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Awede, B ; Berquin, A ; Wuytack, F ; Lebacq, J

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ORIGINAL ARTICLE

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Adaptation of mouse skeletal muscle to a novel functional overload test: changes in myosin heavy chains and SERCA and physiological consequences

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Abstract We have used a new approach to study the effects of overload on skeletal muscle phenotype in mice. The method used avoids any traumatising contact with muscles and the inflammatory reaction that this may provoke. Blocks of lead embedded in silicone were inserted under the skin of the lower part of the back. After 1 month, a 17% hypertrophy was found to have occurred in the tonic soleus muscle, but no change was observed in the fast-twitch extensor digitorum longus (EDL) muscle. The main effects on the contractile properties of the soleus muscle were a decrease in the tetanic relaxation rate and a reduction in the maximal velocity of shortening. Immunohistological analysis of the soleus muscles revealed an increase in the proportion of fibres that express myosin heavy chain (MHC) 1, from 54.2% to 73.9%, with a reduction in the proportion of MHC2a-positive fibres, from 45.8% to 30.2%. These changes were accompanied by an increase in the proportion of fibres that express the slow type of sarcoplasmic reticulum calcium pump (SERCA2a), from 61.8% to 84.7%. In EDL muscles, overload induced only minor changes. Thus, this method of overload affected the soleus muscle in particular. The observed changes in the control of muscle contraction were significantly larger than the changes in typical myofibrillar properties that were observed. These results indicate that there is a temporal dissociation between the relative expression of MHCs and SERCAs.

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Key words Skeletal muscle · Overload · Myosin heavy chain · SERCA · Relaxation time

Introduction

It is well established that the phenotype of adult skeletal muscle is not permanent, but can be modulated by external stimuli as follows: (1) a changing pattern of nerve stimulation (Bárány and Close 1971; Pette and Vrbová 1992) can induce both transformations from slow to fast muscle type and from fast to slow muscle type, (2) hormones (thyroid hormone, androgens and beta-agonists) promote changes towards the fast type in slow muscle (d'Albis et al. 1993; Zhang et al. 1996; Xiaopeng and Larsson 1997), and (3) stretching has also been shown to have an effect (Timson 1990; Golspink et al. 1991).

Physiological changes in fibre phenotype are a response to functional demand: when overall activity changes, structural or biochemical alterations occur, resulting in an adaptation of the muscle properties to the new conditions. Reducing the load results in an increased expression of the fast muscle phenotype, while overloading induces expression of the slow muscle phenotype.

Overload has been induced experimentally by several methods:

1. Denervation of synergistic muscles. In this case, reinnervation can occur after a few weeks.
2. Tenotomy. This consists of severing the tendon of a synergistic muscle (James 1976; Timson 1990). In this case, the resected tendon either re-attaches or adheres to the remaining musculature after 10–14 days, and the re-attached muscle becomes functional again, abolishing the overload (Timson 1990).
3. Ablation of a synergistic muscle has also been used to induce overload. With this method, the overload is stable and can last for 4–6 weeks or more (Baldwin et al. 1982; Timson et al. 1985; Frishknecht and Vrbová 1991; Johnson and Klueber 1991; Frishknecht et al. 1995; Yamaguchi et al. 1996).

These models, especially ablation of a synergistic muscle, result in significant changes in the mass and biochemical constitution of muscle. The removal of synergistic muscles can not be considered as physiological and does not reflect the environmental conditions of the animal. More importantly, the intervention acts locally on, or close to, the muscle of interest and often provokes an inflammatory process. This could interfere with the early paracrine and autocrine signals that are responsible for phenotype modulation, by inducing the secretion of growth factors (epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor). Surgical techniques also remove or modify the sensory innervation of the muscle; this could affect significantly the neural control of muscle development by removing the feedback loop. Therefore, the development of a physiological approach that could redress the insufficiencies of existing methods is required and, if successful, it could constitute a tool for studying the cellular mechanisms of phenotype modulation. In this study, an original approach utilizing overload is presented. The method allows all muscles to remain intact and avoids inflammation due to factors other than increased muscle activity.

The most commonly used marker of myofobrillar phenotype is myosin heavy chain (MHC): it has been shown that overload induces changes towards the slow type (MHC1; Baldwin et al. 1982; Timson et al. 1985; Johnson and Klueber 1991; Yamaguchi et al. 1997). The marker of the control system of muscle contraction is the sarcoplasmic reticulum calcium pump (SERCA): overload induces an increased expression of the slow type (SERCA2a; Kandarian et al. 1994). In single muscle fibres, it has been demonstrated that changes in MHC paralleled those of SERCA (Talmadge et al. 1996). In the work presented here, the changes induced by overload were studied, both in MHC and in SERCA. The results are in agreement with those of previous studies, but suggest a possible temporal dissociation in the changes of MHC and SERCA expression.

Methods

Animals and the overloading protocol

Male NMRI mice (50–60 days of age at the beginning of the protocol) were used. The mice were housed in cages, and were maintained in a light-controlled environment (12:12-h light-dark cycle) and had unlimited access to food and water. The mice were randomly assigned to two groups ($n = 12$ mice/group): control and overloaded. All procedures were performed after the animals had been anaesthetised by subcutaneous injection of a mixture of ketamine hydrochloride 50 mg/ml (Ketalar) and xylazin hydrochloride 2% (Rompun) diluted in water (2 ml Ketalar + 0.5 ml Rompun + 7.5 ml water) at a dose of 0.1 ml/10 g body mass. The mice were kept on a warming bed in order to avoid hypothermia, which can be induced by anaesthesia.

Overload was induced by inserting blocks of lead embedded in silicone underneath the skin of the lower part of the back. First, a short medial incision was made in the skin. After cutaneous detachment, two blocks of lead were inserted, one on each side. The mean size of each block of lead was 18 mm × 9 mm × 6 mm. The mass of the two blocks of lead was about one-third of the body mass of the animal. The incision was stitched with resorbable thread (Vicryl rapide 4/0. Ethicon, France). Total overload was induced in three steps (intervals: 1 week), a set of two lead blocks being added at each step in order to obtain a total overload mass equivalent to the value of body mass at the time of starting the procedure.

Mechanical measurements

After 4 weeks of full overload, the extensor digitorum longus (EDL) and soleus muscles were dissected under anaesthesia for analysis of mechanical performance. After dissection, the mice were killed by cervical dislocation. One muscle of each type was used for studying isometric tetani, the other one for assessing the force/velocity relationship. In a preliminary study performed on a group of 4 mice, both muscles from the two sides were used to assess isometric performance, so that the total number of observations exceeded 12.

Isometric data

The muscles were incubated in a chamber containing Krebs solution (NaCl, 118 mM; $NaHCO_3$, 25 mM; KCl, 5 mM; $MgSO_4$, 1 mM; KH_2PO_4, 1 mM; Glucose, 5 mM; $CaCl_2$, 2.5 mM) that was continously gassed with a mixture of 95% O_2 and 5% CO_2, and maintained at 20°C by water circulation. The muscles were mounted vertically on a force transducer (strain gauge) and stimulated with capacitor discharges of alternating polarity. The force signals were digitised by an Analog Device RTI-800 analogue-to-digital converter under the control of a home-made computer program, and stored on hard disk for analysis. After careful determination of optimal length (l_o, the length at which maximal tension was obtained during a brief isometric tetanus), the twitch, tetanus, force/frequency relationship and fatigue-resistance tests were performed. The adjustment to l_o is important, since relaxation kinetics may vary quite extensively with length.

Twitch parameters

The parameters studied were the rate of rise in tension (measured using the maximal slope of the early part of the tension rise), time-to-peak tension, maximum tension and half-relaxation time (1/2RT), which is the length of time necessary to decrease the twitch tension by 50% from its peak.

Isometric tetani

The soleus muscles were stimulated for a period of 700 ms at 50 Hz, and the EDL muscles were stimulated for 500 ms at 100 Hz, with 3 min of rest between stimulations. The parameters studied were: rate of rise in tension, maximum tension, and relaxation times: $t_{5\%}$, $t_{20\%}$, $t_{50\%}$ (the length of time necessary to obtain a 5%, 20%, 50% decrease in tension, respectively, after the last stimulus of the tetanus).

Force/frequency relationship

The muscles were stimulated for a period of 500 ms at different frequencies, with 2 min rest between tetani; maximum tension was measured.

Fatigue test

Intense activity was induced by a protocol of stimulation adapted from that of Lännergren (Lännergren and Westerblad 1991) and described previously (Deconinck et al. 1998).

Force/velocity relationship

The muscles were mounted horizontally in a thermostated bath (temperature 20°C) containing Krebs solution gassed with 95% O_2-5% CO_2, and attached to an isometric force transducer at one end and an electromagnetic puller at the other end. After determination of l_o, 880-ms tetani at 100 Hz (soleus) or 300 ms at 125 Hz (EDL) were performed; after an initial isometric period of 700 ms (soleus) or 200 ms (EDL), the muscles were allowed to shorten by 1.2 mm (about 10% of l_o) at different constant velocities. Force and length signals were sampled at a rate of 1.8 kHz, digitized through an analog-to-digital conversion card (RTI-815) and stored on floppy disk for later analysis. The data were analysed using an interacting home-made program. Velocities were calculated from the length signals.

At the end of each experiment, muscle length was measured. The muscle bellies were separated from the tendons, gently blotted on filter paper and then weighed. The muscles were then frozen at length l_o in melting isopentane cooled to its freezing point in liquid nitrogen; they were then stored at −80°C.

Immunohistology

Serial 10-μm-thick transverse sections were made from the mid-belly region of the muscles, using a cryostat (Reichert-Jung Cryocut E), and mounted on microscope slide (Superfrost). Immunohistochemical analysis of MHC and SERCA2 was carried out. The anti-MHC antibodies (BAD-5 and SC-71) were obtained by culturing hybridoma cell lines purchased from Deutsche Sammulung von Mikroorganismen und Zellkulturen (DSMZ).

Staining for MHCs was performed according to Gorza (1990): sections were first incubated with one of the two monoclonal antibodies (BAD-5 or SC-71, which react with MHC1 and MHC2a, respectively) at 37°C for 30 min, and then with antimouse IgG-conjugated alkaline phosphatase at room temperature for 1 h. Antibodies were visualised with the aid of 4-nitroblue tetrazolium chloride and 5-bromo4-chloro3-indolyl phosphate. No non-specific labelling was observed. The specificity of the antibodies was verified by immunoblot assay.

SERCA2a immunohistology was performed using the immunoperoxidase technique. After preincubation in H_2O_2, the sections were incubated with an anti-SERCA2a antibody (R15, 1/400 dilution) at 37°C for 1 h, and subsequently with rabbit immunoglobulin and rabbit peroxidase anti peroxidase at room temperature. The antibody labelling was visualised with the aid of diaminobenzidine. Immunostaining for SERCA1 was also performed, but the resulting fibre labelling was not sufficient to enable reliable counting.

The stained sections were photographed on an emulsion for slides (Fujichrome Velvia) using a Zeiss Axiophot microscope fitted with a ×4 objective. This magnification was chosen to allow the capture of pictures of whole sections. The slides were projected onto a horizontal screen (1 m × 66 cm) and the total number of fibres in a cross-section and the number of fibres expressing each type of MHC (1 or 2a) and expressing SERCA2a were counted. The proportions of the different cell types are expressed as percentages of the total number of fibres in the sections.

Statistical analyses

Results are expressed as the mean (SEM). Differences between control and overloaded groups were compared using Student's *t*-test, and the level of statistical significance was set at $P < 0.05$.

Results

Effect of overload on muscle size

Overloading induced a significant increase in the mass of the soleus muscles [15.61 (0.46) mg for the overloaded soleus versus 13.3 (0.58) mg for the control]. The length of the soleus muscles [overload: 11.47 (0.20) mm; control: 11.31 (0.17) mm], and the length [overload: 12.5 (0.26) mm; control: 12.31 (0.19) mm] and mass [overload: 14.38 (0.47) mg; control: 14.24 (0.46) mg] of the EDL muscles were not modified by this treatment.

Effects of overload on isometric contraction

Twitches

The results are given in Table 1. Overload resulted in changes in all twitch parameters except for 1/2RT and total force in the soleus. However, the reduction in the rate of rise in tension for the soleus muscle was the single significant modification ($P < 0.05$). The twitch-to-tetanus ratio was reduced by 16% in both the soleus and EDL muscles.

Tetani

Examples of records of isometric tetani are given in Fig. 1. The most prominent feature of the figure is the difference between the two soleus muscles (control and overloaded) during relaxation: the overloaded muscle relaxed more slowly than the control muscle. The mean tetanus parameters are summarized in Table 2. Overloading induced significant changes only in the soleus muscle. Tetanic force was higher in overloaded than in control muscles. However, tension (force per unit cross-sectional area) was identical in both groups. Thus, in the overloaded soleus, total force was increased in proportion to the muscle mass.

Table 1 Twitch parameters in overloaded (*OL*) and control (*CTR*) soleus and extensor digitorum longus (*EDL*) muscles. Values are means (SEM). (*TTP* Time to peak, *1/2RT* half relaxation time)

Group	*n*	Tension ($kN \cdot m^{-2}$)	Force (mN)	Twitch to tetanus force ratio	Rate of rise in tension ($kN \cdot m^{-2} \cdot s^{-1}$)	TTP (ms)	1/2RT (ms)
Soleus OL	14	24.97 (2.69)	33.31 (3.52)	0.148	*0.72 (0.07)	76.97 (3.14)	94.93 (6.7)
Soleus CTR	16	29.49 (2.77)	33.93 (3.29)	0.176	0.95 (0.11)	70.18 (3.61)	94.94 (8.2)
EDL OL	15	63.50 (7.22)	72.94 (7.10)	0.264	4.28 (0.48)	32.41 (1.00)	40.89 (2.9)
EDL CTR	14	83.34 (12.8)	93.40 (11.26)	0.314	5.94 (0.98)	30.22 (0.95)	36.05 (2.9)

* Significantly different from the CTR condition ($P < 0.05$)

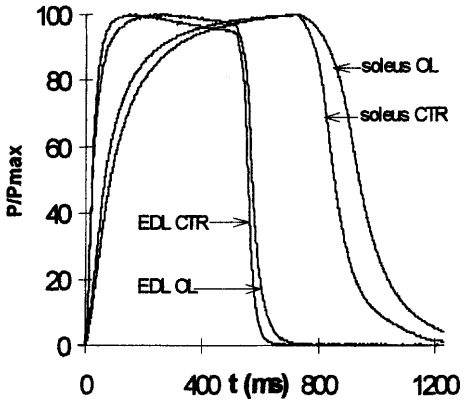

Fig. 1 Records of isometric tetani performed by representative muscles of each of the four groups. Force (P) is expressed as percentage of the maximal isometric force produced by each muscle (P_{max}). (*EDL* Extensor digitorum longus, *OL* overload, *CTR* control, *t* time)

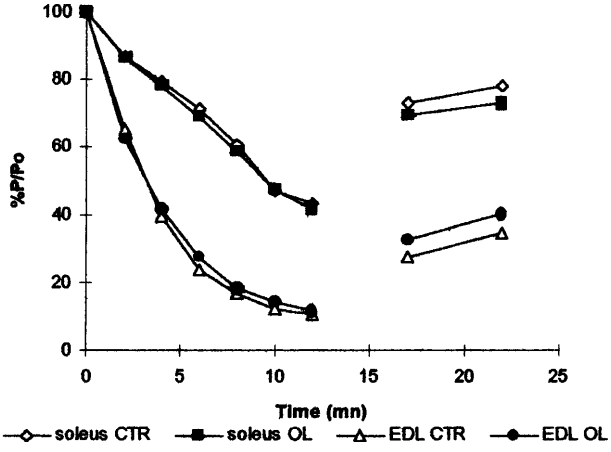

Fig. 2 Maximal isometric force during successive tetani in a fatigue test. Force (P) developed at different times is expressed as a percentage of the force produced at the beginning of the fatigue test ($t=0$; P/P_o). At the end of the fatigue test, two additional tetani were recorded after 5 and 10 min of recovery

All other data showed that overloaded muscles were slower than control muscles: rates of rise in force at the beginning of the tetanus were lower, and the relaxation at the end of the tetanus was prolonged. The largest modifications were observed for the relaxation parameters in the soleus muscles: $t_{5\%}$ and $t_{20\%}$ were increased by about 20%; no further change was observed between $t_{20\%}$ and $t_{50\%}$ of relaxation, the increase observed for $t_{50\%}$ being due to the change observed for $t_{20\%}$.

Force/frequency relationship

The relationship between tetanic force and frequency of stimulation was not affected by overload: no differences were found between overloaded and control EDL and soleus muscles.

Fatigue test

Figure 2 shows the mean results of the fatigue test in the soleus and EDL muscles. No difference was observed between overloaded and control muscles.

Force/velocity relationship

The parameters of the force/velocity relationship found in the four groups of muscles are given in Table 3. The main effect of overload was a reduction of the velocity parameters b (velocity constant) and v_o (maximal velocity) in the soleus muscle; the reduction of v_o was the single significant change.

Effects of overloading on MHC and SERCA

In view of the mechanical results, it can be concluded that overloading altered the contractile properties of the

Table 3 Parameters of the force/velocity relationship in OL and CTR soleus and EDL muscles. Values are means (SEM); $n = 6$ in all cases. (a/P_o Force constant, b velocity constant, v_o extrapolated maximal velocity, *lf* fibre length)

Parameter	Soleus OL	Soleus CTR	EDL OL	EDL CTR
$a/P_o \times 100$	10.4 (0.8)	10.3 (0.18)	31.6 (5.2)	38.3 (6.7)
b (lf · s^{-1})	0.46 (0.03)	0.58 (0.07)	2.81 (0.30)	3.35 (0.51)
v_o (lf · s^{-1})	*4.48 (0.29)	6.37 (0.92)	9.76 (1.31)	9.05 (0.46)

* Significantly different from the CTR condition ($P < 0.05$)

Table 2 Tetanus parameters in OL and CTR soleus and EDL muscles. Values are means (SEM); $n = 16$ for the soleus CTR group, $n = 14$ each for the soleus OL and EDL CTR groups, and $n = 15$ for the EDL OL group. ($t_{5\%}$ Length of time necessary to obtain a 5% reduction in tension after the last stimulus of the tetanus, $t_{20\%}$ length of time necessary to obtain a 20% reduction in tension after the last stimulus of the tetanus, $t_{50\%}$ length of time necessary to obtain a 50% reduction in tension after the last stimulus of the tetanus)

Group	Tension (kN · m^{-2})	Force (mN)	Rate of rise in tension (kN · m^{-2} · s^{-1})	$t_{5\%}$ (ms)	$t_{20\%}$ (ms)	$t_{50\%}$ (ms)
Soleus OL	168.0 (9.3)	223.9 (12.0)	*1.10 (0.09)	*86.8 (5.76)	*149.3 (8.0)	*211.6 (12.0)
Soleus CTR CTR	167.4 (9.4)	195.8 (15.0)	1.46 (0.11)	72.7 (4.49)	124.6 (6.1)	183.8 (9.5)
EDL OL	240.6 (17.0)	277.0 (16.0)	5.37 (0.53)	25.7 (1.20)	48.4 (1.7)	66.3 (2.1)
EDL CTR	265.6 (25.0)	302.3 (22.0)	6.54 (0.88)	23.3 (1.29)	44.7 (1.9)	61.3 (2.5)

* Significantly different from the CTR condition ($P < 0.05$)

soleus muscle in particular. The modifications observed in the relaxation parameters were more obvious (Fig. 1) and more significant than those of the force/velocity relationship. Thus, in addition to the histochemical analysis of soleus muscle fibre types using anti-MHC antibodies, an analysis of calcium pump isoforms (SERCA) using an anti-SERCA2a antibody was also carried out.

Figure 3 shows an example of transverse sections of soleus muscle that have been stained with anti-MHC and anti-SERCA2a antibodies. Table 4 gives the proportions of fibres stained by anti-MHC and anti-SERCA2a antibodies in soleus muscles. In overloaded muscles, the proportion of fibres that express MHC1 was increased at the expense of those containing MHC2a. These data were confirmed by quantitative electrophoretic analysis of MHCs (data not shown). The proportion of fibres that express SERCA2a was also increased.

Table 4 Proportions of fibres stained with antibodies in the soleus muscles. Values are means (SEM); $n = 11$. (*MCH* Myosin heavy chain, *SERCA2a* the slow type of sarcoplasmic reticulum calcium pump)

Group	MHC2a	MHC1	SERCA2a
Soleus OL	*30.2 (3.32)	*73.9 (4.40)	*84.7 (2.62)
Soleus CTR	45.8 (1.42)	54.2 (1.57)	61.8 (1.67)

* Significantly different from the CTR condition ($P < 0.01$)

The generally accepted view is that MHC1 and SERCA2a are expressed in the same fibres. However, the results given in Table 4 show that, in control muscles, the proportion of SERCA2a-positive fibres

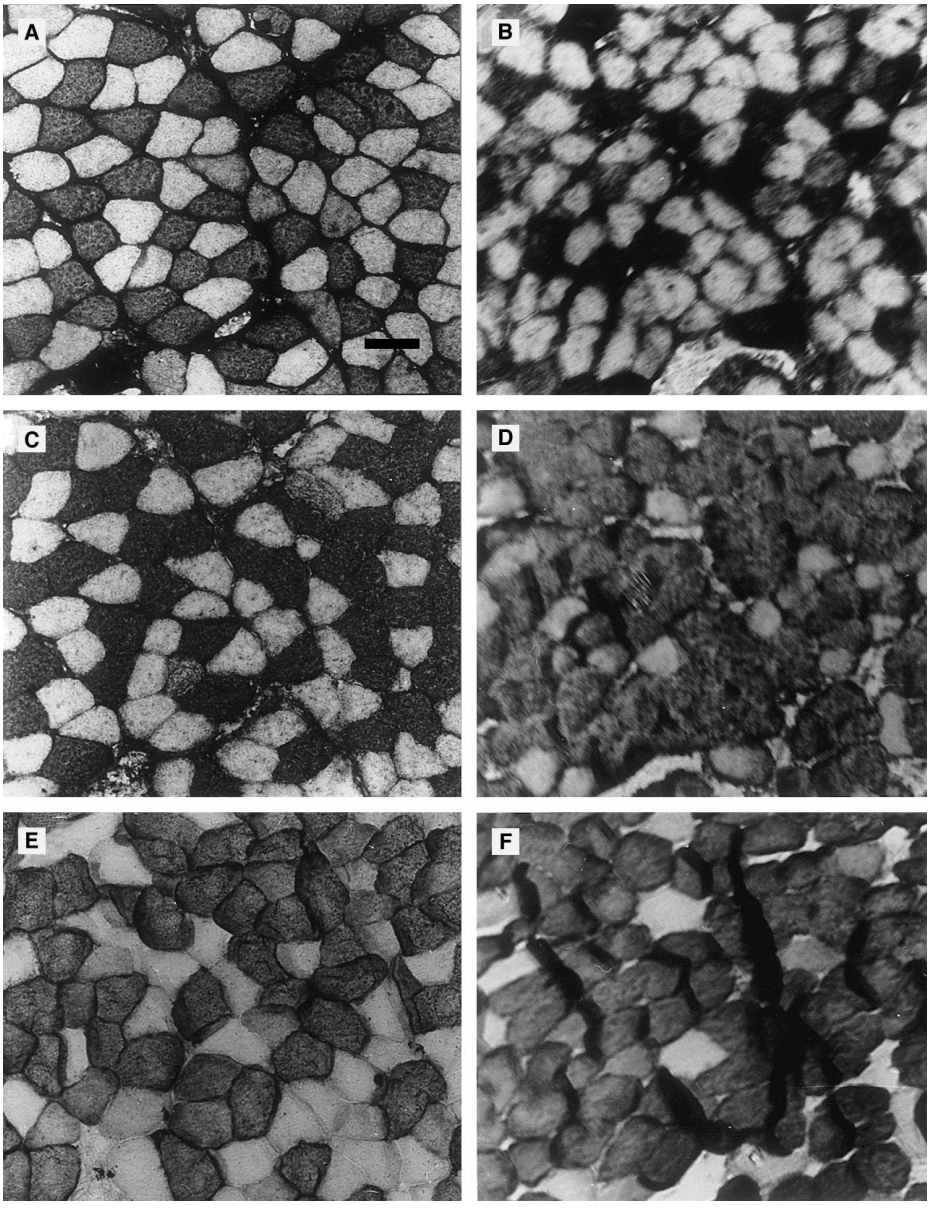

Fig. 3 Transverse sections of overloaded (**B**, **D**, **F**) and control (**A**, **C**, **E**) soleus muscles stained with antibodies directed against myosin heavy chain (MHC) 2a (**A**, **B**), MHC1 (**C**, **D**) and the slow type of sarcoplasmic reticulum calcium pump (SERCA2a) (**E**, **F**). Scale bar in **A**, 50 μm

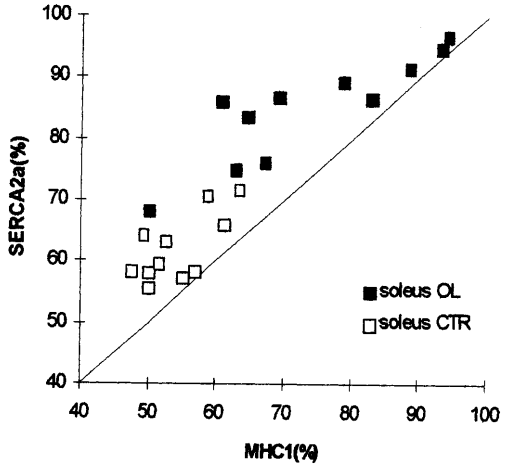

Fig. 4 Correlation between the proportions of fibres expressing MHC1 and SERCA2a in the control (*open squares*) and overloaded (*closed squares*) soleus muscles. The *straight line* drawn on the figure has a unity slope and represents the points where the proportions of MHC1 and SERCA2a are identical. All points fall above the line

exceeded that of MHC1-positive fibres, the difference being significant. The difference was further increased by overload: the change in SERCA2a was larger than that in MHC1. In overloaded muscles, the sum of the proportions of MHC2a and MHC1 exceeded 100%. This observation indicates the existence of hybrid fibres.

Figure 4 shows the correlation between the percentages of fibres expressing MHC1 and SERCA2a. In this figure, each point represents the percentage of fibres that were positive for SERCA2a as a function of the percentage of fibres that were positive for MHC1 in one muscle. A line of unity slope has been drawn on the graph. All points fall above that line. In other words, in all cases, the proportion of SERCA2a-positive fibres was higher than that of MHC1-positive fibres. It should be noted that great care was taken to control the staining in order to obtain the best contrast between positive and negative fibres. Thus, SERCA2a is not strictly expressed in the same fibres as MHC1, since MHC1 is present in type I fibres, and the sum of MHC1 and MHC2a positive fibres is 100% (Table 4), this means that SERCA2a is expressed in some type II fibres.

Figure 4 also shows that for identical values of MHC1 proportions, the corresponding values of SERCA2a proportions were higher in overloaded soleus muscles than in control muscles.

Discussion

In this work we have presented a new approach for studying overload, in which all traumatizing contact with muscles, and the inflammatory reaction that may provoke, was avoided. The overload of mouse hindlimb induced a hypertrophy in the tonic soleus muscle, and phenotypic changes with consequent functional repercussions. The observed changes in the control of the contraction (assessed by the rate of relaxation and the expression of SERCA2a) were more prominent than those of typical myofibrillar properties (estimated by the force/velocity relationship and the expression of MHCs).

Validity of the experimental model

The original approach used to study the effects of overload presents some relevant advantages. The choice of mouse as an animal model in the present study is interesting with regard to the animal size and the fibre type composition of its soleus muscle. The small size of the EDL and soleus muscles allows the measurement of mechanical parameters on isolated muscles in vitro with higher accuracy than in vivo. Indeed, mouse muscles can be kept and stimulated in vitro for several hours without damage. This is not possible in muscles from larger animals, and is a major advantage because only physiological measurements give an insight into the functional consequences within the animal.

With regard to immunohistology, the small size of the muscles also offered the opportunity to count all of the fibres over the entire muscle section. Thus, the problem of area selection was avoided, and accurate measurements of fibre proportions were obtained. In spite of this advantage, the small size of the mouse soleus muscle limits the possibility of performing chemical analyses such as RNA isolation; for this, muscles from several animals must be pooled.

The fibre type composition of the mouse soleus muscle, which is a mixture of only two fibre types (type I and IIA), makes this muscle a suitable model for overloading. This possibility does not exist to the same extent in other commonly used laboratory animals in which the proportion of type I fibre is very high (e.g., rat and rabbit). In those animals, functional overload has been performed preferentially on other muscles.

The procedure developed in this work, which induced phenotypic changes, does not involve any experimental contact with the muscles, thus enabling them to maintain normal nerve and blood supplies. Any local inflammatory process due to surgical trauma was avoided. Moreover, the procedure is less traumatising than others, since only a small incision with cutaneous detachment was sufficient to implant the overloading mass. This new model provides conditions that are closer to physiological conditions than others in which a structural alteration (muscular or nervous) is induced. The dynamic equilibrium that exists between the different muscles of the hindlimb is preserved. No interference occurs with either the local processes that are induced by overload or those involved in mechanisms of phenotype change. The study of the early phenomena, especially the role of the different growth factors involved in the adaptation of skeletal muscle to increased load, is thus made possible. Moreover, the sensory innervation of overloaded muscles is preserved.

Another advantage of our model is reversibility: at any time, the overload mass can be removed and the inverse changes of the different phenomena induced by overload and their time course can be studied.

The present model also gives a quantitative approach to overload, since the overload mass is known, and using a range of masses, a relationship between overload mass and its effects could be found. The magnitude of the overload used may appear to have been very large; however, the behaviour of the overloaded animals was normal and the weights did not seem to limit their activity. However, no quantitative analysis of the biomechanics of locomotion or behavioural study was carried out. This point may well be worth documenting, since mice are active mainly during the night. This model may be compared to the weight-lifting model (Klitgaard 1988) where rats were trained to lift weights of 130%–200% of their body weight. The difference with the latter model is that the weight lifting was not a permanent activity.

A physiologically increased activity is interesting to be noted here: in numerous African tribes, women carry loads equivalent to 70% of their body mass, either on the top of their heads or by a strap across their foreheads, often for several hours (Maloiy et al. 1986). In this case, a more efficient locomotion pattern was adopted, but possible changes within the muscles could not be studied.

Selective hypertrophy of the soleus muscle

The soleus muscles hypertrophied by about 17%. Although this confirms the results found in previous studies using other methods of overload (Ianuzzo et al. 1976; Baldwin et al. 1982; Timson et al. 1985; Frischknecht and Vrbová 1991; Frischknecht et al. 1995; Yamaguchi et al. 1996), the reported increase in muscle mass observed in these studies was larger than that observed in the present work. This may be due to the difference in application of the overload: in the present work, the load was applied to the whole hindlimb and all muscles were kept intact; thus a large portion of the overload was carried by other muscles, and in particular by gastrocnemius muscle.

Functional effects of overload

Overload induced only minor changes in twitch parameters, with the exception of the decrease in the rate of rise in tension. On the contrary, the effects of overload on isometric tetanus were more obvious: the rate of rise in tension and the rate of relaxation were reduced. Relaxation times were increased in overloaded soleus muscles. This increase concerned essentially $t_{5\%}$ and $t_{20\%}$, which reflect the initial processes involved in muscle relaxation (i.e. a decrease in intracellular calcium concentration, dissociation of calcium from troponin C, and re-establishment of the inhibition exerted by troponin I; Gillis 1985). The increase in $t_{5\%}$ could therefore indicate a lower rate of calcium uptake into the sarcoplasmic reticulum by calcium ATPase (SERCA). This modification could involve a change in calcium pump activity that is related to a change in SERCA isoform or regulation of its activity. As expected, immunohistological analysis showed an increase in the proportion of fibres that express SERCA2a, and confirmed the results of previous reports (Kandarian et al. 1994; Talmadge et al. 1996).

Overloading is known to induce higher resistance to fatigue, which is related to a change in the energy supply system (which becomes more oxidative). In the study described here, fatigue resistance remained the same in both groups of soleus muscles, contrary to all previously published data (Roy et al. 1982; Frischknecht and Vrbová 1991). It is possible that changes occured in the energy supplying the metabolic pathways, but to an extent that was insufficient to induce a significant increase in fatigue resistance, since the soleus muscle is composed of fibre types I and IIa, in which metabolic enzyme contents are especially oxidative. As mentioned earlier, the effect that this type of overload has on the biomechanics of locomotion has not been studied.

Biochemical effects of overload

In soleus muscles, the changes induced by overloading in fibres types and myosin composition (increased proportion of type I and reduction of type II proportion) are comparable to those reported in previous studies (Ianuzzo et al. 1976; Timson et al. 1985). The myosin composition of a given muscle determines its force/velocity relationship. In the present study, overloading induced changes in the force/velocity relationship in the soleus muscles, the velocity constant (b) and extrapolated v_o being lower than in control muscles. These changes are in agreement with those of previous reports (Roy et al. 1982).

SERCA changes versus MHC changes

The increase in the proportion of fibres that express SERCA2a found in the present study confirms previously published data (Kandarian et al. 1994; Talmadge et al. 1996). Myosin and SERCA transformations are thus associated. Talmadge et al. showed, in cat plantaris muscle, a high correlation between SERCA2a and MHC1 expression, which was not modified after 3 months of functional overload. In our data, SERCA2a changes were larger than MHC1 changes in all cases, and this was more conspicuous in muscles that contain a relatively low proportion of MHC1-positive fibres (Fig. 4), despite the fact that the overload was applied for at least 1 month. These data suggest that the

adaptative change could be in a transitory state, and that SERCA transformation precedes that of myosin. In the present study, immunostaining of SERCA1 was not performed. It is possible that co-expression of SERCA2a and SERCA1 occurs. Thus, in some type IIa fibres, it is possible that this co-expression occurs when soleus muscles are submitted to an increased load. It may be relevant to note that contrary to the case in other reports, in which the effects of overload were detected in selected areas where MHC1 fibres were more numerous, in the present study whole muscle sections were analysed. Moreover, in our case the soleus muscles were studied and, as discussed earlier, the distributions of fibre types in this muscle are simpler since they contain only type I and type IIa fibres; changes are therefore more easily interpreted.

These data contradict the observation of Mabuchi et al.(1990), who found that in the final stage of chronic stimulation of the rabbit tibialis anterior, changes in myosin preceded those of SERCA. The discrepancy could be due to differences in the aminal species, to different time courses in the adaptation of a muscle to these two treatments (overload and chronic stimulation), and to the type of skeletal muscle, since the soleus is a slow-twitch skeletal muscle, whereas the tibialis anterior is a fast-twitch muscle.

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