The worsening of tibialis anterior muscle atrophy during recovery post-immobilization correlates with enhanced connective tissue area, proteolysis, and apoptosis

Lamia Slimani,1,2 Didier Micol,3 Julien Amat,1,2 Geoffrey Delcros,1,2 Bruno Meunier,3 Daniel Taillandier,1,2 Cécile Polge,1,2 Daniel Béchet,1,2 Dominique Dardevet,1,2 Brigitte Picard,3 Didier Attaix,1,2 Anne Listrat,3 and Lydie Combaret1,2

1Institut National de Recherche Agronomique (INRA), Unité Mixte de Recherche (UMR), 1019 Unité de Nutrition Humaine 63122, Saint Genès Champanelle, France; 2Clermont Université, UFR Médecine, UMR 1019 Nutrition Humaine 63000, Clermont-Ferrand, France; 3INRA, UMR 1213 Herbivores 63122, Saint Genès Champanelle, France

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SKELETAL MUSCLE IS THE MAJOR RESERVOIR of body proteins. Sustained muscle wasting due to immobilization leads to weakening and severe metabolic consequences, which impacts health care costs. This wasting has been investigated thoroughly and results from an imbalance between protein synthesis and breakdown rates but also between apoptotic and regeneration processes (12, 17, 35, 46, 47). By contrast, the mechanisms responsible for muscle recovery after remobilization are poorly defined.

The ubiquitin (Ub)-proteasome system (UPS) is involved in the breakdown of the major contractile proteins in catabolic conditions. Briefly, in this pathway a poly-Ub chain is covalently attached to the substrate by the Ub conjugation machinery, and the targeted protein is then recognized and degraded by the 26S proteasome (4). This pathway is activated after immobilization or unloading in atrophying muscles from animals (2, 25, 35, 45, 46) or humans (23, 28, 41).

The muscle-specific ubiquitin ligases (E3) muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)/atrogin-1 play key roles in disuse-induced-muscle atrophy (7). In addition, MuRF1 is involved in the ubiquitination of the major contractile proteins such as actin and myosins (13, 14, 40).

DNA fragmentation, caspase activities, and the mitochondria-associated apoptotic pathway are also enhanced in immobilized (35, 46) and unloaded (1, 2, 6, 39) atrophying muscles.

Investigating the mechanisms responsible for muscle recovery in the cast immobilization model is very clinically relevant since it better mimics events prevailing with fractures and bed rest-associated immobilization compared with unloading models. However, few groups have investigated muscle recovery pending remobilization. Muscle recovery is delayed after cast removal (46, 47). In addition, tibialis anterior (TA) and gastrocnemius (GA) muscles did not exhibit the same kinetics of recovery. In fact, there is a worsening of the TA atrophy 10 days after cast removal (46). This surprising observation appears to be a distinct feature of the TA, suggesting a more important remodeling of that muscle. This may result from the position of immobilization. In fact, TA and GA muscles are located in the anterior and posterior hindlimb compartment, respectively, and were thus immobilized in a lengthened and shortened position, respectively. Connective tissue thickening that prevailed during immobilization (49) was greater when lengthening was applied (37).

In addition, changes in fiber type composition reported during immobilization and/or recovery may depend on the muscle studied and on its location (16, 22, 37, 50). Finally, the position of immobilization was also previously reported to influence the regulation of muscle protein turnover (21). Both UPS and the mitochondria-associated apoptotic pathways were normalized in GA within 10 days after cast removal (35, 46). To the best of our knowledge, there is no study in the TA

Address for reprint requests and other correspondence: L. Combaret; INRA, UMR 1019 Unité de Nutrition Humaine, 63122 Saint Genès Champanelle, France (e-mail: lydie.combaret@clermont.inra.fr).
investigating the role of these pathways during early recovery periods following cast immobilization. Thus, whether muscle structural alterations and/or the UPS and apoptosis may be involved in the remodeling of the TA during early recovery after cast immobilization remains unknown.

Therefore, we hypothesized that 1) the lengthened TA muscle may exhibit specific modifications of fiber type composition and/or more extensive connective tissue damages compared with the shortened GA muscle during immobilization and 2) the UPS and the mitochondria-associated apoptotic pathway could be involved in the worsening of TA muscle atrophy during early recovery.

MATERIALS AND METHODS

Animals and experimental design. The present study was approved by the Animal Care and Use Committee at the Institut National de la Recherche Agronomique (INRA) and adhered to the current legislation on animal experimentation in France. Six-month-old male Wistar rats (Charles River Laboratories, L’Arbresle, France) were housed individually in controlled environmental conditions (room temperature 22°C; 12:12-h light-dark cycle, light period starting at 8 h), fed ad libitum (46, 47), and given free access to water. After a 3-wk adaptation period, rats were anesthetized with a mixture of ketamine and xylazine (Gibaud). Rats were casted as described previously (34, 35, 46, 47) so that GA and TA muscles were immobilized in a shortened and a lengthened position, respectively. Muscles from the contralateral noncasted leg did not exhibit hypertrophy or atrophy during immobilization due to increased workload or to changes in food intake respectively, as described previously (46), and served as controls. After 8 days of immobilization (18; n = 11), casts were removed and animals (n = 10–11) were euthanized for recovery at 1 (R1), 3 (R3), 6 (R6), 8 (R8), and 10 (R10) days. For convenience, muscles that were immobilized between 10 and 18 during the recovery period are named “remobilized” in this article (R1-R10). A group of noncasted rats was also included (10; n = 11). At the end of the immobilization or recovery periods, animals were euthanized under pentobarbital sodium anesthesia (50 mg/kg ip). GA and TA skeletal muscles were carefully dissected, weighed, and frozen in liquid nitrogen. A central part of one head of GA and TA muscles was fixed at room temperature using a modified method of Tissue-Tek OCT (Sakura Finetek, France) and then frozen in cooled isopentane (−100°C) and stored at −80°C until histological analyses. The remaining part was finely pulverized in liquid nitrogen and stored at −80°C until further analyses.

Histological analyses of muscles. Ten-micrometer-thick TA and GA cross-sections were performed at −25°C using a cryostat (HM500M Microm International) and stained with Picro-Sirius red, which reveals intramuscular connective tissue (19). Observations and image acquisitions were performed using a photonic microscope in bright-field mode (Olympus BX-51, Tokyo, Japan) coupled to a high-resolution cooled digital camera (Olympus DP72) and Cell-D software (Olympus Soft Imaging Solutions, Münster, Germany). For each muscle section, three color images were acquired using a ×10 objective, with each image representing a 879 × 662 μm² field of view. Image analysis was performed using the Visilog 6.9 software (Noesis, France). Briefly, the green component of the initial image was used for higher contrast. Top-hat filtering followed by manual thresholding on gray level allowed segmentation of the connective tissue network (perimysium and endomysium). Measurement of the area of this network was performed by counting the number of pixels in the resulting binary images and was expressed as percent of the total field area. This connective network is potentially discontinuous because of very low thickness of the endomysium in some areas (see Fig. 3). Thus, we applied the watershed algorithm to automatically close this network. When necessary, the network was manually closed. This step results in separating objects corresponding to muscle cells.

RNA extraction and real-time PCR. Total RNA was extracted from TA samples using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined with a Nanodrop ND 1000 spectrophotometer, and the integrity of the RNA was evaluated with a bioanalyzer (Agilent Technologies).

One microgram of RNA was treated with DNase I Amp grade (Invitrogen) prior to cDNA synthesis. Treated RNA was reverse transcribed using random primers and superscript II reverse transcription kit (Invitrogen) according to the manufacturer’s instructions.

Real-time PCR was carried out using the CFX96 Real-Time PCR detection system (Bio-Rad). For PCR reactions, a master mix of the following reaction components was prepared: 3.6 μl of water, 0.7 μl of each forward and reverse primer (10 μM), 7 μl of IQ SYBR Green Supermix (Bio-Rad), and 2 μl of a 50-fold dilution of the reverse-transcribed total cDNA. The reaction mixture was initially denatured at 95°C for 3 min then cycled 40 times. In each cycle, the mixture was kept at 95°C for 10 s plus 60°C for 20 s. The PCR reaction was supplemented by a melting curve analysis process in a temperature range from 55 to 95°C with increments of 0.5°C at each step. A negative control without cDNA template was run with every assay to assess the overall specificity. Primers for rat sequences (Table 1) were used and yielded PCR products 100 bp in size. 18S rRNA was also amplified to normalize for mRNA content. The comparative threshold

<table>
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<tr>
<th>Primer Names</th>
<th>Accession No.</th>
<th>Primer Sense Sequences</th>
<th>Primer Antisense Sequences</th>
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<tr>
<td>Col IA1</td>
<td>NM_053304</td>
<td>5'-CCCTGCTGTGTGAAAGGAT-3'</td>
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Col, collagen; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CstL, cathepsin L; Trim63, muscle RING finger 1; Fbxo32, muscle atrophy F-box/atrogelin-1.

Table 1. Primers used for quantitative RT-PCR analysis
cycle \((2^{\Delta A C})\) method \((33)\), with 18S rRNA as a reference gene, was used to compare the relative mRNA expression between each group, where the relative mRNA abundance was arbitrarily set to 1 for the \(I_0\) group.

**Separation of MyHC isoforms.** Samples of GA and TA from contralateral control and immobilized hindlimbs at each time point were homogenized using a polytron in 10 vol of an ice-cold buffer (pH 7.4) that was freshly prepared \(20 \text{ mM HEPES}, 50 \text{ mM } \beta\text{-glycerophosphate, } 100 \text{ mM potassium chloride, } 50 \text{ mM sodium chloride, } 2 \text{ mM EGTA, } 0.2 \text{ mM EDTA, } 1 \text{ mM dithiothreitol (DTT), } 1 \text{ mM benzamidine, } 0.5 \text{ mM sodium vanadate, } 1 \text{% Triton X-100, } 0.1 \text{ mM phenylmethylsulfonyl fluoride (PMSF), }\) and proteinase inhibitor cocktail \(\text{SIGMA-Aldrich) to distinguish myofiber nuclei from connective tissue nuclei. Hence, the proportion of apoptotic nuclei was quantified by morphometry using a high-resolution digital camera \(\text{Olympus BX-51) coupled with the Visilog 6.9 software (Noesis). Finally, connective tissue and myofiber apoptotic nuclei were localized after images were merged using a dedicated image analysis application developed under the Visilog 6.9 software (Noesis).}

**Apoptotic nuclei quantification.** Ten-micrometer-thick frozen GA and TA cross-sections (cryostat HM500M Microm International) were triple-stained with the TUNEL \(\text{terminal deoxynucleotidyl-mediated dUTP nick-end labeling} \) fluorescent detection kit \(\text{Roche Applied Science, Meylan, France}) green to detect apoptotic nuclei, with Hoechst dye 33258 \(\text{blue} to verify that TUNEL labeling corresponded to nuclei, and then with laminin-2 antibody \(\text{red: Sigma-Aldrich) to distinguish myofiber nuclei from connective tissue nuclei. Brie}fely, apoptotic nuclei were detected using the TUNEL fluorescent detection kit according to the manufacturer’s instructions. For all groups, positive controls were carried out on cross-sections after prior incubation with 0.12 \(\mu\text{g/} \mu\text{l} \text{DNAse I} \) \(\text{Roche Applied Science). Negative controls were incubated using labeling solution instead of TUNEL solution. Then, after antigenic site saturation with 10% goat serum in \(\times 1\text{ PBS, sections were incubated with primary anti-laminin-2a at } 1:200 \text{ dilution to label myofiber basal lamina (1 h, } 37^\circ\text{C), rinsed with } \times 1\text{ PBS (10 min), and then incubated with the secondary antibody conjugated to DyLight 546 (Life Technologies, Saint Aubin, France) at a 1:400 dilution (30 min, } 37^\circ\text{C). Muscle sections were then washed twice with } \times 1\text{ PBS, incubated for 1 min with Hoechst 33258 (1:10,000 in } \times 1\text{ PBS, and mounted with Fluoromount (Sigma-Aldrich). Observations and image acquisitions were performed using a fluorescent microscope \(\text{Olympus BX-51) coupled with a high-resolution cooled digital camera \(\text{Olympus DP72} \) and Cell-D software \(\text{Olympus Soft Imaging Solutions, Münster, Germany). Finally, connective tissue and myofiber apoptotic nuclei were localized after images were merged using a dedicated image analysis application developed under the Visilog 6.9 software (Noesis).}

**Finally, the green image was also filtered and binarized to detect fluorescent dots corresponding to potential apoptotic nuclei. To reduce false-positive detection due to the inherent variability of the TUNEL method \((40)\), only the center of gravity of each fluorescent dot was overlapped and tested for perfect colocalization with both the fiber and connective tissue nuclei. Hence, the proportion of apoptotic nuclei \(\%\) in fiber and in connective tissue was deduced with confidence and for a representative population.

**Fig. 1.** Food intake and body weight of rats during immobilization and recovery. Food intake (A) and body weight (B) are expressed as g/day and g, respectively. Data are means \(\pm SE\) for \(n = 10–11\) rats. Differences during immobilization and recovery were assessed by ANOVA \(*P < 0.05\) vs noncasted controls \(\text{R0}\). \(I_8\), 8 days of hindlimb immobilization; \(R_1\) to \(R_{10}\), recovery period in days after cast removal.

**Fig. 2.** Immobilization-induced muscle atrophy worsened rapidly after cast removal in the tibialis anterior. Muscle atrophy is expressed in \%difference from the contralateral control leg. Values are means \(\pm SE\) for \(n = 10–11\) rats. Statistical differences were assessed by ANOVA. * \(P < 0.05\) vs contralateral controls. Bars with different letters within a given muscle are significantly different.
Measurement of proteasomes and apoptosome activities. Samples of GA and TA from immobilized and contralateral control hindlimbs were homogenized in 10 vol of an ice-cold buffer (pH 7.5) [50 mM Tris-HCl, 250 mM sucrose, 10 mM ATP, 5 mM MgCl₂, 1 mM DTT, and protease inhibitors (10 µg/ml antipain, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 0.2 mM PMSF)]. Apoptosome complexes cosediment with proteasomes (11), and the apoptosome-linked caspase 9 activity coelutes with peptide activities of the proteasome on 10–40% sucrose gradients (11, 46). Therefore, apoptosome complexes and proteasomes were isolated by three sequential centrifugations, as described (18). Briefly, extracts were centrifuged at 10,000 g for 20 min at 4°C. Supernatants were then centrifuged at 100,000 g for 1 h at 4°C. The resulting supernatants were finally centrifuged at 100,000 g for 5 h at 4°C. The resulting protein pellets were resuspended in 150 µl of a buffer containing glycerol (20%), 50 mM MgCl₂, and 5 mM Tris-Cl, pH 7.5 (buffer B). Protein concentration was determined on these resuspended pellets using the Bio-Rad protein assay kit.

The proteasome chymotrypsin- and trypsin-like activities and the apoptosome-linked caspase 9 activity were determined by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC), succinyl-Leu-Leu-Val-Tyr-Arg-AMC (LRR-AMC), and N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (LEHD-AMC) (Enzo Life Sciences), respectively. Five microliters (10–15 µg of proteins) of the resuspended pellets from GA or TA were diluted in 15 µl of buffer B and added to 60 µl of a reaction buffer [50 mM Tris-Cl (pH 8), 11.25 mM MgCl₂, 1.25 mM-DTT, and 0.01 U apyrase] containing either 300 µM LLVY-AMC or 800 µM LRR-AMC to measure the proteasome chymotrypsin- and trypsin-like activities, respectively. To measure the apoptosome-linked caspase 9 activity, 25 µl (~75 µg of proteins) of the resuspended pellets was diluted to 50 µl in buffer B and then incubated with 50 µl of a reaction buffer [100 mM PIPES (pH 6.5), 0.2 mM EDTA, and 5 mM DTT] containing 100 µM LEHD-AMC (Enzo Life Sciences). Preliminary experiments were performed to ensure that the caspase 9 assay does not detect the activity of the caspase site of the proteasome. In addition, pilot experiments were performed with or without inhibitors of the chymotrypsin-like (40 µM MG132; Enzo Life Sciences), the trypsin-like (100 µM MG132), and the caspase 9 (50 µM LEHD-CHO; Enzo Life Sciences) activities to ensure full inhibition. Activities were determined by measuring the accumulation of the fluorogenic cleavage product (AMC) using a luminescence spectrometer FLX800 (Biotek) for 45 min at 380-nm excitation wavelength and 440-nm emission wavelength. The time course for the accumulation of AMC after hydrolysis of the substrate was analyzed by linear regression to calculate activities, i.e., the slopes of best fit of accumulation AMC vs. time.

MuRF1 protein content. The expression of the MuRF1 E3 ubiquitin ligase was assessed by immunoblotting. Seventy-five micrograms of the total protein extract obtained as described above (see separation of MyHC isoforms) was separated by SDS-PAGE on a 12% acrylamide gel (acrylamide-his (29:1)) and then transferred on nitrocellulose membranes (Li-Cor Biosciences, ScienceTec, Courtaboeuf, France). The antibody against MuRF1 (R & D Systems) was used at a 1:200 dilution. Immunoblots were revealed using the Li-Cor Odyssey (Li-Cor Biosciences) procedures after preincubation with a fluorescent-labeled secondary antibody (IRdye 800CW Donkey Anti-Goat IgG; Li-Cor Biosciences) used at a 1:3,000 dilution. The antibody against MuRF1 (R & D Systems) was used at a 1:200 dilution. Immunoblots were revealed using the Li-Cor Odyssey (Li-Cor Biosciences) procedures after preincubation with a fluorescent-labeled secondary antibody (IRdye 800CW Donkey Anti-Goat IgG; Li-Cor Biosciences) used at a 1:3,000 dilution. The densitometry was performed using the Odyssey 2.1 software (Li-Cor Biosciences).

Statistical analysis. Statistical analyses were performed using Statistical Analysis Systems 9.1 procedures (SAS Institute, Cary, NC). Data were analyzed for normality and equal variances. No set of data was transformed for nonnormality distribution. In a first step, data
were analyzed by three-way ANOVA (GLM SAS Procedure) for the fixed effects of muscle (GA, TA), immobilization, and stage of immobilization and recovery (I0 up to R10). A strong interaction “muscle × stage” appeared with nearly all of the variables ($P < 0.001$). Consequently, in a second step, statistical analyses were performed within each muscle (GA or TA) by two-way ANOVA for the effects of immobilization and stage of immobilization/recovery and their interactions. In these analyses, an animal effect (paired effect) nested within the stage of immobilization/recovery was introduced to take into account the fact that the Con and Imm muscles were sampled from the same animal. Multiple comparisons of adjusted means were based on Tukey’s test. Level of significance was set at $P \leq 0.05$.

**RESULTS**

*Animal characteristics.* Food intake decreased by 15% ($P < 0.05$) in casted rats between I0 and I8 and returned progressively to initial I0 values 6 days after cast removal (R6, Fig. 1A). By contrast, body weight slightly decreased by ~7% at R6 compared with I0 ($P < 0.05$; Fig. 1B).

*Gastrocnemius and TA muscle atrophy during immobilization and early recovery.* Figure 2 shows that immobilized GA and TA muscles atrophied by 22 ($P < 0.001$) and 7% ($P < 0.05$), respectively, at I8 compared with contralateral control muscles. Immediately after cast removal, the atrophy stabilized and remained constant in the remobilized GA compared with contralateral controls from R1 to R10 ($P < 0.001$). By contrast, the remobilized TA further atrophied by 15% at R1 ($P < 0.001$) and by 32% at R6 ($P < 0.001$) and then started to improve at R8 and R10 (~26%, $P < 0.001$) compared with contralateral controls. Thus, the worsening of the remobilized TA occurs very early pending cast removal (Fig. 2).

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**Fig. 4.** Changes in mRNA levels for some major collagens, matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (TIMP) in the immobilized and remobilized tibialis anterior muscles. Data were normalized using 18S rRNA and are expressed as fold induction compared with I0 group. Data are means ± SE for $n = 4–5$ rats. *$P < 0.05$ vs. Con; # $P < 0.05$ vs. I0 group; § $P < 0.05$ vs. I8 for the same leg treatment.
Connective tissue area alterations in GA and TA muscles during immobilization and early recovery. We hypothesized that the lengthened TA may exhibit extensive connective tissue damages compared with the shortened GA in the immobilization period. Therefore, we used Sirius red staining (Fig. 3A) to assess such changes in connective tissue (CT) area in both muscles during the whole kinetic studied. CT area increased similarly between immobilized and contralateral control GA and TA at I8 (+22 and +14%, respectively, \( P < 0.05 \)) and remained elevated until R10 in both remobilized muscles (GA: +28%; TA: +32%, \( P < 0.05 \)) (Fig. 3B). By contrast, there were significant differences between muscles compared with I8. In the GA, there was no significant change in the CT area after cast removal between I8 and R10. However, CT area further increased in remobilized TA starting at R6 compared with I8. Thus, an additional thickening of CT prevailed during early recovery in the remobilized TA but not in the GA.

We next investigated changes in mRNA levels for some major collagens, matrix metalloproteinases (MMPs; which degrade collagens) and tissue inhibitors of metalloproteinases (TIMPs) in the TA (Fig. 4). mRNA levels for Col IA1 (i.e., the major component of type I collagen) tended to be elevated in immobilized TA (\( \pm 200\% \), \( P = 0.14 \)) compared with the I0 group and further increased during remobilization at R1, R6, R8, R10.
and R10 5- to 7.5-fold (P < 0.05). Levels of mRNA encoding Col XII, which interacts with type I containing fibrils, also increased in immobilized (I8, +280%, P < 0.05) and remobilized (R1, +500%, P < 0.05) TA compared with the I0 group. Similarly, mRNA levels for Col XVIII and Col IVA1 were elevated in the immobilized TA at I8 (+116 and 92%, respectively, P < 0.05) and remained high in the remobilized TA at R1 (+167 and 62%, respectively, P < 0.05) compared with the I0 group. Col XII, Col IVA1, and Col XVIII mRNA levels returned progressively to basal values at R6 and R10, whereas expression of Col IA1 remained high until R10 (+530%, P < 0.05). mRNA levels for MMP-2 and -14 did not change in the immobilized TA at I8 but increased at R1 (+217 and 192%, respectively, P < 0.05) compared with the I0 group and then returned progressively to basal values at R10. TIMP-1 and -2 mRNA levels increased in the immobilized TA at I8 by ~30- and ~2-fold, respectively, remained elevated in the remobilized TA at R1, and returned to basal levels at R6.

Distribution of MyHC isoforms in GA and TA muscles during immobilization and early recovery. The distribution of MyHC isoforms was investigated after separation of types I, IIb, IIX, and Ila MyHC by SDS-PAGE (Fig. 5A) in TA and GA during immobilization and subsequent recovery. The proportion of these MyHC isoforms did not change in immobilized and remobilized GA compared with contralateral controls, except at the very end of the recovery period studied (R8 and R10) (Fig. 5, B–E).

There were significant differences between immobilized and contralateral control TA for all MyHC isoforms during immobilization and recovery periods, except for type I and type Ila MyHC at R1. In the immobilized TA, the proportion of type IIB MyHC isoforms decreased (~14%, P < 0.05) concomitantly with an increase in type I (+211%), type IIX (+24%), and type Ila (+84%) at I8 compared with contralateral control muscles (Fig. 5, B–E). These changes worsened during recovery, particularly at R6 and R8.

Altogether, these data suggest pronounced and sustained modifications of the TA contractile properties during remobilization.

Regulation of the Ub-proteasome-dependent pathway and autophagy in GA and TA muscle during immobilization and early recovery. To test whether the UPS was involved in the worsening of the TA atrophy after cast removal, we next measured 1) the protein and mRNA expression of the muscle-specific E3 ligases MAFbx/atrogin-1 and MURF1, which polyubiquitinate specific contractile proteins (13, 14, 40), and
2) the chymotrypsin- and trypsin-like activities of the proteasome involved in their breakdown.

Figure 6 shows that MuRF1 protein levels increased in immobilized GA at I8 (+81%, \( P < 0.05 \) vs. contralateral controls) and remained elevated when remobilized until R8 (+40–48%, \( P < 0.05 \) vs. contralateral controls). The increase in MuRF1 protein levels was more pronounced in immobilized TA muscles at I8 (+158%, \( P < 0.05 \) vs. contralateral controls), remained elevated when remobilized at R1 and R3 (+150 and +147%, respectively, \( P < 0.05 \) vs. contralateral controls), and then declined progressively from R6 (+76%, \( P < 0.05 \) vs. contralateral controls) until R10 (not significant). Figure 7 shows that MuRF1 and MAFbx/atrogin-1 mRNA levels also increased at I8 in the immobilized GA (+485 and 270% respectively, \( P < 0.05 \) vs. contralateral controls) and were normalized at R6. By contrast, mRNA levels for MuRF1 and MAFbx/atrogin-1 did not change at I8 in the immobilized TA but increased at R1 (~+90%, \( P < 0.05 \) vs. contralateral controls) and then returned to basal levels at R6.

The proteasome chymotrypsin-like activity increased significantly in remobilized GA muscle only at R1 and R3 (+28 and +46%, respectively, \( P < 0.005 \) vs. contralateral control; Fig. 8A). The trypsin-like activity followed a similar pattern (Fig. 8B).

By contrast, both chymotrypsin- and trypsin-like activities increased markedly in immobilized TA at I8 (+113 and +122% respectively, \( P < 0.001 \) vs. contralateral controls) and remained elevated until R10 (+112 and +67% respectively, \( P < 0.005 \) vs. contralateral controls). Altogether, the UPS was activated very early in remobilized muscles, and this activation was more pronounced and persistent in the TA than in the GA.

The role of autophagy in muscle atrophy and recovery pending immobilization has been assessed by measurement of mRNA levels for cathepsin L, LC3b, and beclin-1. Figure 9 shows that cathepsin L mRNA levels increased in both immobilized GA and TA compared with contralateral muscles at I8 (+300 and 160%, respectively, \( P < 0.001 \) vs. contralateral controls and R1 (+400 and 210%, respectively, \( P < 0.001 \) vs. contralateral controls) and returned to basal levels at R6. mRNA levels for LC3b increased only in the GA at I8 (+350%, \( P < 0.001 \) vs. contralateral controls) and returned to basal levels immediately after cast removal. mRNA for beclin-1 increased at I8 and R1 (~+200%, \( P < 0.001 \) vs. contralateral controls) in the immobilized and remobilized GA, respectively. By contrast, they increased at R6 (+260%, \( P < 0.001 \) vs. contralateral controls) and R10 (+60%, \( P < 0.001 \) vs. contralateral controls) in the remobilized TA.

**Regulation of apoptosis in GA and TA muscles during immobilization and early recovery.** We next investigated the role of the mitochondria-associated apoptotic pathway during early recovery. Figure 10 shows that apoptosome-linked caspase 9 activity increased only in the remobilized GA at R1 (+31%, \( P < 0.05 \) vs. contralateral controls), as did proteasome activities (Fig. 8). In the immobilized TA muscle, this activity was enhanced at I8 (+79%, \( P < 0.05 \) vs. contralateral controls) and remained elevated when remobilized until R8 (+149%, \( P < 0.001 \) vs. contralateral controls), except at R1 (+33%; not significant).

These data suggest that apoptosis was also enhanced to a greater extent in the TA than in the GA during both the immobilization and recovery periods. Thus we investigated whether the proportion of apoptotic nuclei was different in both muscles at I8 (i.e., at the end of the immobilization period) and at R6 [i.e., at the time point of maximal TA atrophy (Fig. 2) and of connective tissue alterations (Fig. 3B)]. It was suggested previously that apoptosis does not occur within fibers undergoing atrophy during disuse but is more likely confined to stromal and satellite cells (10). Therefore, we assessed whether apoptosis may be altered specifically in the extracellular compartment. Myofiber and CT apoptotic nuclei were localized after triple labeling of GA and TA muscle cross-sections. The proportion of apoptotic nuclei did not change either in fiber or in the CT compartment in immobilized GA muscle compared with contralateral controls at I8 or at R6 (data not shown). By contrast, Fig. 11 shows that the proportion of apoptotic nuclei from immobilized (I8) and remobilized (R6) TA increased only within the CT compartment (+158 and +200%, respectively, \( P < 0.01 \) vs. contralateral controls). Altogether, these data suggest a more pronounced and sustained activation of the apoptosome-linked caspase 9 activity as well as an increase of CT apoptotic nuclei in the TA vs. the GA.

**DISCUSSION**

This study aimed to characterize the mechanisms responsible for the worsening of TA atrophy during early recovery following immobilization. We showed that this worsening occurs...
curred very early after cast removal, 2) correlated with a redistribution in MyHC isoforms, and 3) was associated with a sustained activation of both the UPS and apoptosome during early recovery. Furthermore, this worsening was associated with 4) pronounced muscle CT thickening, 5) sustained increased expression of the major fibrillar collagen I, and 6) transient increase of other collagens and markers of CT remodeling. Finally, 7) increased nuclear apoptosis prevailed only in the CT compartment.

We hypothesized that the early and pronounced worsening of TA atrophy after cast removal presumably implies or results from a large remodeling of muscle fibers. Our observations support this hypothesis. Myosins are major contractile proteins, and the proportion of MyHC isoforms is crucial for muscle metabolic and contractile properties. We report that the proportion of the type IIb MyHC isoform decreased, whereas other isoforms increased in the immobilized TA, as reported already in the unloaded TA (15). Our data further show that these changes worsened in the remobilized TA starting at R6, when TA muscle atrophy peaked at a maximum. Altogether, this suggests a transition of muscle fiber metabolic and contractile properties from fast-twitch glycolytic to slow-twitch oxidative, which worsened during early recovery. This transition may influence TA atrophy during immobilization and the subsequent recovery, because fibers expressing mainly type I MyHC seem to be a primary target for proteolysis, at least during unloading (5).

The present study also shows that skeletal muscle atrophy was associated with an increase in muscle connective tissue in both immobilized GA and TA compared with control muscles at the end of the immobilization period. This is in accord with previous studies conducted during cast immobilization or unloading (27, 29, 32, 37). During recovery, an additional thickening of connective tissue was observed only in the remobilized TA muscle. This modification peaked at R6, when TA muscle atrophy worsening was at a maximum. Whether there is a causal relationship between muscle connective tissue thickening and muscle atrophy is unclear. However, the expression of specific collagens and of TIMPs increased in the immobilized TA, without any change in MMP mRNA levels, suggesting both enhanced collagen synthesis and inhibition of collagen breakdown that contributed to collagen deposition. Interestingly, although MMP mRNA levels increased in the recovery period, TIMP-1 and -2 also increased in parallel (Fig. 4). Furthermore, the expression of the major fibrillar collagen I was enhanced during early TA remobilization. Therefore, the thickening of the TA connective tissue during remobilization (Fig. 3B) results from complex modifications of the equilibrium between collagen synthesis and breakdown. Altogether, connective tissue thickening may either impair muscle function and/or alter signal transduction from the extracellular compartment to myofibers. The stiffness of the extracellular compartment may be modified, influencing signal transduction to muscle fibers and proteolytic and apoptotic pathways. In fact, extracellular matrix (ECM) molecules include transmembrane proteins (i.e., integrins) that can associate with scaffold proteins and control intracellular signaling pathways, potentially regulating muscle protein turnover (i.e., PI3K/Akt, p38) and/or apoptosis (3, 9).

Furthermore, we report here that the increased apoptotic nuclei in the TA muscle was restricted to the connective tissue compartment during immobilization. Previous studies sug-
Fig. 9. Changes in mRNA levels for makers of autophagy in tibialis anterior and gastrocnemius muscles during immobilization and after cast removal. mRNA levels for cathepsin L, LC3b, and beclin-1 were measured by quantitative RT-PCR (see MATERIALS AND METHODS) in immobilized and contralateral control gastrocnemius and tibialis anterior muscles. Data were normalized using 18S rRNA, are expressed as % difference from contralateral leg, control gastrocnemius and tibialis anterior muscles. Data were normalized and expressed as % difference from contralateral leg and are means ± SE for n = 4–5 rats. *P < 0.05 vs. contralateral controls.

Fig. 10. Increased and sustained apoptosome-linked caspase 9 activity in the tibialis anterior muscle after cast removal. Apoptosome complexes were partially purified from gastrocnemius and tibialis anterior muscles. Apoptosome-associated caspase 9 activity was measured in immobilized and control gastrocnemius and tibialis anterior muscle extracts, as described in MATERIALS AND METHODS. Data are expressed as % difference from control leg and are means ± SE for n = 10–11 rats. *P < 0.05 vs. contralateral controls.
these E3s increased transiently at R1 in the remobilized TA and were rapidly normalized at R6, whereas protein levels remained elevated until R8. This may result from differences in the half-lives of mRNA and proteins as reported (44) and suggests that posttranslational modifications may play key roles in the control of the activity of the UPS and thus of muscle atrophy. Our data also suggest that autophagy may be enhanced in the GA during immobilization, contributing to muscle atrophy at I8. However, beclin-1 and LC3b, which are required for autophagosome formation (51), did not change in the immobilized TA but increased at the mRNA level only from R6, when the worsening of TA muscle atrophy peaked at a maximum. Altogether, this suggested that autophagy may be induced in the remobilized TA after the large initial remodeling for restoring muscle mass and myofiber integrity, for example, by elimination of deficient organelles (36).

Our data show that the shortened immobilized GA atrophied more than the lengthened immobilized TA at I8, in agreement with previous reports (8, 20, 21, 46). However, there was a greater activation of the proteasome-dependent pathway in the TA than in the GA at I8, but we cannot exclude that the GA atrophy reflected an activation of this pathway at earlier points of immobilization, as reported by Lang et al. (31). The position of immobilization also influences muscle protein turnover. Proteolysis was enhanced to a greater extent in lengthened immobilized muscles compared with shortened immobilized ones (21). This is in agreement with our observations of a much more pronounced activation of the UPS pathway during immobilization in the lengthened TA compared with the shortened GA. Furthermore, protein synthesis is reduced in shortened immobilized muscles but stimulated in lengthened immobilized ones (21, 31). Therefore, the greater atrophy of the GA

Fig. 11. The proportion of apoptotic nuclei increased in the extracellular matrix compartment of the tibialis anterior muscle after cast removal. A: representative images for apoptotic nuclei localization in immobilized tibialis anterior muscle. Triple-labeling of 10-µm cross-sections of the tibialis anterior allows the detection of total nuclei (Hoechst) and of apoptotic nuclei [terminal deoxy- nucleotidyl-mediated dUTP nick-end labeling (TUNEL)] and the visualization of the basal membrane (laminin-2α). Merging the 3 images allows the localization of apoptotic nuclei within the myofibers (left) and the connective tissue (right). B: the proportion of apoptotic nuclei inside or outside myofibers was quantified in triple-labeled cross-sections of tibialis anterior at I0, I8, and R6 (6 days after cast removal) and expressed as % total nuclei. Values are means ± SE for n = 6. *P < 0.05 vs. contralateral controls.
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muscle during immobilization may also result from decreased protein synthesis.

During remodeling both apoptosome and proteasome activities were much more increased in the TA than in the GA, as were protein levels of the muscle-specific E3 ligase MuRF1. These data support that the increased activity of the UPS and perhaps apoptosis contributed to the worsening of the wasting of the remobilized TA, at least up to R6. However, the patterns of changes in MuRF1 levels and proteasome activities are clearly different. Such measurements are static and do not necessarily strictly correlate. Interestingly, MuRF1 protein levels remained highly enhanced at R1 and R3 (Fig. 5), when the remobilized TA dramatically atrophied (Fig. 2), and then decreased when muscle mass stabilized. This is in line with the critical role of MuRF1 in the breakdown of the major contractile proteins (13, 14, 40). The prolonged activation of proteasome activities in the remobilized TA presumably reflected the remodeling of MyHC isoforms since only the proteasome is responsible for the breakdown of myofibrillar proteins. However, there was no strict correlation between the extent of muscle loss and MuRF1 expression or proteasome activities. Altogether, this suggests that changes in protein synthesis may also contribute to the alteration of muscle size.

In conclusion, the position of immobilization influences both overall rates of protein turnover and structural properties of skeletal muscle (21, 37). TA and GA muscles are located in the anterior and posterior hindlimb compartment, and they were immobilized in a lengthened and shortened position, respectively. We clearly show for the first time that the TA further atrophies very rapidly upon remobilization, pending sustained activation of the UPS and/or apoptosis. Our data show that during the immobilization period muscle lengthening prevented muscle loss but impacted the subsequent muscle recovery because of sustained alterations in proteolysis, in apoptosis, and in extracellular matrix structure and composition. Whether manipulating the UPS and/or apoptosis may improve muscle recovery in such conditions remains to be demonstrated experimentally.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS


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