Unilateral immobilization: a simple model of limb atrophy in mice

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Abstract

We describe a new, simple immobilization protocol designed to induce muscle atrophy in lower hindlimb muscles in mice. Hindlimbs of C57BL6 mice were unilaterally immobilized for 7 days. Morphological analysis revealed that immobilization resulted in a significant reduction in muscle fiber size in both the extensor digitorum longus (EDL) and tibialis anterior (TA). By contrast, no significant changes in muscle fiber type content, as measured by NADH-TR staining, were detected. At the molecular level, expression of the muscle-specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 genes was induced following immobilization in all the muscles examined. The characteristics of this model lend themselves to muscle atrophy research; its availability in mice may be instrumental for future studies on therapeutic interventions of muscle atrophy using transgenic and mutant mice strains.

Key Words: Muscle atrophy, muscle disuse, unilateral immobilization, MuRF1, atrogin-1

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Skeletal muscle atrophy can be defined as a wasting or decrease in muscle mass as a result of surgery, immobilization, non-weight-bearing or an extended period of bed rest, as well as of disease and aging.

Regardless of the inciting event, skeletal muscle atrophy is characterized by a decrease in myofiber size, protein content, force production and fatigue resistance, and thus dramatically affects quality of life and increases morbidity and mortality. Myofiber size is regulated at two different levels: first, the cytoplasmic volume associated with individual nuclei is regulated through the balance between protein synthesis and degradation; second, the number of myonuclei within an individual myofiber determines the protein content and cytoplasmic volume in individual myofibers.

Recent studies have focused on diverse molecular cascades that control the balance of dynamic anabolic and catabolic reactions and thus determine the level of muscle proteins. Essentially, all atrophic conditions share an imbalance of this system, which leads to the activation of ubiquitin ligases. MAFbx/atrogin-1 and MuRF1, two muscle specific E3 ubiquitin ligases, are now considered as muscle atrophy markers whose expression is strongly up-regulated in most, if not all, experimental models of muscle loss [4,5,8].

Many studies on muscle wasting rely on animal models in which muscle homeostasis is affected by altered systemic conditions, such as cancer associated cachexia, diabetes and fasting, or by the removal of nerve supply through denervation or spinal cord isolation [7]. Although these models may be suitable for studying disease-induced muscle atrophy, the concurrent presence of systemic alterations may make it more difficult to dissect the muscle-specific mechanisms leading to atrophy occur. By contrast, a model of muscle disuse may prove very useful in studies on therapeutic interventions of muscle atrophy using transgenic and mutant mouse strains. Although hindlimb suspension simulates human disuse atrophy, this technique appears to be more suited to studies on microgravity than on disuse [6]. Bilateral cast immobilization, another model of muscle atrophy, is instead complex and invasive, which may cause stress-induced systemic alterations in the mouse [3]. In this paper we describe a simple model of unilateral hindlimb immobilization in mice; we show that this protocol induces significant muscle atrophy, as revealed by both the morphological and molecular analysis. We thus propose it as a reliable model for muscle disuse in mice.

Materials and Methods

Animals

10-week-old C57BL6 male mice were used throughout the study. The animals were housed in the accredited animal facility room in our department, at controlled temperature (22°C) and humidity (≈50%), in a 12h light/dark cycle. All procedures were conducted in conformity with the institutional guidelines that are

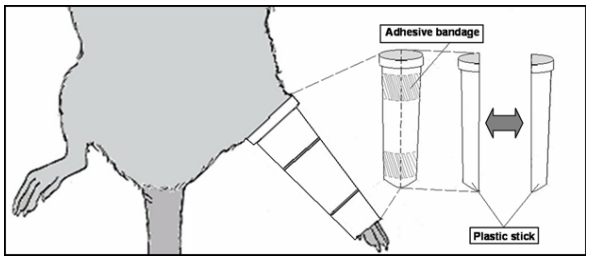

Fig. 1 *Unilateral hindlimb immobilization. Drawing of the method used to immobilized mouse hindlimb: a plastic stick was placed over and under the limb and fixed with a medical adhesive bandage*

in compliance with national and international laws and policies.

Unilateral immobilization.

Mice were anesthetized with a 1:1 concentration of Zoletil and Rompum. Randomly, one hindlimb was immobilized with a plastic stick placed over and under the limb and fixed with a medical adhesive bandage. Animals were monitored on a daily basis for chewed plaster, abrasions and problems with ambulation. The animals were free to move and ate and drank ad libitum. The immobilization procedure prevented movement of the immobilized leg alone. After 7 days the animals were sacrificed and the EDL and the TA muscles were removed from both hindlimbs, the contralateral, non-immobilized leg being used as an internal control.

Fasting.

Age- and sex-matched mice were fasted for 48h; the animals were then sacrificed and the EDL and the TA muscles were removed.

Morphological analysis.

Myofiber cross-sectional area (CSA) was determined in cryosections (7µm) of the EDL and the TA muscles stained with haematoxylin/eosin (H/E) staining. Digital pictures were obtained with a CCD camera and acquired using the Axio Vision 2.1 software. Myofiber CSA was measured in all myofibers within each section, using the Scion Image software (Beta 4.0.2 version, Scion Corporation, Federick; MD). For NADH-TR staining, cryosections were incubated for 15 min with a mixture containing NADH, nitrotetrazodium blue in Tris (pH 7.4) at 37°C, as described [1]. The number of dark and light stained myofibers was determined in the entire section and expressed as percentage of the total number of myofibers. Modified Gomori trichrome staining of cryosections was used to determine connective tissue composition.

Statistical analysis.

Statistical analysis was conducted using the t test. The criterion for statistical significance was * $p \leq 0.05$.

RT-PCR.

Total RNA extraction and cDNA synthesis were performed as described [2]. The following PCR protocol was applied: 7 min at 94°C, followed by 28 (atrogin-1) or 35 (MurF1) or 25 (GAPDH) cycles of PCR (30 sec at 94°C, 30 sec at annealing temperature, 60 sec at 72°C) and a final 10-minute extension step at 72°C. PCR products were separated on 1% agarose gel containing ethidium bromide. The following primers were used for PCR amplifications: atrogin-1: fw AAC

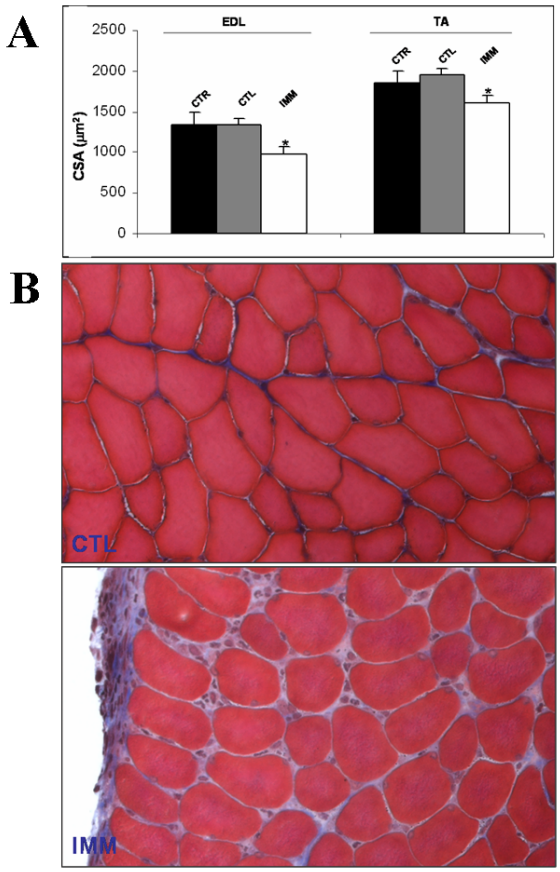

Fig. 2. *Seven-day immobilization induces muscle atrophy.*
*A) Muscle fibre CSA was measured in H/E - stained cryosections of the EDL or TA, as indicated, derived from: untreated mice (CTR); contralateral non-immobilised limb (CTL) or the hindlimb immobilised for 7 days (IMM). *$p < 0.05$*
B) Representative images of Gomori trichrome stained crysections of TA derived from the contralateral non-immobilised hindlimb (CTL) or from the 7 days immobilised hindlimb (IMM).

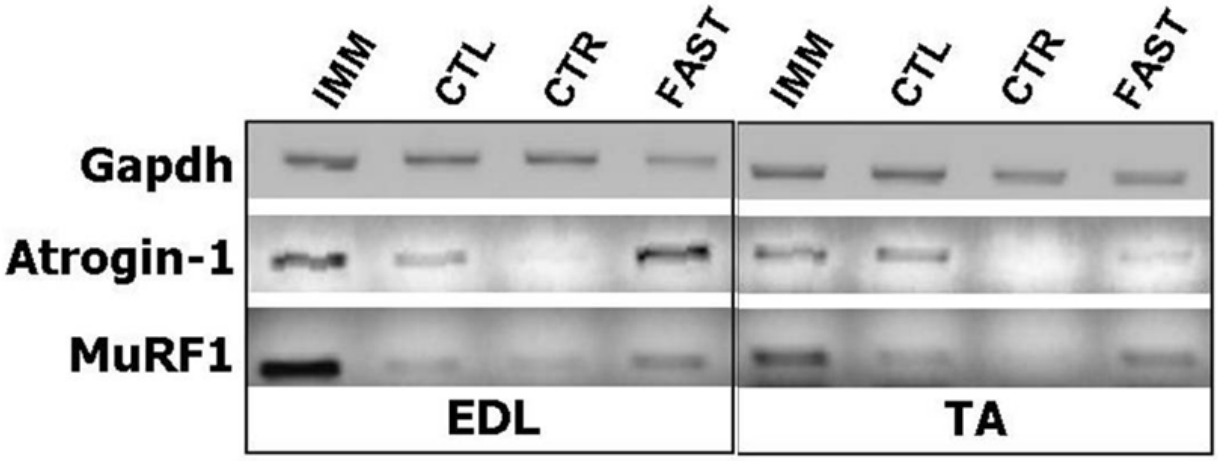

Fig. 3. *Seven-day immobilization induces the expression of the muscle specific E3 ubiquitin ligases. RT-PCR analysis of the expression of MAFbx/atrogin-1 and MuRF-1 in EDL and TA as indicated, derived from: hindlimb immobilised for 7 days (IMM), contralateral non-immobilised limb (CTL) or untreated mice (CTR); muscles from mice fasted for 48hrs (FAST) were also analysed as a positive control. The reactions were normalised for GAPDH expression.*

ATG TGG GTG TAT CGG and rev TCT TGA GGG GAA AGT GAG (annealing temperature: 51.7°C, expected band 464bp); MuRF-1: fw GTT AAA CCA GAG GTT CGA G and rev ATG GTT CGC AAC ATT TCG G (annealing temperature 52.5°C, expected band 342bp); GAPDH: fw ATG TTC CAG TAT GAC TCC ACT CAC G and rev GAG TTG CTG TTG AAG TCG CAG GAG ACA A (annealing temperature: 64°C, expected band 700bp).

Results and Discussion

Unilateral immobilization was assessed as in Figure 1. After seven days, the EDL and TA were dissected from both the immobilized hindlimb and the contralateral hindlimb (used as internal control) of the mice subjected to immobilization, as well as from sex- and age-matched control mice, which were not subjected to immobilization. Figure 2A shows that myofiber CSA, as determined by H/E staining on crysections, was significantly lower in both these muscles following immobilization than in the same muscles from the contralateral and control mice hindlimbs. The decrease in CSA following immobilization in our study is in keeping with data obtained by means of other inciting events to induce muscle atrophy [4]; by contrast, hindlimb suspension, which is the most widely used animal model for muscle disuse, has been reported to yield a hypertrophic response in the EDL during the first week of suspension [9]. This suggests that unilateral immobilization is a more reliable model for muscle disuse. To determine whether immobilization induced an increase in intra-muscular connective tissue as a result of the myofiber reduction, cryosections were stained with the modified Gomori trichrome staining; as shown in Figure 2B, an increase in intra-fibre connective tissue in the immobilized muscle, if compared with the contralateral muscle, was observed.

Table 1. *The percentage of glycolitic (light) or oxidative (dark) fibres was counted in cryosections of both the EDL and the TA, derived from: untreated mice (CTR); contralateral non-immobilised limb (CTL) or the hindlimb immobilised for 7 days (IMM).*

Fiber Type content

	EDL		TA	
	% Gly	% Ox	% Gly	% Ox
CTR	47,4 ± 2,40	52,5 ± 2,40	40,5 ± 2,02	59,4 ± 2,02
CTL	50,4 ± 5,50	49,5 ± 5,50	40,6 ± 2,65	59,3 ± 2,65
IMM	44,9 ± 4,82	55,0 ± 4,82	41,0 ± 3,68	58,9 ± 3,68

No morphological signs of fibre necrosis or regeneration were observed. Taken together, these results demonstrate that muscle atrophy was specifically induced in muscles derived from the limb immobilized for seven days. Moreover, nomorphological adaptation was observed in muscles derived from the contralateral hindlimb, which suggests that immobilization did not alter systemic processes.

As muscle atrophy is often accompanied by alterations in fiber type content, crysections from both the EDL and TA (immobilized and contralateral) were stained for NADH transferase; as shown in Table 1, no changes in the percentage of glycolitic (light) and oxidative (dark) fibres were observed after 7 days of immobilization in either muscle, if compared with the contralateral muscle from treated mice or muscles from untreated mice. These results suggest that immobilization does not induce any fibre metabolism changes, though it should be borne in mind that seven days of immobilization may not be enough to determine such changes. A longer period of immobilization is required to clarify whether the metabolic changes often observed in muscle wasting models are due to muscle specific adaptation or whether they are a result of systemic (induced) alterations.

To verify whether the observed muscle atrophy was due to the up-regulation of the E3-ubiquitine ligases, and consequently to increased protein degradation, expression of the muscle-specific ubiquitin ligases MAFbx /atrogin-1 and MuRF1 was analysed by RT-PCR. RNA was extracted from the EDL and TA isolated from the immobilized and contralateral hindlimbs of the treated mice, as well as from the hindlimbs of the untreated mice. As a positive control, muscles from mice which had been fasted for 48 hours were also used. As shown in Figure 3, the expression of both MAFbx/atrogin-1 and MuRF1 was markedly induced in both muscles derived from the immobilized hindlimbs, though at different levels; the level of expression was comparable to, if not higher than, the level of expression observed in the same muscle after fasting. Same results have been obtained using the Soleus muscle (not shown). Surprisingly, both markers were also slightly expressed in muscles derived from the contralateral hindlimb of the treated mice, whereas no expression of any of those markers was, as expected, observed in the control muscles derived from the untreated mice. Taken together, these results indicate that the mere immobilization of limb movement activates proteasome mediated protein degradation, as a result of muscle adaptation, regardless of the functional activity of the single muscle; interestingly, muscles from the contralateral (freely movable) limb sensed the general condition, consequently up-regulating, though to a lesser extent, the same atrophic markers, which did not, however, induce any morphological changes. This finding may be due to the fact that the mouse has to adapt to the reduced movement during the first days of immobilization, though this may only be clarified by a longer period of immobilization.

Concluding remarks

In conclusion, as this simple model of unilateral immobilization induces muscle atrophy, regardless of the functional activity of the single muscles, and does not dramatically modify mice welfare, we believe it may be highly suited to studies on muscle atrophy. In addition, this model may be also applied for studies on muscle reload after disuse, although further experiments are needed to verify this aspect.

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