Effect of the Elimination of Neural Influences in the Rat Soleus Muscle During Unweighting

Maurice Falempin and Soumeya Fodili

Laboratoire de Physiologie des Structures Contractiles, Bâtiment SN4, Université des Sciences et Technologies de Lille I, Villeneuve D'Ascq Cedex France

Abstract

Morphological, contractile, histochemical and electrophoretic properties of the rat soleus (SOL) muscle were studied after 2 weeks in hindlimb unweighting conditions (HU) and after 2 weeks of HU associated with a complete elimination of the neuromuscular activity induced by chronic perfusion of tetrodotoxin (TTX) to the sciatic nerve (HU-TTX). HU induced decreases in the (SOL) weight, muscle cross sectional area (MCSA), fibre cross sectional area (FCSA) of type I, IIa and IIc fibres, force output (twitch and tetanic tension), time to peak tension (TPT) during the twitch, percentage of type I fibres and percentage of myosin heavy chain (MHC) type I isoform. After the elimination of the neuromuscular activity (HU-TTX), muscular atrophy and a decrease in the force output were always observed but the TPT, the fibre type composition and the electrophoretic profile of the myosin isoforms of the soleus muscle were maintained at normal values. These results may suggest that certain alterations in the soleus muscle observed after HU could be in relation with the modification of the neuromuscular activity.

Key words: unweighting, tetrodotoxin, muscle atrophy, contractile activity, fibre type, myosin heavy chain isoforms, soleus.

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Rodent hindlimb unweighted (HU) is a ground based model successfully used to mimic the changes in skeletal muscle properties that occur with spaceflight. For most parameters evaluated, slow twitch muscles were more affected than fast twitch ones. Many reports have shown that HU led to pronounced structural atrophy [25], loss of twitch and tetanic tensions [14, 15, 37], changes in fibre type percentage of slow-twitch fibres [12, 19], in proteinic metabolism [22, 33] and in capillary density [12]. Moreover, mechanical measurements of contractile speed have shown that the slow-twitch soleus muscle acquires faster contractile properties during unweighting [15, 21, 31, 37].

Among all the mechanisms suggested as a trigger for the muscular changes, a modification in the neuromuscular activity can be considered since the structural and functional integrity of a skeletal muscle depends on the presence and normal function of the nervous support [27]. We therefore speculated that the slow to faster muscle type transformation observed during HU could be a consequence of the changes in the nervous activity observed by several authors [1, 2, 27]. Consequently, the aim of this study was to compare changes in morphological, histochemical and mechanical properties of the rat soleus muscle after two weeks of HU and after two weeks of inactivity induced by the chronical perfusion of tetrodotoxine (TTX) during HU. The physiological experiments reported here show that chronical inactivity induced by TTX can prevent the decrease in the soleus isometric contraction time and maintain the normal fibre type composition and the electrophoretic profiles of myosin heavy chain isoforms despite HU conditions.

Materials and Methods

Animal care

Nineteen male Wistar rats weighing 240 to 250 g were divided randomly into three groups: control (n = 8), hindlimb unweighted (HU, n = 5), hindlimb unweighted implanted with a TTX pump (HU-TTX, n = 6). They were housed individually in conventional plastic cages and had access to pellet food and water *ad libitum*. The rats were acclimatized at a 23°C room temperature and with a 12: 12 light: dark cycle for one week before use in the experi-

ments. The maintenance conditions of the animals and the experiments received authorizations from both the Agricultural and Forest Ministry and National Education Ministry (Veterinary service of health and animal protection; authorization 03805).

Suspension protocol

Both unweighted groups (HU and HU-TTX) were subjected to a suspension period of 14 days, a period long enough for the soleus to develop maximal atrophy [14]. The suspension model used was the tail suspension model introduced by Morey [24]. Briefly, the tail of each rat was washed, cleaned, dried and wrapped in an anti-allergic orthopedic tape adhesive plaster. This cast, covering less than half the tail was secured to an overhead swivel mounted at the top of the cage permitting free 360° rotation by the animals. The rats were unweighted at about a 30° head down angle to mimic fluid shifts consecutive to weightlessness.

Chronic TTX disuse

TTX was chronically delivered to the sciatic nerve by a system consisting in an osmotic mini-pump (Alzet osmotic mini-pump, model 2002, Alga corp, Palo Alto, CA) and connecting silastic tubing and cuff. The tubing and the osmotic pump were filled with TTX (500 µg/ml solution diluted in sterile 9‰ saline). The osmotic mini-pump held a volume of 225 µl. TTX was perfused continuously at a rate of 0.5 µl / hr for the suspension period.

The implantation protocol

In the HU-TTX group, surgery was performed 24 h before suspension. The rats were anaesthetized with pentobarbital sodium (35 mg/kg ip) and the left sciatic nerve was exposed at the thigh under aseptic conditions. The cuff connected to the pump was carefully slipped around the nerve and fixed with two surgical knots. The osmotic pump was placed subcutaneously along the lumbosacral vertebral column. The overlying muscles and skin incisions were sutured closely.

In order to detect any specific effect of the osmotic pump and the cuff, four rats were implanted with sham osmotic pumps filled with sterile 9‰ saline, and unweighted. It appeared that (after 2 weeks) the morphological and histochemical properties and electrophoretic profiles of the sham group were similar to those of the HU group.

Measurement of isometric contractile properties

For the final experiments, the soleus muscle of the rats of the three groups was dissected and care was taken to keep the blood supply intact. The hindlimb of the rats was immersed in a bath filled with mammalian Ringer's solution, oxygenated and kept at 36 ± 0.5°C. To prevent uncontrolled movements of the hindlimb and to respect isometric recording conditions, the knee and the foot were rigidly fixed. A short thread connected the distal muscle tendon to a transducer (ENTRAN, model ELP 1200 - 0.393 mV/g, band pass 3 KHz) which recorded isometric twitch and tetanic tensions at optimal muscle length for twitch tension. The soleus muscle was stimulated directly through two platinium plates placed close to the muscle surface, one on each side, using pulses 0.5 ms long and supramaximal voltage (GRASS S88). The output from the transducer was recorded on an electrostatic chart (ANKERSMIT WR 7900), sampled (AXON INSTRUMENTS, 125 KHz LABMASTER DEMA TL1-125-Interface) and analysed on line with a computer (LEANORD 386 - 6/25 MHz). Isometric maximum tension parameters included single twitch tension (P_t), maximum tetanic tension (P_0). Three parameters were used to characterize the speed-related properties and the typing of the muscle in unweighting conditions. First, the time to peak tension (TPT), which is the time between force onset and peak during the isometric twitch; second, the ratio of the subtetanic tension at 20 Hz relative to the tetanic tension (P_{20}/P_0), this ratio is used as an indicator of the type of a muscle; third, the fatigue index FI which was defined after the application of the fatigue test of Burke et al. [6], also used by other investigators in HU conditions [20, 37], FI being generally connected with the type of a muscle in relation to its metabolism.

At the end of the contractile testing, the rats received an over-dose of sodium pentobarbital and the soleus muscle was dissected and weighed (MWW).

Histochemical and morphological analyses

After mechanical studies, muscles of the control, HU and HU-TTX groups were frozen in isopentane precooled to its freezing point by liquid N_2 and stored at - 80°C until analysis. At the midbelly, the muscle was cut perpendicular to its longitudinal axis into serial 10 µm thin cross sections in a cryostat microtome at - 30°C. The muscular fibre types were classified following the method used by Desplanches et al. [12]. Briefly, sections were incubated in a buffer (pH 9.4) with 18 mM $CaCl_2$, and 2.7 mM ATP at 37°C for 20 min. A preincubation was carried out in acid buffer (acetic acid 50 mM) with 25 mM $CaCl_2$ (pH 4.4) for 4 min at 25°C. This histochemical method allowed the distinction of three types of soleus fibres (I, IIa, IIc). With the myosin ATPase reaction, intermediate type IIc fibres displayed an intermediate behaviour to pH sensitivity between type I and IIa fibres. Distribution was expressed as the number of fibres of each type relative to the total number of fibres. Measurements were made on 250 fibres from each section. The cross sectional area of each type of fibres (FCSA) and of each muscle (MCSA) were automatically measured (SAMBA ALCATEL, TITN, France).

The analysis of myosin heavy chain isoforms by SDS-PAGE

The left soleus muscle was subjected to the electrophoretic analysis of myosin heavy chain (MHC) isoforms. MHC isoforms were separated by SDS-PAGE on a 6% polyacrylamide gel, with high glycerol concentration (40%) using the method described by Carraro and Catani [7], improved by Danieli-Betto et al. [11]. The qualitative and quantitative analyses were made possible by compar-

ing the intensity of staining of the relative amounts of MHC isoforms with Coomassie Blue on the electrophoretic bands, using an image processor (SAMBA, Alcatel TTTN, France) connected with "gelpro" software.

Statistical analysis

The results were analysed using a one-way ANOVA. Student's t test was used as a post hoc test to examine statistical significance set at the 95% level of confidence between the groups. All the results are presented as mean ± SEM.

Results

Preliminary remarks

The extent of paralysis in the HU-TTX rats was checked by:

- the loss of the toe spreading reflex response and insensitivity to plantar reflex (twice daily);
- the maintenance of the paralysed hindpaw in a neutral position without any scratching movements of the lombar coat;
- and, during the final experiment, verification of the loss of motor response of the soleus muscle after electrical stimulation of the sciatic nerve above the cuff at a strength five times greater than needed to induce movement when applied below the cuff. This procedure never produced any contractile response when previously assessed toe spreading was absent.

Morphometric measurements

Quantitative data are reported in Table 1. Following the 14 days of suspension, with or without TTX perfusion to the sciatic nerve, no significant difference was observed between the body weight (BW) of the CON, HU and HU-TTX rats. In contrast, the mean soleus muscle wet weight (MWW) was significantly smaller in the HU (-37%) and HU-TTX (- 19%) in comparison to the CON rats. The soleus-to-body weight ratio also declined in the HU and HU-TTX rats by 33% and 25% respectively. There was a marked decrease in the MCSA for the HU and the HU-TTX rats, 50% and 52%, respectively. HU had also an important effect on the soleus fibre sizes. Soleus muscle type I, IIa, IIc fibre mean cross-sectional areas were reduced significantly to 57%, 56% and 70% of the control values, respectively. The same decreases were observed in the HU-TTX conditions except for type IIc fibres.

Force and fatigue-related properties

HU significantly decreased the absolute Pt (g) and Po (g) of the soleus muscle by 47% and 52%, respectively (Table 2). Relative to the muscle weight, the tetanic tension was significantly decreased by 26% comparatively to the CON value. P_t and P_0 values of the HU-TTX and HU rats were not significantly different. However, the force generated per milligram of muscle (P_0/MWW) decreased by 50% when TTX was associated with HU. The soleus FI was influenced neither by HU nor by HU-TTX (Table 2). When Po was expressed relatively to MCSA (P_0/MCSA), the ratio was significantly decreased only in HU conditions by 43% comparatively to the CON value.

Speed-related properties

HU resulted in a significant decrease (17%) in TPT (Table 2). Subtetanic tension at 20 Hz (P_{20}) expressed relative to Po was significantly lower in the HU group than in CON, indicating a pattern more typical of a "faster" muscle. On the contrary, when TTX was associated with HU, TPT and the P_{20}/P_0 values were similar to those of CON.

Analysis of fibre-type composition by myosin ATPase staining

In CON soleus, 86% of the muscle fibres were of type I, 7% of type IIa and 7% of type IIc (Table 3). HU decreased the percentage of type I fibres to 73% with a simultaneous

Table 1. Morphometric characteristics of control, hindlimb unweighted and hindlimb unweighted perfused rats.

Group	BW g	MWW mg	MWW/BW mg/g	MCSA mm^2	FCSA Type I μm^2	FCSA Type IIa μm^2	FCSA Type IIc μm^2
CON	303	145.0	0.48	13.8	3343	3067	3070
n=8	±7	±6.6	±0.02	±1.2	±336	±414	±410
HU	290	90.4*	0.32*	6.8*	1419*	1352*	908*
n = 5	± 4	± 3.3*	± 0.01	± 0.5	± 104	± 178	± 90
HU-TTX	331	118.0**	0.36**	6.6*	1556*	1525*	1270**
n=6	± 22	±9.4	±0.01	±0.4	±80	±206	±116

BW = Body weight; MWW = Muscle wet weight; MCSA = Muscle cross sectional area; FCSA = Fiber cross sectional area.
Values are means ± SEM.
* significantly different from control. ** significantly different from HU.

Table 2. Contractile properties of control, hindlimb unweighted and hindlimb unweighted perfused rats.

Group	P_t g	P_0 g	P_0/MWW g/mg	P_0/MCSA g/mm^2	TPT ms	P_{20}/P_0 %	FI %
CON	32.4	168.6	1.18	12.9	33.4	59	88
n=8	±1.6	±9.3	± 0.08	±1.4	±0.8	±3	±2
HU	17.2*	79.4*	0.87*	7.3*	27.7*	38*	88
n=5	±0.8	±6.8	±0.04	±1.0	±1.4	±7	±4
HU-TTX	16.7*	67.0*	0.59*	10.9**	33.0	58**	76
n=6	± 0.8	± 4.6	± 0.08	± 1.2	± 2.5	± 5	±7

P_t = Twitch tension; P_0 = Tetanic tension; TPT = Time to peak tension; P_{20} = Tetanic tension developed at 20 Hz; FI = Fatigue index. Values are means ± SEM.
* significantly different from control. ** significantly different from HU.

increase in type IIa fibres. On the contrary, HU-TTX induced no change in fibre type distribution since the percentages of type I, type IIa, and type IIc fibres were identical to CON values. The results are illustrated on Figure 1.

Myosin HC isoforms analysed by SDS-PAGE

Two types of MHC isoforms, MHC I and MHC IIA-IIX were observed in the control soleus muscles (Table 3); their mean percentages were 95.2% and 4.8%, respectively. After hindlimb unweighting, the percentage of the slow type MHC I isoform had significantly decreased (15%) comparatively to the control and a concomitant increase in fast type MHC IIa-IIx isoform was observed. After HU-TTX, these modifications in the percentage of myosin MHC isoforms were not observed. The mean percentages of MHC I and MHC IIa-IIx were 94.4% and 5.6%, respectively, values not statistically different from the control values.

Discussion

Muscular atrophy

The model of unweighting leads to muscle atrophy with a preferential effect on slow twitch muscles [15, 28, 37]. The muscular atrophy observed in this study after 14 days of HU is in agreement with these earlier results. The loss of muscle mass can be associated with an important decrease in fibre cross sectional area of type I, IIa, IIc fibres. This decline in the soleus fibre size has usually been explained by a decrease in myofibril proteins [33]. The mechanism evoked in the reduced content of proteins may include an increase in protein degradation rates, decreased synthesis rates of muscle protein, or a combination of both [4]. Atrophy of rat soleus also occurred after chronic TTX treatment during the HU period presumably due to reduced rates of synthesis and/or increased degradation rates of muscle proteins since the type I, IIa, IIc FCSA also decreased. However a relative discrepancy between the de-

Table 3. Percentage of fibre type composition and percentage of myosin heavy chain isoforms of control, hindlimb unweighted and hindlimb unweighted perfused rats.

Group	fibre type composition % Type I	Type IIa	Type IIc	MHC isoforms % Type I	Type IIa-IIx
CON	85.9	7.3	6.8	95.2	4.8
	± 1.6	± 2.3	± 0.9	± 1.4	± 1.5
HU	73.3*	20.2*	6.5	80.6*	19.2*
	± 2.6	± 0.6	± 2.2	± 2.8	± 2.4
HU-TTX	85.4**	8.5**	6.2	94.4**	5.6**
	± 2.9	± 3.5	± 2.2	± 2.9	± 1.9

MHC = myosin heavy chain. Values are means ± SEM. * significantly different from control. ** significantly different from HU.

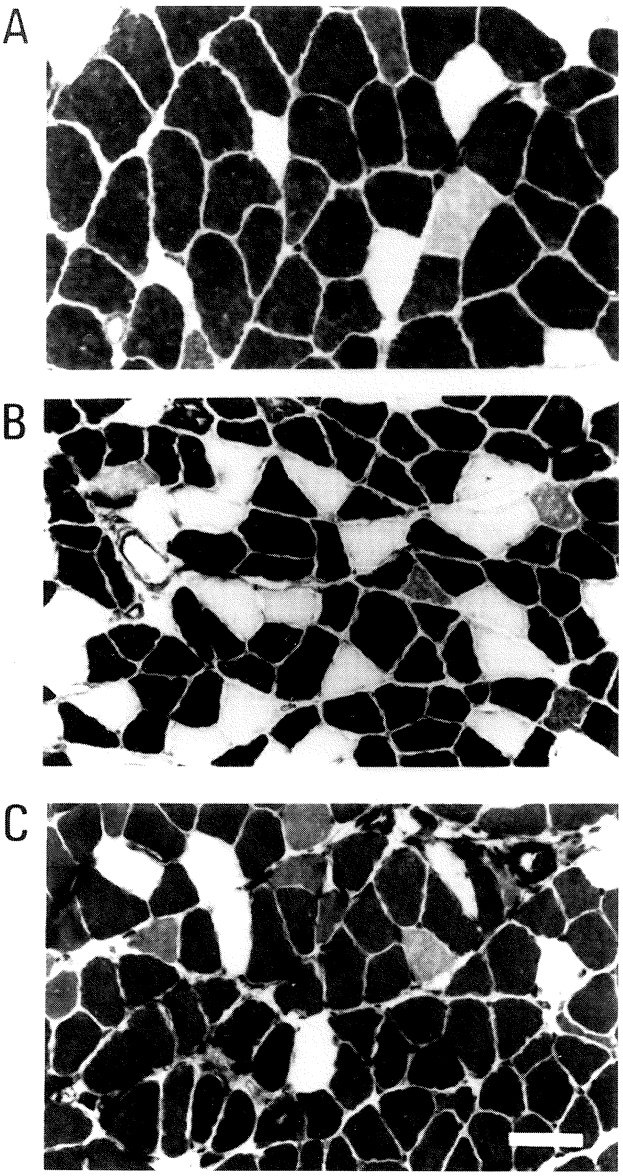

Figure 1. Cross sections of soleus from control (A), hindlimb unweighted (B) and hindlimb unweighted perfused with TTX (C) rats, stained for myofibrillar ATPase. Only the sections stained after pre-incubation at pH 4.4 are shown. Bar = 100 μm.

crease in the whole muscle weight and the decrease in fibre size suggested that there were disproportionate decreases in fibre volume relative to extrafibre components. On the other hand, the variations in the degree of atrophy of the muscle wet weight can be dependent on the length of the muscle during the disuse and it is well known that stretch is thought to be an important regulator of muscle size [17]. The difference in the muscle length in HU (soleus in shortened position) and in HU-TTX (soleus in neutral position) conditions had to be considered to explain the differences observed in the degree of atrophy.

Tension generating capacity

As in many other studies [13, 15, 23] HU markedly decreased the ability of the soleus muscle to generate tension. Absolute Po was 52% lower than for control values and this decrease was lower (26%) when expressed comparatively to MWW and lower (43%) when expressed per muscle CSA. It can be suggested that the force decrease was correlated both to the absolute loss of myofibril proteins [33] - that implied a decrease in the number of cross bridges [30] - and to the loss of force developed by each cross bridge. In the present study, superimposition of neural inactivation with TTX further compromised this functional deficit. Deficits in muscle weight, muscle or fibre cross sectional area were always lower than the decrease in the maximal tension developed by the HU-TTX soleus. The loss in the ability of the muscle to generate tension per muscle weight was unclear. In the HU-TTX muscles, the maintenance of the P_0/MCSA ratio suggested changes in the extrafibre components relatively to the total volume of fibre . The decrease in the force output could also be explained by the fact that the decrease in the contractile protein concentration was enhanced by the removal of the nerve impulse activity. The effects of TTX either on myofibril function or sarcoplasmic reticulum properties [35] or Ca^{++} activation characteristics of myofibril ATPase [16] could also be additive to the changes induced by unloading alone.

Speed-related properties

With regard to the decreased contraction time and the P_{20}/P_0 ratio, the soleus in HU conditions was transformed toward a faster muscle as already reported by others [23, 37]. Our results showed significant decreases in TPT and in the ratio P_{20}/P_0 values. These changes in the mechanical properties of HU soleus were well correlated with changes in the fibre type composition and changes in the percentage distribution of myosin heavy chain isoforms. In this study a significant increase in the ratio of fast-twitch type IIa fibres was observed as already described in other works [12, 26, 32]. Moreover, we also observed an increase in the percentage distribution of MHC IIa-IIx with a concomitant decrease in the MHC I isoform. On the contrary, blockage of the nerve impulse activity by TTX during HU prevented these modifications. The fibre type composition and the distribution of MHC in the rat soleus muscles was identical in the control and the HU-TTX groups. As the fibre type composition of a muscle is greatly dependent on the motoneuron activity [27], it can therefore be postulated that the modifications observed in the soleus neuromuscular activity by several authors [1, 2, 28] might be a cause of some alterations occurring during HU.

Since 1) the major difference between the HU and HU-TTX groups was the presence or the absence of a modified motor nervous activity and 2) it has been established by cross-reinnervation [5, 9, 10] or by electrostimulation experiments [8, 36] that the fibre type composition and the speed properties, are in great part under the nervous in-

fluence, our results obtained after TTX perfusion during HU suggested that the modifications of the motoneurons activity observed by others [1, 2, 28], may have determined the change in some muscle characteristics during HU. These mechanical alterations of HU and HU-TTX muscles are therefore consistent with changes in the histochemical and electrophoretic characteristics and are in agreement with the fact that the phenotypic expression of some contractile properties of rat soleus muscle is under neural control.

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Address correspondence to:

M. Falempin, Laboratoire de Physiologie des Structures Contractiles, Bâtiment SN4, Université des Sciences et Technologies de Lille I, 59655 VILLENEUVE D'ASCQ CEDEX FRANCE, tel. (33) 20 43 40 90, fax (33) 20 43 68 88.

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