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Ambient hypoxia enhances the loss of muscle mass after extensive injury

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Abstract

Hypoxia induces a loss of skeletal muscle mass and alters myogenesis in vitro, but whether it affects muscle regeneration in vivo following injury remains to be elucidated. We hypothesized that hypoxia would impair the recovery of muscle mass during regeneration. To test this hypothesis, the soleus muscle of female rats was injured by notexin and allowed to recover for 3, 7, 14, and 28 days under normoxia or hypobaric hypoxia (5,500 m) conditions. Hypoxia impaired the formation and growth of new myofibers and enhanced the loss of muscle mass during the first

7 days of regeneration, but did not affect the final recovery of muscle mass at 28 days. The impaired regeneration under hypoxic conditions was associated with a blunted activation of mechanical target of rapamycin (mTOR) signaling as assessed by p70^{S6K} and 4E-BP1 phosphorylation that was independent of Akt activation. The decrease in mTOR activity with hypoxia was consistent with the increase in AMP-activated protein kinase activity, but not related to the change in regulated in development and DNA response 1 protein content. Hypoxia increased the mRNA levels of the atrogene muscle ring finger-1 after 7 days of regeneration, though muscle atrophy F box transcript levels remained unchanged. The increase in MyoD and myogenin mRNA expression with regeneration was attenuated at 7 days with hypoxia. In conclusion, our results support the notion that the enhanced loss of muscle mass observed after 1 week of regeneration under hypoxic conditions could mainly result from the impaired formation and growth of new fibers resulting from a reduction in protein synthesis and satellite cell activity.

Keywords

Muscle regeneration; Akt/mTOR pathway; AMPK; Satellite cells; MRF

Introduction

Low O₂ availability (hypoxia) is a physical stressor that leads to cell adaptations under physiological and pathological conditions. Ambient hypoxia is known to reduce skeletal muscle mass in humans after high-altitude expeditions [35] and disrupt the rate of muscle growth in young rodents [1, 11]. Moreover, muscle atrophy and weakness are observed in several chronic diseases associated with hypoxemia (low arterial O₂ pressure), such as chronic obstructive pulmonary disease (COPD), heart failure, and peripheral arterial disease [7, 13, 28], which may lead to impaired locomotion and disability, that can contribute to increased mortality and morbidity.

Skeletal muscle mass is determined by the balance between protein synthesis and degradation, which is finely controlled by specific signaling pathways [15]. A primary regulator of protein synthesis is the PI3K (phosphatidylinositol 3-kinase)/Akt/mTOR (mechanical target of rapamycin) signaling pathway. Activation of mTOR signaling leads to an increase in the rate of protein synthesis by stimulating ribosomal biogenesis and translation initiation through phosphorylation of p70^{S6K} and eIF-4E binding protein (4E-BP1), respectively. The alteration of muscle mass in rodents after severe hypoxia is associated with the downregulation of mTOR signaling, while hypoxemia in COPD patients has also been reported to cause a decrease in mTOR activity [11]. The mechanism through which hypoxia disrupts the mTOR signaling pathway remains poorly understood.

As the master sensor of cellular energy status, AMP-activated protein kinase (AMPK) has been shown to repress mTORC1 (mTOR complex 1) activity [18]. One possible scenario is that under hypoxic conditions, AMPK inhibits mTOR signaling in an effort to restore the energy status of the cell by shutting down energy-consuming processes such as protein synthesis [19]. In support of this idea, AMPK activation was shown to inhibit mTOR signaling in HEK293 (human embryonic kidney 293) cells in response to hypoxia [26].

Whether such a mechanism is operative in vivo within skeletal muscle exposed to hypoxia remains to be examined. The hypoxia-inducible factor REDD1 (regulated in development and DNA response 1) has also been shown to repress mTOR activity by facilitating the inhibitory function of TSC2 (tuberous sclerosis complex 2) on mTOR [9]. It was recently shown in the skeletal muscle that the downregulation of mTOR signaling in response to hypoxia exposure correlated with an increase in REDD1 protein content [11]. Hypoxia could also increase protein degradation in skeletal muscle, as evidenced by the transcriptional activation of MAFbx (muscle atrophy F box) and MURF1 (muscle ring finger-1) [4, 22], two atrogenes often used to assess the activity of the ubiquitin–proteasome system (UPS) that is activated under catabolic conditions [2]. Altogether, hypoxia could shift the balance between protein synthesis and degradation toward a catabolic state.

Muscle damage has been observed in diseases associated with cellular hypoxia, such as COPD [39] and ischemia [16]. COPD patients with preserved muscle mass appeared to be in a more regenerative state as indicated by a greater prevalence of centrally located myonuclei [39]. These findings suggest that hypoxia-induced atrophy could be associated with muscle damage and regeneration and that an impairment of satellite cell function could contribute to the loss of muscle mass in hypoxia. The question arises as to whether satellite cell dysfunction might play a role in the atrophic response to ambient hypoxia. Satellite cells, which are localized between the sarcolemma and the basal membrane, are considered the primary stem cell in adult skeletal muscle [29]. Following muscle damage, these cells become activated, proliferate, fuse, and differentiate to form new multinucleated muscle fibers or repair damaged fibers. These myogenic cells seem to respond to the O₂ availability, at least in vitro. Indeed, hypoxia leads to an alteration of myogenesis in both cell lines and primary myoblasts, through inhibition of myoblast differentiation and myotube growth [10, 25, 42]. This adaptation is associated with the downregulation of the myogenic regulatory factors (MRFs) MyoD and myogenin [10]. During myogenesis and regeneration, MyoD is involved in satellite cell activation and early differentiation, while myogenin is expressed later and stimulates terminal differentiation [6]. The hypoxia-induced alteration of myoblast differentiation is also associated with a downregulation of the mTOR signaling [34]. This pathway is required during myogenesis and muscle regeneration since it facilitates myoblast differentiation and myotube growth [14] and stimulates protein synthesis in regenerated muscles [30]. To the best of our knowledge, there is only limited information on the influence of hypoxia on myogenesis and satellite cell function in vivo [27].

In the present study, we investigated whether hypoxia exposure could influence the recovery of muscle mass after extensive injury. We hypothesized that hypobaric hypoxia would impair the recovery of muscle mass during regeneration. Thus, we compared the recovery of muscle mass after 3, 7, 14, and 28 days following notexin-induced degeneration of the soleus muscle in rats exposed to either normoxia or hypobaric hypoxia. We determined if the regenerative response under hypoxic conditions affected Akt/mTOR signaling and whether or not the expression of the metabolic (AMPK) and hypoxia-induced (REDD1) factors was modulated under such conditions. We also studied on the changes in protein levels of MyoD and myogenin as markers of satellite cell activity and assessed the involvement of the UPS through expression of the muscle-specific ubiquitin ligases MAFbx and MURF1 [36].

Material and methods

Animals

Seventy-eight female rats of Wistar Han strain initially weighing 180–200 g were purchased from Charles River Laboratories (L'Arbresle, France). Because gender differences have been previously reported on the adaptive responses of rats to hypoxia [41], female rats, which are more tolerant to hypoxia than males, were used in the present study. 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 study 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 in the animal facility, rats were randomly assigned to either normoxic (N, $n=38$) or hypoxic (H, $n=40$) groups. Initially, animals in the H group were exposed for 7 days to barometric pressure equivalent to 5,500 m of altitude (barometric pressure=370 mmHg; $PIO_2=79$ mmHg) in a hypobaric chamber. The objective of this hypoxia pre-acclimation was to minimize the deleterious effect of hypoxia on food intake and body mass during the subsequent experiment. Indeed, our preliminary data revealed that food intake and body mass severely decreased during the first 24–48 h of hypoxia exposure (about 45–60 and 6–7 %, respectively; data not shown). In an effort to minimize the effect of reduced food intake on the early regenerative response, we exposed H groups to 1 week of ambient hypoxia prior to the induction of muscle regeneration by notexin injection. The length of this acclimation period was based on the recovery of food intake; our preliminary data revealed that animals progressively recovered their food intake during hypoxia exposure, which was stabilized after 1 week to a level 20–25 % lower than the one observed in normoxic animals (data not shown). After this pre-acclimation period of hypoxia exposure, the rate of body mass growth was relatively similar in both H and N groups (Table 1).

In the morning of the eighth day (called d0 as the start of the experiment), degeneration of the left soleus muscle was induced by notexin injection in N and H animals in the surgical facility. After a postsurgery recovery period of 6–8 h in normoxia, the H animals were rehoused in the hypobaric chamber at the same barometric pressure as previously described. The chamber was opened daily to assess the body mass and refill water dispensers. The retention period did not exceed 30–45 min. The animals were then anesthetized for tissue sampling and killed 3, 7, 14, and 28 days (d3, d7, d14, and d28, respectively) after the notexin injection ($n=9$ –10 animals in hypoxia and normoxia at each time).

Notexin injection

Rats were anesthetized with sodium pentobarbital (50 mg kg^{-1} ip), and left soleus muscle degeneration was induced by notexin injection (0.2 mL, $10\text{ }\mu\text{g/mL}$) isolated from snake venom (*Notechis scutatus*, Latoxan, France) directly into the belly of the muscle surgically exposed, as previously described [12]. Because the sham surgery does not induce any alteration in muscle tissue, no surgical procedure was done on the right soleus muscle, which served as an intact control.

Tissue and blood collection

Animals were anesthetized with sodium pentobarbital (80 mg kg⁻¹ ip). Blood was withdrawn from the abdominal aorta into a heparinized syringe, and a portion of the blood was analyzed for hematocrit. Regenerating (Reg) and intact (Int) soleus muscles were carefully dissected and weighed. Soleus muscles were slightly stretched to approximately resting length, frozen in liquid nitrogen, and stored at -80 °C for later use. Animals were subsequently euthanized by lethal injection of sodium pentobarbital.

Histomorphometric analysis

Serial transverse sections (14 µm thick) were cut from the mid-belly portion of the soleus muscle in a cryostat microtome maintained at -20 °C and were stained with hematoxylin and eosin to visualize the nucleus and cytoplasm. To determine the whole cross-sectional area (CSA), photographs of the entire muscle were taken at low magnification. Moreover, at least 20 photographs at high magnification, covering the entire muscle section, were used to determine the mean cross-sectional area from about 400 fibers (FCSA). The fiber density was determined by counting the number of fibers per field (three to four fields at low magnification) to assess the involvement of satellite cells in muscle regeneration. To estimate the proportion of contractile tissue that composed the regenerated muscles, we calculated an index of contractile tissue (ICT) by multiplying FCSA with fiber density. Analyses were performed with a light microscope computerized image-analysis system (Lucia 5, Laboratory Imaging, Prague, Czech Republic).

Protein isolation and immunoblot analysis

Protein isolation and immunoblot analysis were processed on intact and regenerated soleus muscles as previously described [4]. Briefly, protein concentration was determined by the bicinchronic acid method (Roche/Hitachi 912 Instrument; Roche Diagnostic, Mannheim, Germany). Equal amounts of total protein (25 or 50 µg) were subjected to SDS-PAGE using a Mini Protean II System (Biorad, France) and transferred onto nitrocellulose membranes. The equal amount of protein loading between lanes was confirmed by Ponceau staining. A standardized amount of protein prepared from a pool of Int muscles (only for REDD1 protein assessment) or Reg muscles (for the other proteins) 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 purchased from Cell Signaling Technology (Beverly, MA, USA) [Akt1 (1:1,000, no. 2938), phospho-Akt^{Thr308} (1:500, no. 4056), p70^{S6K} (1:1,000, no. 2708), phospho-p70^{S6K} Thr389 (1:1,000, no. 9234), 4E-BP1 (1:1,000, no. 9452), phospho-4E-BP1^{Thr70} (1:1,000, no. 9455), eEF2 (1:2,000, no. 2332), phospho-eEF2 (1:1,000, no. 2331), AMPKα (1:500, no. 2532), phospho-AMPKα^{Thr172} (1:500, no. 2535)], Santa Cruz Biotechnology (Heidelberg, Germany) [myogenin (1:250; sc-12732)], Protein Tech Group (Chicago, IL) [REDD1 (1:750; no. 10638-1-AP)], or from BD Biosciences (Pont de Claix, France) [MyoD (1:1,000; no. 554130)]. After incubation with horseradish peroxidase-conjugated antibody [donkey anti-rabbit IgG (1:10,000, sc 2313) or goat anti-mouse (1:10,000, sc 2005) from Santa Cruz Biotechnology; rabbit IgG TrueBlot, (1:8,000, for REDD1 analysis), or mouse IgG TrueBlot (1/2,000, for myogenin analysis) from eBioscience (San Diego, CA)], proteins were detected by chemiluminescence. The

immunoblot images in Figs. 2, 3, and 4, which represent the mean change in protein levels, are composites of original lanes from samples that were processed in parallel during the same immunoblot experiment. Due to the number of groups ($n=16$), our wish to randomise the sample loading order and the number of wells available per gel (one for the ladder, seven for the samples, and one for the internal standard), the immunoblot images are derived from three blots. The time of exposure for each blot was adjusted if necessary to obtain a similar signal intensity for the internal standard (standardized amount of protein prepared from a pool of Reg or Int muscles), thereby allowing the comparison of the inter-blot changes in protein levels.

RNA isolation and cDNA synthesis

Transverse sections of the soleus muscle (8–10 mg) were disrupted (50 mg/mL) in 50 % RLT/2 β -mercaptoethanol (10 %) lysis buffer (Qiagen, Courtaboeuf, France) and 50 % sterile water with a Mixer Mill MM300 (Rescht, Haan, Germany) (30 Hz, 4 min; 1 mm ceramic beads ($\times 2$)). Tissue homogenate (130 μ L) was incubated for 10 min at 55 °C with proteinase K (10 μ L) (Qiagen) before optimal tissue lysis (750 μ L of QIAzol lysis reagent (Qiagen) +150 μ L of chloroform; centrifugation was at 12,000 $\times g$ for 15 min at 4 °C). After an additional centrifugation step (450 μ L of the aqueous phase+isovolume of chloroform), total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen) with an additional DNase step according to the manufacturer's instructions using a QIAcube system (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 as previously described [4].

Real-time qPCR and quantification

Primer design and optimization were conducted as previously described [4]. The following forward and reverse primers were used to assess MURF1 (NM_080903) and MAFbx (NM_133521) mRNA: MURF1 (F: 5'-GGGAACGA CCGAGTTCAGACTATC-3'; R: 5'-CCTTCACC TGGTGGCTGTTTTC-3') and MAFbx (F: 5'-GCTTGTG CGATGTTACCCAAGAA-3'; R: 5'-TGAAAGTGA GACGGAGCAGCTCT-3'). qPCR was performed with LightCycler FastStart DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany), using 0.25 μ L of cDNA in a 20- μ L final volume, 4 mM MgCl₂, and 0.4 μ M (for MURF1) or 0.8 μ M (for MAFbx) of each primer. qPCR was performed using LightCycler (Roche Applied Science) for 50 cycles at 95 °C for 20 s, 54 °C (for MAFbx) or 56 °C (for MURF1) for 5 s, and 8 s at 72 °C. Quantification cycles (Cq) were ascertained using the second-derivative maximum method (LightCycler Software v 3.5, Roche Applied Sciences). All Cq values were within the standard curve.

The method of target mRNA normalization was based on the results of a previous study from our laboratory [5]. Briefly, the normalization factors (NFs) determined from four reference genes (Hprt1, Arbp, Tbp, Tnfr1) were significantly higher in Reg than Int muscle, especially at day 3 (data not shown). Moreover, although the NF was roughly constant between Reg-N and Reg-H muscle at each time, the pairwise variations obtained from geNorm analysis [40] were above the threshold (0.15) at both days 3 and 28 (data not shown). Thus, we used an alternative method for mRNA normalization; mRNA levels were

determined per unit mass of tissue by multiplying the exponential conversion of the Cq values, corrected for amplification efficiency, by the total RNA concentration [23].

Statistical analysis

All data are presented as mean $\pm$ SE. Morphometric, immunoblot, and qPCR data were analyzed using three-way ANOVA to determine the main statistical effects of regeneration, environment and time, and interactions between these factors. For histomorphometric analysis, statistical comparisons between Reg-N and Reg-H groups were performed using unpaired *t* test, and statistical comparisons between Int muscle (day 7) and Reg muscle (days 3, 7, and 28) in both normoxia and hypoxia were performed using one-way ANOVA. When ANOVA revealed a significant interaction, a Newman–Keuls post hoc test was performed to identify the source of significance with $p < 0.05$.

Results

Specific effect of hypoxia on body growth

As shown in Table 1, body mass was 6 % lower in rats subjected to the 1-week acclimation period prior to the start of experiment (d0) compared to that in N rats; this difference in body mass remained similar throughout the experiment (d28). Tibia length, a marker to estimate the body size, did not change between N and H groups (data not shown). These results indicate that ambient hypoxia does not impair the body growth in young rats and that the acclimation period was beneficial in minimizing the deleterious effect of hypoxia on body mass. As expected, hematocrit values significantly increased from 42 % in N rats to 56 % in H rats at d28.

Hypoxia enhances the loss of muscle mass during the early regenerative phase

A global effect of time, environment (normoxia vs hypoxia), and regeneration was observed in the soleus muscle mass (Table 1). The soleus mass decreased following notexin injection, reaching a minimum at d7. Thereafter, the Reg muscle mass progressively recovered, but remained lower than the Int muscle mass at d28 (21 and 23 % less in N and H groups, respectively). Hypoxia negatively affected the muscle mass, but only during the first week of the experiment (environment $\times$ time interaction). The mass of the intact soleus was lower in H than in N at d7 (14 %), while only a tendency was observed at d3 (12 %, $p = 0.07$). The soleus muscle mass was more impaired by the notexin injection in H than in N rats (regeneration $\times$ environment interaction). A reduced muscle mass was only observed in Reg compared to Int muscles at d3 for hypoxic animals (30 %), while the decreased mass in Reg muscle compared to Int muscle was higher in hypoxia than in normoxia at d7 (54 vs. 35 %, respectively). Moreover, the soleus muscle mass was lower in Reg-H than in Reg-N at both d3 and d7 (41 and 40 %, respectively). Between d7 and d14, the gain of mass was higher in Reg-H than in Reg-N muscle (72 and 13 %, respectively), such that no significant difference was observed in muscle mass between the groups from d14. These results were also confirmed when muscle mass was normalized to the tibia length (Table 1).

Morphometric analysis of the soleus muscle was carried out during the early (d3 and d7) and final (d28) phases of muscle regeneration. These analyses were only performed at d7 for Int

muscle since this was the only time point associated with a significant difference in muscle mass between N and H rats (Table 1). As expected, histological inspection of Int muscles showed no difference in the gross morphology of myofibers of both H and N groups (Fig. 1). At d3, injured muscle primarily consisted of necrotic cells undergoing phagocytosis. By d7, all necrotic fibers appeared to have been replaced by regenerated fibers in N rats, whereas injured muscles contained regenerated fibers, some remaining necrotic fibers, and larger areas of fibrosis in H animals. The decreased ICT values in Reg-H muscle compared to Reg-N muscle (72 %) supports the notion of increased fibrosis during regeneration under ambient hypoxia (Table 2). At d28, some regenerated fibers still possessed central nuclei in both H and N groups, despite the large cross-sectional areas. At this time, most of fibrosis disappeared in the N group, but not in the H group. This was supported by similar ICT values between Reg-N muscle at d28 and Int-N muscle at d7, whereas ICT values remained 19 % lower in Reg-H than in Reg-N muscles at d28 (Table 2).

Muscle CSA and mean FCSA changes paralleled those observed for the muscle mass (Table 2). Notexin induced a significant decrease in CSA, with minimal values observed at d7 in both N and H groups. The diameter of regenerated fibers was the smallest at d7 and progressively increased until d28; however, CSA and FCSA were not completely restored in Reg muscle at this final time point. Hypoxia slightly decreased CSA in Int muscle at d7 (13 %) and dramatically decreased this parameter in injured muscle at d3 (30 %). Mean FCSA was lower in Reg-H than Reg-N muscle at d7 (37 %), while no difference was shown at d28. At d7, the fiber density (number of fibers per microscopic field) was only significantly increased in injured muscle from N rats (178 % in N, 38 % in H, NS) (Table 2). The fiber density declined in Reg-N muscles between d7 and d28, reaching similar values as those observed in Reg-H group. At d28, this parameter remained higher in Reg than in Int muscle in both N and H groups. Between d7 and d28, the gain of CSA is higher in Reg-H than in Reg-N muscle (182 and 39 %, respectively). This result is consistent with the increase in FCSA during this period for Reg-H muscles (182 %), but not for Reg-N muscle (104 %). The lowest increase in CSA compared to FCSA in Reg-N muscle between d7 and d28 is mainly explained by the decrease in fiber density across this period (37 %).

Hypoxia impairs mTOR signaling independent of Akt during regeneration

Akt phosphorylation on Thr308 was increased in Reg muscles at both d3 and d7, returning to baseline at d14 (Fig. 2(A1)). Ambient hypoxia did not alter Akt^{Thr308} phosphorylation levels. Akt1 protein levels, the main Akt isoform expressed in skeletal muscle [31], paralleled the changes in Akt phosphorylation status (Fig. 2(A2)). The ratio of Akt^{Thr308} to total Akt1 was elevated in response to muscle injury, without any effect of ambient hypoxia (global effect of regeneration; Fig. 2(A3)).

Activation of the mTOR signaling was assessed by measuring the phosphorylation status of two direct targets, p70^{S6K} and 4E-BP1. p70^{S6K} phosphorylation (Thr389) was significantly increased at d3 and remained elevated until d7 in Reg muscle, in comparison to Int muscle (Fig. 2(B1)). p70^{S6K} phosphorylation was lower in Reg-H than Reg-N muscle at d3 (44 %) and decreased in Reg-N muscle between d3 and d7, becoming slightly lower than in Reg-H muscle at this time. p70^{S6K} protein levels were increased at d3 in Reg muscles and until d7

in Reg-H muscles, but were not significantly affected by hypoxia (Fig. 2(B2)). A significant increase in the ratio of phosphorylated to total p70^{S6K} occurred in injured muscle at both d3 and d7, that was totally blunted by ambient hypoxia (regeneration×environment interaction, see Fig. 2(B3)). 4E-BP1 phosphorylation (Thr70) was only transiently increased at d3 after notexin administration in normoxia (Fig. 2(C1)). At this time, 4E-BP1 phosphorylation was 47 % lower in Reg-H than in Reg-N muscle. The content of total 4E-BP1 was not affected during regeneration in normoxia, whereas it was significantly reduced in Reg-H muscle, except at d7 (Fig. 2(C2)). The ratio of phosphorylated to total 4E-BP1 was only increased in injured muscles at d3, independent of environmental conditions (Fig. 2(C3)).

Translation elongation was assessed by measuring the expression of the eukaryotic elongation factor 2 (eEF2), which has been shown to be controlled by p70^{S6K} through eEF2 kinase [8] and inhibited by AMPK [24]. eEF2 phosphorylation on Thr56 was only increased after notexin injection at d3 in hypoxic animals (regeneration×time×environment interaction, Fig. 2(D1)). At this time, eEF2 phosphorylation (Thr56) levels were slightly higher in Reg-H than in Reg-N muscle (24 %). Total eEF2 content peaked at d3 in injured muscle and then progressively decreased in Int muscle level by d14 (see Fig. 2(D2)). The phosphorylated-to-total eEF2 ratio was markedly lowered in Reg muscle at d3 and progressively returned to baseline levels (Fig. 2(D3)). Hypoxia exposure failed to affect the phosphorylated-to-total eEF2 ratio.

Hypoxia-induced AMPK activation during muscle regeneration

AMPK phosphorylation on Thr172 increased in injured muscle at d3 in normoxia and until d7 in hypoxia (Fig. 3a). Hypoxia increased AMPK phosphorylation in Int muscle at d3 and enhanced the increase in phosphorylated AMPK following notexin injection. The content of total AMPK remained relatively constant over the time course in both Reg and Int groups (Fig. 3b). Ambient hypoxia led to an 89 % increase in the ratio of phosphorylated to total AMPK in Reg muscles at d3 (Fig. 3c).

REDD1 expression was completely blunted in injured muscle during the first week of regeneration and then increased from d14, though still remained at a lower level of expression compared to Int muscle (Fig. 3d). Moreover, hypoxia increased REDD1 content (global effect), but the increase was only significant in Int muscle at d14 and d28 (63 and 74 %, respectively).

Hypoxia alters MyoD and myogenin expression during regeneration

MyoD protein expression increased during regeneration until d14 in both N and H rats (Fig. 4a). MyoD protein content remained constant from d3 through d14 in Reg-H muscle, whereas MyoD expression peaked at d7 in Reg-N muscle, being 78 % higher than in Reg-H muscle at this time.

The content of myogenin protein increased in injured muscles at both d3 and d7 in N rats, but only at d3 in H rats (Fig. 4b). Hypoxia blunted the regeneration-induced increase in myogenin expression. Myogenin protein levels were 42 and 68 % lower in Reg-H than Reg-N muscle at d3 and d7, respectively.

Hypoxia increased MURF1 mRNA expression

MURF1 and MAFbx mRNA expression was decreased during the early phase of regeneration (Fig. 5a, b). Hypoxia induced a 238 and 211 % increase in MURF1 mRNA levels in Int muscle at d3 and d7, respectively, while a more modest (88 %) increase in MURF1 expression was observed in Reg muscle only at d7 (Fig. 5b).

Discussion

This study was designed to test the hypothesis that hypobaric hypoxia would impair the recovery of the skeletal muscle mass after extensive injury by assessing the response of markers of muscle regeneration and signaling pathways known to be involved in muscle growth. Our results show that (1) hypoxia alters the formation and growth of new fibers and enhances the loss of muscle mass after initial injury but does not affect the long-term recovery of muscle mass; (2) the early hypoxia-induced impairment of mTOR activation during regeneration is independent of Akt but concomitant with AMPK activation; (3) ambient hypoxia impairs the MyoD and myogenin activation during the early phase of regeneration; and (4) hypoxia increases the expression of MURF1 mRNA, but not MAFbx mRNA in Reg muscle at d7. Taken together, these results provide evidence that hypoxia alters the early stage of muscle regeneration, delays, but does not affect the overall recovery of muscle mass at middle or long term. The enhanced loss of muscle during the initial phase of regeneration appears to mainly result from an alteration in the formation and growth of new fibers, which was likely due to a reduced rate of protein synthesis and to impaired satellite cell activation, proliferation and/or differentiation.

Chronic hypoxia was previously shown to alter skeletal muscle mass in humans [35] and animals [1, 11, 22]. However, its influence on satellite cells, the primary myogenic cells involved in muscle repair after extensive injury, and the recovery of muscle mass are largely unknown in vivo. Recently, it was reported that ischemia induced by femoral ligation resulted in a decrease in MyoD and myogenin expression [27]. However, it is difficult to elucidate whether this alteration of the myogenic program was the result of hypoxia and/or an impairment of nutrient supply. To investigate the putative role of hypoxia on skeletal muscle repair after extensive injury, we used the model of notexin-induced muscle degeneration, a model known to cause a rapid and extensive fiber necrosis while sparing satellite cells, followed by a complete regenerative process [6, 21]. Although this model is characterized by a drastic regenerative response that is not observed in physiological conditions [6], it is highly reproducible and well suited to determine whether environmental hypoxia could modulate the regenerative response and affect the activity of the satellite cells.

It is currently known that hypoxia alters myogenesis in vitro, by inhibiting myogenic cell differentiation and myotube growth [10, 25, 42]. Here, we show for the first time that hypoxia impairs the development and growth of new fibers during the early regenerative step after extensive injury, leading to an enhancement of the loss of muscle mass. The mTOR signaling, which both promotes protein synthesis, myoblast differentiation, and myotube growth during muscle regeneration [14, 30], was disrupted by hypoxia in myogenic cell culture [34] and in intact skeletal muscle [11]. Results of the present study are in agreement with these findings by showing that hypoxia impairs the mTOR activity in Reg muscle at d3,

as evidenced by a lower level of active p70^{S6K} and a lower content of phosphorylated 4E-BP1. Interestingly, we observe a slight but significant increase in eEF2 phosphorylation by hypoxia in Reg muscle at d3 which suggests a moderate reduction of its biological activity. This finding may result from the reduced p70^{S6K} activation and increased AMPK phosphorylation, two kinases known to control the eEF2 activity [8, 24]. However, the marked increase in total eEF2 content in injured muscle, independent of hypoxia, suggests that its increased activity during the regenerative response is mainly explained by the increased expression of eEF2, and finally, hypoxia exposure could have a limited role on eEF2 activity.

Here, we show that hypoxia represses mTOR activity independent of Akt during the early step of regeneration. This finding is in accordance with our recent study that suggested a dissociation between Akt and mTOR during overload-induced muscle hypertrophy in hypoxia [4]. The metabolic sensor AMPK and the hypoxia-induced factor REDD1 were previously reported to impair mTOR signaling in hypoxic culture cells [9, 26]. An increased expression of REDD1 protein was recently observed in the skeletal muscle of growing rats exposed to severe hypoxia [11], and our results in intact soleus muscles agree with this finding. However, hypoxia-related increase in REDD1 protein content was blunted in regenerating muscles. This evidence strongly suggests that the stimuli associated with the regenerative response prevail over the hypoxic signal. One of the main findings of the present study is that hypoxia increases AMPK phosphorylation during early regeneration simultaneously with impaired mTOR activity. This finding supports the notion that hypoxia stimulates AMPK activity during the early step of regeneration, most likely due to a disruption of the energetic status, leading to the impairment of mTOR activation. Interestingly, AMPK phosphorylation is transiently increased by hypoxia in Int slow-twitch soleus muscle, while no change was reported in fast-twitch plantaris muscle during exposure to a similar level of hypoxia [4]. This finding suggests that slow-twitch muscles are more sensitive than fast-twitch muscles to ambient hypoxia.

Hypoxia exposure was reported to disrupt myogenesis in vitro by repressing myoblast differentiation and myotube growth [10, 25, 42], while the influence of hypoxia on myogenic cell proliferation is more controversial [10, 25, 34]. Here, we show for the first time in vivo that ambient hypoxia exerts a differential control on MRF expression, myogenin, a marker of myogenic differentiation, being decreased during the first week of regeneration under ambient hypoxia, and MyoD, a marker of proliferation and early differentiation, being decreased only at d7. These results, which are consistent with the lower fiber density and fiber size observed at d7 in Reg-H muscle, suggest that hypoxia impairs MRF expression that could negatively affect satellite cell proliferation and differentiation, leading to a delay in the formation and growth of new myofibers.

The process of muscle regeneration is characterized by a cycle of degeneration/regeneration [6]. In particular, the injection of notexin, a phospholipase A₂ toxin, results in the hydrolysis of the lipids of the sarcolemma, depolarization of the muscle fibers, and cellular proteolysis [20]. The final stages of degeneration are mainly determined by the phagocytosis related to the inflammatory response and the activation of Ca²⁺-dependent proteases [17, 20]. Because the objective of this study was to investigate on the influence of ambient hypoxia on the

recovery of muscle mass after extensive injury, we did not focus our attention on the molecular events related to the degeneration step. The reduced muscle mass and CSA observed in Reg-H muscle at d3, a time point characterized by the absence of new fibers (Fig. 1), supports the concept of an accelerated degradation of necrotic fibers during the early degeneration in ambient hypoxia. Although it remains to be elucidated, the presence of remaining necrotic fibers at d7 in Reg-H muscle provides evidence of a delay in the complete degradation of injured fibers in hypoxic animals, suggesting that ambient hypoxia could modify the degenerative response after notexin injection. In this study, we only assessed the mRNA levels of the muscle-specific ubiquitin ligases MURF1 and MAFbx, two atrogenes that reflect the activity of the ubiquitin–proteasome system (UPS), an essential proteolytic system during skeletal muscle atrophy [2]. We show that the expression of these atrogenes is partly blunted at d3 in injured muscle, suggesting that the UPS does not contribute to the degeneration step induced after notexin injection. Interestingly, hypoxia leads to an increase in the mRNA content of MURF1 in both Reg and Int muscles, without changes in MAFbx mRNA levels. Although we cannot exclude the possibility that hypoxia regulates the gene expression at the posttranscriptional level [32], our finding suggests that the transcription of these two atrogenes could be differentially regulated during hypoxia. The lack of concomitant regulation of both MURF1 and MAFbx by hypoxia may not be explained by the forkhead family of transcription factors, known to activate these two atrogenes [38], but could be influenced by other factors [3]. The hypoxia-induced increase in MURF1 mRNA expression after 7 days of regeneration suggests that MURF1 could contribute to impair the formation and growth of new fibers at d7 by promoting the increase in myofibrillar protein breakdown. Moreover, the marked induction of MURF1 by hypoxia in intact muscle at d7 supports the notion that the moderate and transient loss of muscle mass observed in these muscles is mainly a result of increased proteolysis.

In contrast to our initial hypothesis, we show that hypobaric hypoxia delays the regenerative response but does not impair the recovery of muscle mass. The delay in transcript upregulation after endurance exercise in hypoxia [37] and in muscle hypertrophy in response to functional overload [4] supports the idea that reduced oxygenation delays but does not prevent the muscle adaptive response. Surprisingly, the rate of growth in Reg muscle is even higher between d7 and d14 in hypoxia than in normoxia. This result may be due to the higher expression of active p70^{S6K} at d7 in Reg muscle during hypoxia exposure. Moreover, a decreased expression of total 4E-BP1 independent of its phosphorylation was observed in Reg-H muscle between d14 and d28. This result suggests that the content of unphosphorylated 4E-BP1, which acts as a translational repressor [33], was reduced by hypoxia. Although it remains to be elucidated, this change could promote protein translation during the late step of regeneration.

In conclusion, our results provide strong evidence that hypoxia stimulus leads to a delay during the first step of regeneration but does not affect the late recovery of muscle mass. The early loss of muscle mass observed in injured muscle (d3) during hypoxia exposure may be explained by an accelerated degeneration. However, the enhanced loss of muscle mass induced by hypoxia at d7 results mainly from an alteration in the formation and growth of new fibers, which could be due to impaired proliferation and differentiation of satellite cells and/or reduction of the protein synthesis flux. The hypoxia-induced alteration of the mTOR

signaling during the initial steps of muscle regeneration could be related to the negative control of AMPK, while increased MURF1 mRNA likely contributes to increase the myofibrillar protein breakdown. However, hypoxia exposure does not prevent the recovery of muscle mass. The present results have potential relevance in hypoxemic patients, and both the altered growth of new fibers and increased fibrosis after full myofiber regeneration support the notion that hypoxia could impair the contractile function.

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Fig. 1.

Cross sections of the soleus muscles stained with hematoxylin and eosin. Morphological data are presented in Table 2. All pictures are visualized at the same magnification ($\times 20$). Bars = 100 μm . *Int-N*, intact soleus muscle from normoxic rats; *Int-H*, intact soleus muscle from hypoxic rats; *Reg-N*, regenerated soleus muscle from normoxic rats; *Reg-H*, regenerated soleus muscle from hypoxic rats

Fig. 2.

Analysis of the Akt/mTOR signaling during muscle regeneration. Phosphorylated and total levels of Akt (*A1* and *A2*, respectively), p70^{S6K} (*B1* and *B2*, respectively), eukaryotic initiation factor-4E-BP1 (*C1* and *C2*, respectively), and eEF2 (*D1* and *D2*, respectively) during soleus regeneration from normoxic and hypoxic rats. The phosphorylated-to-total protein ratios were also quantified (Akt^{Thr308}/Akt1, *A3*; p70^{S6K} Thr389/p70^{S6K}, *B3*; 4E-BP1^{Thr70}/4E-BP1, *C3*; eEF2^{Thr56}/eEF2, *D3*). Immunoblot pictures are composites of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are means±SE. Legends as in Fig. 1. The *asterisk* indicates significant difference from the respective Int group. The *number sign* indicates significant difference from the respective normoxic group. The *dollar sign* indicates significant difference from the previous time for the same group

Fig. 3.

Analysis of the endogenous repressors of the mTOR signaling during muscle regeneration. Phosphorylated and total levels of AMPK (**a** and **b**, respectively) and the ratio of phosphorylated AMPK^{Thr172} to AMPK (**c**). REDD1 protein levels (**d**). Immunoblot pictures are composites of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are means $\pm$ SE. Legends as in Fig. 1. The *asterisk* indicates significant difference from the respective Int group. The *number sign* indicates significant difference from the respective normoxic group. The *dollar sign* indicates significant difference from the previous time for the same group

Fig. 4.

Protein levels of the myogenic regulatory factors MyoD (**a**) and myogenin (**b**) during muscle regeneration. Immunoblot pictures are composites of original lanes from muscle samples that were derived at the same time and processed in parallel. Values are means $\pm$ SE. Legends as in Fig. 1. The *asterisk* indicates significant difference from the respective Int group. The *number sign* indicates significant difference from the respective normoxic group. The *dollar sign* indicates significant difference from the previous time for the same group

Fig. 5. mRNA levels of the muscle-specific ubiquitin ligases MAFbx (**a**) and MURF1 (**b**) during muscle regeneration. Values are means $\pm$ SE. Legends as in Fig. 1. The *asterisk* indicates significant difference from the respective Int group. The *number sign* indicates significant difference from the respective normoxic group. The *dollar sign* indicates significant difference from the previous time for the same group

Table 1

Body and soleus masses

		d0	d3	d7	d14	d28
Body mass (mg)	N	196.0±2.0	193.0±2.0	202.4±2.4 ^c	210.9±2.6 ^c	225.0±3.0 ^c
	H	184.9±1.9 ^b	188.9±1.8	199.3±2.3 ^c	202.3±2.2 ^b	213.8±2.3 ^{b,c}
Soleus mass (mg)	Int-N		85.4±4.4	89.2±2.6	91.7±2.3	91.2±3.4
	Int-H		75.0±2.3	76.8±2.7 ^b	95.9±2.8	95.7±3.3
	Reg-N		89.6±9.0	58.1±2.3 ^{a,c}	65.9±1.9 ^a	72.3±2.7 ^a
	Reg-H		52.6±2.7 ^{a,b}	35.1±1.8 ^{a,b,c}	60.2±2.5 ^{a,c}	73.5±2.8 ^{a,c}
	Int-N		24.8±1.2	24.9±0.7	25.7±0.6	24.6±0.9
Normalized mass of the soleus (mg cm ⁻¹ of tibia)	Int-H		21.8±0.7	21.5±0.7	26.7±0.7	26.5±1.1
	Reg-N		26.0±2.6	16.2±0.6 ^{a,c}	18.4±0.5 ^a	19.8±0.8 ^a
	Reg-H		15.3±0.8 ^{a,b}	9.9±0.5 ^{a,b,c}	16.8±0.6 ^{a,c}	19.9±0.7 ^a

Values are means±SE. The time point d0 represents the start of the experiment. Before d0, H groups were exposed to a 7-day period of acclimation in ambient hypoxia (see “Material and Methods”)

N normoxic animals, H hypoxic animals, Int-N intact soleus muscle from normoxic rats, Int-H intact soleus muscle from hypoxic rats, Reg-N regenerated soleus muscle from normoxic rats, Int-H regenerated soleus muscle from hypoxic rats

^aSignificantly different from the respective Int muscles

^bSignificantly different from the respective normoxic group

^cSignificantly different from the previous time for the same group

Table 2

Histological analyses

	CSA (mm ²)	FCSA (μm ²)	Fiber density (number per field)	ICT value
d7				
Int-N	7.0±0.2	2,654±146	162±11	1.00±0.05
Int-H	6.1±0.1 ^b	2,508±127	155±13	0.91±0.07
d3				
Reg-N	6.7±0.3	–	–	–
Reg-H	4.7±0.3 ^{a,b}	–	–	–
d7				
Reg-N	3.3±0.6 ^{a,c}	818±26 ^a	447±25 ^a	0.85±0.04 ^a
Reg-H	1.7±0.7 ^{a,c}	512±39 ^{a,b}	214±11 ^b	0.24±0.01 ^{a,b}
d28				
Reg-N	4.6±0.1 ^{a,c}	1,670±111 ^{a,c}	283±21 ^{a,c}	1.09±0.02 ^c
Reg-H	4.8±0.4 ^c	1,445±151 ^{a,c}	266±25 ^a	0.88±0.03 ^{b,c}

Values are means±SE. ICT value is calculated by multiplying FCSA with the fiber density and is expressed in relative to the group Int-N at day 7

CSA cross section area, FCSA fiber cross-sectional area, ITC index of contractile tissue, Int-N intact soleus muscle from normoxic rats, Int-H intact soleus muscle from hypoxic rats, Reg-N regenerated soleus muscle from normoxic rats, Int-H regenerated soleus muscle from hypoxic rats

^aSignificantly different from the respective Int muscles at d7

^bSignificantly different from the respective normoxic group

^cSignificantly different from the previous time for the same group