Distinct responses of protein turnover regulatory pathways in hypoxia- and semistarvation-induced muscle atrophy

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Hypoxemia and chronic respiratory failure have also been associated with decreased appetite and malnutrition (7, 43, 55). The contribution of lowered energy intake to hypoxia-induced muscle atrophy has been investigated at high altitude (34), but the effects of normobaric hypoxia on energy intake and signaling pathways regulating protein turnover in skeletal muscle have not been studied earlier to our knowledge. Protein turnover is the balance between protein synthesis and protein degradation.

Key regulatory elements of protein synthesis are the mammalian target of rapamycin complex 1 (mTORC1) (32) and endoplasmic reticulum (ER) stress signaling (45). Activation of mTORC1 signaling stimulates protein synthesis through phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (ElF4EBP1, or 4E-BP1) at threonine (Thr) residues 37 and 46. This initial phosphorylation primes 4E-BP1 for further phosphorylation at serine (Ser) 65 and Thr70, whereupon it dissociates from ElF4E, thereby making it available for mRNA translation (13, 14). In addition, mTORC1 can regulate mRNA translation through activation of ribosomal protein S6 kinase-β1 (P70S6K1) at phosphorylation site Thr389. This initial phosphorylation is required to allow full activation of the kinase by an additional phosphorylation at Thr229 catalyzed by phosphoinositide-dependent kinase 1 (22).

ER stress inhibits general protein synthesis through the phosphorylation of ElF2α by the unfolded protein response (UPR) at phosphorylation site Ser51. Phosphorylation of ElF2α reduces but does not completely shut down global protein synthesis, and translation of selected mRNAs may even be increased (27, 28).

Proteasomal and lysosomal protein degradation play important roles in muscle catabolism. Tripartite motif containing 63, E3 ubiquitin protein ligase (TRIM63, or MURF-1) and F-box protein 32 (FBXO32, or ATROGIN-1) are rate-determining enzymes of the proteasomal protein degradation machinery in skeletal muscle. Increased mRNA expression of these genes can be used as a marker for the activation of these pathways (15, 16). Furthermore, these genes are positively regulated by hypoxia at high-altitudes results in muscle atrophy in men and animals (5, 11, 21, 23), it is still under debate whether hypobaric hypoxia induces other physiological effects than normobaric hypoxia (39, 40). It is not clear whether findings from hypobaric hypoxia studies related to skeletal muscle loss can be translated directly to the situation of patients with COPD who experience normobaric hypoxia and also show muscle atrophy. We therefore have chosen a normobaric hypoxia mouse model to investigate the effects of hypoxia on skeletal muscle mass.

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the transcription factor Forkhead box O1 (FOXO1) (26, 30, 38). Increased expression of BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) and microtubule-associated protein 1 light chain-3B (MAP1LC3B) has been used to demonstrate activation of lysosomal protein degradation (3, 47). The expression of the respective genes, BNip3 and Map1lc3b, is also subject to regulation by the UPR and hypoxia-inducible factor 1α (HIF-1α) (46, 54).

Protein kinase B (PKB, or AKT) functions as a central regulator of both protein synthesis and degradation. AKT is activated through the phosphorylation of Thr308 by 3-phosphoinositide-dependent protein kinase 1 (PDK1), which then facilitates phosphorylation of Ser473 by a complex of proteins containing mTORC2 (9). Activated AKT can inhibit FOXO1 activity and subsequent transcription of markers of proteasomal protein degradation, such as Murf1 and Atrogin-1 (52).

Furthermore, activated AKT can activate mTORC1 and thus stimulate mRNA translation through inactivation of the inhibitors tuberous sclerosis complex 1 and 2. We have measured the expression of relevant genes and the activation status of these key proteins of several pathways involved in protein synthesis and degradation to assess the effect of hypoxia on the activity of protein turnover signaling pathways.

Patients with COPD may experience, in addition to chronic mild hypoxia, episodes of acute severe hypoxia during exacerbations. Both mild and severe episodes of hypoxia may contribute to the muscle atrophy observed in these patients. To model the adaptive effects of long-term chronic hypoxia and the activation of signaling pathways during acute severe episodes within a feasible experimental time range, we chose severe hypoxic conditions for 21 days in our mouse model. We hypothesize that normobaric hypoxia induces muscle atrophy, which is not completely attributable to semistarvation. Furthermore, hypoxia-induced muscle atrophy involves a coordinated increase in muscle proteolysis signaling and a decrease of regulatory signals for protein synthesis.

MATERIALS AND METHODS

Animals and tissue collection. Twelve-week-old C57BL/6J male mice (Charles River Laboratories International, Wilmington, MA) (n = 72) were randomly divided into three groups: normoxia (N), normoxia pair-fed to hypoxic animals (PF) and normobaric hypoxia (H). All mice were housed in experimental chambers at 21°C with a 12:12-h dark/light cycle. Mice received standard chow (V1534-000 Ssniff R/M-H; Ssniff Spezialdiäten, Soest, Germany) and water ad libitum. After 5 days of acclimatization, 48 mice were exposed to 21% O2 (N group) and 24 to normobaric hypoxia (H group). With the use of the proOX system P110 (Biosphere, Lacona, NY), oxygen levels were reduced in a stepwise manner to 12% (day 1), 10% (day 2), and finally 8% (61 mmHg) on day 3, which was then maintained for the remainder of the experiment. To control for the effects of reduced food intake during hypoxia, 24 mice of the normoxia group received the amount of food consumed by the hypoxic mice (PF group). Eight mice per condition were killed on days 2, 4, and 21. Abdominal aortic blood was collected under general anesthesia with isofluorane (IsoFlu; Abbott Laboratories, Abbott Park, IL) at an oxygen pressure corresponding to the respective experimental condition. Tissues were isolated and either frozen immediately in liquid nitrogen, fixed in formalin, or frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, Netherlands) for further analysis. Tissue weights were corrected for body weight at the start of the experiment. All experimental procedures were approved by the Committee for Animal Care and Use of Maastricht University.

Blood parameters. Abdominal aortic blood was collected into a 1-ml syringe (Becton Dickinson, Breda, Netherlands) coated with heparin (Leo Pharma, Breda, Netherlands). Oxygen levels and pH were measured immediately using the ABL 510 Blood Gas Analyzer (Radiometer Benelux, Zoetermeer, Netherlands), and blood cell count was determined with the Coulter Ac T Diff hematology Analyzer (Beckman Coulter, Krefeld, Germany).

Western blotting. Tissue was ground to powder using an N2-cooled steel mortar. The powder (~50 mg) was lysed in 1 ml lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3PO4, 2 mM activated Na3VO4, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 100 mg/mL glycerol, 17.5 mM β-glycerophosphate (all chemicals from Sigma-Aldrich Chemie, Zwijndrecht, Netherlands), and protease inhibitors (Complete; Roche Nederland, Woerden, Netherlands)). Crude lysates were incubated on ice for 30 min, followed by 10-min centrifugation at 16,000 g. Total protein concentration of the supernatant was determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL) according to manufacturer’s instructions. Samples were analyzed by Western blot, briefly, 12.5 μg of protein per lane were separated on a CriterionTM XTR Precast 4–12% Bis-Tris gel (Bio-Rad Laboratories, Veenendaal, Netherlands) and transferred to a nitrocellulose transfer membrane (Bio-Rad Laboratories) by electroblotting. The membrane was stained with Ponseau S solution (0.2% Ponseau S in 1% acetic acid; Sigma-Aldrich Chemie) to control for protein loading. The membrane was blocked for 1 h at room temperature in 5% (wt/vol) nonfat dried milk (Campina, Zaltbommel, Netherlands) dissolved in TBS-Tween-20 (0.05%). Nitrocellulose blots were washed in TBS-Tween-20 (0.05%) on a rocking platform for 5 min, followed by overnight incubation at 4°C with primary antibodies (E2, E2a: no. 9722; p-E2a(Ser51): no. 3398; AKT: no. 4685; p-AKT(Ser473): no. 9271; P70S6K1: no. 9202; p-P70S6K1(Ser389): no. 9205; 4E-BP1: no. 9452; p-4E-BP1(Ser65): no. 5460; FOXO1: no. 2880; p-FOXO1(Ser256): no. 9461; GSK-3β(P): no. 9315; p-GSK-3β(Thr21): no. 9336; L83HYPOXIA-INDUCED MUSCLE ATROPHY MODULATES PROTEIN TURNOVER SIGNALING L83
Hypoxia induces hypoxemia, acidosis, and polycythemia. Partial pressure of oxygen in blood (Pao2), pH, oxygen saturation of hemoglobin (SaO2), and red blood cell (RBC) count were determined in blood collected from the abdominal aorta. Pao2 of the normoxic and pair-fed groups remained around 125–140 mmHg, whereas, under hypoxic conditions, Pao2 was decreased to 54 mmHg after day 2 and to 33 mmHg on day 21 (PaSO2 day 21 vs. day 2, P < 0.01; day 21 vs. day 4, P < 0.05) (Fig. 1C). An increase of RBCs to ~150% of control was observed in the hypoxic group after 21 days (RBC day 21 vs. day 2, P < 0.01; day 21 vs. day 4, P < 0.01) (Fig. 1D). Reduced food intake alone had no or only minor effects on Pao2, pH, SaO2, and RBC count.

Results

1. RT-qPCR primer sequences

<table>
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<tr>
<th>Gene</th>
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<th>Reverse Primer (5’-3’)</th>
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<td>TCAGCGGCGCTTCTTGTAGT</td>
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hypoxic mice still had lower body weights than normoxic animals (~96% and ~90% for the PF and H group, respectively). To assess the contribution of adipose tissue to the observed loss in body weight, we determined the weight of the epididymal fat pad (Fig. 2C). Semistarvation alone did not affect fat pad weight, whereas hypoxia caused a significant reduction, which developed most markedly during the first 4 days (~71% of N group) but continued thereafter, resulting in a loss of ~50% of N group on day 21 (day 21 vs. day 2, P < 0.01). The weight difference between groups is thus in part attributable to the reduced fat mass in hypoxic animals. Under hypoxia, gastrocnemius muscle weight rapidly decreased to 92% of control within 4 days (day 4 vs. day 2, P < 0.05) (Fig. 2D). On day 21, muscle weight was decreased to 88% of control (day 21 vs. day 2, P < 0.01; day 21 vs. day 4, P < 0.05). Gastrocnemius weight in the PF group was unchanged on day 4 but had declined to 95% of control on day 21 (day 21 vs. day 4, P = 0.09). The rapid, initial muscle atrophy following exposure to hypoxia is, therefore, independent of the decreased food intake, whereas the reduction of muscle weight during chronic hypoxia is partially attributable to the lowered food intake. These data indicate that the decrease in body weight and the muscle atrophy in response to hypoxia include a hypoxia-specific component.

Hypoxia activates UPR but does not inhibit regulatory effectors of protein synthesis. It was shown previously in vitro that hypoxia induces the phosphorylation (inactivation) of EIF-2α (45). We therefore analyzed the phosphorylation status of EIF-2α in control mice (normoxic or pair fed) and mice exposed to hypoxia. Contrary to expectations, quantification of

and gradually recovered to ~80% of the control intake on day 10 to remain constant thereafter (Fig. 2A). To distinguish specific effects of hypoxia from those mediated by the reduced food intake, a normoxic pair-fed group (PF group) was included that received the same amount of food as consumed by the H group. Mice in the PF group lost less body weight during the first 4 days (~91%) of N group) than mice exposed to hypoxia (~85% of N group) and continued to lose weight until day 7 (Fig. 2B). Normoxic mice gained little weight throughout the experiment (~0.1 g/wk), whereas pair-fed and hypoxic mice showed a higher but comparable weight gain (~0.5 g/wk) after the initial weight loss. After 21 days, both pair-fed and

Fig. 1. Hypoxia induces hypoxemia, acidosis, polycythemia, and differential expression of hypoxia-sensitive genes in skeletal muscle. Mice (n = 6–8 per group) were exposed to normoxia or hypoxia, or we pair-fed hypoxic mice for 21 days. Blood was sampled from the abdominal aorta at the indicated time points and the following parameters were determined: PaO2 (A), pH (B), SaO2, (C), and red blood cell (RBC) concentration (D). mRNA expression of marker genes was measured in the gastrocnemius muscle including Glut1 (E) and Ca-9 (F). G: luciferase expression in C2C12 cells stably transfected with a hypoxia-responsive element reporter plasmid (n = 6) and cultured at an ambient oxygen concentration of 4%. H: expression of Ca-9 and Glut1 in C2C12 myotubes exposed to an ambient oxygen concentration of 4% (n = 3). Relative expression of mRNAs was corrected for 18S RNA concentration and normalized to normoxic values of the corresponding time points. Significant differences between groups at a given time point are indicated. *P ≤ 0.05, **P ≤ 0.01, or ***P ≤ 0.001. Trends are indicated by the specific P value (n = 6–8 per group).

Fig. 2. Decreases in body, muscle, and adipose weight following hypoxia indicate a hypoxia-specific component. Mice were exposed to normoxia (N) or hypoxia (H), or to normoxia with pair-feeding (PF) for hypoxic mice. Mice were killed at the indicated time points, and tissue weights were determined.

A: food intake per animal per day. B: body weight as the percentage of the start weight. Significant differences (P < 0.05) between groups H and N are indicated by $ and between groups H and PF by a #. C: epididymal fat pad weight corrected for start body weight expressed as the percentage of the normoxic value for the corresponding time point. Significant differences (P < 0.05) between groups H and N are indicated by $ and between groups H and PF by a #. D: gastrocnemius muscle weight corrected for start body weight expressed as the percentage of the normoxic value for the corresponding time point. Significant differences between groups at a given time point are indicated by asterisks. *P ≤ 0.05, **P ≤ 0.01, or ***P ≤ 0.001. Trends are indicated by the specific P value (n = 6–8 per group).
phosphorylated (Ser51) and total EIF-2α protein revealed a decrease in the degree of phosphorylation in hypoxic vs. pair-fed and normoxic animals, suggesting a permissive rather than inhibitory effect of hypoxia on protein synthesis (Fig. 3, A and G). Despite the reduced phosphorylation of EIF-2α, mRNA expression of its downstream target activating transcription factor 4 (Atf4) was elevated by hypoxia at days 4 and 21 (Fig. 3B). Increased ATF4 protein expression induces upregulation of downstream targets such as protein phosphatase 1, regulatory subunit 15A (PPP1R15A, or GADD34), which is part of a complex that dephosphorylates EIF-2α (33). We indeed found increased mRNA expression of Gadd34 at days 4 and 21 (Fig. 3C), in agreement with an increase of ATF4 protein concentrations. The acute increase in Gadd34 expression could be mimicked by food restriction alone, whereas the later increase was attributable to hypoxia.

When tested in vitro, hypoxia also induced an approximately twofold upregulation of Atf4 and Gadd34 mRNA in differentiated myotubes (Fig. 3D), indicating that Atf4 and Gadd34 can be directly induced by hypoxia in muscle. Thus, despite activation of downstream targets of the UPR in muscle in response to hypoxia, the low phosphorylation levels of EIF-2α suggest a permissive state for mRNA translation.

**Hypoxia increases mTOR-related facilitation of protein synthesis.** Increased protein synthesis requires an increased phosphorylation of the markers 4E-BP1 and P70S6K1 (32). Food restriction reduced protein synthesis as shown by a transient decrease of 4E-BP1 (Ser65) and P70S6K1 (Thr389) phosphorylation (Fig. 3, E–G). Remarkably, phosphorylation levels of these proximal regulators of mRNA translation were maintained during the acute response to hypoxia (day 4) and even increased (4E-BP1) at day 21 (late response). Total protein content of 4E-BP1 remained unchanged (Fig. 3, E and G), whereas that of P70S6K1 had decreased on day 21 (Fig. 3, F and G). These data show that 4E-BP1 and P70S6K1 phosphorylation was maintained or even increased in response to hypoxia.
Hypoxia affects AKT signaling during the acute and chronic response. AKT plays a major role in the regulation of muscle metabolism, as it can both stimulate protein synthesis via mTOR and inhibition of GSK-3β, and prevent protein degradation through the inhibitory phosphorylation of FOXO1 (4, 48, 52). Short-term hypoxia decreased the absolute amount of phosphorylated AKT (Ser473) and increased the concentration of total AKT, so that the degree of phosphorylation of AKT (ratio p-AKT/total AKT) decreased (Fig. 4, A and F). Long-term hypoxia further increased the abundance of total AKT, without further affecting the degree of phosphorylation. In contrast, p-AKT was strongly increased in muscle of pair-fed mice, suggesting that hypoxia counteracted the activation of AKT that was induced by food restriction. Although not significantly different in all instances from control conditions, the phosphorylation status of the downstream substrates of AKT, that is GSK-3β (Fig. 4, B and F) and FOXO1 (Fig. 4, C and F), corresponded with the alterations in AKT phosphorylation and therefore likely reflected AKT activity. In addition, total FOXO1 protein levels were increased in response to chronic hypoxia. The increased FOXO1 protein concentration corresponded with the rapid increase in Foxo1 mRNA expression during hypoxia (Fig. 4D). Hypoxia also increased Foxo1 mRNA expression in cultured muscle cells, indicating a cell-autonomous response to hypoxia (Fig. 4E).

**Hypoxia activates proteasomal and lysosomal protein degradation.** Gene expression levels of markers of proteasomal and lysosomal protein degradation were determined. Hypoxia caused a rapid and transient increase in the expression of the proteasomal degradation gene Murf1 mRNA, which was completely mimicked by food restriction (Fig. 5A), whereas the increased expression of Atrogin-1 mRNA was only in part dependent on reduced food intake on days 2 and 4 of the hypoxia treatment (Fig. 5B). Only Atrogin-1 was upregulated in response to chronic hypoxia. Expression of Bnip3 and Map1lc3b mRNAs, which are markers of lysosomal protein degradation, was increased in response to hypoxia (Fig. 5, C and D). Semistarvation only accounted for the initial, but not the chronic, increase of Map1lc3B and Bnip3 expression. A cell-autonomous effect of hypoxia was only apparent on Bnip3 mRNA expression in C2C12 myotubes (Fig. 5E), suggesting that Murf1, Atrogin-1, and Map1lc3B expression in hypoxia-exposed skeletal muscle is regulated through an indirect mechanism. Together, these data showed an increased expression of genes of the proteasomal and the lysosomal protein degradation pathways, suggesting increased protein degradation during the acute and chronic response to hypoxia.

**DISCUSSION**

To address the potential contribution of hypoxia to the development of muscle wasting associated with COPD, signal-
Hypoxia-induced reduction of food intake. Malnutrition is a prevalent problem in chronic respiratory diseases. Because a negative energy balance affects muscle mass, we measured food intake in our study. The present findings show that, in agreement with literature (43), hypoxemia is an important causal factor in reduced energy uptake, as hypoxia induced a dramatic but transient reduction in food intake in our model. Even though food intake slowly recovered, it remained lower than in the normoxic group throughout the experiment, stabilizing at around 80% of the control intake. A similar decrease in food intake has been described earlier with milder normobaric hypoxia (11% O₂) (29). Decreased appetite was also reported in rats and healthy volunteers exposed to hypobaric hypoxia and also led to reductions in body weight and muscle mass (7, 55). Similar to the expression of HIF-1α marker genes (Glut1 and Ga-9) (10, 58), the food-intake pattern suggested an acute (up to 4 days) and a chronic phase in the adaptation to hypoxia.

Acute effects of hypoxia. Subsequent comparisons to a normoxic group of mice, pair fed to the hypoxic animals, allowed us to separate the contributions of reduced food intake from the specific effects of hypoxia on muscle atrophy. Food restriction during the acute phase alone resulted in an acute inhibition of regulatory steps of protein synthesis in muscle, such as decreased phosphorylation of 4E-BP1 and P70S6K1. This observation is in line with previous reports showing suppressed mTORC1 signaling and protein synthesis in response to food restriction (10a, 42). Although inhibition of mTOR activity has been demonstrated during hypoxia in vitro (2), we did not find inhibition of mTOR signaling in vivo, as phosphorylation of 4E-BP1 and P70S6K1 was maintained in hypoxic mice in contrast to pair-fed normoxic mice. Apparently, hypoxia suppresses the inhibitory effects of semistarvation on mTOR signaling. This effect of protein synthesis was also reflected in the hypoxia-induced decrease of EIF-2α phosphorylation and suggested that cap-dependent translation remains active under
hypoxia even though similar or even increased expression of downstream targets of the stress pathway were found. In addition, an increased expression of genes involved in proteosomal or lysosomal protein degradation (Murf1, Atrogin-1, Map1lc3b, and Bnip3) by hypoxia was observed, suggesting an enhanced muscle protein breakdown as described previously (12, 44, 46, 59). Whereas expression of Murf1 and Map1lc3b mRNA was entirely dependent on food restriction during the acute response, expression of Bnip3 and Atrogin-1 mRNAs was further increased by hypoxia, suggesting a hypoxia-specific effect. Bnip3 expression was also increased in hypoxia-exposed myotubes, suggesting direct regulation by oxygen deficiency. Indeed, the Bnip3 promoter contains a functional HRE (3), supporting the notion that HIF-1α stabilization occurred during hypoxia in skeletal muscle in vivo. Despite induction of increased proteolytic capacity, as shown by Murf1, Atrogin-1, Map1lc3b, and Bnip3 expression and suppressed protein synthesis signaling, semistarvation did not result in muscle weight loss during the acute phase. Acute hypoxia, on the other hand, induced an even greater loss of body weight than mere food restriction alone, and this was also reflected in muscle- and fat-pad weight loss. In summary, hypoxia had a strong catabolic effect during the acute phase of hypoxia, whereas food restriction did not affect muscle weight. Despite differences on muscle mass, acute hypoxia and food restriction both increased regulatory cues of proteosomal and lysosomal protein degradation. In addition, hypoxia relieved suppression of protein synthesis signaling by reduced food intake.

Chronic effects of hypoxia. As expected, the partial recovery of food intake led to a normalization of regulatory steps in protein synthesis and protein degradation. Accordingly, pair-fed animals did not differ from their normoxic controls with respect to parameters of mTORC1 signaling, lysosomal or proteosomal protein degradation signaling, or stress kinase signaling. Pair-fed animals did show increased phosphorylation of AKT and downstream targets, which we interpret as a relief of mTOR signaling, and a suppression of the induction of genes involved in protein degradation. Together, this could result in a partial recovery of muscle mass relative to the initial muscle mass loss. Despite the slow regain in body weight and the altered regulation of protein turnover, pair-fed animals did show a residual decrease in muscle weight at the end of the chronic phase.

Interestingly, fat tissue weight remained constant. Hypoxic animals, on the other hand, continued to lose both muscle and adipose tissue despite similar rates of body weight gain. Accordingly, they did not recover from the muscle loss during the acute phase of hypoxia. The sustained activation status of protein synthesis and degradation and the continuous loss of tissue weight, compared with control and pair-fed animals, may reflect an elevated protein turnover and subsequent higher energy consumption under sustained hypoxic conditions. Although future studies should address actual protein synthesis and degradation rates and compare them to the observed changes in protein turnover signaling, elevated protein turnover rates in response to hypoxia described by Chaudhary et al. (6) correspond with the increased expression of genes involved in protein turnover that we report here.

Conclusion. Hypoxia-induced atrophy involves food intake- and hypoxia-dependent effects (Fig. 6). We could also distinguish acute and chronic adaptive responses to hypoxia. Acute, severe hypoxia but not food restriction alone led to a rapid loss of muscle and fat tissue mass. During chronic hypoxia, a mild, continuous loss of muscle and fat mass was observed. Overall, hypoxia counteracted the inhibitory effects of reduced food intake on protein synthesis signaling and induced the expression of genes involved in proteosomal and lysosomal protein degradation. As expected, normobaric hypoxia-induced muscle atrophy was indeed partially a result of reduced food intake. Furthermore, contrary to our hypothesis, normobaric hypoxia-induced muscle atrophy involves activation of protein synthesis pathways in addition to increased expression of genes involved in protein degradation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
HYPOXIA-INDUCED MUSCLE ATROPHY MODULATES PROTEIN TURNOVER SIGNALING

AUTHOR CONTRIBUTIONS


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