

Disuse Induced-Atrophy and Contractile Impairment of Human Skeletal Muscle Fibres

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Abstract

In this study we described the effect of four months of disuse and immobilization on Myosin Heavy Chain (MHC) isoform composition and contractile properties of single fibres in human muscles. We compared muscle samples collected from the vastus lateralis of three groups of two subjects: 1) subjects (60-70 y old) who underwent to the replacement of a total knee prosthesis after four months with the leg immobilized in extended position, 2) control adult subjects (30-40 y old) sampled by needle biopsy and 3) control aged subjects (60-70 y old) who underwent to knee surgery for various reasons. We found in immobilized muscles an increase in MHC-2X, a decrease of slow MHC and the expression of a developmental MHC isoform, indication of fibre regeneration. The proportion of hybrid fibres, i.e. fibres containing two or more MHC isoforms, was increased. Slow fibres showed significant reductions in cross sectional area and in specific tension (force/cross sectional area) developed during maximal isometric contractions. Unloaded shortening velocity was, however, not modified. No alteration was detectable in fast 2A fibres when compared with corresponding fibres of adult and old controls. Slow fibres seemed, therefore, selectively affected by the combination of immobilization and disuse examined in this study.

Key words: disuse, immobilization, muscle atrophy, myosin isoforms.

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A period of time without weight bearing can deeply modify the structure and the function of skeletal muscles. Animal studies using the hind limb suspension model or several models of immobilization show that removal of mechanical load produces atrophy and contractile alterations, more evident in the slow muscle soleus than in fast muscles as extensor digitorum longus [7, 14, 19]. The different response can be explained taking into account the greater anti-gravitary activity of the soleus. In the last four years two experimental models of muscle unloading, bed rest [1, 11, 21] and space flight [20], have been studied in details in adult healthy human beings. The results obtained have shown that muscle disuse bring not only to the decrease of fibre size, but also to the decrease of specific tension (force per cross sectional area) development. An increase in unloaded shortening velocity of slow fibres has been also found in some studies [20, 21].

Beside space flight and bed rest experiments, long periods of muscle disuse in relation to disease or traumatic

injuries of the joints or of the bones are a relatively common experience for human beings. A detailed knowledge of the structural and functional alterations which occur in muscle fibres when mechanical load is chronically removed may be of help to plan preventive countermeasures or to define rehabilitative protocols. It must be also taken into account that the majority of the people who are forcibly immobilized due to joint or bone lesions are elderly. For those people the interruption of the weight bearing function of the muscles superimposes on conditions where skeletal muscles are more prone to functional and structural alterations, due to reduced physical activity, loss of motor neurons and decline of hormonal levels.

On this ground it seems of interest to study the functional and structural alterations of muscle fibres after a long period of disuse and lack of mechanical load in elderly people. In this study we analyzed muscle fibres of vastus lateralis collected during the surgical interventions for total knee arthroplasty. The patients who

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needed replacement of the prosthesis were immobilized in extended leg position for about four months before the intervention. The muscle fibres from these patients were compared with fibres obtained from bioptic samples of vastus lateralis of healthy adult volunteers and with fibres obtained from the same muscle of patients who underwent knee surgery keeping their locomotory ability. The results showed that slow fibres were selectively affected by atrophy and contractile impairment.

Methods

Muscle biopsy and fibre preparation

Muscle fibres examined in this study were obtained from the vastus lateralis (distal third) of six subjects: 1) two young healthy controls (both males, age 30-40 years) who joined as volunteers to this study, 2) two old subjects (one male and one female, 60-70 years) who underwent knee surgery for various reasons which did not abolish their walking ability and 3) two old subjects (females 60-70 years) who underwent the replacement of total knee prosthesis after a period of about 4 months of complete knee immobilization with the leg in extended position.

The study was approved by the ethical committee of the Department of Anatomy and Physiology, University of Padova. Muscle sampling was carried out by dr. Carmine Carlizzi Naccari at the Hospital Casa di Cura Columbus, Milano.

A portion of each bioptic sample was quickly immersed in ice cold skinning solution (see below). This portion was then divided in several small bundles (about 100 fibre segments each) which were pinned on the Sylgard covered bottom of a Petri dish filled with 1:1 mixture of skinning solution and glycerol. The samples could be stored at -20°C for several days but in no case more than three weeks.

On the day of the experiment a bundle was transferred to a dish containing skinning solution at about 10°C and, under a stereo-microscope (10-60 x magnification), single fibres were manually dissected. At the end of the dissection the skinning solution was replaced with a new one containing 1% Triton X-100 to ensure membrane solubilization. After 1 hour the dish was refilled with a solution without Triton. Then, segments of about 2 mm length were cut from the fibres, light aluminum clips were applied at both ends of the segments and the segments were transferred to the experimental set up.

Solutions

Skinning, relaxing, pre-activating and activating solutions were prepared according to Bottinelli et al. [3].

Experimental setup

The experimental setup has been described in detail in previous papers [2, 3]. Briefly, fibre segments were mounted between the hook of a force transducer (AE

801, Akssjeselkapet Mikroelektronikk, Horten, Norway) and the shaft of the electromagnetic puller (model 101 vibrator, Ling, Royston, UK). The force transducer, the puller and the aluminum plate in which seven chambers were milled, were placed on the stage of an inverted microscope (Axiovert 10, Zeiss, Germany). One chamber was large (volume 0.5 ml), contained relaxing solution and was used for fibre mounting. The other chambers were smaller (70 µl) and contained pre-activating and activating solutions. Two mm above the small chambers a long cover slip was suspended by means of a movable arm. Drops of solution (70 µl) were kept in the gap between the bottom of the chambers and the cover slip. The fibre segments could be moved from one drop to the next one just by sliding between the aluminum plate and the cover glass. The electromagnetic puller was driven by a feed back circuit which allowed either to keep constant the length of the fibres or to produce fast length changes of predetermined amplitude. Temperature was kept constant by circulating a mixture of water and ethylene glycol in channels milled in the aluminum plate. All experiments were carried out at 12°C.

A stereomicroscope was fitted above the chambers to view (magnification 10-60x) the fibres during mounting and during activation. As the floor of the chambers was made by cover slips, the fibres could be seen at 320x through the eyepieces of the inverted microscope. Moreover a videocamera fitted to the camera tube of the microscope and connected with a computer allowed to view the specimen at 1000x on a TV monitor and to stored digitized images of the fibres during the experiments.

The signals from the force and displacement transducers were visualized on the screen of a storage oscilloscope (Mod 5113, Tektronix, Beaverton, Oregon, USA) and on a chart recorder (WR3701, Graphtek, Japan). The signals after A/D conversion (interface CED 1401plus, Cambridge, UK) were fed into a personal computer and stored in the hard disk. The software Spike2 (CED, Cambridge, UK) was used for data storage, recall and analysis.

Experimental procedure

The fibre segment was mounted between the force transducer and the puller in the large chamber containing relaxing solution. While the segment was in this chamber, sarcomere length (at 320 x), diameters (at 320x) and segment length (at 60 x) were measured. From at least three diameters cross sectional area was calculated, assuming a circular shape. Sarcomere length was adjusted to 2.5 µm.

The fibre was then transferred to the pre-activating and then to the activating solution. The slack test procedure [6] was used to measure unloaded shortening velocity (V_0). Briefly, the fibre was immersed in activating solution (pCa = 4.6) and, once tension was fully de-

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veloped, it was quickly shortened so that it remained slack for several ms before redeveloping force at the final length. The fibre was then returned to the pre-activating solution and relaxation occurred. The fibre was then re-lengthened to initial length. In each fibre this sequence of operations was repeated 4-6 times and, during subsequent activations, shortenings between 5% and 15% of segment length were applied. The amplitude of the length change (ΔL) applied was plotted against the time (Δt) required to take up the slack. V_0 was obtained as the slope of the regression line of ΔL versus Δt . The intercept of the regression line was the series compliance. Maximal isometric tension (P_0) was measured just before the first shortening manoeuvre.

The appearance of irregularity in the striation pattern which was continuously controlled on the TV monitor or a decrease of tension development by more than 10% between the first and the last maximal activation were considered reasons to discard a fibre.

Fibre typing and myosin isoform identification

Fibre typing was based on the use of myosin heavy chain (MHC) isoforms as molecular markers. At the end of the mechanical experiment each fibre segment was immersed in a small test tube containing 20 μ l of Laemmli solution and stored at -20°C . MHC isoform composition of each segment was later on determined by polyacrylamide gel electrophoresis. Fragments of the bioptic samples were also solubilized in Laemmli solution and analyzed by gel electrophoresis. The electrophoretic method used was derived from that of Danieli-Betto, Zerbato and Betto [4] and was described in detail by Stienen et al [18]. Three major bands in the region of MHC isoforms corresponding to MHC-1, MHC-2A and MHC-2X (previously identified as MHC-2B [17]) were separated. A fourth band corresponding to a MHC migrating with velocity greater than MHC-2A was detected in some cases (see Figure 1). Immunoblotting with monoclonal antibodies BA-F8 and SC-71 specific to MHC-1 and, respectively, to MHC-2A was in some case performed to confirm isoform identification. The antibodies were kindly donated by Prof. S. Schiaffino (Padova, Italy). Their preparation and characterization has been described in previous papers [15]. The fourth band was identified as a developmental isoform on the basis of the migration velocity of embryonic and neonatal isoforms in rat [13, 16]. In relation to the presence of one or two bands in the MHC region fibres were classified in one of the following groups: 1, 1-2A, 2A, 2A-2X, 2X, 1-2A-2X and regenerating. The distribution of the four isoforms identified in the bioptic samples was quantified by computerized gel electrophoresis.

Statistical analysis

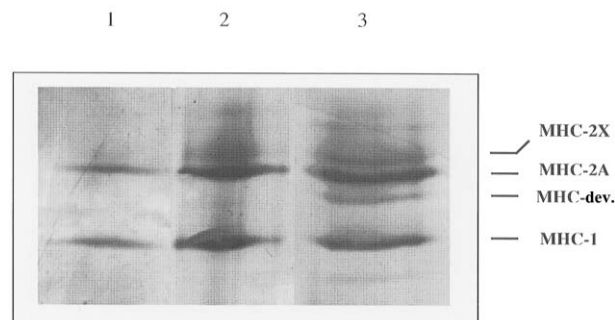


Figure 1. Examples of electrophoretic separation of MHC isoforms in the biopsy samples of vastus lateralis muscle. Lane 1: adult control subject. Lane 2: old control subject. Lane 3: immobilized atrophic muscle. MHC-dev denotes a developmental isoform of MHC.

Data were expressed as means and standard errors. Statistical significance of the differences between means was assessed by variance analysis followed by Student-Newman-Keuls test. A probability of less than 5% was considered significant.

Results

The distribution of MHC isoforms in the biopsy samples of vastus lateralis showed large variations between the three groups of subjects. As can be seen in Figure 1 and 2, in the vastus lateralis of the two adult healthy subjects MHC slow (i.e. MHC-1) and MHC-2A were expressed in approximately equal parts. MHC-2X was virtually not expressed. In the vastus lateralis of control old subjects the distribution was different as slow MHC increased and a significant component of MHC-2X appeared. In the atrophic muscles the proportion of slow MHC was reduced and that of MHC-2X increased. Furthermore a fourth MHC isoforms, likely a developmental isoform, appeared.

Single fibres were dissected from the biopsy samples and characterized as regard to their cross sectional area, isometric tension during maximal activation and unloaded shortening velocity. Fibres were then identified and grouped on the basis of their MHC isoform composition. The distribution of the 171 fibres dissected from the biopsy samples and characterized as regard to their MHC isoform composition is reported in Table 1. The percentage of various fibre groups generally corresponded to the MHC isoform percent distribution shown in Figure 2. It is worth, however, to underline that the number of hybrid fibres, i.e. fibres containing two or more MHC isoforms was higher in old control subjects than in adult controls and became even higher in immobilized atrophic muscles.

The functional comparison between fibres was restricted to the two more abundant groups: the slow fibres containing MHC-1 and the fast 2A fibres. The average values of the parameters measured during me-

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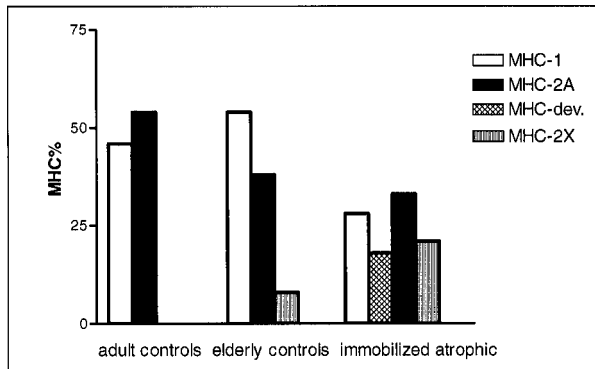


Figure 2. Distribution of MHC isoforms in the biopsy samples of vastus lateralis muscle of the three groups of subjects. Each column is the average of two values, each obtained in one subject as the average of at least three densitometric measurements. MHC-dev. denotes a developmental isoform of MHC.

chanical experiments are reported in Figure 3. As can be seen, cross sectional area was significantly reduced in old control subjects compared to adult controls for both slow and fast 2A fibres. Atrophic muscles showed a significant size reduction only for slow fibres and not for fast 2A fibres. Maximal isometric tension was not reduced in old controls compared to adult controls for both slow and fast 2A fibres. A significant reduction was, on the other hand, detected in slow fibres of atrophic muscle. Finally, unloaded shortening velocity did not show any significant reduction in old controls or in atrophic immobilized subjects when compared to adult controls. In all three groups unloaded shortening velocity was three-four times higher in fast 2A fibres than in slow fibres.

Discussion

Table 1. Fibre type composition of the samples of vastus lateralis from the three groups of subjects. Fibre groups were formed by classifying fibres on the basis of their MHC isoform composition. The number of fibres dissected for each group is indicated in brackets. Dev. indicates the presence of a developmental myosin isoform.

fibre type	adult controls (n = 57)	elderly controls (n = 75)	immobilized atrophic (n = 39)
slow-1	70%	44%	34%
1-2A	5%	24%	17%
2A	24%	22%	31%
2A-2X	0	9%	7%
2X	1%	0%	0%
1-2A-2X	0	1%	3%
1-2A-2X-dev.	0	0%	3%
1-2A-dev.	0	0%	5%

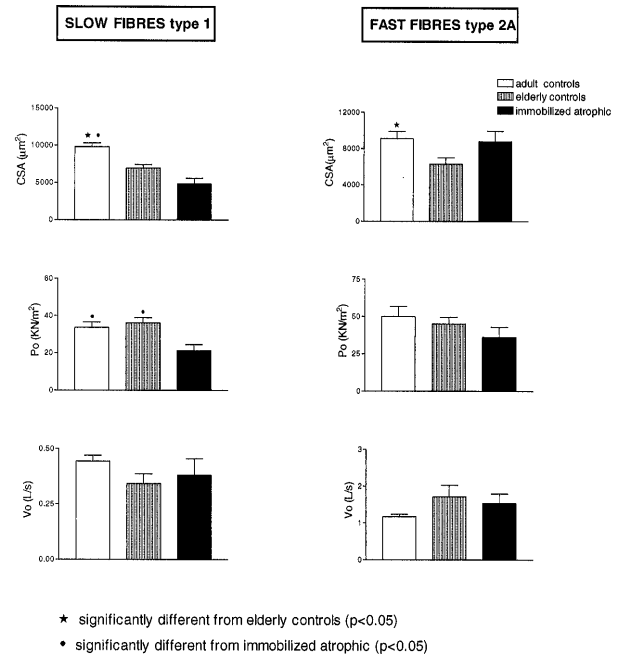


Figure 3. Comparison of slow and fast (2A) fibres from the three groups of subjects. Each column represents mean and standard error of values obtained in single fibres. The following number of fibres were studied: adult controls: 40 slow fibres and 14 fast 2A fibres; old controls: 33 slow fibres and 17 fast 2A fibres; immobilized atrophic subjects: 13 slow fibres and 12 fast 2A fibres.

The purpose of this study was to investigate the effect of chronic unloading and immobilization on myosin isoform composition and contractile properties of single fibres of human muscle. Muscle samples were collected from vastus lateralis during knee surgery in two elderly patients who needed replacement of a total knee prosthesis and had their leg immobilized in extended position for about four months. The controls were represented by two adult healthy men who underwent needle biopsy and by two old people who underwent surgery of the knee for various reasons which did not impair their walking ability.

Slow and fast 2A fibres were dominant in the vastus muscle of the two adult controls. MHC-2X was barely detectable at the electrophoresis and very few fibres containing this isoform were found. The picture is consistent with our previous observations on vastus lateralis of healthy young adults [8]. An increase in slow MHC expression and in slow fibres and an increase in MHC-2X was observed in old controls, in agreement with previous studies [9]. The comparison between adult and old controls also revealed that the proportion of hybrid fibres increased with aging, as previously reported [10]. Cross sectional area showed a significant decrease. This aging related atrophy was also in agreement with previ-

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ous reports [9, 12]. No changes in the contractile parameters (isometric tension and unloaded shortening velocity) were found. This is in contrast with the finding of Larsson and coworkers [12] who showed a decrease in both parameters with aging of human muscle fibres.

Immobilization and unloading were associated with a further decrease of slow MHC expression and increase in fast MHC-2X. These changes indicate a slow to fast transition similar to that observed in unloaded muscle of rats [19] as well as of human beings (bed rest and space flight [5]). Interestingly in both patients a fourth MHC isoform, which might be identified as developmental on the basis of its migration velocity [13] appeared. The presence of a developmental isoform is suggestive of fibre regenerative processes, which in turn might imply that in immobilized and unloaded muscles necrosis or apoptosis occur.

Only slow fibres and not fast fibres showed a reduction of cross sectional area. The same slow fibres also showed a reduction in the ability to develop tension during maximal isometric activation in agreement with the finding of lower tension development by atrophic fibres after bed rest or space flight [11, 20, 21]. At variance of the results of Larsson and coworkers [11] who observed also a decrease in unloaded shortening velocity (V_0) and Widrick and coworkers [20, 21] who reported an increase in V_0 , no change in V_0 was observed in slow fibres after immobilization and unloading.

In conclusion the present study provide a demonstration that in elderly people chronic unloading and immobilization of a muscle lead to a slow to fast transition in myosin expression and to a selective impairment of slow fibres characterized by a reduction in size and a decrease in tension development. This response reminds the changes produced in the animal model of hindlimb suspension and suggests that different models of muscle unloading might produce distinct structural and functional alterations.

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References

- [1] Andersen JL, Gruschy-Knudsen T, Sandri C, Larsson L, Schiaffino S: Bed rest increases the amount of mismatched fibers in human skeletal muscle. *J Appl Physiol* 1999; 86: 455-60.
- [2] Bottinelli R, Betto R, Schiaffino S, Reggiani C: Unloaded shortening velocity and myosin heavy chain and alkali light chain isoform composition in rat skeletal muscle fibres. *J Physiol* 1994; 478: 341-349.
- [3] Bottinelli R, Canepari M, Pellegrino MA, Reggiani C: Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. *J Physiol* 1996; 495: 573-586.
- [4] Danieli-Betto D, Zerbato E, Betto R: Type 1, 2A and 2B myosin heavy chain electrophoretic analysis of rat muscle fibers. *Biochem Biophys Res Commun* 1986; 138: 981-987.
- [5] Edgerton VR, Zhou M-Y, Ohira Y, Klitgaard H, Jiang B, Bell G, Harris B, Saltin B, Gollnick PD, Roy RR, Day MK, Greenisen M: Human fiber size and enzymatic properties after 5 and 11 days of space flight. *J Appl Physiol* 1995; 78: 1733-1739.
- [6] Edman KAP: The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. *J Physiol* 1979; 291: 143-159.
- [7] Gardetto PR, Schluter JM, Fitts RH: Contractile function of single muscle fibers after hindlimb suspension. *J Appl Physiol* 1989; 66: 2739-2749.
- [8] Harridge SDR, Bottinelli R, Canepari M, Pellegrino MA, Reggiani C, Esbjornsson M, Saltin B: Whole muscle and single fibre contractile properties and myosin heavy chain isoforms in humans. *Pflugers Arch* 1996; 432: 913-920.
- [9] Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnor P, Saltin B: Function, morphology and protein expression of ageing skeletal muscle: a cross sectional study of elderly men with different training background. *Acta Physiol Scand* 1990a; 140: 41-54.
- [10] Klitgaard H, Zhou M, Schiaffino S, Betto R, Salviati G, Saltin B: Ageing alters the myosin heavy chain composition of single fibres from human skeletal muscle. *Acta Physiol Scand* 1990b; 140: 55-62.
- [11] Larsson L, Li X, Berg HE, Frontera WR: Effects of removal of weight-bearing function on contractility and myosin isoform composition in single human skeletal muscle cells. *Pflugers Arch* 1996; 432: 320-8.
- [12] Larsson L, Li X, Frontera WR: Effects of aging on shortening velocity and myosin isoform composition in single human skeletal muscle cells. *Am J Physiol* 1997; 272: C638-49.
- [13] Pette D, Staron RS: Cellular and molecular diversity of mammalian skeletal muscle fibers. *Rev Physiol Biochem Pharmacol* 1990; 116: 1-76.

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- [14]Reiser PJ, Kasper CE, Moss RL: Myosin subunits and contractile properties of single fibers from hypokinetic rat muscles. *J Appl Physiol* 1987; 63: 2293-2300.
- [15]Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lomo T: Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Motil* 1989; 10: 197-205.
- [16]Schiaffino S, Reggiani C: Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 1996; 76: 371-423.
- [17]Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L, Schiaffino S: Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscles. *Am J Physiol* 1994; 267: C1723-C1728.
- [18]Stienen GJM, Kiers J, Bottinelli R, Reggiani C: Myofibrillar ATPase activity in skinned human skeletal muscle fibres: fibre type and temperature dependence. *J Physiol* 1996; 493: 299-307.
- [19]Thomason DB, Booth FW: Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* 1990; 68: 1-12.
- [20]Widrick JJ, Knuth ST, Norenberg KM, Romatowski JG, Bain JL, Riley DA, Karhanek M, Trappe SW, Trappe TA, Costill DL, Fitts RH: Effect of a 17 day spaceflight on contractile properties of human soleus muscle fibres. *J Physiol (Lond)* 1999; 516: 915-30.
- [21]Widrick JJ, Romatowski JG, Bain JL, Trappe SW, Trappe TA, Thompson JL, Costill DL, Riley DA, Fitts RH: Effect of 17 days of bed rest on peak isometric force and unloaded shortening velocity of human soleus fibers. *Am J Physiol* 1997; 273: C1690-1699.