Electrostimulation during hindlimb unloading modulates PI3K-AKT downstream targets without preventing soleus atrophy and restores slow phenotype through ERK

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Submitted 30 November 2009; accepted in final form 22 November 2010

Dupont E, Cieniewski-Bernard C, Bastide B, Stevens L. Electrostimulation during hindlimb unloading modulates PI3K-AKT downstream targets without preventing soleus atrophy and restores slow phenotype through ERK. Am J Physiol Regul Integr Comp Physiol 300: R408–R417, 2011. First published November 24, 2010; doi:10.1152/ajpregu.00793.2009.—Our aim was to analyze the role of phosphatidylinositol 3-kinase (PI3K)-AKT and MAPK signaling pathways in the regulation of muscle mass and slow-to-fast phenotype transition during hindlimb unloading (HU). For that purpose, we studied, in rat slow soleus and fast extensor digitorum longus muscles, the time course of anabolic PI3K-AKT-mammalian target of rapamycin (mTOR) and catabolic PI3K-AKT-forkhead box O (FOXO), and MAPK signaling pathway activation after 7, 14, and 28 days of HU. Moreover, we performed chronic low-frequency soleus electrostimulation during HU to maintain exclusively contractile phenotype and so to determine more precisely the role of these signaling pathways in the modulation of muscle mass. HU induced a downregulation of the anabolic AKT, mammalian target of rapamycin, 70-kDa ribosomal protein S6 kinase, 4E-binding protein 1, and glycogen synthase kinase-3β targets, and an upregulation of the catabolic FOXO1 and muscle-specific RING finger protein-1 targets correlated with soleus muscle atrophy. Unexpectedly, soleus electrostimulation maintained 70-kDa ribosomal protein S6 kinase, 4E-binding protein 1, FOXO1, and muscle-specific RING finger protein-1 to control levels, but failed to reduce muscle atrophy. HU decreased ERK phosphorylation, while electrostimulation enabled the maintenance of ERK phosphorylation similar to control level. Moreover, slow-to-fast myosin heavy chain phenotype transition and upregulated glycolytic metabolism were prevented by soleus electrostimulation during HU. Taken together, our data demonstrated that the processes responsible for gradual disuse muscle plasticity in HU conditions involved both PI3K-AKT and MAPK pathways. Moreover, electrostimulation during HU restored PI3K-AKT activation without counteracting soleus atrophy, suggesting the involvement of other signaling pathways. Finally, electrostimulation maintained initial contractile and metabolism properties in parallel to ERK activation, reinforcing the idea of a predominant role of ERK in the regulation of muscle slow phenotype.

mitogen-activated protein kinase pathway; low-frequency stimulation; glycolytic metabolism; myosin heavy chain isoform

SKELETAL MUSCLES ARE ABLE TO ADAPT to a large variety of disuse conditions (immobilization, microgravity, bed rest, or nerve injury), leading to a remarkable plasticity, mainly characterized by a reduction in fiber diameter, protein content, and force, accompanied by increased fatigability and insulin resistance (3, 5, 10, 11, 27). Disuse muscle plasticity has been extensively studied in the rat model of hindlimb unloading (HU) (25, 26). Using this model, we focused on two main changes in slow postural muscle, such as soleus, that have significant repercussions on functional properties: 1) progressive decrease in muscle mass (muscle atrophy) linked to elevated proteolysis and/or reduced synthesis; and 2) partial and progressive shift from slow to fast isoforms of myosin heavy chain (MHC) protein due to modifications in the gene expression of contractile proteins associated with oxidative to glycolytic metabolism (40). By contrast, fast-type muscles such extensor digitorum longus (EDL) are generally not affected by disuse conditions (31).

Over the past few years, considerable progress has been made toward identifying the signaling pathways involved in skeletal muscle plasticity (35, 46). Here, we focused our attention on phosphatidylinositol 3-kinase (PI3K)-AKT and MAPK signaling pathways that might potentially mediate the time course of disuse muscle plasticity, i.e., mass and phenotype changes induced by HU conditions.

Indeed, PI3K-AKT pathway is known to play a pivotal role in activating both muscle protein synthesis and degradation process (12, 20, 33). PI3K, activated by insulin or growth factors, induces AKT phosphorylation, which controls the activation of two targets relevant to protein synthesis: mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3β (GSK-3β). mTOR promotes enhanced translation through its targets 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), a repressor of the cap-binding protein eukaryotic initiation factor (eIF) 4E (41). GSK-3β, also involved in glycogen synthesis, is a negative regulator of protein synthesis through the inhibition of the eIF2B by decreasing its capacity for ribosome recycling and thereby reducing protein synthesis at the initiation step (12). While largely described to promote muscle growth, the deactivation of AKT pathway could also be involved in the regulation of muscle atrophy (12, 20). A third downstream target of PI3K-AKT pathway is forkhead box O (FOXO) transcription factors, a subgroup of the forkhead family of transcription factors. AKT phosphorylates FOXO (1, 3, or 4), leading to the exclusion of phosphorylated FOXO proteins from the nucleus and the inhibition of atrogenes transcription, i.e., atrogin-1 (also known as muscle atrophy F-box) and muscle-specific RING finger protein-1 (MuRF1) (4, 34, 33). Atrogin-1 and MuRF1, two muscle-specific ubiquitin ligases, may play a role in the initiation and maintenance of accelerated proteolysis (4, 46).

MAPK pathway activation leads to the phosphorylation of cytoplasmic and/or nuclear proteins and is partly responsible...
for the regulation of cellular proliferation and differentiation by modulation of gene expression (8). It is composed of three families of parallel kinases, including ERK, JNK, and p38. Even if the specific role of these kinases in skeletal muscle plasticity is not well understood, some studies indicate that MAPK pathway could be implicated in the regulation of MHC isoform expression (14, 35).

Then, the overall aim of the present work was to define activation patterns of signaling targets of both PI3K-AKT and MAPK pathways during disuse muscle plasticity. The primary objective was to follow the evolution of different key markers of these signaling cascades, as a function of the duration of disuse, to identify the potential role of the markers at the onset and/or during the development of the disuse. For this purpose, we analyzed in slow soleus and fast EDL muscles the time course of muscleatrophy (muscle mass), contractile phenotype (MHC isoform transitions), and metabolic profile after 7, 14, and 28 days of HU and measured in parallel the expression and activation states of AKT, GSK-3β, mTOR, p70S6K, and 4E-

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Experimental procedures

Animals and muscles. Fifty adult male Wistar rats (280–320 g) were randomly divided into five groups: C (control); HU-7, HU-14, and HU-28 (HU for 7, 14 and 28 days, respectively); and HU-14 + ES (HU + electrostimulation, both for 14 days). The animals were housed under temperature- and light-controlled conditions (23°C, 12:12-h light-dark cycle) and received standard rodent chow and water ad libitum. The food and water availabilities were daily controlled. The day before the end of the experiment, the rats were fasted. So, the day of death, all of the rats were in the same nutritional state. For all groups, soleus (slow-type postural muscle) and EDL (fast-type muscle) were quickly removed from animals anesthetized with pentobarbital sodium (60 mg/kg ip); muscles were weighed and immediately frozen in liquid nitrogen. Then they were powdered in a steel stain mortar and stored at −80°C until analysis. All procedures described below were approved by both the Agricultural and Forest Ministry and the National Education Ministry (Veterinary service of health and animal protection, authorization 59–00996).

HU. HU was performed using the tail suspension model (25, 26). Briefly, the tail of each rat was cleaned, dried, and wrapped in antiallergic adhesive plaster. This cast was secured to an overhead swivel that permitted 360° rotation and allowed the rats to walk freely on their forelimbs to have access to food and drink water. The rats were suspended by the tail at a 30° head-down angle to avoid a contact of the hindlimbs with the ground.

Chronic electrostimulation. Rats of the HU-14 + ES group were anesthetized with pentobarbital sodium (60 mg/kg ip) and prepared for sterile implantation into the right hindlimb of stimulating electrodes around the tibial nerve (one of the terminal branches of the sciatic nerve that innervates, in particular, the soleus muscle). Two electrodes (Teflon-coated multistranded steel wire, A-M Systems) were placed inside a 5-mm-long cuff ligatured around the nerve so that the distal ends of the wires, the insulation of which had been removed, were in contact with the tibial nerve. The two wires were run under the skin to the middle of the back, and then into a flexible iron fixed on the back skin. This protecting tube was rigidly locked with the swivel at the top of the cage, and the wires were connected to a rotating contact. After 1 day of recovery, the rats were unloaded, and electrostimulation started immediately. The electrostimulation pattern (20 Hz on for 10 s and off for 20 s) was chosen according to its closest imitation of the firing pattern of individual soleus motor units during normal motor behavior (22, 44). The stimulus (stimulator A360, WPI) was always bipolar, with a duration of 0.2 ms and an intensity of 0.2–0.4 mA, producing visible repetitive plantar flexion movements. Such chronic stimulation was performed during the whole 14-day unloaded period. No complications or infections resulting from the surgical or stimulation procedures were encountered. The animals did not exhibit any indication of discomfort or pain during the chronic electrostimulation.

Contractile phenotype determination. Muscle contractile phenotype was determined through analysis of MHC composition. Slow (MHC I) and fast (MHC IIa, IIx, and IIb) isoforms were separated by one-dimensional SDS-PAGE, as previously described (43). Myofibrillar proteins were extracted from 10 mg of muscle powder in a buffer containing 20 mM Tris, pH 7.4, 4 mM EGTA, 10 mM EDTA, 100 mM glycerophosphate, 5 mM Na3VO4, 15 mM Na2P2O7, 25 mM NaF, 15 μg/μl leupeptin, 15 μg/μl aprotonin, 15 μg/μl pepstatin. The samples were homogenized with an ultrasonic cell disrupter, rotated for 60 min at 4°C, and centrifuged at 13,000 g for 10 min at 4°C. The protein...
concentration of the supernatant was determined by a Bradford assay using bovine serum albumin as standard. Samples were then diluted in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue), and heated 3 min at 95°C until analyses.

**Protein identification using mass spectrometry analysis.** Fifty micrograms of proteins extracted from C, HU-14, and HU-14 + ES were resolved on 10% SDS-PAGE. The gel was sensitized with sodium thiosulfate and silver stained. Quantification of proteins was performed using QuantityOne software to determine their variation of expression level between each group. Bands corresponding to proteins differentially expressed were carefully excised from the gel. Gel pieces were first destained using 30 mM potassium ferricyanide/100 mM sodium thiosulfate solution and washed extensively with ultra-pure water. Gel pieces were shrunk by two acetonitrile (ACN) baths, and dried under speed-vacuum. Proteins were reduced at 56°C for 30 min with 10 mM DTT in 0.1 M NH4HCO3, shrunk with ACN and dried, and then submitted to alklylation in 55 mM iodoacetamide in 0.1 M NH4HCO3 for 20 min at room temperature in the dark. Gel pieces were washed with 0.1 M NH4HCO3 for 15 min and were then dehydrated and shrunk with ACN in a vacuum centrifuge.

For trypsin “in-gel” digestion, gel pieces were rehydrated in the digestion buffer containing 50 mM NH4HCO3, 5 mM CaCl2, and 5–12.5 ng/µl of trypsin at 4°C for 20 min. The excess of digestion buffer containing trypsin was removed, and the gel pieces were covered with the digestion buffer without trypsin. Digestion was performed overnight at 37°C.

After digestion, peptides were extracted from the gel pieces. After addition of 25 µl of 25 mM NH4HCO3, gel pieces were shaken for 15 min, and the supernatant was collected. Two successive extractions were performed with 45% ACN/0.1% trifluoroacetic acid (TFA) for 20 min with shaking, and a last one with 95% ACN/0.1% TFA. All supernatants were pooled together and dried in a speed-vacuum. Samples were desalted using Zip-Tip® Pipette Tips just before their analysis on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. Binding and washing of proteins were directed against specific sites as follows: Thr37/46 on FOXO1, Thr180/Tyr182 on p38, Ser473 on AKT, Ser9 on GSK-3β, Ser2448 on mTOR, Thr173 on p70S6K, Thr389 on JNK1/2, Thr1462/Tyr1465 on MuRF1, Thr202/Tyr204 on ERK1/2, Thr536/Tyr545 on JNK1/2, Thr810/Tyr816 on GSK-3β, Ser448 on p538, and Ser211 on myosin heavy chain. Elution was performed with 20% ACN/0.1% trifluoroacetic acid (TFA) for 50 min at room temperature in the dark. Gel pieces were washed with 0.1 M NH4HCO3 for 15 min and were then dehydrated and shrunk with ACN in a vacuum centrifuge.

Peptide mass fingerprint spectra were recorded in reflectron positive ion mode. Mass spectrometry analysis was carried out by immunoblot analysis. Fifty microliters of 10 mg/ml of trypsin at 4°C for 20 min. The excess of digestion buffer containing trypsin was removed, and the gel pieces were covered with the digestion buffer without trypsin. Digestion was performed overnight at 37°C.

After digestion, peptides were extracted from the gel pieces. After addition of 25 µl of 25 mM NH4HCO3, gel pieces were shaken for 15 min, and the supernatant was collected. Two successive extractions were performed with 45% ACN/0.1% trifluoroacetic acid (TFA) for 20 min with shaking, and a last one with 95% ACN/0.1% TFA. All supernatants were pooled together and dried in a speed-vacuum. Samples were desalted using Zip-Tip® Pipette Tips just before their analysis on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. Binding and washing of peptides on Zip-Tip column were carried out in 0.1% TFA in water. Elution was performed with 2 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 45% ACN/0.1% TFA directly by spotting on the target plate.

Protein identification was carried out using peptide mass fingerprinting on a MALDI-TOF mass spectrometer (Voyager DE-STR PRO). Peptide mass fingerprint spectra were recorded in reflection positive ion mode. On average, 150–200 laser shots per spectrum were acquired for each spectrum. Each spectrum was internally calibrated using the monoisotopic mass of the fragments resulting from trypsin autodigestion at 842.5100, 1045.5642, and 2211.1046 Da. Proteins were identified using Protein Prospector (http://prospector.ucsf.edu/), Profound (http://prowl.rockefeller.edu/), and Mascot (http://www.matrixscience.com/) software from National Center for Biotechnology Information and Swiss-Prot databases. The following parameters were used: *rattus norvegicus* species; one missed cleavage site; mass tolerance setting of 50 ppm; and carbamidomethylation of cysteine and partial chemical modifications such as oxidation of methionine. The criteria used to accept identifications included the extent of sequence coverage, the number of matched peptides, the percentage of recovered sequence, the Mowse probability score, the mass accuracy and whether the protein appeared as the top candidate, and the correlation between theoretical and experimental molecular weight. Otherwise, the identification was not considered valid. Validation of proteins identified by mass spectrometry analysis was carried out by immunoblot analysis (see below).

**Western immunoblot analyses.** Samples containing 5 µg [for GADPH, pyruvate kinase (PK), lactate dehydrogenase (LDH), and adenylate kinase 1 (AK1)], 20 µg (for ERK, JNK, p38, AKT, FOXO1, MuRF1, p70S6K, 4E-BP1 detection), or 40 µg (mTOR detection) of proteins were resolved on 10% SDS-PAGE (except for mTOR: 6–10% linear gradient and for 4E-BP1 and AK1: 18%). The proteins were transferred at 200 mA for 2 h onto a 0.2-µm nitrocellulose membrane. Each membrane was processed in three sequential steps: 1) antibody against the phosphorylated form of the protein; 2) antibody against the whole expression level of the protein; and 3) antibody against a housekeeping protein (α-actin) to check that equal amounts of proteins were loaded on the gel. This process was performed for each antibody, except for nonphosphorylated MuRF1, GADPH, PK, LDH, and AK1, for which only steps 2 and 3 were realized. Between each steps, membrane were stripped with Western Re-probe buffer (Agro-Bio). The efficiency of the stripping was tested by incubation with the secondary antibodies and a revelation by ECL. The membrane was then rehybridized with appropriate antibody after blocking.

More precisely, membranes were blocked with 5% BSA or nonfat dry milk in TBST (Tris-buffered saline/0.05% Tween-20) for 2 h at room temperature. Primary antibodies were incubated overnight at 4°C at a dilution of 1:1,000 for step 1 or 1:2,000 for step 2. Membranes were washed three times in TBST and incubated with specific horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After 5 × 10 min washes in TBST, immunoreactivity was detected using enhanced chemiluminescence (PerkinElmer) on hyperfilms Biomax MR (GE Healthcare).

All antibodies were purchased from Cell Signaling Technology, except for GADPH, LDH, and AK1 (Abcam). The antibodies recognizing the phosphorylated form of proteins were directed against specific sites as followed: Thr37/46 on FOXO1, Tyr202/Tyr204 on ERK1/2, Thr180/Tyr182 on JNK1/2, Thr1480/Tyr1485 on p38, Ser173 on AKT, Ser49 on GSK-3β, Ser2448 on mTOR, Thr173 on p70S6K, Thr389 on 4E-BP1, and Thr17/46 on FOXO1.

All blots were scanned, and densitometric analysis of the bands was conducted using GS-800 Imaging densitometer and QuantityOne software. For all groups, protein expression in experimental groups was compared with their basal expression in the C group. Phosphospecific signal was normalized to the total signal to estimate the ratio of activated marker. For MuRF1 and metabolic enzymes, total signal was normalized to the α-actin signal to estimate protein expression.

**Statistical analyses.** Results are presented as means ± SE. Significance of intergroup differences was examined using Student’s t-test or one-way ANOVA when appropriate. Post hoc analysis was performed using Bonferroni’s test. For all statistical analyses, the level of significance was set at P < 0.05.

**RESULTS**

**HU induced progressive soleus muscle atrophy.** Changes in body weight, muscle weight, and muscle weight-to-body weight ratio (MW/BW) after 7, 14, and 28 days of HU, and after 14 days of HU associated with ES are shown in Table 1. Overall, body weight during HU was smaller than that of C (aged-matched at 14 days). Indeed, a significant body weight decrease is generally observed during the first week of HU, but thereafter the growing showed the same time course of C rats (13, 16).

The ratio between muscle weight (mg) and body weight (g) (MW/BW in mg/g) was chosen as an indicator of muscle atrophy. For the slow soleus (Table 1), MW/BW significantly decreased for all of the HU groups compared with C ones (−28% for HU-7, −46% for HU-14, and −63% for HU-28, P < 0.001). Soleus atrophy can be qualified as progressive since significant differences appeared between each HU period (−25% between HU-7 vs. HU-14, P < 0.001, and −31% between HU-14 vs. HU-28, P < 0.001). Chronic electrostimulation of the soleus via the tibial nerve did not prevent atrophy since I) the MW/BW for HU-14 + ES was significantly
Table 1. Effects of hindlimb unloading and chronic electrical stimulation on rat body weight and soleus and EDL muscle weights

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Body Weight, g</th>
<th>Soleus Muscle Weight, mg</th>
<th>Soleus Weight-to-Body Weight Ratio, mg/g</th>
<th>EDL Muscle Weight, mg</th>
<th>EDL Weight-to-Body Weight Ratio, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>311 ± 8</td>
<td>123 ± 5</td>
<td>0.4 ± 0.01</td>
<td>130 ± 6</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>HU-7</td>
<td>286 ± 7</td>
<td>82 ± 2**</td>
<td>0.29 ± 0.01***</td>
<td>121 ± 4</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>HU-14</td>
<td>290 ± 8</td>
<td>65 ± 3***§§</td>
<td>0.22 ± 0.01***§§</td>
<td>133 ± 4</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>HU-28</td>
<td>340 ± 4$$^\alpha$$ΔΔ</td>
<td>50 ± 2***</td>
<td>0.15 ± 0.01***ΔΔ</td>
<td>152 ± 3$$^\alpha$$ΔΔ</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>HU-14 + ES</td>
<td>299 ± 4</td>
<td>70 ± 2***</td>
<td>0.23 ± 0.01***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Hindlimb unloading (HU) was performed for 7 (HU-7), 14 (HU-14), and 28 days (HU-28), and electrical stimulation (ES) was delivered during 14 days of HU (HU-14 + ES). The level of soleus muscle atrophy was determined by the ratio of the muscle weight (mg) relative to the body weight (g). C, control; EDL, extensor digitorum longus. Significant differences are referenced as follows: comparison with C group (*P < 0.05, **P < 0.01, ***P < 0.001); comparison between HU-7 and HU-14 groups (§§P < 0.01, §§§P < 0.001); comparison between HU-14 and HU-28 groups ($$$P < 0.01, ΔΔΔP < 0.001).

As expected, muscle atrophy induced by HU was specific of the muscle type (slow vs. fast) since the MW/BW in the fast EDL remained constant whatever the HU duration (Table 1).

Chronic electrostimulation of soleus muscle prevented slow-to-fast MHC phenotype transition. Figure 1A shows SDS-PAGE representative of MHC isoform expression in soleus of C, HU-14, and HU-14 + ES. Relative contents of the fast and slow isofoms are presented in Fig. 1B for all of the experimental groups. MHC I, the predominant isoform in C soleus muscle (94%), progressively decreased between HU-7 and HU-28 (to 81 and 65%, respectively, P < 0.001). This reduction was in favor of a significant increase in MHC IIa at HU-7 (from 6 to 14%, P < 0.01), and the appearance of MHC IIld/x at HU-7 (5%, P < 0.05) and MHC IIb at HU-14 (2%, P < 0.05). The proportion in these two latter isoforms continuously increased to reach 16 and 8% at HU-28 (P < 0.001), whereas MHC IIa decayed (10%, nonsignificant compared with C). Moreover, we demonstrated a beneficial effect of the chronic electrical stimulation on the maintenance of soleus isoform distribution (Fig. 1, A and B). Indeed, in HU-14 + ES rats, only MHC I (87%; nonsignificant vs. C rats, and P < 0.01 vs. HU-14 rats) and MHC IIa (13%) were detected, whereas MHC IIld/x (11%, P < 0.001) and IIb (2%, P < 0.05) were found in HU-14. By consequence, soleus electrostimulation during HU maintained a MHC phenotype profile similar to C.

For C EDL, the predominantly expressed isoforms were MHC IIb (50%), MHC IIld/x (28%), and MHC IIa (18%). MHC I was expressed at very low levels (5%). Nonetheless, we did not observe significant modifications in this MHC isoform distribution for all HU time points (data not shown).

Glycolytic metabolism upregulation induced by HU was prevented by chronic electrostimulation of soleus. Figure 2 presents SDS-PAGE expression profiles (A) and relative contents (B) of proteins in C, HU-14, and HU-14 + ES groups. Bands selected on gel were identified by MALDI-TOF mass spectrometry analysis (Table 2). Validation of selected identified proteins by immunoblots is shown in Fig. 3.

We identified five upregulated enzymes involved in glycolytic metabolism upon HU: phosphoglucosemutase 1 (band 1), which catalyzes the reversible isomerization of 3-phosphoglycerate to 2-phosphoglycerate; PK (band 2), which catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP, yielding one molecule of pyruvate and one molecule of ATP; GAPDH (band 3), which catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate; LDH B (band 4), which catalyzes the reversible NAD-dependent interconversion of pyruvate to lactate; and cytosolic malate dehydrogenase (band 5), which catalyzes in the cytosol the reversible conversion of malate to oxaloacetate (oxaloacetate serving as substrate for phosphoenolpyruvate carboxykinase and so leading to pyruvate). We also identified AKI
(band 6), which is an important enzyme of nucleotide metabolism that reversibly converts 2 ADP into ATP and AMP.

According to Figs. 2 and 3, we observed a global ~1.5- to 2.5-fold significant increase in the expression of the identified metabolic proteins after 14 days of HU. Interestingly, chronic low-frequency electrical stimulation during HU allowed the maintenance of their expression close to C level.

**PI3K-AKT-mTOR protein synthesis pathway was downregulated in the soleus muscle by HU.** Figure 4 shows representative Western blots and relative contents of phosphorylated/whole expression level of key components of the PI3K-AKT-mTOR pathway in soleus. We showed a significant decrease of phosphorylated (P)-AKT (−64%, $P < 0.001$), P-GSK-3β (−44%, $P < 0.001$), and P-mTOR (−40%, $P < 0.001$) after 7 days of HU compared with C rats. This downregulation of key factors of the PI3K-AKT pathway was maintained after 14 and 28 days of HU ($P < 0.001$). As expected, the decrease in mTOR phosphorylation was associated with a decreased phosphorylation of its downstream targets p70S6K and 4E-BP1 in soleus muscle. From 7 days of HU, we measured a strong decrease (−96%, $P < 0.001$) of p70S6K phosphorylation, and, as mTOR regulation, the decrease was maintained for 14 and 28 days of HU ($P < 0.001$). Three isoforms of 4E-BP1, α-, β-, and γ-forms, have been described. According to their phosphorylated states, the higher phosphorylated form γ migrates slower than β and α, respectively (9). 4E-BP1 phosphorylation shift (calculated as $\gamma/\alpha + \beta + \gamma$ forms) was determined in P-4E-BP1 and 4E-BP1 Western blots, and we detected lower γ-4E-BP1 expression from 7 days (86 and 58%, respectively, $P < 0.001$) to 28 days of HU ($P < 0.001$).

**PI3K-AKT-FOXO protein degradation pathway was upregulated in the soleus muscle by HU.** Figure 4 also shows representative Western blots and relative contents of two components of the PI3K-AKT-FOXO pathway, FOXO1 and MuRF1. The decrease in AKT phosphorylation was correlated with a decrease in FOXO1 phosphorylation that was relatively constant from 7 days of HU (−57%, $P < 0.01$, compared with C) to 28 days of HU ($P < 0.01$). The expression of FOXO...
Table 2. Detailed list of differentially expressed proteins between C, HU-14, or HU-14 + ES soleus

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Accession No.</th>
<th>Protein Identification</th>
<th>Experimental Molecular Mass, kDa</th>
<th>Theoretical Molecular Mass, kDa</th>
<th>No. Matched Peptides/Total Peptides</th>
<th>Probability Score</th>
<th>Sequence Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P38652</td>
<td>Phosphoglucomutase 1</td>
<td>62.4</td>
<td>61.4</td>
<td>20/89</td>
<td>3.41E+12</td>
<td>35.6</td>
</tr>
<tr>
<td>2</td>
<td>P11980</td>
<td>Pyruvate kinase, isozymes M1/M2</td>
<td>57.7</td>
<td>57.8</td>
<td>26/76</td>
<td>5.11E+15</td>
<td>41.8</td>
</tr>
<tr>
<td>3</td>
<td>P04797</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>34.8</td>
<td>35.8</td>
<td>13/43</td>
<td>8.10E+8</td>
<td>34.2</td>
</tr>
<tr>
<td>4</td>
<td>P42123</td>
<td>L-lactate dehydrogenase B</td>
<td>33.5</td>
<td>36.6</td>
<td>14/64</td>
<td>1.70E+7</td>
<td>32.6</td>
</tr>
<tr>
<td>5</td>
<td>O88989</td>
<td>Malate dehydrogenase, cytosolic</td>
<td>32.6</td>
<td>36.4</td>
<td>8/39</td>
<td>3.56E+5</td>
<td>23.1</td>
</tr>
<tr>
<td>6</td>
<td>P39069</td>
<td>Adenylate kinase isozyme 1</td>
<td>21.8</td>
<td>21.5</td>
<td>9/88</td>
<td>3.10E+6</td>
<td>45.9</td>
</tr>
</tbody>
</table>

Numbers were assigned to polyepitidal spots identified on gel from Figure 2. Accession numbers were obtained from Swiss-Prot database. Probability scores result in the database interrogation on MS-Fit from Protein Prospector (http://www.expasy.ch/). Assignments were made according to UniProt release 2010_07, which consists of UniProtKB/Swiss-Prot Release 2010_07 of 15-Jun-10 (517,802 entries) and to UniProtKB/TrEMBL Release 2010_07 of 15-Jun-2010 (11,109,684 entries).

downstream target, MuRF1, was normalized to α-actin signal. HU induced a progressive increase in MuRF1 expression (~6.4 fold for HU-7, P < 0.05, and ~24.8-fold for HU-28, P < 0.001).

Soleus electrical stimulation strongly modulates synthesis and degradation of PI3K-AKT pathways in HU rats. The global downregulation of the P-AKT, P-GSK-3β, and P-mTOR was not compensated by chronic electrical stimulation, since their phosphorylation levels in HU-14

ERK phosphorylation was maintained by soleus electrical stimulation. The MAPK pathway activation has also been examined (Fig. 5). HU induced a significant and stable decrease in ERK phosphorylation in soleus for all of the HU periods (~42% for HU-7, P < 0.05, ~50% for HU-14, P < 0.01, and ~53% for HU-28, P < 0.01 vs. C). Chronic electrical stimulation of the unloaded soleus allowed the maintenance of ERK phosphorylation to C level, since ERK activation for HU-14 + ES was not significantly different from C, and significantly increased compared with HU-14 (+95%, P < 0.01).

p38 and JNK phosphorylations were upregulated by HU in soleus muscle. We observed an increase in p38 phosphorylation overall in the time course of HU in soleus (Fig. 5), with a maximal peak at HU-14 (+369%, P < 0.001). At HU-28, there was a tendency to return to the p38 activation level obtained at HU-7, a value significantly higher (P < 0.005) than in C rats. Chronic electrostimulation of the HU soleus had no specific effect on p38 phosphorylation, since its level in HU-14 + ES remained equivalent to that of HU-14. Here, it should be noticed that a slower migrating band, possibly p38α, could be detected in the HU groups (7). Since this p38 isoform was not expressed in C soleus muscle, densitometric analysis was only performed on the faster and predominant migrating band, p38α.

Phosphorylation of JNK was generally not detectable in C muscles. However, as shown in Fig. 5, the phosphorylation

Fig. 3. Immunoblot analysis of metabolic protein expression in C, HU-14, and HU-14 + ES soleus muscles. A: representative Western blots showing the expression of total forms of GAPDH, pyruvate kinase, lactate dehydrogenase, and adenylate kinase 1 in C, HU-14, and HU-14 + ES rats. B: protein expression in HU-14 and HU-14 + ES groups was measured by densitometric analysis and was compared with their basal expression in the C group. Actin was used as an internal standard. Data are expressed as au. Comparison with C group (*P < 0.05, ###P < 0.001); comparison between HU-14 and HU-14 + ES groups (#P < 0.05, ##P < 0.01, ###P < 0.001).
level progressively increased until HU-14 (+317%, $P < 0.001$, from C rats) and returned to basal rate at HU-28 ($P < 0.001$, from HU-14 rats). It seems that chronic electrostimulation of the HU soleus had no specific influence on JNK activation level since it remained similar to the one of HU-14.

**DISCUSSION**

The general interest of this study was to analyze the role of PI3K-AKT and MAPK intracellular pathways in regulating muscle atrophy and fiber-type transitions in a model of func-
tional atrophy. By using HU over 7, 14, and 28 days and chronic low-frequency electrostimulation during one period of disuse (14 days), we demonstrated that PI3K-AKT pathway was implicated in the regulation of muscle mass in unloading conditions, but was not the only pathway involved in the atrophy process, since electrostimulation could not prevent mass atrophy while it restores the activation of this pathway. Moreover, soleus chronic electrostimulation during HU preserved contractile phenotype and metabolism profile characteristics of slow fibers and, in parallel, ERK phosphorylation level similar to that of C rats. These data reinforced the predominant role of ERK in the regulation of muscle slow phenotype.

**Regulation of muscle mass by PI3K-AKT and MAPK pathways.** We focused our study on the temporal regulation of PI3K-AKT and MAPK pathways in atrophied soleus during 4 wk of HU.

PI3K-AKT pathway is known to play a key role in the balance between protein synthesis and degradation (12, 33, 46). In soleus, we showed a downregulation of the anabolic PI3K-AKT-mTOR pathway through the reduced phosphorylation of AKT, mTOR, p70S6K, and 4E-BP1 during HU. Furthermore, HU decreased GSK-3β phosphorylation and could be at the origin of eIF2B inhibition (12). Our findings are in agreement with other studies following 10 or 14 days of HU or limb immobilization (9, 39). The reduced phosphorylation of p70S6K and 4E-BP1 and the inhibition of eIF2B could explain decreased protein synthesis during HU, as described by Ref. 17. Moreover, HU led to the upregulation of the catabolic PI3K-AKT-FOXO pathway. Indeed, reduced phosphorylation of AKT was followed by the activation of FOXO1 in the nucleus and the increased expression of MuRF1, promoting protein breakdown (34, 46). Overall, regulation of the PI3K-AKT pathway contributed to the loss of muscle protein in unloaded soleus. Moreover, progressive muscle atrophy process observed here could be correlated with the evolution of MuRF1 expression (4), which was progressive during the 4 wk of HU.

Chronic low-frequency electrostimulation was performed on the soleus during the 14 days of HU. We showed a downregulation of AKT, GSK-3β, and mTOR phosphorylation in HU-14 ES rats. However, the activation of p70S6K, 4E-BP1, and FOXO1, as well as the expression of MuRF1, were restored to C values in HU-14 ES. These data suggest that PI3K-AKT downstream targets may be activated by contractile activity induced by electrostimulation protocol via PI3K-AKT-independent mechanisms (30, 45). As a matter of fact, mechanisms through which mechanical activity regulates signaling pathways remain largely unidentified. Targets of these pathways could be specifically influenced by electrostimulation protocols, i.e., modes of contractile activity (acute vs. chronic), stimulation patterns, duration/number of contractions, and length of stimulation protocol, fiber, and/or muscle type (2, 42). Furthermore, upregulation of p70S6K and 4E-BP1 and restoration of FOXO1 and MuRF1 to C levels were both ineffective to prevent soleus atrophy. This intriguing result was also supported by study of Ref. 32, which suggested that electrostimulation downregulated MuRF1 in denervated muscles, but failed to reduce muscle atrophy. Thus our data clearly demonstrate that PI3K-AKT pathway, although implicated in the regulation of muscle mass, was not the only signaling pathway involved in the atrophy process.

MAPK pathway was also shown to be implicated in the regulation of muscle mass (15, 23, 36). Although a previous study (28) hypothesized that ERK was not implicated in muscle mass regulation, Shi et al. (36) reported that in vitro
ERK inhibition decreased myotube size and protein content. Additionally, ERK inhibition upregulated gene transcription of atrogin-1 and MuRF1 and downregulated AKT phosphorylation and its downstream kinases, GSK-3β and p70S6K. Interestingly, we demonstrated the same events during the 4 wk of HU, and we hypothesized that ERK could induce soleus atrophy through activation of PI3K-AKT pathway. Nevertheless, soleus electrostimulation during HU restored ERK phosphorylation to C level without reducing muscle atrophy, suggesting the development of atrophy in HU could not be directly attributed to ERK. Concerning the other members of the MAPK cascade, JNK has been shown to negatively regulate PI3K-AKT pathway through the phosphorylation of the insulin receptor substrate-1 (15, 21), and p38 was demonstrated to stimulate protein degradation through atrogin-1 in skeletal muscle (23). Consistent with the elevated phosphorylation of p38 and JNK, both in HU-14 and HU-14 + ES, soleus atrophy could result from the interaction between these kinases and the PI3K-AKT pathway.

Regulation of soleus MHC phenotype by MAPK and PI3K-AKT pathways. Skeletal muscles are composed of a heterogeneous population of fiber types characterized by their contractile and metabolic properties. In agreement with previous studies (38, 40), we demonstrated a transition from slow-to-fast myosin fiber types in slow-twitch soleus associated with metabolic changes, including a shift toward an increased expression in glycolytic enzymes, as well as in energy metabolism-related proteins (18, 37).

Previous experiments demonstrated the key role of MAPK pathway in the regulation of MHC phenotype (14, 28, 35). Here, the decreased ERK phosphorylation in unloaded soleus could trigger the phenotype transition from slow-to-fast MHC isoforms (appearance of MHC IIx at HU-7, and of MHC IIb at HU-14), as well as the acquisition of enzyme markers of glycolytic metabolism since ERK cascade was demonstrated 1) to upregulate slow muscle fiber expression and to downregulate MHC IId/x and IIb isoform expression (14, 28, and 2) to downregulate the glycolytic enzyme PK (14). Interestingly, we showed that chronic electrostimulation during 14 days of HU maintained ERK phosphorylation level similar to C rats and initial slow contractile phenotype in soleus muscle. Moreover, we demonstrated, for the first time, that electrostimulation prevented glycolytic metabolism upregulation in unloaded soleus. By consequence, these data strongly supported that ERK could be implicated in the regulation of contractile phenotype and glycolytic metabolism profile observed during muscle disuse conditions.

p38 phosphorylation was strongly increased in HU, as reported after 10 days of limb immobilization or 2 wk of HU (9, 15). Furthermore, p38 could regulate the quantitative expression of fast MHC IId/x and IIb isoforms, as previously suggested by Ref. 24. However, MHC IId/x and IIb were not expressed in HU-14 + ES, although p38 was highly phosphorylated, suggesting that expression of fast MHC isoforms in unloaded soleus could be regulated through other mechanisms. According to Ref. 1, JNK may be involved in the activation of MHC IIa promoter. Nevertheless, MHC IIa expression was clearly dissociated from the time course of JNK activation in unloaded soleus, excluding a key role of JNK in the MHC phenotype regulation.

Finally, it is generally reported in literature that fiber-type regulation is independent of PI3K-AKT pathway (29, 39). However, FOXO1 was shown to negatively regulate type I fiber gene expression in mice (19). Interestingly, in our study, FOXO1 was downregulated during the 4 wk of HU in parallel to MHC I decreased expression. Moreover, FOXO1 was restored to C level by soleus electrostimulation during HU concomitantly with the maintenance of slow MHC phenotype. Accordingly, the involvement of PI3K-AKT-FOXO pathway in fiber-type regulation during HU remains to be clearly demonstrated, and further experiments have to be performed to confirm this hypothesis.

Influence of HU on fast EDL muscle properties. The data obtained on EDL muscle provide evidence that the regulation of PI3K-AKT and MAPK signaling pathways and structural/phenotypical muscle changes are closely associated during HU conditions. Indeed, in fast EDL, muscle mass and MHC composition were unchanged in HU conditions (from 7 to 28 days of HU). Moreover, immunoblot analyses performed on PI3K-AKT and MAPK pathways revealed that expression and activation of these signaling pathways remained stable in EDL, whatever the HU duration (data not shown). All together, our data suggested that the observed changes in disuse conditions only concerned slow soleus muscle and, therefore, are muscle specific.

Perspectives and Significance

The processes responsible for gradual skeletal muscle plasticity induced by a time course of HU seem to involve PI3K-AKT and MAPK signaling pathways. However, the implication of these processes is probably more complex than what has been previously hypothesized, since we showed that soleus chronic electrostimulation during HU restored PI3K-AKT and ERK pathway activation to C level without counteracting the decrease in mass. Our data clearly demonstrated that muscle atrophy in unloaded soleus is a highly ordered adaptive event that is probably controlled by interactions between PI3K-AKT, MAPK, and other signaling pathways. Further experiments should be conducted by using specific electrostimulation protocols to better understand the specific involvement of other catabolic pathways (AMPK, calcineurin, nuclear factor-κB, or myostatin signaling cascades), contributing to the regulation of muscle plasticity during HU.

GRANTS

This work was supported by the Centre National d’Etudes Spatiales (no. 9024) and grants from Association Française contre les Myopathies (no. 11817).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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