type 1 than in type 2A and 2X fibres, whereas no difference was observed among fast (2A and 2X) fibres. More relevant for the purpose of the present study was the comparison between corresponding fibre types in the different subject

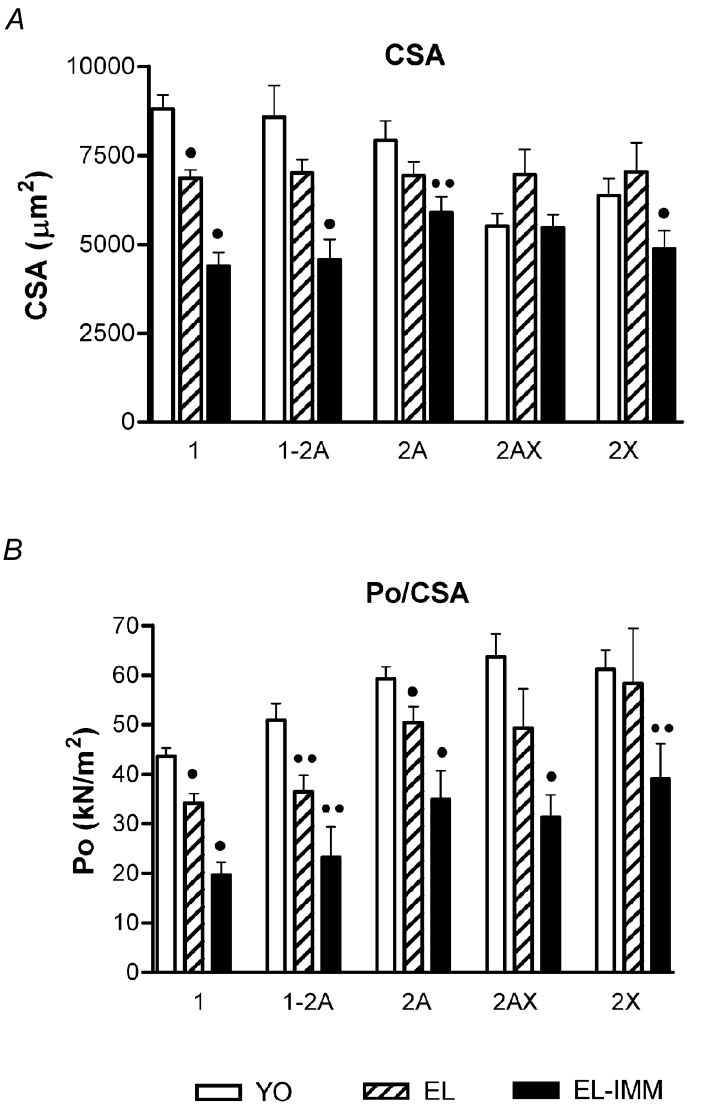

Figure 5. Cross-sectional area (CSA; *A*) and specific force (P_o/CSA; *B*) of the single muscle fibres used for mechanical experiments from young (YO), elderly (EL) and elderly immobilized (EL-IMM) subjects

CSA is expressed in μm^2 and P_o/CSA in kN m^2. The height of the bars represents the mean values (± S.E.M.). The numbers of fibres studied per type and per group were: 143 (YO), 128 (EL) and 28 (EL-IMM) for type 1; 20 (YO), 32 (EL) and 13 (EL-IMM) for type 1–2A; 75 (YO), 64 (EL) and 21 (EL-IMM) for type 2A; 19 (YO), 8 (EL) and 20 (EL-IMM) for type 2XA; 9 (YO), 5 (EL) and 8 (EL-IMM) for type 2X. • Significantly different from all the other subject groups, •• significantly different from YO, $P < 0.05$.

groups. P_o/CSA was significantly lower in all fibre types from EL-IMM than in corresponding fibre types from YO. The decrease in P_o/CSA was larger in type 1 fibres (−55 %), than in type 2A (−42 %) and type 2X (−37 %) fibres in EL-IMM. The P_o/CSA of type 1 and 2A fibres was significantly lower in EL than in YO, with a larger decrease in type 1 (−22 %) than in type 2A (−16 %). Therefore, P_o/CSA appeared to decrease in the order YO → EL → EL-IMM, although this trend reached full statistical significance for type 1 and 2A fibres only. To assess the variability of the P_o/CSA of single fibres between the two EL-IMM subjects, P_o/CSA values for type 1, 2A and 2AX fibres from the two EL-IMM subjects were compared and found to be: 18.9 ± 8.0 (n = 13) and 21.0 ± 18.8 kN m^{-2} (n = 15) for type 1, 38.0 ± 31.7 (n = 11) and 31.7 ± 19.8 kN m^{-2} (n = 10) for type 2A, and 29.2 ± 22.3 (n = 7) and 34.3 ± 16.6 kN m^{-2} (n = 13) for type 2AX. Type 1–2A and 2X fibres were not sufficiently numerous to enable a reliable comparison between the two subjects. The variability was very low and fibres from both EL-IMM subjects were weaker than fibres from YO and EL.

The P_o/CSA of type 1–2AX (22.8 ± 3.0 kN m^{-2} n = 6) and 1–2–neo (17.6 ± 3.6 kN m^{-2} n = 3) fibres was, like CSA, somewhat lower than the P_o/CSA of the other fibre types from EL-IMM, although no statistical analysis was performed due to the small number of these unusual hybrids.

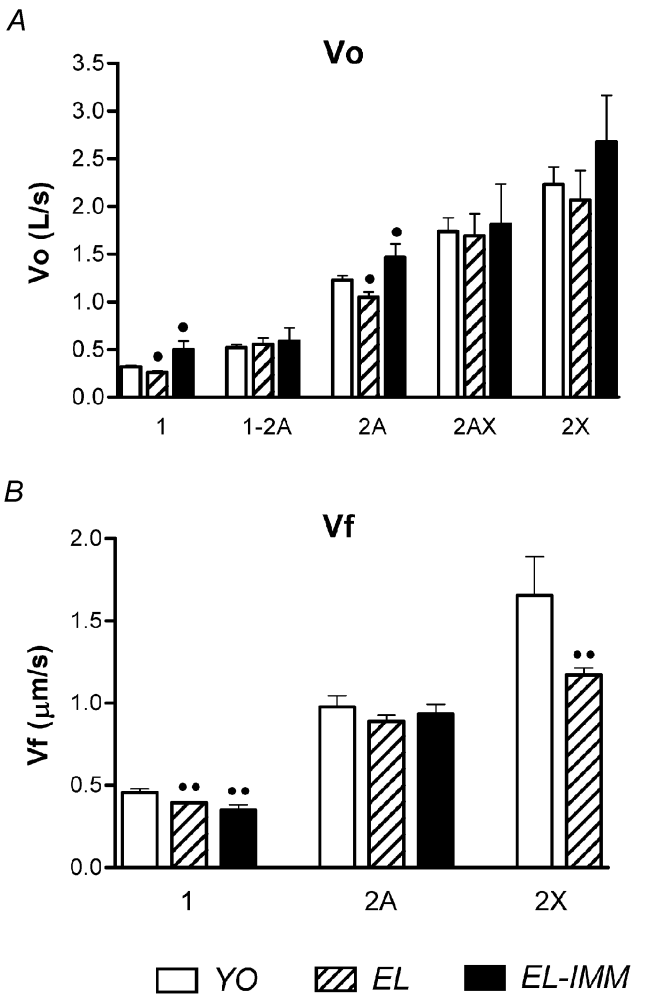

Figure 6. Maximum shortening velocity (V_o) of single muscle fibres (*A*) and actin sliding velocity (V_f) on pure myosin isoforms extracted from single muscle fibres (*B*) of young (YO), elderly (EL) and elderly immobilized (EL-IMM) subjects

V_o is expressed in segment length per second (L s^{-1}) and V_f in μm s^{-1}. V_o was determined at 12 °C and V_f at 25 °C. The numbers of fibres analysed for V_o per type and per group are the same as those reported in Fig. 5. The numbers of samples studied for V_f per myosin isoform and per group were: 20 (YO), 30 (EL) and 6 (EL-IMM) for myosin 1; 15 (YO), 20 (EL) and 12 (EL-IMM) for myosin 2A; 6 (YO), 8 (EL) for myosin 2X. The height of the bars represents mean values (± S.E.M.). • Significantly different from all the other subject groups, •• significantly different from YO, $P < 0.05$.

Maximum shortening velocity of single muscle fibres and actin sliding velocity on myosin

Figure 6*A* reports the mean values of V_o for the whole population of fibres studied. As expected on the basis of previous observations (Bottinelli & Reggiani, 2000), V_o increased in the order type 1 → 2A → 2X in all subject groups. More relevant is the comparison of corresponding fibre types in the different subject groups. V_o of type 1 and 2A fibres from EL was significantly lower than V_o of type 1 and 2A fibres from both YO and EL-IMM. V_o of type 1 and 2A fibres from EL-IMM was significantly higher than V_o of corresponding fibre types from EL and YO. No statistically significant difference was seen among type 2AX and 2X fibres. Therefore, although a general trend towards a lower V_o in EL and a higher V_o in EL-IMM could be seen for all fibre types, it reached statistical significance only in type 1 and type 2A fibres. To assess the variability in V_o of single fibres between the two EL-IMM subjects, V_o values of corresponding pure fibre types from the two subjects were compared and found to be: 0.43 ± 0.39 (n = 13) and 0.58 ± 0.56 L s^{-1} (n = 15) for type 1, 1.42 ± 0.71 (n = 10) and 1.49 ± 0.79 L s^{-1} (n = 11) for type 2A. Type 2X fibres were not sufficiently numerous to enable comparison between the two subjects. V_o variability was very low and fibres from both subjects had higher V_o values than fibres from YO and EL.

V_o values of type 1–2AX and 1–2–neo fibres were 1.48 ± 0.85 (n = 6) and 0.53 ± 0.51 L s^{-1} (n = 3), respectively. These values are difficult to interpret due to the complex interplay between different isoforms expressed in the same fibre and to the variable content of the different MHC isoforms in such fibres. However, it appears that the presence of MHC-neonatal decreases the velocity of shortening of the fast adult MHC isoforms, and suggests that MHC-neonatal is a slow isoform.

To clarify whether the differences in V_o could be due to the MLC isoform content of the fibres, MLC analysis was performed on 80 type 1 fibres (50 from YO, 15 from EL and 15 from EL-IMM) and on 40 type 2A fibres (20 from YO, 10 from EL and 10 from EL-IMM). All type 1 fibres from all three subject groups contained only slow MLC isoforms (MLC1 s and MLC2s), all type 2A fibres from all subject groups contained only fast MLC isoforms (MLC1F, MLC3f and MLC2f). MLC distribution, therefore, could not account for the lower V_o in EL or the higher V_o in EL-IMM.

The results reported so far suggest that V_o of single muscle fibres containing the same MHC isoform was lower following ageing and higher following immobilization than in controls. Indeed, as V_o was found to be higher in single fibres from EL-IMM than in single fibres from YO, it appears that immobilization more than counteracted the effect of ageing on V_o. To clarify whether the observed changes in V_o reflected changes in properties of myosin itself, pure myosin isoforms were extracted from single fibres and studied using IVMA. Muscle samples were from the three YO, the three EL and the two EL-IMM subjects who underwent surgical biopsy. Surgical biopsies provided larger samples, which enabled additional analyses to be performed.

Figure 6*B* reports the mean values of the velocity of sliding of actin (V_f) on pure myosin isoforms from the three subject groups. V_f significantly increased in the order myosin 1 → 2A → 2X within each subject group, consistent with the order of increase in V_o of single fibres (Fig. 6*A*). V_f on myosin 1 and 2X was significantly lower in EL than in YO. The difference in V_f among 2A myosins from YO and EL did not reach statistical significance. The lower V_f values on specific myosin isoforms in EL than in YO is consistent with the lower V_o of single fibres from EL (Fig. 6*A*). Interestingly, whereas V_o of single fibres was higher in EL-IMM than in YO and EL (Fig. 6*A*), V_f values were lower in EL-IMM than in YO and very similar in EL-IMM and EL.

Myosin concentration in single fibres

The P_o/CSA of single fibres was observed to be lower in EL and even more so in EL-IMM than in YO subjects. To clarify the mechanism underlying this phenomenon, the myosin concentration was determined in a total of 71 single muscle fibres from the three YO, the three EL and the two EL-IMM subjects who underwent to surgical biopsy.

The myosin concentration in type 1 fibres significantly decreased in the order YO (215 ± 84 μM (± S.D.), n = 15) → EL (160 ± 47 μM, n = 15) → EL-IMM (89 ± 36 μM, n = 8). The myosin concentration of type 2A fibres was significantly lower in EL-IMM (101 ± 39 μM, n = 12) than in YO (184 ± 82 μM, n = 11), whereas no statistically significant difference was observed between EL-IMM and EL (147 ± 60 μM, n = 10), and between YO and EL. No statistically significant difference was observed between type 1 and 2A fibres from the same subject group. As a large population of single fibres were studied from each of the subjects whose fibres were used for myosin quantification, it was possible to relate the myosin concentration of type 1 and 2A fibres to the P_o/CSA of the corresponding fibre types of the same subjects. Figure 7 shows that the P_o/CSA values were linearly related to myosin concentration. The slope of the regression was significantly different from zero for both type 1 (0.185 ± 0.006, P = 0.023), and type 2A fibres (0.314 ± 0.041, P = 0.028). The difference between the slopes of type 1 and 2A fibres was statistically significant (P = 0.012). The intercept with the force axis was very close to zero for both regression lines.

DISCUSSION

In the present study, the structure and function of single skeletal muscle fibres from young controls (YO), elderly subjects (EL) and elderly immobilized subjects (EL-IMM) were studied. The main results are as follows.

Myosin concentration was found to be lower in single muscle fibres from EL and especially from EL-IMM

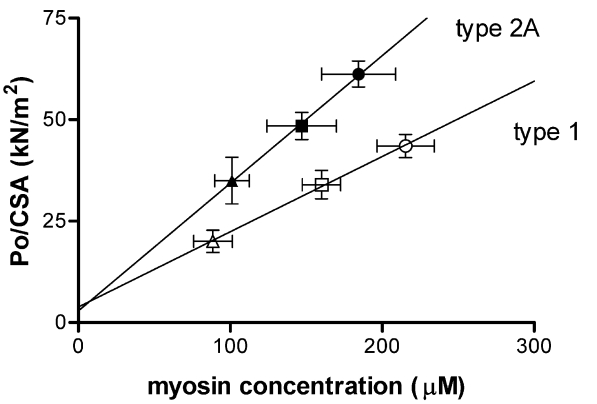

Figure 7. Myosin concentration of type 1 and 2A single muscle fibres of YO, EL, and EL-IMM subjects plotted *versus* mean P_o/CSA values of type 1 and 2A fibres from the same subjects

The numbers of fibres used for determination of myosin concentration per fibre type and per group are reported in the text. The number of fibres plotted for P_o/CSA per type and per group were: 60 (YO), 49 (EL) and 28 (EL-IMM) for type 1; 45 (YO), 28 (EL) and 21 (EL-IMM) for type 2A. Error bars are ± S.E.M. The slope of the regression line for both type 1 (0.185 ± 0.006, P = 0.023) and type 2A fibres (0.314±.041, P = 0.028) was statistically different from zero,(P < 0.05). Open symbols indicate type 1 fibres, filled symbols type 2A fibres. Circles, fibres from YO subjects; squares: fibres from EL subjects; triangles, fibres from EL-IMM subjects.

subjects than in single muscle fibres from YO subjects. A linear relationship between loss of specific force and myosin concentration was found.

Immobilization affected maximum shortening velocity of single muscle fibres, but did not affect the velocity at which myosin propelled actin filaments in IVMA, i.e. an 'uncoupling' occurred between V_o of single fibres and V_f on isolated myosin.

MHC isoform and fibre type distribution

The very clear fast muscle phenotype of EL-IMM subjects, with type 2X representing ~45 % of total MHCs and MHC-1 only ~20 %, and hybrid type 2AX fibres representing 30 % of the entire single fibre population studied, confirms and extends previous observations on vastus lateralis muscle following 3 weeks of knee immobilisation in young subjects (Hortobagyi *et al.* 2000) and on paraplegic subjects (Round *et al.* 1993). Other models of decreased neuromuscular activity in humans (bed rest, spaceflight, hindlimb suspension) have failed to show such a shift towards fast fibre types (Edgerton *et al.* 1995; Berg *et al.* 1997; Fitts *et al.* 2001). Moreover, EL-IMM subjects showed a preferential atrophy of slow muscle fibres (Fig. 5*A*). This effect has previously been observed in small mammals (Baldwin, 1996), but not in humans following spaceflight (Edgerton *et al.* 1995; Fitts *et al.* 2001), bed rest (Berg *et al.* 1997) and immobilization (Hortobagyi *et al.* 2000). It appears that unloading and disuse can determine the 'default' fast muscle phenotype to emerge in humans, as well as in small mammals, if the duration and extent of decreased neuromuscular activity are sufficient.

Although, in elderly subjects, a slower phenotype would be expected on the basis of the preferential denervation of fast motor units (Campbell *et al.* 1973; Lexell, 1995), a slower phenotype, a faster phenotype or no change in phenotype at all have been observed (Klitgaard *et al.* 1990; Aoyagi & Shephard, 1992). Such lack of consistency can be explained by the findings that, with ageing, skeletal muscle can be affected by both disuse and partial denervation of muscle fibres which have opposite effects on muscle phenotype. Disuse would determine a shift towards a faster phenotype (see above), whereas denervation would affect more fast motor units (Campbell *et al.* 1973). Moreover, factors such as the physiological and pathological condition of the subjects and their exercise habits might be relevant. In the population of sedentary elderly subjects enrolled in the present study, disuse might have predominantly affected myosin isoform content, determining the 2A → 2X shift.

The expression of MHC-neonatal and the presence of unusual, albeit infrequent (3–5 %), hybrids (1–2AX and 1–2–neonatal) has not been shown before in aged muscle and following immobilization. Re-expression of MHC-neonatal could be induced by degeneration and regeneration (Williams *et al.* 2001), and/or denervation (Schiaffino *et al.* 1988). Denervation and re-innervation are known to occur in ageing (Campbell *et al.* 1973; Lexell, 1995), and necrosis and regenerating satellite cells have been shown in human skeletal muscle following bed rest (Hikida *et al.* 1989). The observation that, in cross-sections of muscle bundles, fibres containing MHC-neonatal were relatively small (Fig. 2*B*) is consistent with the possibility that they were regenerating fibres.

Size and force of single muscle fibres

In EL and EL-IMM subjects the loss of force of single muscle fibres exceeded the decrease in CSA, i.e. P_o/CSA was lower than in YO. The only fibre types in which this did not happen were type 2AX and 2X fibres from EL subjects. The observation of a lower P_o/CSA with ageing is consistent with the results of two previous works (Larsson *et al.* 1997; Frontera *et al.* 2000). No previous data are available on the function of single muscle fibres following strict immobilization. The results from previous works on the P_o/CSA of single muscle fibres following disuse are not consistent: lower values were obtained in some studies (Larsson *et al.* 1996; Widrick *et al.* 1999), but not in others (Widrick *et al.* 2001). In the present work, P_o/CSA was very clearly lower following immobilization and P_o/CSA variability between the two EL-IMM subjects was low, probably due to the strict immobilization protocol and to the synergistic effects of ageing and immobilization.

The decrease in P_o/CSA could be due to either a lower number of strongly bound acto-myosin interactions during maximal activation or a lower force generated by one such interaction or both phenomena. The correlation between myosin concentration in single fibres and P_o/CSA of single fibres from the same samples (Fig. 7) strongly suggests that a major determinant of the lower P_o/CSA in aged muscle and especially following immobilization is the lower number of acto-myosin interactions.

The absolute values of myosin concentration in single fibres from YO (215 μM in fast, type 2A fibres and 185 μM in slow, type 1 fibres) are consistent with estimates of myosin concentration in psoas muscle fibres of the rabbit (He *et al.* 1997) and with a recent determination of myosin concentration in single fibres of the rabbit (184 μM in psoas and 144 μM in soleus fibres) performed using an approach similar to the one used here (Tikunov *et al.* 2001). No difference in myosin concentration between fast and slow human fibres was observed in the present study, which is at variance with the lower concentration found in slow fibres in rat (Geiger *et al.* 2000) and rabbit (Tikunov *et al.* 2001). As discussed in detail in the Methods, the procedure used to extract myosin from single fibres (Geiger *et al.* 2000) did not guarantee that 100 % of myosin was actually extracted and quantified. However, the very good agreement between the values of myosin

concentration found here and those reported by Tikunov *et al.* (2001), who carefully verified complete extraction, suggests that most, if not all myosin was actually extracted. Moreover, since we were aware of the issue, myosin extraction was performed using exactly the same approach and timing in all fibres to ensure that the results could be used for comparative purposes.

No previous determinations of myosin concentration in single muscle fibres of elderly or immobilized subjects exist, and hardly any information is available on possible changes in the protein content of human muscles following ageing and unloading. However, the lower myosin concentration in single fibres in EL-IMM is consistent with the data of Larsson *et al.* (1996), obtained using a semi-quantitative approach, which suggested that the myosin content of muscle fibres at the end of a 6 week period of bed rest was lower, and with the significant decline in whole body protein synthesis rates shown in humans following more than 3 months of spaceflight (Stein *et al.* 1999) and 2 weeks of bed rest (Ferrando *et al.* 1996). The lower myosin concentration in fibres from EL subjects is consistent with the decrease in the rate of MHC synthesis in elderly humans previously reported (Balagopal *et al.* 1997; Hasten *et al.* 2000), but not with a determination of MHC content of cross-cryosections of muscle bundles (Marx *et al.* 2002).

Besides the decrease in myosin concentration, alternative explanations for the decrease in P_o/CSA with ageing and disuse have been proposed. It has been suggested that in single intact fibres of old mice an uncoupling between the dihydropyridine receptors and the ryanodine receptors could produce submaximal activation and lower specific force (Renganathan *et al.* 1997). However, in skinned fibres that lack plasma membrane and are directly and maximally activated by exposure to solutions containing calcium in saturating concentrations, alterations in excitation–contraction coupling cannot account for the decreaase in P_o/CSA. More relevant is the observation that maximally activated skinned fibres of elderly rats have a reduced fraction of myosin heads in the strong-binding structural state (Lowe *et al.* 2001). The latter finding is not incompatible with a decrease in myosin concentration playing a major role in affecting P_o/CSA. It might well be that both a decrease in myosin concentration and a reduction in the fraction of strongly bound myosin heads affect P_o/CSA in skinned fibres. The decrease in P_o/CSA following spaceflight has been suggested to depend on the decrease in thin filament density (Riley *et al.* 2002). However, in the same population of fibres no statistically significant decrease in P_o/CSA in relation to control fibres has been shown (Widrick *et al.* 2001; Riley *et al.* 2002). Moreover, reduced thin filament density has been shown in single fibres that maintained almost normal force following bed rest (Widrick *et al.* 1997; Riley *et al.* 1998) and spaceflight (Widrick *et al.* 1999; Riley *et al.* 2000). The decrease in thin filament density is therefore unlikely to be the major determinant of a decrease in P_o/CSA.

Maximum shortening velocity and actin sliding velocity

As expected (Bottinelli & Reggiani, 2000), V_o was significantly affected by the MHC isoform content of the fibres, increasing in the order type 1 → 2A → 2X in all subject groups. However, differences between corresponding fibre types from YO, EL and EL-IMM subjects cannot be explained on the basis of the MHC isoform composition. Analysis of the MLC isoform composition of the fibres failed to show a role of MLC isoform content in determining the observed V_o differences, in agreement with previous observations in young adults (Larsson & Moss, 1993) and in muscle fibres following disuse (Widrick *et al.* 1999) and ageing (Larsson *et al.* 1997).

The observations that V_o of type 1 and 2A fibres is lower in EL than in YO and of a general trend towards a lower V_o in type 2AX and type 2X fibres (Fig. 6*A*) strengthen the idea that ageing affects V_o of both slow and fast fibres (Larsson *et al.* 1997), and is at variance with the observation that in elderly men V_o decreases only in type 2A fibres (Krivickas *et al.* 2001). To assess whether the changes in V_o of single fibres were due to a change in the properties of the myosin molecule, pure myosin isoforms were extracted from single fibres and studied in IVMA (Canepari *et al.* 1999; Pellegrino *et al.* 2003). The velocity of sliding of actin on myosin in IVMA, in fact, solely depends on the properties of the myosin molecule, as sarcomere structure is lost and the other myofibrillar proteins are not present (Canepari *et al.* 1999; Hook *et al.* 2001). The lower V_f on myosin 1 and 2X in samples from EL and the trend towards a lower V_f on myosin 2A confirm previous findings on human myosin 1 (Hook *et al.* 2001) and extends them to fast myosins. Ageing appears to alter the function of a given myosin isoform through a direct effect on the myosin molecule, suggesting that myosin can change properties without changing isoform type (Bottinelli, 2001). It has recently been suggested that glycation of myosin might be involved in such phenomenon (Ramamurthy *et al.* 2001).

In this work, for the first time, V_o of single muscle fibres and V_f on isolated myosin were studied in elderly subjects following a long period of immobilization. In agreement with previous observations in young subjects following bed rest (Widrick *et al.* 1997) and spaceflight (Widrick *et al.* 1999; Fitts *et al.* 2001; Widrick *et al.* 2001), V_o of type 1 and 2A fibres was found to be higher in EL-IMM than in EL subjects and a similar trend was also found for type 2X fibres. Indeed, immobilization more than counteracted the effect of ageing on V_o, i.e. V_o of single fibres was higher in EL-IMM than in YO subjects. Interestingly, V_f on myosin 1 and 2A from EL and from EL-IMM subjects, was

similar, suggesting that immobilization did not change the properties of the myosin molecule. As V_f was unaffected by immobilization, the higher V_o of single muscle fibres following immobilization cannot be attributed to a change in myosin properties. The uncoupling between V_o of single fibres and V_f on the myosin from the same fibres is surprising. A strict relationship between the two parameters has been recently observed by analysing a large number of fibre types and myosin isoforms in different species (Pellegrino *et al.* 2003). The uncoupling between V_o and V_f suggests that, following immobilization, a mechanism able to modulate V_o independently of a change in myosin properties operates. Interestingly, a mechanism of this kind, consisting of an increase in the distance between thin and thick filaments due to a disproportionate loss of thin filaments, has recently been suggested to account for the higher V_o of single fibres following bed rest and spaceflight in young subjects (Widrick *et al.* 1999; Riley *et al.* 2002).

Conclusions

The present data suggest that with ageing and following immobilization the decrease in muscle power, which shows as muscle weakness, might depend not only on a quantitative mechanism (loss of mass), but also on qualitative mechanisms, i.e. loss of the intrinsic capacity of muscle fibres to develop force (Fig. 5*B*) and, in aged muscle, a slowing of shortening velocity (Fig. 6*A*).

A major determinant of the decrease in P_o/CSA of skinned muscle fibres following immobilization is probably a decrease in myosin concentration (Fig. 7). With ageing, a decrease in the percentage of strongly bound acto-myosin interactions (Lowe *et al.* 2001), and, *in vivo*, alterations of excitation–contraction coupling (Renganathan *et al.* 1997) can contribute to muscle weakness as well.

Immobilization and ageing have synergistic effects on P_o/CSA and myosin concentration in single fibres. Immobilization has specific effects on V_o of single fibres and probably on MHC isoform expression, thus modifying the structure and function of ageing skeletal muscles.

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