Hypoxia transiently affects skeletal muscle hypertrophy in a functional overload model

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PLASTICITY IS A FUNDAMENTAL design feature of muscle tissue that enables mammalian species to respond to specific challenges of the environment. Hypertrophic growth of skeletal muscle is one expression of muscle malleability in response to increased load-bearing work. It is now well established that gene expression is regulated at multiple steps, including transcription, posttranscriptional processing, and finally translation of mRNA messenger (mRNA) into proteins. Mechanical factors involved in muscle growth mainly regulate the translation processes that are determined by the activation of intracellular signaling pathways. Protein synthesis is under the control of the Akt/mTOR signaling pathway (45). When active, mTOR enhances the translation efficiency by activating the eukaryotic initiation factor-4E complex, which is inhibited by the eIF-4E binding protein (4E-BP1). Moreover, mTOR increases the translation capacity and enhances the ribosomal biogenesis via the translation regulator P70S6K.

Skeletal muscle mass is tightly regulated by the synergy between protein synthesis and degradation, two concomitant processes that are determined by the activation of intracellular signaling pathways. Protein synthesis is under the control of the PI3K (phosphatidylinositol 3-kinase)/Akt/mTOR (mammalian target of rapamycin) signaling pathway (45). When active, mTOR enhances the translation efficiency by activating the eukaryotic initiation factor-4E complex, which is inhibited by the eIF-4E binding protein (4E-BP1). Moreover, mTOR increases the translation capacity and enhances the ribosomal biogenesis via the translation regulator P70S6K. The control of muscle mass also depends on proteolytic influences, in which the ubiquitin-proteasome system (UPS) is known to be involved in protein degradation (26). Two atrogenes, muscle atrophy F box (MAFBx) and muscle ring finger-1 (MURF1), are often used to assess the UPS activity and have been shown to be overexpressed in numerous catabolic conditions (8). In addition, myostatin, a transforming growth factor-β (TGFβ) member, has been reported to be a negative regulator of muscle mass (3), presumably through a downregulation of the Akt/mTOR pathway (49).

Functional overload induced by the surgical removal of synergistic muscles is a relevant model to study muscle growth in rodents, since chronic mechanical strains induce a marked muscle hypertrophy and increase the size of all myofibers (5). The compensatory hypertrophy observed in this model involves activation of the Akt/mTOR signaling pathway (9, 35). However, the effects of mechanical overload on markers of selected catabolic pathways, such as myostatin signaling or expression of the muscle-specific ubiquitin-ligases MAFBx and MURF1, remain controversial and have to be elucidated.

Low-oxygen availability (hypoxia) could negatively affect muscle mass. Muscle atrophy has already been observed in humans after expeditions in high altitude (44), while a reduction in muscle growth has been shown in rodents chronically exposed to hypobaric hypoxia (7, 16). In vitro studies showed that the Akt/mTOR pathway could be inhibited by hypoxia exposure (12, 27, 31), but to our knowledge, only few in vivo studies reported that prolonged hypoxia exposure altered this signaling pathway in skeletal muscles (16). Myostatin, a mediator of muscle atrophy, has been shown to partly account for the hypoxia-induced effects on muscle mass (20). Moreover, although the UPS was stimulated in hypoxic heart (43), it remained unaffected by hypoxia in skeletal muscle, at least after chronic exposure (16). Thus, the effects of ambient hypoxia on both anabolic and catabolic signaling pathways need to be carefully examined in skeletal muscle during hypoxia exposure.

The mechanisms by which hypoxia could minimize the mTOR pathway activity are poorly understood. One explana-

tion could be related to the hypoxia-induced alteration of the energy homeostasis. The AMPK complex, which is now considered as the main sensor and regulator of energy balance at the cellular level (18), is activated under conditions of metabolic stress (19). Recent studies revealed that AMPK activity inhibits mTORC1 (mTOR complex 1) signaling, directly by phosphorylating raptor, or indirectly by phosphorylating an upstream repressor of mTOR, tuberous sclerosis complex 2 (TSC2) (18). The activation of AMPK in response to hypoxia exposure in human embryonic kidney 293 cells has been shown to inhibit mTOR and its downstream effectors 4E-BP1, P70S6K, and eukaryotic elongation factor 2 (eEF2) (31), but the activation of AMPK in skeletal muscle during hypoxia exposure and its function on the regulation of the Akt/mTOR signaling need to be clarified.

Moreover, some hypoxia-inducible factors, such as regulated in development and DNA damage response 1 (REDD1) or BCL2 and adenovirus E1B 19-kDa-interacting protein 3 (BNIP-3), could be potentially involved in the inhibition of the mTOR signaling. REDD1 contributes to the dissociation of the TSC2/14–3-3 complex, a mechanism leading to mTOR inhibition during acute hypoxia in vitro (14). It has been recently shown that REDD1 expression increased in skeletal muscle during chronic hypoxia, in parallel with an alteration of muscle growth and a down-regulation of the mTOR pathway (16). BNIP-3 is a proapoptotic protein that belongs to the Bcl-2 superfamily (54). In response to a hypoxia exposure, BNIP-3 inhibits the ability of Rheb, a Ras-related small GTPase, to activate mTOR, (29). However, the exact relevance of BNIP-3 expression on the control of muscle mass during exposure to ambient hypoxia remains to be examined.

In the current study, we investigated the main signaling pathways determining the muscle growth mass under a situation of challenge between both positive (muscle functional overload) and negative (hypoxia exposure) influences. We hypothesized that hypobaric hypoxia could impair the hypertrophic response of skeletal muscle. Thus, we examined whether the overload-induced muscle hypertrophy may be altered by hypoxia, due, in part, to the activation of metabolic (i.e., AMPK) and/or hypoxia-induced factors (i.e., REDD1 and BNIP-3). To test this hypothesis, we compared the response of muscle mass at days 5, 12, and 56 after functional overload of plantaris muscle in rats exposed to either normoxic or hypobaric hypoxia (hypoxia exposure was imposed 3 days after surgical procedure). Moreover, we assessed the involvement of the ubiquitin proteasome pathway, by studying expression of the muscle-specific ubiquitin-ligases MAFBx and MURF1 (45). Although activation of satellite cells is likely involved in the early hypertrophic response of functional overloaded muscle (47), we focused our attention in the present study on the effects of hypoxia on intracellular signaling pathways involved in muscle protein synthesis and degradation.

**MATERIAL AND METHODS**

**Animals**

Seventy-seven female rats of Wistar Han strain initially weighing 170–180 g were purchased from Charles River Laboratories (L’Arbresle, France). Because sex differences have been previously reported on the adaptive responses of rats to hypoxia, with females being more tolerant to hypoxia than males, female rats were used in the present study (53). All animals were housed one 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.

**Experimental Design**

After 7 days of acclimatization to the animal facility, rats were randomly assigned to either control (Ct) or overload (Ov) groups. Mechanical overload of plantaris muscles was accomplished by the bilateral removal of its synergists, the soleus and the gastrocnemius. After 3 days of recovery, Ct and Ov animals were both divided into two main subgroups: normoxic (N) and hypoxic (H) groups (n = 38, n = 39, respectively). This recovery period was set to avoid an excessive weakness that results from the surgical removal of plantaris agonist muscles in Ov animals and to minimize the risk of death after acute exposure to hypoxia. OvH and CtH rats were housed in a hypobaric chamber and exposed to barometric pressure equivalent to 5,500 m altitude (barometric pressure = 370 mmHg; PO2 = 79 mmHg). The chamber was opened daily to measure food consumption and refill water dispensers. The retention period did not exceed 30–45 min. Animals in each subgroup (CtN, CtH, OvN, and OvH) were then anesthetized for tissue sampling and killed 5, 12, and 56 days after the beginning of the experiment, i.e., 2, 9, and 53 days after hypoxia exposure for H groups (n = 6–8 at each time).

**Surgical Procedure**

Rats of the Ov groups were anesthetized with pentobarbital sodium (50 mg/kg ip), and compensatory overload was accomplished by the bilateral removal of its synergists, as previously described (6). Briefly, a small incision was made to the posterior lower limb, and the entire soleus was carefully removed, as well as the major portion of the gastrocnemius. Particular attention was made to ensure that plantaris neural and vascular supplies were not damaged before anepinephrosis, and the skin was independently sutured. A sham operation was systematically performed in control nonoverloaded hindlimbs, which consisted of separating tendons of the soleus and gastrocnemius muscles from that of the plantaris muscle.

**Tissue and Blood Processing**

Animals were anesthetized with pentobarbital sodium (80 mg/kg ip). Blood was withdrawn from the abdominal aorta into a heparinized syringe, and a portion of the blood was analyzed for hematocrit (Hct). Plantaris muscles were carefully dissected and weighed. Left plantaris muscles were slightly stretched to recoil passively to a roughly resting length and frozen in isopentane cooled to its freezing point (−160°C) by liquid nitrogen. The distal half portions of right plantaris muscles were immediately frozen in liquid nitrogen and stored at −80°C for later use. The proximal half portions of right plantaris muscles were immediately placed in 0.5 ml of RNAlater (Ambion, Austin, TX), maintained at +4°C for 24 h and then stored at −20°C until use. Animals were subsequently euthanized by lethal injection of pentobarbital sodium.

**Histomorphometric Analyses**

For each experimental group at day 12, serial transverse sections (14 μm thick) were cut from the third of the proximal part of plantaris muscles in a cryostat microtome maintained at −20°C and were stained with hematoxylin and eosin to visualize the nucleus and the cytoplasm. To determine the whole 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 cross-sectional area of at least 400 fibers (FCSA). The number of myofibers exhibiting internal nuclei, indicative of myofiber regeneration (28), was recorded...
in at least 20 fields. The number of nuclei per myofiber (assessed on at least 400 fibers) and the number of myofibers per muscle were also determined to assess the potential involvement of satellite cells in muscle hypertrophy. Analyses were performed with a light microscope computerized image-analysis system (Lucia 5; Laboratory Imaging, Prague, Czech Republic).

Analysis of the Distribution of Immature Myosin Heavy-Chain Isoforms

Muscles removed at days 5 and 12 were subjected to the analysis of myosin heavy-chain (MHC) isoforms, as described previously (48), to examine the additive effect of hypoxia on the putative enhanced expression of embryonic and neonatal MHC isoforms during functional overload. Myosin was extracted from small sections of —80°C frozen muscles (15—25 mg) in 10 volumes of buffer solution (0.3 M NaCl, 0.1 M NaH2PO4, 0.05 M Na2HPO4, 0.01 M Na2PO3, 1 mM MgCl2, 6 mM H2O, 10 mM EDTA, 1.4 mM 2-β-mercaptoethanol, pH = 6.5). Electrophoresis was performed using a Mini Protein III system (Bio-Rad, Marne-la-Coquette, France) with 8% and 4% acrylamide-bis (50:1) separating and stacking gels, respectively, containing 0.4% SDS. Myofibril samples were denatured with the SDS incubation medium, according to the method of Laemmli. Gels were run at constant voltage (72 V) for 31 h and then silver-stained (2). The MHC protein isoform bands were scanned and quantified using a densitometer GS 800.

Protein Isolation and Immunoblot Analyses

Initial —80°C frozen muscles samples were homogenized at 4°C in 15 vol buffer [50 mM Tris·HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM sodium fluoride, 120 mM okadaic acid, 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 50 mM glycerophosphate, 10 μl/ml activated sodium orthovanadate, 3 μl/ml protease inhibitor cocktail (set III, EDTA-free, Calbiochem, Darmstadt, Germany), and 3 μl/ml phosphatase inhibitor cocktail (set II, Calbiochem)]. Homogenates were centrifuged at 12,000 g for 20 min at 4°C. Protein concentration was determined by the BCA method. Equal amounts of total protein (50 or 75 μg) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Hybond C-extra, Amersham Pharmacia Biotech, Orsay, France). A standardized amount of protein prepared from overloaded plantaris was also applied on each gel to serve as an internal standard for comparison across blots. Membranes were incubated overnight at 4°C with primary antibodies coming from Cell Signaling Technology (Beverly, MA) [mTOR (1:500, no. 2,972), phospho-mTORSer2448 (1:500, no. 2,971), Akt1 (1:500, no. 2,938), phospho-AktThr308 (1:500, no. 4,056), phospho-AktSer473 (1:500, no. 9,271), P70S6K (1:1,000, no. 2,708), phospho-P70S6KThr389 (1:1,000, no. 9,234), rpS6 (1:1,000, no. 2,217), phospho-rpS6Ser240/244 (1:1,000, no. 4,838), 4E-BP1 (1:1,000, no. 9,452), phospho-4E-BP1Thr70 (1:1,000, no. 9,455), EF2 (1:2,000, no. 2,332), phospho-EF2 (1:1,000, no. 2,331), AMPKα (1:500, no. 2,352), phospho-AMPKαThr172 (1:500, no. 2,353)] from Santa Cruz Biotechnology (Heidelberg, Germany), [myostatin (1:200; sc-34781)] from Protein Tech Group (Chicago, IL) [REDD1 (1:750; no. 10,638—1-AP), or from Abcam (Cambridge, MA)] [BNP-3 (1/1,000; no. 109,362)]. Incubation with corresponding secondary antibodies [anti-goat (1:10,000, sc 46034)], or from eBioscience (San Diego, CA) [myostatin (1:200; sc-34781)] from Protein Tech Group (Chicago, IL), [REDD1 (1:750; no. 10,638—1-AP)], or from eBioscience (San Diego, CA) [BNP-3 (1/1,000, sc 46034)], or from eBioscience (San Diego, CA) [myostatin (1:200; sc-34781)]. Blots were then exposed to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech), and protein expression was determined by the ratio of sample band intensity to the internal standard band intensity by densitometry using the densitometer GS 800 driven by Quantity One 4.6.1 software (Bio-Rad, Marne-la-Coquette, France).

RNA Isolation and cDNA Synthesis

Muscle samples were disrupted (100 mg/ml) with a Mixer Mill MM300 (Retsch, Haan, Germany) in RLT lysis buffer, 1% β-mercapto-ethanol (Qiagen, Courtaboeuf, France) following the manufacturer’s instructions [30 Hz, 2 min; 3-mm ceramic beads (×3)]. After optimal tissue lysis [isovolume QIAzol lysis reagent (Qiagen) + 1/5 vol chloroform], total RNA was isolated using RNeasy Lipid Tissue Mini-kit (Qiagen, Venlo, The Netherlands) with additional DNase step, according to the manufacturer’s instructions using a Qiagene (Qiagen). The total RNA concentration and purity were assessed by measuring the optical density (230, 260, and 280 nm) with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcription was carried out in a 20-μl reaction volume from 800 ng, using the Reverse Transciptase Core Kit (Eurogentec, Seraing, Belgium), with 50 μg oligo (dT) 15 primer and RNase inhibitor (4 UI), according to the manufacturer’s instructions. The same number of samples within each experimental group was simultaneously processed, to minimize intergroup variations (extraction and reverse transcription steps). All samples were stored at —80°C until further analysis.

Primer Design

Primer design and optimization, concerning dimerization, self-priming, and melting temperature were carried out using MacVector software (Accelrys, San Diego, CA). Primers were designed in intron flanking to exclude amplification of genomic DNA. Primers were then assessed for specificity, using Blast algorithm (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), with search parameters adjusted for a short input sequence. Primer optimization was carried out as described previously (41). Specificity of the PCR amplification was checked comparing recombinant DNA with unknown products, by melting curve analysis, as described previously (41). Selected forward and reverse primer sequences and characteristics are listed in Table 1.

Real-Time Quantitative PCR and Quantification

Quantitative PCR (qPCR) was carried out with LightCycler Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany). Primers were designed in intron flanking to exclude amplification of genomic DNA. Primers were then assessed for specificity, using Blast algorithm (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), with search parameters adjusted for a short input sequence. Primer optimization was carried out as described previously (41). Specificity of the PCR amplification was checked comparing recombinant DNA with unknown products, by melting curve analysis, as described previously (41). Selected forward and reverse primer sequences and characteristics are listed in Table 1.

Target mRNA quantification was performed as previously described (11). Briefly, the use of reference genes was ruled out for the comparison of J) Ov and Ct groups, and 2) Ov groups between day 5 and 12, since normalization factors (NFs) were not constant between these groups. For these between-group comparisons, the mRNA levels were determined per unit mass of tissue, multiplying the exponential conversion of the Cq values, corrected for amplification efficiency, by the total RNA concentration (21). Otherwise, constant NFs were found between all of the Ct groups and between OvH and OvN groups at both days 5 and 12, using four reference genes [Tbp, Hprt1(a), Arbp, Pol2ra]. On the basis of the geNorm analysis (50), we selected these four genes since their pairwise variations were below the threshold (0.15) that requires the inclusion of an additional control gene. For these groups, relative quantification of mRNA was performed, using multiple reference genes, as previously described (11).
RESULTS

Body and Muscle Masses

The lower values of body mass observed in Ovq rats compared with Ct animals at day 3, support a specific effect of the surgical ablation of synergistic muscles on the body growth rate (Table 2). The sham operation failed to significantly affect the body mass growth, whereas the bilateral surgical excision of plantaris synergist muscles led to a mean decrease in body mass (8 and 15 g in 3 days in normoxic and hypoxic rats, respectively; data not shown). Since body mass of OvH rats was lower than that of OvN animals at day 5, an additional effect of ambient hypoxia on the body growth rate was sug-

Table 2. Morphological data

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 12</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>179.1 ± 3.8</td>
<td>183.3 ± 3.2</td>
<td>199.0 ± 2.4$</td>
<td>240.4 ± 6.5$</td>
</tr>
<tr>
<td>CH</td>
<td>181.3 ± 1.8</td>
<td>170.4 ± 2.5#</td>
<td>181.9 ± 2.2#</td>
<td>215.9 ± 4.2#</td>
</tr>
<tr>
<td>OvN</td>
<td>171.3 ± 1.3*</td>
<td>170.0 ± 2.5*</td>
<td>181.5 ± 2.2*</td>
<td>221.3 ± 3.2*</td>
</tr>
<tr>
<td>OvH</td>
<td>166.5 ± 2.9*</td>
<td>158.5 ± 2.6#</td>
<td>173.8 ± 1.8$</td>
<td>212.9 ± 1.8$</td>
</tr>
<tr>
<td>Plantaris mass, mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td></td>
<td>169.9 ± 7.8</td>
<td>201.9 ± 10.0</td>
<td>224.1 ± 7.4</td>
</tr>
<tr>
<td>CH</td>
<td></td>
<td>164.0 ± 4.3</td>
<td>173.5 ± 8.1</td>
<td>207.3 ± 8.7</td>
</tr>
<tr>
<td>OvN</td>
<td></td>
<td>253.0 ± 7.1*</td>
<td>336.5 ± 16.8*$</td>
<td>422.4 ± 26.8*$</td>
</tr>
<tr>
<td>OvH</td>
<td></td>
<td>222.2 ± 5.8#</td>
<td>302.7 ± 11.9*$#</td>
<td>424.6 ± 15.0*$#</td>
</tr>
<tr>
<td>Normalized plantaris mass, mg·100 g⁻¹·body mass⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>88.6 ± 3.6</td>
<td>92.1 ± 3.0</td>
<td>93.3 ± 2.7</td>
<td>93.3 ± 2.7</td>
</tr>
<tr>
<td>CH</td>
<td>95.3 ± 1.8</td>
<td>93.0 ± 2.7</td>
<td>95.5 ± 3.1</td>
<td>95.5 ± 3.1</td>
</tr>
<tr>
<td>OvN</td>
<td>137.2 ± 4.2#</td>
<td>167.1 ± 7.0*$</td>
<td>187.8 ± 11.5*$</td>
<td>187.8 ± 11.5*$</td>
</tr>
<tr>
<td>OvH</td>
<td>132.9 ± 3.6*</td>
<td>161.0 ± 5.4*$</td>
<td>196.3 ± 6.0*$</td>
<td>196.3 ± 6.0*$</td>
</tr>
</tbody>
</table>

Values are means ± SE. CN, control nonoverloaded plantaris muscle from normoxic rats; CH, control nonoverloaded plantaris muscle from hypoxic rats; OvN, overloaded plantaris muscle from normoxic rats; OvH, overloaded plantaris muscle from hypoxic rats. *Significantly different from the respective Ct group. #Significantly different from the respective normoxic group. $Significantly different from the previous time for the same group.
gested (environment × overload interaction), specifically, early after hypoxia exposure (Table 2). Moreover, the body mass gain recovered quickly and was roughly similar for each experimental group between day 12 and day 56 (~20% for both N and H groups).

As expected, hypoxia exposure led to increased Hct values. Reference values observed in normoxic rats at day 56 (42.5 ± 0.9%) reached 55.5 ± 3.4% and 61.5 ± 1.6% in Ct-H and Ov-H groups, respectively (global effect of environment).

Plantaris muscle mass was significantly affected by functional overload, time, and environment (Table 2). The Ov-induced muscle hypertrophy was shown as early as day 5 in both H and N groups, and it increased progressively up to day 56 (overload × time interaction). Considering parallel changes in the total protein content of plantaris muscles and muscle mass (data not shown), it emerges that edema likely accounts for approximately 25–40% of the Ov-induced muscle hypertrophy, without specific effect of hypoxia. When normalized to body mass, hypoxia failed to affect plantaris muscle mass.

Hypoxia exposure transiently minimized the Ov-induced hypertrophy. At day 5, plantaris muscle mass was 12% lower in OvH than in OvN rats, and the Ov-induced muscle hypertrophy was lower in hypoxia than in normoxia (35% and 49%, respectively). Hypoxia did not affect the Ov-muscle growth rate between days 5 and 12, and Ov-H muscles remained 10% lower than in N animals at day 12. At day 56, there was no difference in plantaris muscle mass between OvH and OvN animals.

Histological and Morphological Aspects of Overloaded Muscles

Histomorphometric analyses were only performed at day 12 to assess entire plantaris CSA, mean FCSA and the number of myofibers per muscle (Table 3). The muscle CSA was higher in both OvN and OvH groups than in their respective Ct groups. As illustrated in Fig. 1A, parallel changes were shown in mean FCSA (global effect of overload). The Ov-induced muscle hypertrophy was minimized by hypoxia (global effect of environment on CSA and mean FCSA). Indeed, mean FCSA was 17% lower in OvH than in OvN groups. These results paralleled the changes reported in plantaris muscle mass at day 12. The total number of myofibers per muscle and the number of nuclei per myofiber were unaffected by overload and hypoxia exposure (Table 3).

Food Intake

Mean absolute daily intakes are reported for the first 4 wk of hypoxia exposure (Table 4). Compared with normoxic rats, the mean daily food intakes were 39% and 35% lower during the first week for Ct and Ov groups, respectively. The hypoxia-induced decrease in mean absolute food intake was minimized during the 3 following weeks, and reached in mean 23% and 15% for Ct and Ov groups, respectively.

Markers of Myofiber Regeneration

We determined the number of fibers with internal nuclei to assess the extent of mechanical overload-induced muscle damage. Only a negligible number of internal myonuclei was observed (about 1 for 200 myofibers) in both OvH and OvN muscles, especially in peripheral areas, without any effect of ambient hypoxia.

Similarly, the developmental MHC isoform distribution was assessed during the early stages of overload-induced hypertrophy (Fig. 1B). The embryonic MHC isoform was not detected in Ov muscles in either N or H animals. In contrast, the neonatal MHC isoform was detected in both OvN and OvH muscles using the SDS-PAGE analysis, but its relative amount did not exceed 5% of the total MHC isoforms and was not affected by ambient hypoxia.

As hypoxia only minimized the Ov-induced hypertrophy until day 12 (Tables 2 and 3), modulation of intracellular pathways was only assessed at day 5 and day 12.

Table 3. Histological analyses

<table>
<thead>
<tr>
<th>Day 12</th>
<th>CSA, mm²</th>
<th>FCSA, μm²</th>
<th>Number of Myofibers per Muscle</th>
<th>Number of Nuclei per Myofiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>CtN</td>
<td>9.4 ± 0.2</td>
<td>1911 ± 102</td>
<td>3639 ± 551</td>
<td>2.49 ± 0.15</td>
</tr>
<tr>
<td>CtH</td>
<td>7.5 ± 0.5</td>
<td>1668 ± 11</td>
<td>3412 ± 245</td>
<td>2.45 ± 0.08</td>
</tr>
<tr>
<td>OvN</td>
<td>18.0 ± 1.5</td>
<td>2765 ± 150*</td>
<td>3922 ± 484</td>
<td>2.54 ± 0.05</td>
</tr>
<tr>
<td>OvH</td>
<td>15.2 ± 0.6*</td>
<td>2293 ± 151*#</td>
<td>3622 ± 118</td>
<td>2.53 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. CSA, cross section area; FCSA, fiber cross sectional area. *Significantly different from the respective Ct group. #Significantly different from the respective normoxic group.
Phosphorylation of Akt, mTOR, and Downstream Effectors

Akt phosphorylations on Thr\(^{308}\) and Ser\(^{473}\) were enhanced by Ov, especially at day 5, in both N and H animals (overload × time interaction) (Fig. 2, A1 and A2). Akt\(^{Thr308}\) and Akt\(^{Ser473}\) levels were not affected by hypoxia exposure. Changes in total Akt1 protein levels, the main Akt isoform expressed in skeletal muscle, paralleled the variations of the phosphorylated proteins (Fig. 2A3). As for the phosphorylated forms, hypoxia per se failed to alter Akt1 protein levels. The ratios of both Akt\(^{Thr308}\) and Akt\(^{Ser473}\) to Akt1 were higher in Ov than in Ct muscles, without effect of hypoxia (global effect of overload; Fig. 2A4 and A5).

Functional overload, time, and environment affected phosphorylated mTOR (mTOR\(^{Ser2448}\)) (Fig. 2B1). mTOR\(^{Ser2448}\) levels were higher in Ov than in Ct muscles at day 5 in N and H animals, and no significant differences were found between OvN and OvH groups. In contrast, the Ov-induced increase in mTOR\(^{Ser2448}\) was markedly reduced by hypoxia at day 12 (69% compared with OvN; overload × time × environment interaction). Total mTOR protein levels showed similar alterations (Fig. 2B2). A significant increase of the ratio of phosphorylated to total expression of mTOR occurred in overloaded muscles at day 5 (global effect, Fig. 2B3), whereas hypoxia exposure failed to increase the proportion of active mTOR in Ov muscles at day 12.

Table 4. Daily food intake after the 1st, 2nd, 3rd, and 4th week of hypoxia exposure

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CtN</td>
<td>18.0 ± 0.5</td>
<td>19.2 ± 0.5</td>
<td>20.4 ± 0.4$</td>
<td>19.4 ± 0.5</td>
</tr>
<tr>
<td>CtH</td>
<td>10.8 ± 0.2#</td>
<td>15.6 ± 0.4$</td>
<td>16.4 ± 0.3#</td>
<td>15.1 ± 0.5#</td>
</tr>
<tr>
<td>OvN</td>
<td>15.1 ± 0.8*</td>
<td>18.0 ± 0.6$</td>
<td>18.6 ± 0.7*</td>
<td>18.0 ± 0.5</td>
</tr>
<tr>
<td>OvH</td>
<td>11.9 ± 0.5#</td>
<td>15.7 ± 0.3#</td>
<td>15.4 ± 0.3#</td>
<td>15.1 ± 0.3#</td>
</tr>
</tbody>
</table>

Values are means ± SE. Absolute food intake values are given in grams per day. *Significantly different from the respective Ct group. #Significantly different from the respective normoxic group. $Significantly different from the previous time for the same group.

Fig. 2. Phosphorylated (A1 and A2) and total (A3) levels of Akt and mammalian target of rapamycin (mTOR) (B1 and B2) in response to overload in plantaris muscles from normoxic and hypoxic rats. The phosphorylated-to-total protein ratios were also quantified (Akt\(^{Ser473}/Akt1\), A4; Akt\(^{Thr308}/Akt1\), A5; mTOR\(^{Ser2448}/\)mTOR, B3). Immunoblot pictures are compositions of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are means ± SE. *Significantly different from the respective control (Ct) group. #Significantly different from the respective normoxic group. $Significantly different from the previous time for the same group.
Phosphorylated P70S6K levels were higher in Ov than in Ct muscles (global effect; Fig. 3A1). The Ov-induced increase in P70S6K Thr389 was minimized by hypoxia exposure, at both days 5 and 12 (environment/H11003 overload interaction). P70S6K Thr389 levels were lower in OvH than in OvN rats (39% and 77%, at days 5 and 12, respectively). Changes in total P70S6K levels were roughly similar to those observed in P70S6K Thr389 (Fig. 3A1 and A2). Hypoxia did not significantly alter the proportion of active P70S6K (Fig. 3A3), but the key finding was that absolute quantity of phosphorylated P70S6K in OvH samples markedly decreased, especially at day 12.

Global effects of overload, time, and environment were shown in rpS6 phosphorylation (Fig. 3B1). rpS6Ser240/244 levels were significantly higher in Ov than in Ct groups at both days 5 and 12 (environment × overload interaction). P70S6K Thr389 levels were lower in OvH than in OvN rats (39% and 77%, at days 5 and 12, respectively). Changes in total P70S6K levels were roughly similar to those observed in P70S6K Thr389 (Fig. 3A1 and A2). Hypoxia did not significantly alter the proportion of active P70S6K (Fig. 3A3), but the key finding was that absolute quantity of phosphorylated P70S6K in OvH samples markedly decreased, especially at day 12.

Global effects of overload, time, and environment were shown in rpS6 phosphorylation (Fig. 3B1). rpS6Ser240/244 levels were significantly higher in Ov than in Ct groups at days 5 and 12 and decreased in Ov groups at day 12 in both normoxic and hypoxic rats (overload × time interaction). The Ov-induced increase in rpS6Ser240/244 was minimized by hypoxia exposure (environment × overload interaction), and rpS6Ser240/244 levels were 38% lower in OvH than in OvN at day 5. Total rpS6 levels were significantly affected by environment, overload, and time, but the post hoc analysis did not reveal any significant differences between N and H muscles (Fig. 3B2). The ratio of phosphorylated to total rpS6 was higher in Ov than in Ct groups at both days 5 and 12 (global effect of overload; Fig. 3B3). This ratio was also lower in OvH than in OvN at day 5 (26%, P < 0.05).

4E-BP1 Thr70 and total 4E-BP1 levels were only increased by overload at day 5 (overload × time interaction) (Fig. 3C1 and C2). The Ov-induced increase in 4E-BP1 Thr70 levels were about twofold in N and H muscles at day 5. Exposure to hypoxia had no effect on phosphorylated and total 4E-BP1 levels. Functional overload positively affected the ratio of phosphorylated to total 4E-BP1 (global effect; Fig. 3C3).

eEF2 phosphorylation. At day 5, eEF2 Thr56 levels were significantly higher in OvH than in both OvN (67%) and CtH animals (71%) (overload × environment interaction) (Fig. 4A). The increased levels of eEF2 Thr56 in OvH group convey a decreased activity of this elongation factor. Surprisingly, eEF2 Thr56 levels increased in all of the muscles at day 12, except in OvH muscles, such as no between-group differences were observed at day 12. Total eEF2 levels were
not affected by time, overload, or hypoxia (Fig. 4B). Hypoxia exposure increased the ratio of phosphorylated to total eEF2 in Ov muscles at day 5, whereas overload decreased this ratio at day 12 (overload $\times$ time interaction), especially in hypoxic rats (25% and 44% less than in nonoverloaded muscles, in normoxic and hypoxic rats, respectively, Fig. 4C).

Endogenous Repressors of mTOR

**AMPK phosphorylation.** Global effects of overload and time were found in AMPK phosphorylation (Fig. 5A). AMPK$^{Thr172}$ levels were significantly higher in Ov than in Ct groups only at day 5 (overload $\times$ time interaction), whereas exposure to hypoxia had no effect on phosphorylated AMPK levels. Total AMPK levels were affected by both overload and environment, but the post hoc analysis did not reveal any significant differences between experimental groups (Fig. 5B). The ratio of phosphorylated to total AMPK was increased by Ov at day 5 in N and H muscles, while these changes were only observed in N muscles at day 12 (global effect of overload; Fig. 5C). This ratio was not affected by hypoxia exposure.

**REDD1 protein.** Functional overload decreased REDD1 protein levels (global effect), but differences were only significant in hypoxic rats at both days 5 and 12 (Fig. 5D). Moreover, hypoxia increased REDD1 protein levels (global effect), but these hypoxia-induced changes were only significant in Ct muscles at both days 5 and 12.

**BNIP-3 mRNA and protein.** Functional overload did not affect BNIP-3 mRNA levels in N groups, while it enhanced BNIP-3 mRNA levels in H muscles at day 5 by 41% (Fig. 5E). Hypoxia strongly increased BNIP-3 mRNA levels at day 5, but differences between N and H animals were more pronounced in Ov than in Ct muscles (96% and 45%, respectively). BNIP-3 protein levels failed to parallel mRNA changes. The

Fig. 4. Phosphorylated (A) and total (B) levels of eukaryotic elongation factor 2 (eEF2). The phosphorylated eEF2$^{Thr56}$/eEF2 ratio was quantified (C). Immunoblot pictures are compositions of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are means ± SE. *Significantly different from the respective Ct group. #Significantly different from the respective normoxic group. $Significantly different from the previous time for the same group.

Fig. 5. Analysis of the endogenous repressors of the mTOR signaling. Phosphorylated (A) and total (B) levels of AMPK and the ratio of phosphorylated AMPK$^{Thr172}$ to AMPK (C). Regulated in development and DNA damage response 1 (REDD1) protein levels (D), mRNA (E) and protein (F) levels of BCL2 and adenovirus E1B 19 kDa-interacting protein 3 (BNIP-3). Immunoblot pictures are compositions of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are expressed as means ± SE. Legends as in Fig.1. *Significantly different from the respective Ct group. #Significantly different from the respective normoxic group. $Significantly different from the previous time for the same group.
BNIP-3 protein content was markedly increased by functional overload (global effect), but it remained unaffected by hypoxia. (Fig. 5F).

**Myostatin mRNA and Protein**

There was a global effect of overload that decreased both myostatin mRNA and protein levels (Fig. 6, A1 and A2). Myostatin transcript levels were 42% lower in Ov than in Ct muscles at day 5 in N animals, and 44% lower at day 12 in H rats. Myostatin protein levels were only lower at day 5 in OvH muscles, compared with CtH muscles. Hypoxia significantly affected myostatin at its transcriptional (global effect assessed for only Ct groups) and translational levels. The hypoxia-induced significant changes were only observed for the protein in Ct groups at day 5.

**Changes in MURF1 and MAFbx mRNA Levels**

MAFbx mRNA levels were not affected by overload, whereas hypoxia significantly increased its levels at day 5 in both Ct and Ov animals (81% and 84%, respectively) (Fig. 6B1). Hypoxia exposure did not affect MURF1 transcript levels in Ct animals. In contrast, the abundance of MURF1 mRNA was 38% higher in hypoxic than in normoxic rats in Ov muscles at day 5 (Fig. 6B2).

**DISCUSSION**

The present study was designed to test the hypothesis that hypoxia negatively affects the hypertrophic response of skeletal muscle to functional overload and investigate the response of the main signaling pathways involved in the control of muscle mass. Our results show that 1) hypoxia exposure transiently minimized the Ov-induced muscle hypertrophy until day 12; 2) hypoxia lowered the overload-induced activation of the mTOR/P70S6K, independently of Akt phosphorylation; 3) any potential endogenous repressors of mTOR (AMPK, REDD1, and/or BNIP-3) could explain the hypoxia-induced impairment of the mTOR signaling in Ov muscles; 4) hypoxia exposure specifically deactivated eEF2, a key component of protein elongation, in Ov muscles at day 5, and 5) hypoxia increased the mRNA levels of the muscle-specific E3 ubiquitin-ligases MAFbx, and MURF1, especially in Ov muscles at day 5. Taken together, these results provide evidence that hypoxia exposure leads to a transient alteration of the Ov-induced muscle hypertrophy that could be due at least partly to an alteration of both the translation capacity and elongation process, as well as increased content of muscle-specific ubiquitin ligases.

Although largely discussed, the decreased body and muscle growth rate reported during chronic hypoxia exposure have been often explained by the hypoxia-induced hypophagia (7, 16). As previously suggested, the putative inhibitory effects of moderate food restriction on the hypertrophic response to functional overload would result from activation of AMPK, REDD1, and/or BNIP-3, three of well-known mTOR inhibitors (33, 34, 39). However, preliminary results showed that normoxic rats pair-fed an equivalent quantity of food to that consumed by hypoxic animals did not show any alteration in the Ov-induced muscle hypertrophy, AMPKThr172 phosphorylation, REDD1 protein expression, and levels of BNIP-3 mRNA and protein (data not shown). Taken together, these findings suggest that the putative effects of ambient hypoxia on muscle hypertrophy are independent of the hypoxia-induced decrease in food intake.

Satellite cells have been shown to be activated in response to functional overload (1, 24, 25, 47), but their specific role during the hypertrophic process is very complex and still discussed. Satellite cells are early activated to repair the damaged myofibers submitted to mechanical overload (47), whereas the recruitment of new myonuclei to growing myofibers occurs at later times to preserve an adequate nuclear domain and contributes to muscle hypertrophy (38). The only subtle appearance of the neonatal MHC isoform and the lack of embryonic MHC isoform in Ov muscles, as well as the negligible presence of very small caliber, presumably nascent fibers, with internal nuclei suggest that the contribution of satellite cells to myofiber reparation during the early hypertrophic process is moderate and not affected by hypoxia exposure. Because of the deleterious effect of hypoxia on muscle hypertrophy was only observed during the early period of functional overload, we focused our attention on the molecular control of protein synthesis and degradation.

Studies in cultured cells reported that the Akt/mTOR intracellular pathway could be altered by hypoxia (12, 27, 31). However, only few works reported such alterations in vivo in either humans (51) or rodents (16). We presently show that ambient hypoxia induced a lower activation of mTOR in Ov muscles at day 12, expressed by the ratio of phosphorylated to
total expression of mTOR, and a decreased absolute quantity of active P70S6K. Consistent with previous data in vivo (16), the hypoxia-induced depression of mTOR signaling did not significantly influence 4E-BP1 activity. The elongation factor eEF2, is shown to be controlled by P70S6K through eEF2 kinase (13) and inhibited by AMPK (23). The lack of change in AMPK phosphorylation in hypoxic rats suggests that the increased proportion of phosphorylated eEF2, and then decreased eEF2 activity in OvH muscles at day 5 could be rather explained by the impairment of P70S6K activation. In contrast, the increased ratio of phosphorylated to total eEF2 in Ov muscles at day 12, especially in hypoxic animals, could represent a biological response that offsets the initial inhibition of the elongation step. Ultimately, our findings show that the negative effect of hypoxia on hypertrophic response to overload could be due at least partly to a transient inhibition of the mTOR/P70S6K signaling that mainly could affect the ribosomal biogenesis (rpS6) and the translation elongation (eEF2).

One interesting finding of this study was the attenuation of the overload-induced activation of P70S6K and rpS6 in hypoxic animals at day 5, regardless of mTOR influence. This apparent dissociation between mTOR and P70S6K phosphorylation is in disagreement with the general view that mTOR activates P70S6K via a linear signaling sequence (17). However, experimental evidence supports the notion that full and sustained P70S6K activation requires additional inputs by mTOR-independent mechanisms, such as PKC (22) or phosphoinositide-dependent protein kinase activation (42). Therefore, results of the present study suggest the notion that components of the Akt/mTOR/p70S6K pathway are not exclusively under the control of their respective upstream kinase, but they could also depend on specific activators/repressors.

Here, we show that hypoxia did not reduce Akt phosphorylation until day 12 in OvH muscles, whereas it markedly repressed mTOR and P70S6K. Several regulatory factors can modulate mTOR signaling independently of Akt, but this regulation is poorly documented during hypoxia exposure, especially in skeletal muscle. AMPK is known to impair mTORC1 by activating its upstream repressor TSC2 (18), but AMPK phosphorylation remained unaffected by hypoxia in Ov muscles. Thus, our results do not support a putative role of altered cellular energy status in mTOR inhibition. Once phosphorylated by Akt, TSC2 is sequestered by 14–3–3 factor (32) and is blunted. However, a recent in vitro study demonstrated that REDD1 dissociates the TSC2/14–3–3 complex and consequently inhibits mTORC1 in hypoxia (14). Here, we showed that functional overload minimized the specific effect of hypoxia on REDD1 expression. Although REDD1 has been suggested to explain the impairment of muscle mass in growing rats exposed to severe hypoxia (16), our findings do not support this idea during overload-induced muscle hypertrophy. We showed that hypoxia early increased the BNIP-3 mRNA levels, a hypoxia-induced factor recently identified as an inhibitory regulator of mTOR by interacting with Rheb (29). However, the lack of variation in BNIP-3 protein levels excludes the possibility that this factor could be involved in the control of muscle hypertrophy under hypoxic conditions. Our finding suggests that other regulatory factors may exist in hypoxia to reduce the mTOR activity. Intriguingly, BNIP3 protein levels are markedly increased in Ov muscles, without any changes of the mRNA, suggesting a posttranscriptional regulation of this factor. For AMPK, we hypothesize that BNIP-3 overexpression may contribute to prevent muscle overgrowth, resulting from excessive activation of the Akt/mTOR/p70S6K pathway by mechanical load.

Here, we report that mRNA levels of the E3 ubiquitin ligases MURF1 and MAFbx were increased by ambient hypoxia in overloaded plantaris muscles. Increased MURF1 and MAFbx mRNA levels, as shown in the present study in OvH muscles, do not necessarily result from increased transcription of target genes but would also result from posttranscriptional events acting at a level of mRNA stability. Indeed, hypoxia has been shown to regulate gene expression also through specific mechanisms that enhance the mRNA stability (40). Altogether, it remains to be elucidated whether the RNA availability of these two atrogenes is due to its transcription by the forkhead family of transcription factors (Foxo) (46) or other influences (10, 30) or is dependent on posttranscriptional events. Moreover, recent data showed that these two atrogenes have different functions in the control of muscle mass, MURF1 enhancing specifically the proteolysis of myofibrillar protein and MAFbx contributing to reduce muscle protein synthesis (4). Here, we showed that myostatin, another negative regulator of muscle growth (3), was early activated by hypoxia in Ct muscles, but this upregulation was minimized by functional overload. This finding confirms the role played by hypoxia on myostatin expression (20), but it rejects its involvement in explaining the inhibitory effect of hypoxia on muscle hypertrophy.

In conclusion, we showed that hypoxia exposure transiently minimized muscle hypertrophy in the functional overload model. The early inhibitory effect of hypoxia could be caused by an impairment of protein translation, probably due to an alteration of the ribosomal biogenesis and the elongation translation, as well as by activation of the two muscle-specific ubiquitin ligases MURF1 and MAFbx, known to be upregulated in catabolic states. Upregulation of MURF1 and MAFbx in catabolic states could contribute to the increase in myofibrillar protein breakdown and to depress protein synthesis, respectively. However, long-term hypoxia exposure cannot counteract the specific effect of mechanical overload on muscle hypertrophy.

Perspectives and Significance

This original work represents a significant advance in the understanding of the intracellular pathways involved in the control of muscle mass in hypoxia. Such results could have medical relevance for patients suffering from chronic obstructive pulmonary diseases (COPD). Skeletal muscle atrophy and weakness in these patients are partly attributed to hypoxia (52) and are associated with an activation of REDD1 (16) and myostatin (20). Our preliminary data support the notion that anabolic signals related to functional load are able to counteract the increased expression of these two repressors of muscle mass during hypoxia exposure. Because these two negative regulators of muscle mass are repressed after low-intensity resistance exercise (15), this type of exercise could be recommended in COPD patients to improve muscle mass and prevent muscle atrophy.

GRANTS

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