Recovery of skeletal muscle mass after extensive injury: positive effects of increased contractile activity

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Richard-Bulteau H, Serrurier B, Crassous B, Banzet S, Peinnequin A, Bigard X, Koulmann N. Recovery of skeletal muscle mass after extensive injury: positive effects of increased contractile activity. Am J Physiol Cell Physiol 294: C467–C476, 2008. First published December 12, 2007; doi:10.1152/ajpcell.00355.2007.—The present study was designed to test the hypothesis that increasing physical activity by running exercise could favor the recovery of muscle mass after extensive injury and to determine the main molecular mechanisms involved. Left soleus muscles of female Wistar rats were degenerated by notexin injection before animals were assigned to either a sedentary group or an exercised group. Both regenerating and contralateral intact muscles from active and sedentary rats were removed 5, 7, 14, 21, 28, and 42 days after injury (n = 8 rats/group). Increasing contractile activity through running exercise during muscle regeneration ensured the full recovery of muscle mass and muscle cross-sectional area as soon as 21 days after injury, whereas muscle weight remained lower even 42 days postinjury in sedentary rats. Proliferator cell nuclear antigen and MyoD protein expression went on longer in active rats than in sedentary rats. Myogenin protein expression was higher in active animals than in sedentary animals 21 days postinjury. The Akt-mammalian target of rapamycin (mTOR) pathway was activated early during the regeneration process, with further increases of mTOR phosphorylation and its downstream effectors, eukaryotic initiation factor-4E-binding protein-1 and p70S6K, in active rats compared with sedentary rats (days 7–14). The exercise-induced increase in mTOR phosphorylation, independently of Akt, was associated with decreased levels of phosphorylated AMP-activated protein kinase. Taken together, these results provided evidence that increasing contractile activity during muscle regeneration ensured early and full recovery of muscle mass and suggested that these beneficial effects may be due to a longer proliferative step of myogenic cells and activation of mTOR signaling, independently of Akt, during the maturation step of muscle regeneration.

ADULT SKELETAL MUSCLE has the remarkable capacity to regenerate after injury, mainly due to satellite cells, which represent a population of adult myoblasts that remain normally quiescent (34). Following muscle damage and the initial step of necrosis and phagocytosis of injured myofibers, satellite cells are activated and then rapidly proliferate, fuse, and differentiate to form new myofibers or to repair damaged ones (1). This sequence of cellular events is accurately regulated, especially by myogenic regulatory factors (MRFs), which are members of a family of basic helix-loop-helix proteins that act as transcription activators and regulate the transcription of muscle-specific genes (29, 42). MyoD acts as an early MRF mainly involved in satellite cell activation and proliferation, whereas myogenin is a late-acting MRF, expressed during differentiation (3). The last step of muscle regeneration consists of the maturation step leading to functional muscle, which includes the recovery of both the mature phenotype and muscle mass.

Among the numerous growth factors involved at the onset of the regeneration process, IGF-I is known to stimulate satellite cell proliferation and differentiation and to increase muscle protein synthesis (16, 41). IGF-I activates the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway, which is necessary and sufficient to induce skeletal muscle hypertrophy (20). Akt phosphorylation regulates the catabolic pathway by preventing the induction of muscle-specific ubiquitin ligases such as atrogin-1 [also known as muscle atrophy F box (MAFbx)] and muscle ring finger 1 (MuRF1) (44). Akt also activates the anabolic pathway by phosphorylating the mammalian target of rapamycin (mTOR) (37); mTOR then stimulates translation initiation via the activation of translation regulators p70S6K and the eukaryotic initiation factor-4E (eIF-4E) complex, after phosphorylation of eIF-4E-binding protein-1 (4E-BP1), one of the main translational inhibitors (19).

Cell growth depends on a high rate of protein synthesis and consequently requires a high level of cellular energy. The major sensor of cell energy status is AMP-activated protein kinase (AMPK), which is activated in response to low cellular energy and then downregulates energetically demanding processes, such as muscle protein synthesis, through mTOR inhibition (4, 24, 27). Thus, mTOR appears to have an important and central function in integrating a variety of signals, from those related to the cellular energy state to those dependent on the presence of growth factors, peptides, or nutrients, resulting in the control of muscle mass by the regulation of cell numbers and sizes (40). Moreover, the Akt/mTOR signaling pathway has been involved in the regeneration process, and its activation is known to increase the muscle fiber size in regenerating rat skeletal muscle (36, 38).

IGF-I is also able to activate specific MAPK pathways, such as the Ras-Raf-MEK-ERK pathway, which is crucial in mitosis-competent cells for cell proliferation and cell survival (2, 35). Another MAPK protein, namely, p38, has been shown to play an important role in cultured cells to induce terminal muscle cell differentiation (30). However, the role played by ERK and p38 pathways during the regeneration process following extensive muscle damage is still unknown and needs to be examined.
The recovery of muscle mass after severe muscle damage is a very long process. After myotoxic-induced muscle degeneration in rats, >56 days are needed to recover muscle mass under control conditions (15). The maturation of regenerating fibers appears to be under the influence of numerous external factors, among them neural influence (38, 46) and mechanical loading (13). Because muscle contractile activity involves biological signals related to both motor nerve activity and mechanical stress (17), it is logical to speculate that physical activity should have beneficial effects on muscle growth. This putative beneficial effect of contractile activity has been previously examined during regeneration after either grafting (47) or myotoxic-induced muscle degeneration (14). Using these two different means of muscle degeneration, results were controversial, and the specific effects of increased contractile activity on the recovery of muscle mass remain to be examined carefully. In the present study, we hypothesized that increased muscle contractile activity by running exercise could favor the recovery of muscle mass after extensive injury, and our objective was to determine the main molecular mechanism(s) involved.

To test this hypothesis, we compared the recovery of muscle mass on days 5, 7, 14, 21, 28, and 42 after notoxin-induced degeneration of soleus muscle in rats either kept sedentary (Sed) or exercising daily [active (Act)] as soon as 3 days after muscle injury. To highlight the intracellular mechanisms involved in the regeneration process and recovery of muscle mass, we studied the time course of changes in protein levels of PCNA as a marker of initial cell proliferation and MRFs (i.e., MyoD and myogenin). Moreover, we examined the phosphorylation states of the Akt-mTOR, p38, and ERK1/2-MAPK pathways and of the α-catalytic subunit of AMPK. We also analyzed the transcript levels of atrogin transcription factors MuRF1 and MAfbx.

**MATERIAL AND METHODS**

**Animals**

Female Wistar rats initially weighing 180–200 g were purchased from Charles River Laboratories (L’Arbresle, France). Animals were housed two per cage in a thermoneutral environment (22 ± 2°C) on a 12:12-h photoperiod and were provided with food and water ad libitum. This investigation was carried out in accordance with the Helsinki Accords for Human Treatment of Animals During Experimentation and was approved by the local Animal Ethics Committee. Female rats were used in this study because, in contrast to males, they increase their food intake to compensate for the increased energy expenditure caused by running exercise. The body weight growth rate of exercised female rats was similar to that of Sed rats, and it is then possible to compare muscle masses of freely eating exercised (Act) and Sed rats of the same body weight. Moreover, many previous studies have shown that in rodents, females run more easily than males.

**Experimental Design**

All rats underwent 5 days of treadmill acclimatization (10 min/day, 12 m/min). After 24 h of rest, on day 0 of the experiment, degeneration of the left soleus muscle was induced by notoxin injection. After 3 days of recovery, animals were divided in two main groups: Act and Sed (n = 48 rats/group). Act rats were submitted to practice a daily forced treadmill running exercise 5 days/wk and were placed into individual cages for voluntary exercise. Sed rats were kept in a normal cage without any programmed activity. Eight animals of each group were then anesthetized for tissue sampling and killed 5, 7, 14, 21, 28, and 42 days after the initial muscle injury.

**Notoxin Injection**

Rats were anesthetized with pentobarbital sodium (60 mg/kg), and left soleus muscle degeneration was induced by notoxin injection (0.2 ml, 10 μg/ml) isolated from snake venom (Notechis scutatus; Latoxan, France) directly into the belly of the muscle surgically exposed, as previously described (15). Because the blank surgery did not induce any specific alteration in muscle tissue, no surgical procedure was done on the right soleus muscle, which served as an intact control. The effects of physical conditioning were then studied in regenerating soleus muscle (Reg muscle) compared with contralateral intact noninjured muscles (Int muscle).

**Voluntary and Forced Exercise Procedures**

Act rats were placed into individual plastic cages (26 cm width × 50 cm height × 40 cm length) with access to purpose-built running wheels. Wheels were attached vertically to a freely rotating shaft (30 cm diameter and 8 cm width) inserted into an incremental position encoder (GHMS, Idéacod-Automation SA, Strasbourg, France), producing a current proportional to the running speed. The voltage output was demodulated through a series of resistors and sent to a dual-channel chart recorder. Electronic signals were stored in a computer and provided measures of the total distance run per hour and daily. A constant load was attached to the wheel so that a torque of 0.04 Nm was necessary to overcome wheel inertia. The torque was maintained at this value throughout the experiment.

The forced running exercise was performed on a treadmill (Medical Developpement, Tecmachine, Andrézieux-Bouthéon, France) 5 days/wk, with a progressive increase of speed (from 10 to 30 m/min), time (from 1 to 2 h), and slope (from 5% to 8%).

**Tissue Processing**

Animals were anesthetized with pentobarbital sodium (90 mg/kg) administered intraperitoneally. Reg (i.e., left) and Int (i.e., right) soleus muscles were excised, cleaned of adipose and connective tissue, and weighed. Muscles were immediately frozen in liquid nitrogen. All samples were stored at −80°C until analyses were performed.

**Histomorphometric Analyses**

Serial transverse sections (12 μm thick) were cut from the midbelly portion of soleus muscles in a cryostat microtome maintained at −20°C and stained with hematein, eosin, and safran (HES) to visualize the nucleus, cytoplasm, and adipose tissue at each time of recovery. To determine the whole muscle cross-sectional area (CSA), photographs of the entire muscle were taken at low magnification. Moreover, 20–30 photographs at high magnification, covering the entire muscle section, were used to determine the CSA of at least 1,000 fibers in each muscle sample on days 21 and 42 for both Int and Reg muscles. The total number of myofibers was calculated by dividing whole muscle CSA per the mean of the fiber CSA (FCSA) for each muscle on days 21 and 42. Analyses were performed with a light microscope computerized image-analysis system (Lucia 5, Laboratory Imaging, Prague, Czech Republic).

**Protein Isolation and Immunoblot Analyses**

Muscles (10–20 mg) were lysed with the appropriate buffer, and homogenates were all centrifuged at 15,000 g for 15 min at 4°C. Equal amounts of muscle protein (12 μg for PCNA; 50 μg for myogenin, Akt, mTOR, and 4EB-P1; and 75 μg for MyoD, p70S6k, and AMPK) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond C-Extra, Amersham Pharmacia Biotech, Orsay, France). A standardized amount of protein prepared from intact soleus...
muscle was also applied on each gel to serve as an internal standard for comparison across blots. Membranes were incubated overnight with the appropriate target antibody and then for 2 h with the corresponding horseradish peroxidase-conjugated antibody. Washed blots were subjected to the ECL Western Blotting Detection Reagent kit (ECL, Amersham Pharmacia Biotech) and then exposed to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech). The relative protein expression was determined by the ratio of sample band intensity to the internal standard band intensity using densitometry using the densitometer GS 800 driven by Quantity One 4.6.1 (Bio-Rad, Marne-la-Coquette, France).

To determine the phosphorylation state of specific markers of some intracellular signaling pathways, both phosphorylated and total proteins were studied on the same blot, once using the antibody against the phosphorylated protein and, after membranes had been stripped [with 2% SDS, 12.33% of 0.5 M Tris (pH = 6.7), and 0.072% 2β-mercapto-ethanol for 30 min at 50°C], once again using the antibody against the total protein.

**Immunoblot Antibodies**

The following antibodies were used: mouse monoclonal antibodies against myogenin [1:500, sc-12732 (F5D), Santa Cruz Biotechnology, Heidelberg, Germany]; PCNA (1:500, Ab-1 Clone PC10, NeoMarkers Intermich, Monachuon, France); MyoD (1:500, N°554130 clone MoAb 5.8A, BD Pharmingen, BD Biosciences, Pont de Claix, France); and rabbit polyclonal antibodies from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, France) against phosphorylated p38 on Thr180/Tyr182 (1:500, no. 9211), total p38 (1:1,000, no. 9101), phosphorylated ERK1/2 on Thr202/Tyr204 (1:1,000, no. 9101), total ERK1/2 (1:1,000, no. 9102), phosphorylated Akt on Ser473 (1:500, no. 2532), total Akt (1:1,000, no. 9272), phosphorylated mTOR on Ser2448 (1:1,000, no. 2971), total mTOR (1:1,000, no. 2972), phosphorylated p70S6K on Thr389 (1:500, no. 9205), total p70S6K (1:500, no. 9202), phosphorylated 4E-BP1 on Thr70 (1:1,000, no. 9271), total 4E-BP1 (1:1,000, no. 9252), phosphorylated p38 on Thr180/Tyr182 (1:500, no. 9211), total p38 (1:1,000, no. 9101), phosphorylated Akt on Ser473 (1:500, no. 2532), total Akt (1:1,000, no. 9272), phosphorylated mTOR on Ser2448 (1:1,000, no. 2971), total mTOR (1:1,000, no. 2972), phosphorylated p70S6K on Thr389 (1:500, no. 9205), total p70S6K (1:500, no. 9202), phosphorylated 4E-BP1 on Thr70 (1:1,000, no. 9455), total 4E-BP1 (1:1,000, no. 9452), phosphorylated AMPK-α on Thr172 (1:1,000, no. 2531), and total AMPKα (1:1,000, no. 2532). Incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:2,000, 1:2,500, and 1:5,000 for myogenin, PCNA, and MyoD, respectively, sc-2005, Santa Cruz Biotechnology) or with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:10,000, sc-2313, Santa Cruz Biotechnology) was performed.

**mRNA Isolation and RT Reaction**

Frozen muscle samples of 10 mg were disrupted in 50 volumes of TRizol (Eurogentec, Seraing, Belgium) with Mixer Mill MM300 (Rescht, Haan, Germany) for 2 x 30 s (30 Hz). tRNA was isolated from an adapted protocol as previously described (6): an additional step of isovolume chloroform extraction and two additional ethanol washes were performed. The total amount of RNA was measured with a nanospectrophotometer (Nanodrop). RT was carried out in a 10-f final volume from 400 ng of tRNA solution using the Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium) with 50 µM oligo (dT) 15 primer and RNase inhibitor (2 UI) according to the manufacturer’s instructions.

**Table 1. RT-PCR primers and conditions of utilization**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession Number</th>
<th>Primers</th>
<th>Primer Concentration, µM</th>
<th>Hybridization Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle ring finger 1</td>
<td>NM_080903</td>
<td>Forward: 5’-GGGAAAGGCGGTTGTTGATC-3’</td>
<td>0.4</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CTTTCACTGTTGTTGTTGTTT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle atrophy F box</td>
<td>NM_133521</td>
<td>Forward: 5’-GCTTTGATGGTTCACCCAAGA-3’</td>
<td>0.5</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-TGAAGTTGAGAAGGAGAGCTCCT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Primer Design**

Oligonucleotide primers used in this study were designed with MacVector software (Accelrys, San Diego, CA) as previously described (39) and synthesized at Eurogentec (Table 1). Primers for internal control genes were as previously described (42). Specificities of the PCR amplification were documented with LightCycler melting curve analysis. Melting peaks obtained from either the RT product or specific recombinant DNA were identical.

**Real-Time Quantitative PCR**

PCR was carried with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) from 2 µl of cDNA (1:12 dilution) in a 20-µl final volume [4 mM MgCl₂ and specific primer concentrations (shown in Table 1)]. PCR was performed using a LightCycler (Roche Applied Science) for 45 cycles at 95°C for 20 s, primer-specific temperatures (shown in Table 1) for 5 s, and a final step of 10 s at 72°C. Crossing point values were calculated from LightCycler software version 3.5 (Roche Applied Science) using the second derivative maximum method. Quantification was achieved using a pool of all the cDNA samples as a calibrator, according to the comparative threshold cycle method (42). Values obtained for each target gene were compared with values of three internal control genes [cyclophilin A (CycA), GAPDH, and β-actin], and normalization was then done by calculating the geometric average of these three values. The expression stability of six potential reference genes [β-actin, acidic ribosomal phosphoprotein P0 (ARBP), CycA, GAPDH, hypoxanthine guanine phosphoribosyl transferase (HPRT), and MAPK-14] was initially assessed using GENORM software (45). Gene stability ranking from least to most stable genes was HPRT, MAPK-14, ARBP, CycA, and GAPDH or β-actin. Pairwise variations of CycA, GAPDH, and β-actin were below 0.15, the threshold below which the inclusion of an additional control gene is not required. CycA, GAPDH, and β-actin were therefore used for the normalization of all target genes.

**Statistical Analysis**

All data are presented as means ± SE. Data were analyzed using three-way ANOVA to determine the main statistical effects of recovery over time, injury, and activity and interactions between those factors. When appropriate, differences between groups were tested with a Newman-Keuls post hoc test, especially to compare values measured during muscle regeneration with those observed in contralateral noninjured muscles. Statistical significance was accepted at P < 0.05.

**RESULTS**

**Running Distances**

During the first week after muscle injury (days 3–7), the average daily running distance was 933 ± 180 m for voluntary and forced exercise, respectively (Fig. 1). The mean daily distance increased to 3,231 ± 320 and 4,359 ± 578 m for voluntary wheel running and to 2,029 ± 306 and...
Histological and Morphological Aspects of Regenerated Muscles

Histological aspects of Int muscles were similar in Sed animals (Fig. 3A) and Act animals (Fig. 3B). By day 7 of regeneration, all necrotic fibers appeared to have been replaced by regenerated myofibers (Fig. 3, C and D). The size of these fibers was still smaller than that of uninjured fibers, and most of the regenerating fibers showed central nuclei. By 21 days after injury, the regeneration process was in progress, and numerous small-diameter fibers were present, especially in Sed animals (Fig. 3, E compared with F). By 42 days after toxin injection, the polyhedric aspect of normal fibers was restored in Reg-Act muscles, whereas myofibers remained irregular with variable diameters and larger spaces between fibers and fibrosis in Reg-Sed muscles (Fig. 3, G compared with H).

The three-way ANOVA evidenced a global effect of activity ($P < 0.01$) and injury ($P < 0.001$) on values of muscle CSA measured on days 21 and 42. Whole muscle CSA was 30% lower in Reg muscles compared with Int muscles, but only in Sed rats throughout the experiment ($P < 0.05$; Table 2). The corresponding mean FCSA was also lower than in Int muscles and in a more important extent than whole muscle CSA (68% and 63% on days 21 and 42, respectively, $P < 0.001$), with an associated increase in the number of fibers (133% and 83% on days 21 and 42, $P < 0.001$, respectively). In contrast, CSA values of whole Reg muscles were similar to those of Int muscles in Act rats on days 21 and 42, although the mean FCSA remained lower than in the Int-Act group ($P < 0.05$). During recovery from extended injury, running activity increased both muscle CSA [24%, not significant (NS), and 32%, $P < 0.05$, on days 21 and 42, respectively] and mean FCSA (63%, $P < 0.05$, and 21%, NS, on days 21 and 42, respectively) with a lower number of fibers only on day 21 (23%, $P < 0.05$; Table 2).

Body and Muscle Weights

The mean body weights of rats were similar in the two groups when muscle degeneration was induced by notexin injection. There was a global effect of time on the body weight of animals, consistent with the expected growth rate ($P < 0.001$). Physical activity had no significant effect on the body weights of the rats (data not shown).

There was no specific effect of running activity on Int soleus muscle weight. The Reg soleus muscle weight was normalized and expressed as the ratio of the weight of Reg muscle to the weight of contralateral Int muscle. Reg muscles exhibit 33% and 36% decreases in the normalized weight on day 5 for Sed and Act rats, respectively ($P < 0.001$; Fig. 2). There was a global effect of time and activity ($P < 0.001$) on the normalized Reg muscle weight, with a significant time $\times$ activity interaction ($P < 0.001$). Muscular activity enhanced the recovery of Reg muscle weight so that Reg-Act muscles rapidly grew up and had returned to control values on day 21. In contrast, Reg-Sed muscles did not completely recover weight values similar to Int muscles, even 42 days after initial injury (17% less than Int-Sed muscles, $P < 0.01$). The normalized weight of Reg muscles was higher in Act rats than in Sed rats from days 21 to 42 ($P < 0.05$; Fig. 2).
Evaluation of Proliferative and Differentiation Steps of the Regenerative Process

PCNA protein is longer expressed in regenerating muscles of active rats. PCNA protein expression is a marker of cell proliferation. It was markedly increased on day 5 after myotoxic injection compared with Int muscles (Fig. 4A). PCNA protein levels decreased rapidly and had returned to control values on day 14 in Sed animals. Although PCNA expression was lower in Reg-Act animals than in Reg-Sed animals on day 7 (P < 0.05), it decreased more slowly in Act rats than in Sed rats, and the content of PCNA protein was 7.5-fold higher in Reg-Act muscles than in Reg-Sed muscles on day 14 (P < 0.05). MyoD protein levels increased on day 14 in regenerating muscles of active rats. MyoD expression is commonly used as a reliable marker of satellite cell activation. Although it can be

Table 2. Morphological characteristics of soleus muscle

<table>
<thead>
<tr>
<th></th>
<th>Int-Sed</th>
<th>Int-Act</th>
<th>Reg-Sed</th>
<th>Reg-Act</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle cross-sectional area, mm²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>3.33±0.28</td>
<td>3.73±0.21</td>
<td>2.42±0.27*</td>
<td>3.00±0.23</td>
</tr>
<tr>
<td>Day 42</td>
<td>3.73±0.22</td>
<td>4.05±0.12</td>
<td>2.58±0.19*</td>
<td>3.40±0.28†</td>
</tr>
<tr>
<td><strong>Fiber cross-sectional area, μm²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>2.595±74</td>
<td>2.421±128</td>
<td>817±62*</td>
<td>1,332±72†</td>
</tr>
<tr>
<td>Day 42</td>
<td>2,686±103</td>
<td>2,651±252</td>
<td>1,006±45*</td>
<td>1,222±40*</td>
</tr>
<tr>
<td><strong>Total number of fibers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>1,279±92</td>
<td>1,552±79</td>
<td>2,991±277*</td>
<td>2,310±263†</td>
</tr>
<tr>
<td>Day 42</td>
<td>1,400±91</td>
<td>1,571±98</td>
<td>2,565±155*</td>
<td>2,772±192**‡</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Muscle and fiber cross-sectional areas were determined after hematein-eosin-safran staining by analyzing pictures covering the entire muscle. Data are shown for days 21 and 42 of intact (Int)-sedentary (Sed), Int-active (Act), regenerated (Reg)-Sed, and Reg-Act soleus muscles. More than 1,000 fibers were analyzed. *Significantly different from the Int group; †significantly different from the Sed group; ‡significantly different from the previous time for the same group.
expected that MyoD peaked earlier than 5 days after muscle injury, in the present experiment, MyoD protein expression was still markedly increased in Reg muscles compared with Int muscles of both Act and Sed rats (global effect of injury, \( P < 0.001 \); Fig. 4B). MyoD was expressed at the same level from day 5 to 21 after muscle injury in Sed animals (\( P < 0.05 \) compared with Int muscles). In Act rats, MyoD protein levels of Reg muscles increased on day 14 compared with values measured on day 7 (\( P < 0.05 \)) and had returned to levels measured in the Int-Act group.

Myogenin protein levels increased on day 21 in regenerating muscles of active rats. Myogenin is a protein known to be expressed during the terminal differentiation program. As expected, myogenin protein levels were higher in Reg muscles than in Int muscles (global effect, \( P < 0.001 \); Fig. 4C). There was a marked effect of time (global effect, \( P < 0.001 \)), with an interaction with muscle injury (\( P < 0.001 \)). As a result of this time \( \times \) injury interaction, the increased myogenin expression in Reg muscles was only observed on day 5 (\( P < 0.001 \) compared with Int muscles). Thereafter, myogenin protein levels decreased as soon as day 7. Moreover, a significant increase in myogenin protein levels occurred in Act muscles compared with Sed muscles (global effect, \( P < 0.01 \)).

MAPK Phosphorylation

Total p38 protein expression was lower in Reg muscles than in Int muscles, mainly during the first step of regeneration (global effect of injury, \( P < 0.001 \)), whereas total ERK1/2 protein expression increased during muscle regeneration (global effect of injury, \( P < 0.001 \)), with no difference between Sed and Act groups (data not shown).

There were no changes in phosphorylation levels of both p38 and ERK1/2 MAPKs over time in Int muscles of either Sed or Act animals (Fig. 5). A global time effect was shown on the p38 phosphorylation level (\( P < 0.01 \)), with a significant interaction with muscle injury (\( P < 0.01 \)). Moreover, there was an interaction between activity and muscle injury (\( P < 0.001 \)), so that the increase in phosphorylated p38 expected on day 14 in Reg muscles was only observed in Sed rats. This interaction also resulted from lower phosphorylated p38 values on day 5 in the Reg-Act group compared with the Reg-Sed group (\( P < 0.05 \)).

There was a global effect of both time and injury in the muscle content of phosphorylated ERK1/2 (\( P < 0.001 \)), with an interaction between these factors (\( P < 0.001 \)). In contrast with Int muscles, an early increase in ERK1/2 phosphorylation occurred during muscle regeneration on days 5 and 7 (\( P < 0.001 \)), which declined progressively (Fig. 5B). Moreover, the initial increase in ERK1/2 phosphorylation reported early during muscle regeneration was less in Act rats than in Sed rats (\( P < 0.05 \)), whereas ERK1/2 phosphorylation was higher in Reg-Act muscles than in Reg-Sed on day 21 (\( P < 0.05 \)).

Phosphorylation of Akt, mTOR, and its downstream effectors p70S6K and 4E-BP1. The expression of total Akt protein was increased in Reg muscles of both Sed and Act rats in the early times of regeneration (global effect of injury, \( P < 0.001 \), and significant time \( \times \) injury interaction, \( P < 0.001 \)). Akt phosphorylation on Ser473 decreased with time after day 5 postinjury, but only in Reg muscles (global effects of time and injury, \( P < 0.001 \), and significant interaction, \( P < 0.001 \)). Phosphorylated Akt levels were not affected by running activity (data not shown). As a consequence of both changes in total and phosphorylated Akt, the ratio of phosphorylated to total Akt was increased in Reg muscles compared with Int muscles from days 5 to 14 without any difference between Sed and Act rats and then decreased and returned to basal levels earlier in Act rats than in Sed rats (\( P < 0.05 \) on day 21; Fig. 6A).

No effects of time, injury, or activity were found on the total amounts of mTOR, 4E-BP1, and p70S6K protein (data not shown). Phosphorylated mTOR levels were higher in Reg muscles than in Int muscles (global effect, \( P < 0.001 \); Fig. 6B), decreased sharply on day 7 in Reg-Sed muscles, and remained slightly higher than in Int muscles over time. In contrast, Reg muscles from Act rats exhibited high phosphorylated mTOR levels until day 14 compared with Sed animals (46% and 67% on days 7 and 14, respectively, \( P < 0.05 \)) and then decreased to values measured in Int muscles from day 21.

Both time and injury affected phosphorylated 4E-BP1 (global effects, \( P < 0.001 \)), with a significant interaction between these factors (Fig. 6C), so that a transient increase occurred on day 7 in Reg muscles of both Sed and Act groups and similar levels to those of Int muscles were recovered from 14 days after the initial injury. Only a slight global effect of activity was detected (\( P < 0.05 \)), with higher levels of phosphorylated 4E-BP1 in the Reg-Act group compared with the Reg-Sed group on days 7 and 14 (30% and 37%, respectively, \( P < 0.05 \)).

Phosphorylated p70S6K levels increased only in Reg muscles as soon as day 5 after muscle injury and then decreased to
recovery levels similar to Int muscles on day 21 (global effect of time and injury, \( P < 0.001 \); Fig. 6D). However, p70\(^{65k}\) phosphorylation was higher in the Reg-Act group than in the Reg-Sed group on day 14 (50\%, \( P < 0.01 \)).

**MurF1 and MAFbx mRNA.** Activated Akt is known to decrease the activity of FOXO transcription factors, leading to a decrease in the transcription of MurF1 and MAFbx atrogin genes. We showed that MurF1 and MAFbx mRNA in Reg-Act muscles mirrored Akt phosphorylation (Figs. 7 and 6A, respectively). MurF1 and MAFbx mRNA levels were very low on day 5 (\( P < 0.05 \) compared with Int muscles) and increased regularly thereafter in Reg groups to recover to transcript levels observed in Int muscles on day 21. In Reg muscles, MurF1 and MAFbx mRNA levels were higher in Act rats than in Sed rats on day 21 (81\% and 159\%, respectively, \( P < 0.05 \)).

**AMPK phosphorylation.** There was a global effect of time and injury on the expression of total AMPK protein levels (\( P < 0.001 \)) and a significant time \( \times \) injury interaction (\( P < 0.001 \); data not shown). Total AMPK was low at the beginning of muscle regeneration and increased steadily to a similar extent in Sed and Act groups.

A global effect of time and injury (\( P < 0.001 \)) affected AMPK phosphorylation (Fig. 8), with a significant interaction (\( P < 0.001 \)). Results of the three-way ANOVA showed that AMPK phosphorylation increased regularly over time, but only in Reg muscles. AMPK phosphorylation was low on day 5 (~55\% of values measured in Int muscles) and then increased steadily over time in Sed rats to reach levels measured in Int muscles on day 42. There was also a global effect of activity (\( P < 0.001 \)) that resulted in 15\% lower AMPK phosphorylation values in Int muscles of Act rats compared with those measured in Sed rats (\( P < 0.05 \)). Moreover, in contrast to Sed rats, AMPK phosphorylation remained low until day 14 in Act animals (\( P < 0.05 \)), increased sharply thereafter (\( P < 0.05 \)), and had returned to control values by day 21. At this time
point, phosphorylated AMPK levels were higher in the Reg-Act group than in the Reg-Sed group (29%, \( P < 0.05 \)).

**DISCUSSION**

The present study was designed to test the hypothesis that increasing contractile activity during the regeneration process may have beneficial effects on the recovery of muscle mass after extensive muscle injury and to investigate the intracellular mechanisms involved in this process. The main results were the following: 1) increased contractile activity through running exercise during muscle regeneration ensured full recovery of soleus muscle mass as soon as 21 days after notexin-induced muscle degeneration; 2) the proliferation step of regeneration, estimated by levels of PCNA and MyoD protein expression, went on longer in Act rats than in Sed rats; 3) the Akt-mTOR pathway was activated early during the regeneration process, with a further increase of mTOR phosphorylation and its downstream effectors in Act rats compared with Sed rats; and 4) this specific effect of running exercise on the activation of the mTOR signaling pathway during muscle regeneration was associated with a concomitant decrease in the phosphorylation state of AMPK. Taken together, the results of this study provide evidence that increasing contractile activity during muscle regeneration leads to the early and full recovery of muscle mass after extensive injury. Moreover, these beneficial effects could be due to a longer proliferative step of myogenic cells and activation of mTOR signaling, independently of Akt, during the maturation step of muscle regeneration.

Skeletal muscle regeneration appears to be an interesting and fruitful model to study the molecular mechanisms involved in the control of skeletal muscle growth and the modulation of muscle mass. In the present study, we used notexin-induced soleus muscle degeneration, a well-defined model of muscle injury known to cause a rapid and extensive myofiber necrosis sparing satellite cells, followed by a complete and synchronous regenerative process (3, 22). Using this reproducible model of muscle injury, we have previously shown that the recovery of muscle weight is a long process, only partially achieved 56 days after muscle injury (15). This result is consistent with the present study since 42 days after initial muscle injury, Reg soleus muscle weight remained 20% lower than that of Int muscles in Sed animals. The full recovery of muscle weight only 21 days after injury in Act rats appeared then as a striking result, all the more noteworthy as morphological data clearly showed that this was achieved by the development of myofibers, with less fibrosis development than in Reg muscles of Sed rats. These results strengthen previous observations that suggested that physical activity ensured to limit the appearance of connective tissue in Reg muscles (23).

Such positive effects of running exercise on the recovery of muscle mass could be explained at least partly by a specific influence of contractile activity on the size of the myogenic cell pool available for regeneration and/or increased activity of signaling pathways involved in the control of muscle mass. In the present experiment, we used PCNA expression to examine the cellular activity during the proliferating step, as previously suggested (11). Although we know that PCNA is not specific to satellite cells, it has been previously used as a biological marker to follow the entry of satellite cells into the cell cycle in primary mass cultures (25). Under our conditions, PCNA expression could be relevant to cellular events related to the proliferation of satellite cells, other myogenic cells, and/or infiltrating nonmyogenic cells. However, the higher expression of PCNA observed in Reg muscles from Act rats on day 14 compared with Sed muscles was associated with higher levels of MRF expression, MyoD on day 14 and then myogenin on day 21, suggesting that at least part of proliferating cells may have myogenic capacities. These findings may suggest that muscle precursor cells were activated and then proliferated to a larger extent in Act animals and are consistent with an expansion of the myogenic cell pool necessary for myofiber formation. It has been shown that a single bout of unaccustomed high-intensity exercise was able to activate satellite cells (7), while evidence of cell proliferation exists early after a single bout of voluntary running (5). The exercise training program used in the present study associated two different activities, voluntary wheel running activity, which consists of bouts of high speed running (9), and imposed treadmill running, which forces the animal to run for a sustained period despite its natural running behavior. Both activities have probably contributed to activate satellite cells (7, 8) and promote stem cell proliferation and myogenesis (5). These effects of running exercise on satellite cell activation, their proliferation, and reentry in the growth cell cycle are consistent with the faster recovery of muscle mass reported in running rats and suggest that running exercise may influence the early phase of muscle regeneration through the activation and proliferation of satellite cells.

Myogenesis is a dynamic process controlled by members of MRFs, in association with specific transcription cofactors such as members of the MEF2 family (33). Although it could be expected that MyoD and myogenin peaked earlier, the present experiments show that both MRFs were still increased in Reg muscles 5 days after muscle injury, with further elevated levels under running conditions on days 14 and 21 for MyoD and myogenin, respectively. The exercise-induced increase in MyoD during the late and terminal steps of differentiation is consistent with the increased expression of muscle-specific genes and the fast recovery of muscle mass shown in exercised animals. Moreover, specific ERK1/2 and p38 MAPKs have been shown to have an important role in the proliferation and early differentiation in cultured cells (33, 35). We showed in the present study that the early steps of muscle regeneration were associated with high levels of phosphorylated ERK1/2, which returned to basal values on day 14. Surprisingly, ERK1/2 protein levels were lower in Act rats than in Sed rats on day 5, but the increased levels of this activated MAPK observed 21 days after muscle injury could favor the late steps of muscle differentiation through motor nerve activity (36). Although p38 MAPK activity plays an essential role in muscle differentiation (33), p38 phosphorylation in Act rats had returned to levels reported in Int-Sed muscles on day 14. This finding is surprising and not consistent with the positive effects of exercise on muscle mass recovery. Nevertheless, because it has been shown that constitutive activation of p38 MAPK induced interstitial fibrosis, at least in the heart (31), this early decrease to normal values may have protected Reg-Act muscles against fibrosis, as attested by their histomorphological aspects.

In addition to molecular events related to the activation, proliferation, and differentiation of myogenic cells, regenerat-
ing skeletal muscle growth is also under the control of the PI3K-Akt pathway (36), with mTOR as the major effector (38). In the present study, Akt and mTOR phosphorylation were strongly and early increased during regeneration, whereas the expression of atrogen transcription factors MurF1 and MAFbx decreased in mirror, in both Sed and Act rats. The decreased expression of MurF1 and MAFbx, two genes that encode proteins required for ubiquitin-ligase activity (26), is consistent with a slowdown of protein degradation during muscle growth. Interestingly, MurF1 and MAFbx mRNA levels had returned to control levels 21 days after muscle injury in Act rats, when muscle weights had recovered values similar to those measured in noninjured muscles. However, whereas the level of Akt phosphorylation was the same in Sed and Act rats, phosphorylation of mTOR and its downstream targets 4E-BP1 and p70S6K on day 14 were higher in Act rats than in Sed rats. These results suggest, for the first time, that during muscle regeneration, physical activity increased the activation of mTOR, independently of Akt.

There is now experimental evidence that AMPK activation inhibits mTOR signaling, without a significant alteration of Akt activity (27, 48). One of the main results of the present study was that increased mTOR phosphorylation in Act rats during regeneration was associated with decreased levels of phosphorylated AMPK. These low levels of phosphorylated AMPK may have acted as a permissive effect on mTOR phosphorylation and then activation of its downstream targets (48). Independently of changes in AMPK activity, other factors have been found to control mTOR signaling, among them nutrients, especially amino acids such as leucine (21, 28). Whether or not physical activity during muscle regeneration affected nutrient availability or induced changes in other factors known to control mTOR phosphorylation needs to be examined in further studies.

Another interesting result of this study was the decreased phosphorylation level of AMPK in skeletal muscle from Act rats. It has been extensively reported that AMPK phosphorylation increased in skeletal muscle at the end of a single bout of exercise, before returning to basal values after 1 h of recovery (43). The basal activity of AMPK increased in human skeletal muscle in response to endurance training, at least 15 h after the last exercise bout (18). This is a surprising result because increased basal AMPK activity would slow down the increase in muscle protein synthesis commonly reported during the recovery from endurance exercise (10). Although there are discrepancies between our results and those reported previously in human muscle (18), the decreased AMPK phosphorylation reported in the present study after running exercise may have played a physiological role during this period in enhancing the activity of the mTOR pathway, particularly in Reg muscles.

In conclusion, the results from the present study demonstrate that increasing contractile activity through both voluntary and forced running exercise ensured the full recovery of the soleus muscle mass after notexin-induced degeneration in only 21 days and avoided the development of fibrosis. These data suggest that in rats, physical exercise may enhance the recruitment and proliferation of myogenic cells and favor the activation of mTOR signaling throughout the maturation step of muscle regeneration. Moreover, the present results suggest that increased mTOR phosphorylation in Act rats results likely from the control by AMPK. Although we showed that increasing contractile activity is an efficient strategy to recover muscle mass after severe muscle damage, it remains to be elucidated whether or not this active strategy may have also beneficial effects on the recovery of muscle phenotype and function.

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