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Chronic normobaric hypoxia does not alter markers for protein synthesis in human skeletal muscle

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ABSTRACT

Exposure to chronic hypoxia, environmental or pathological, results in loss of skeletal muscle mass due to an imbalance between muscle protein synthesis and degradation. Although, the molecular mechanisms behind hypoxia-induced muscle atrophy are still poorly understood, certainly *in vivo*. We hypothesized that chronic normobaric hypoxia (14.1% O_2, 15 days) would induce a negative net protein balance in resting human skeletal muscle by inhibiting the mTORC1 pathway, a key regulator of protein synthesis. To test this hypothesis, eight male subjects lived 15 days in a hypoxic hotel (Victoria University, Australia). From each subject, a muscle biopsy was taken from m.vastus lateralis before (PRE) and after (POST) 15 days of hypoxia. Western Blot and RT-PCR were used to determine protein and mRNA expression, respectively, of key signaling molecules in the mTORC1 and HIF pathway. Chronic hypoxia did not alter key markers in the mTORC1 pathway: phosphorylation status of Akt, mTOR, $p70^{S6K1}$ and 4E-BP1 was not modified in response to hypoxia. At the same time, expression of REDD1 and phosphorylation of AMPK, two factors known to be up-regulated in hypoxia and to inhibit the mTORC1 pathway, were unaffected. However, protein expression of HIF-1α, the main regulator of hypoxic responses, decreased parallel with a decrease in VEGF mRNA expression, a target gene of HIF-1α. Thus, in contrast to our hypothesis, markers for protein synthesis were not altered by chronic hypoxia. Therefore, reduced protein synthesis does not seem to be the underlying mechanism for hypoxia-induced muscle wasting.

Keywords: hypoxia, protein synthesis, mTORC1, AMPK, HIF-1α

INTRODUCTION

Hypoxia, induced by environmental conditions such as high altitude or by pathological conditions such as chronic obstructive pulmonary disease (COPD) (3), is a state of lowered oxygen tension at the tissue level. Whatever the origin of hypoxia, skeletal muscle cells can adapt to the lowered oxygen tension (40). Many studies have shown that chronic hypoxia, defined as hypoxic conditions for several days (15), results in a reduction of skeletal muscle mass (29,41,47). This muscle atrophy is due to an imbalance between protein synthesis and protein breakdown but the exact underlying molecular mechanisms remain unclear. It is likely that reduced protein synthesis and/or increased protein degradation contribute to hypoxia-induced muscle atrophy.

Most research on the regulation of protein metabolism by hypoxia focuses on protein synthesis, which is under the control of the Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway (25,54) (Fig. 1). Activated mTORC1 phosphorylates p70 ribosomal S6 protein kinase 1 ($p70^{S6K1}$) and 4E binding protein 1 (4E-BP1), making it unable to bind to and inhibit eukaryotic translation initiation factor 4E (eIF4E) thereby promoting the formation of the mRNA cap-binding complex eIF4F (25). These two downstream targets are key regulators in cap-dependent translation initiation, translation elongation and ribosome biogenesis, resulting in up-regulation of protein synthesis (25). Upstream targets of mTORC1, that can modify mTORC1 activity, will cause an effect on tuberous sclerosis complex 2 (TSC2), a GTPase activating protein (GAP) that can form a complex with TSC1 and subsequently inhibit mTORC1. This inhibition is obtained because activated TSC2 interacts with Ras homolog enriched in brain (Rheb), a G protein, and converts active Rheb-GTP to inactive Rheb-GDP. However, only active Rheb-GTP can activate mTORC1, which, in turn, can up-regulate protein synthesis (54). An important upstream target of mTORC1 is Akt, which causes inhibition of TSC2 through TSC2/14-3-3 inhibitory proteins association, allowing the accumulation of Rheb-GTP and thus activation of mTORC1.

A catabolic state, like hypoxia, can decrease protein synthesis *in vitro* through inhibition of the mTORC1 pathway (37). The primary mediator for hypoxic responses is hypoxia inducible factor 1 (HIF-1), an oxygen-sensitive transcription factor which triggers targets genes that induce adaptive responses in order to cope with oxygen stress (27,33,39). HIF-1 is a heterodimer that consists of an oxygen-sensitive α subunit (HIF-1α) and a constitutively expressed β subunit (HIF-1β) (27,40). In normoxia, HIF-1α is post-transcriptionally hydroxylated by prolyl hydroxylases (PHD), allowing the von Hippel-Lindau (VHL) E3 ligase complex to interact with HIF-1α. HIF-1α protein is subsequently polyubiquitilated and degraded by the 26S proteasome. However in hypoxia, HIF-1α protein is not

hydroxylated and degraded due to inhibition of PHD, which requires oxygen. HIF-1α translocates to the nucleus and dimerizes with HIF-1β to form the HIF-1 complex. This HIF-1 complex associates with hypoxia responsive elements (HRE) in the nucleus and ultimately triggers the transcription of target genes, among others vascular endothelial growth factor (VEGF), a major regulator of angiogenesis. Stabilization of HIF-1α in hypoxia induces expression of regulated in development and DNA damage responses 1 (REDD1) (9). REDD1 binds to 14-3-3 proteins resulting in TSC2/14-3-3 complex dissociation (8,16), TSC2 activation and consequently mTORC1 inhibition (Fig. 1). Chronic hypoxia can also inhibit mTORC1 activity through another, HIF-independent mechanism. Chronic hypoxia promotes cellular energy starvation and consequently activation of AMP-activated protein kinase (AMPK), a sensor of energetic status of the cell. AMPK phosphorylates and activates TSC2, leading to mTORC1 inhibition (39) (Fig. 1).

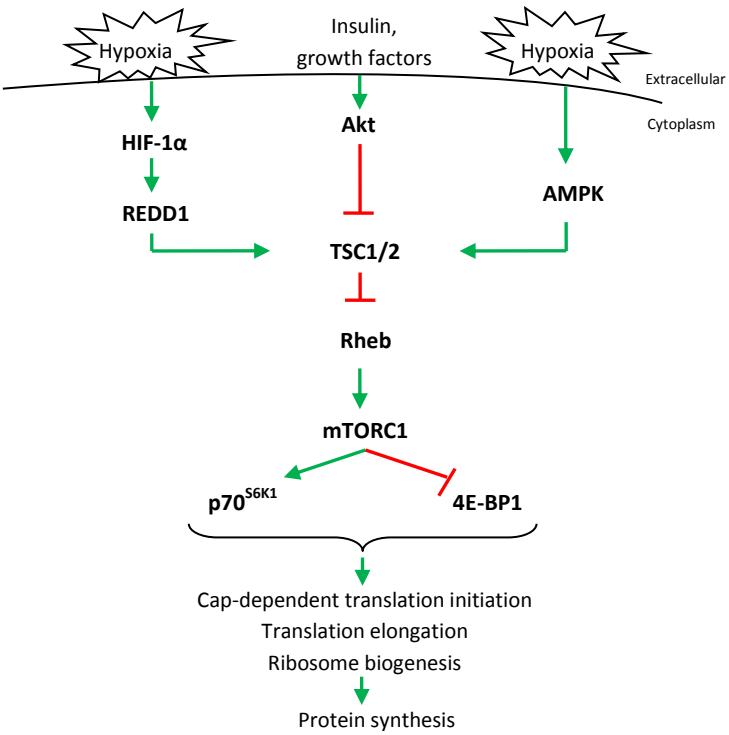


Fig. 1. Simplified diagram of the Akt/mTORC1 pathway, the main regulator of protein synthesis in human skeletal muscle and the influence of hypoxia.
Under stimulation of insulin or growth factors, Akt becomes activated and inhibits TSC2 through TSC2/14-3-3 association, which leads to the accumulation of Rheb-GTP and subsequently mTORC1 activation. Activated mTORC1 phosphorylates $p70^{S6K1}$ and 4E-BP1, who, in turn, stimulate cap-dependent translation initiation, translation elongation and ribosome biogenesis, resulting in up-regulation of protein synthesis. Hypoxia can inhibit the mTORC1 pathway via two distinct mechanisms: 1) activation of AMPK, an energy sensor of the cell and 2) expression of REDD1, induced by HIF-1α. Both AMPK and REDD1 activate TSC2, that, in turn, converts Rheb-GTP to Rheb-GDP, resulting in inhibition of mTORC1 and thus protein synthesis.

Several *in vitro* studies suggest that decreased protein synthesis, in order to spare oxygen, is the key underlying mechanism for skeletal muscle loss in hypoxic conditions (2,35,39). Studies in COPD patients even report that the reduction in protein synthesis is a direct effect of pathological chronic hypoxia (20,32). These findings have been confirmed in *in vivo* studies using rats, exposed to chronic hypoxic conditions (11,20). One study exposed male rats to hypobaric hypoxia (6300m) for 3 weeks. Muscle mass was decreased, independently of hypophagia, and was associated with an increase in REDD1 expression, its association with 14-3-3 partners and a down-regulation of the Akt/mTORC1 pathway (20). Another study, exposing female rats to hypobaric hypoxia (5500m) for 12 days, reported that chronic hypoxia lowered the overload-induced activation of mTORC1/$p70^{S6K1}$, increased REDD1 protein levels but did not activate AMPK (11). However, contrasting results have also been reported (12,28). Male rats exposed for 3, 7 or 14 days to hypobaric hypoxia (7620m) showed an increase in protein synthesis. However, this was accompanied by a higher increase in protein degradation, resulting in muscle atrophy (12). Furthermore in humans, exposed to hypobaric hypoxia (4559m) for 7-9 days, skeletal muscle loss was associated with an increased myofibrillar protein synthesis (28). The conflicting results may be due to different hypoxic conditions like altitude and duration. It is also possible that indirect markers of protein synthesis (11,20) not always represent an accurate reflection of how protein synthesis (12,28) is really modified (26).

Based on the aforementioned studies, it still remains unclear whether decreased protein synthesis, regulated by the mTORC1 pathway, is the underlying mechanism for hypoxia-induced muscle atrophy. Besides, studies examining the effect of hypoxia on protein synthesis in human are limited. Against this background, the purpose of this study is to investigate the effect of chronic environmental hypoxia on markers of muscle protein synthesis in resting human skeletal muscle in order to determine if decreased protein synthesis is the underlying mechanism for hypoxia-induced muscle wasting. Based on previous studies, we hypothesize that chronic environmental hypoxia would induce a negative net protein balance by inhibiting protein synthesis, regulated by the mTORC1 pathway.

MATERIALS AND METHODS

Subjects

Eight young male subjects participated in the study (Table 1). They were all healthy, recreationally-active and non-smokers. Subjects were excluded if they had any cardiovascular (e.g., high blood pressure, diabetes and arrhythmias) or pulmonary disease (e.g., asthma, bronchitis, etc.).

All subjects gave written informed consent before the start of the study, which was approved by the Victoria University human research Ethics Committee and in conformity with The Declaration of Helsinki.

Table 1. *Subject characteristics (n=8)*

Age	25 $\pm$ 5 years
Weight	89.5 $\pm$ 23.4 kg

Data are expressed as mean $\pm$ SD for age and weight.

Study design

The study was conducted in the hypoxic facility at the Institute of Sport, Exercise, Active Living (ISEAL), Footscray Park Campus, Victoria University, Australia. The eight participants lived 15 days in a normobaric hypoxic hotel at a simulated altitude of 3200m above sea level (F_iO_2 14.1%). Temperature and humidity were controlled and maintained constant during the study. During the first 3 days, the subjects were gradually exposed to the simulated altitude of 3200m. The first day, they lived at an altitude of 2500m (F_iO_2 15.4%), the second day at 2800m (F_iO_2 14.9%), the third day at 3000m (F_iO_2 14.5%) and afterwards at the altitude of 3200m (F_iO_2 14.1%). From each subject, two muscle biopsies were taken during supine rest at normoxia. The first biopsy was taken approximately one week before entering the hypoxic hotel (PRE) and the second biopsy immediately after living 15 days at 3200m altitude (POST).

Chronic hypoxia results in sleep deprivation, reduced physical activity and less appetite leading to reduced energy intake. These factors indirectly induce a catabolic state. To evaluate the effect of chronic hypoxia *per se* on muscle protein synthesis, these mentioned confounding factors must be controlled. One month prior to the start of the study, physical activity levels and diet of each subject were recorded during one week of the normal life in normoxia. Habitual physical activity levels were assessed by wearing a small accelerometer (Sensewear) on the upper arm. During their stay in the hypoxic hotel, subjects continuously wore the same accelerometer on their upper arm. Subjects had to maintain their previously recorded physical activity levels by checking the quantity of energy expenditure every day and adapt their level of physical activity when necessary. For this, the hypoxic

hotel was equipped with a treadmill and some dumbbells. Dietary habits during normal life were assessed using dietary-recall questionnaires. Based on the questionnaire and an interview, a nutritionist created a personal diet for each subject, which replicated the composition and total calories of the habitual diet. Two weeks prior to the start of the study and during their stay in the hypoxic hotel, every subject was required to follow his individually-designed diet. All the food and ingredients were provided.

Muscle biopsy

After sterilizing the skin and injecting a local anaesthesia (5ml, 1% Xylocaine) into the skin and fascia, a small incision was made and a muscle sample (80-120 mg) was taken from the left m. vastus lateralis (1/3 distal), using a 5-mm Bergström needle. Muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Western Blot

Proteins were extracted by homogenizing the frozen muscle tissue 3 times 5 seconds with a Polytron mixer in a lysis buffer (1:10, w/v) [50 mM Tris-HCL pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 0.1% Triton-X 100 and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were centrifuged during 10 min at 10 000 g (4°C) and supernatants were stored at -80°C. The DC protein assay kit (Bio-Rad laboratories, Nazareth, Belgium) was used to determine protein concentration.

Equal amounts of protein (25-70µg) from each sample were mixed with Laemmli sample buffer and separated by SDS-PAGE. Following electrophoresis (40mA for 1h), proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (80V for 2h). Membranes were then blocked with TBST, containing 5% non-fat milk, during 1h at room temperature. Afterwards, membranes were incubated overnight at 4°C with one of the following primary antibodies (1:1000): phospho-Akt Ser^{473}, Akt pan, phospho-mTOR Ser^{2448}, total mTOR, phospho-$p70^{S6K1}$ Thr^{389}, total $p70^{S6K1}$, phospho-4E-BP1 $Thr^{37/46}$, total 4E-BP1, phospho-AMPK Thr^{172}, total AMPK, phospho-ACC Ser^{79}, total ACC, HIF-1α (Cell Signaling), REDD1 (Proteintech), HIF-2α (Novus Biologicals) and total α-tubulin (Sigma). Next morning, membranes were washed 3 times 10 min with TBST and incubated with horseradish peroxidase-conjugated anti-mouse (1:10 000) or anti-rabbit (1:5000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium) for 1h at room temperature. Following an additional 3 washes, chemiluminescence detection was performed. Membranes were scanned and quantified using Genesnap and Genetools softwares. When phosphorylation status of a protein was determined, phosphorylated proteins were normalized with respect to their total forms. Otherwise, protein

expression was normalized with respect to the loading control α-tubulin, which was not significantly different between the measurement before and after 15 days of hypoxia.

RNA extraction, reverse transcription and real-time quantitative PCR analysis

Total RNA from 20 mg of frozen muscle tissue was extracted using TRIzol reagent (Invitrogen, Vilvoorde, Belgium). RNA quality and quantity were evaluated using Nanodrop spectrophotometry (Thermo Scientific, Erembodegem, Belgium). Subsequently, RNA was reverse transcribed to generate cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) consistent with manufacturer's instructions. cDNA samples were stored at -20°C until further analysis. Quantitative PCR was performed with a Power SYBR Green-based master mix (Applied Biosystems). Amplification and detection were carried out using the ABI PRISM 7300 (Applied Biosystems). Primers were developed for human HIF-1α, HIF-2α, MyoD, proliferating cell nuclear antigen (PCNA), REDD1 and VEGF (Table 2). The procedure consisted of 40 repeating cycles. Each cycle included 3 thermal conditions: denaturation during 30 sec at 95°C, annealing during 30 sec at 58°C and extension during 30 sec at 72°C. All the reactions were executed in triplicate. Data were normalized using the Vandesompele method (56).

Table 2. *Primer sequences*

	Forward Primer	Reverse Primer
HIF-1α	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG AAC
HIF-2α	AAG CTG AAG CGA CAG CTG GAG TAT	GTA CAT TTG CGC TCA GTG GCT TGT
MyoD	TGC CAC AAC GGA CGA CTT CTA TGA	AAG TGC GAG TGC TCT TCG GGT TT
PCNA	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
REDD1	TGA GGC ACG GAG TGG GAA	CAG CTC GAA GTC GGG CAA
VEGF	TTT CTG CTG TCT TGG GTG CAT TGG	ACC ACT TCG TGA TGA TTC TGC CCT

HIF-1α, hypoxia inducible factor 1 alpha; HIF-2α, hypoxia inducible factor 2 alpha; PCNA, proliferating cell nuclear antigen; REDD1, regulated in development and DNA damage responses 1; VEGF, vascular endothelial growth factor.

Statistical analysis

Values obtained before (PRE) and after (POST) living 15 days in hypoxia were compared to evaluate the effect of chronic hypoxia on markers of protein synthesis and the HIF pathway. First, a Kolmogorov-Smirnov test was used to check for normal distributions. In case of normal distributions, a parametric student paired t-test was used, otherwise a non-parametric Wilcoxon signed-rank test was used to test the statistical significance of differences between PRE and POST values. For each individual, PRE and POST values are visualized, along with mean±SEM. All analyses were performed in SAS Enterprise Guide 5.1. A P value <0.05 was considered significant.

RESULTS

Effect of chronic hypoxia on markers of protein synthesis

Protein synthesis in the skeletal muscle is mainly regulated by the Akt/mTORC1 pathway. Chronic hypoxia had no effect on phosphorylation of Akt (Fig. 2A) and mTOR (Fig. 2B). Also the phosphorylation status of the two main downstream targets of mTORC1, $p70^{S6K1}$ (Fig. 2C) and 4E-BP1 (Fig 2D), was not different between PRE and POST hypoxia exposure.

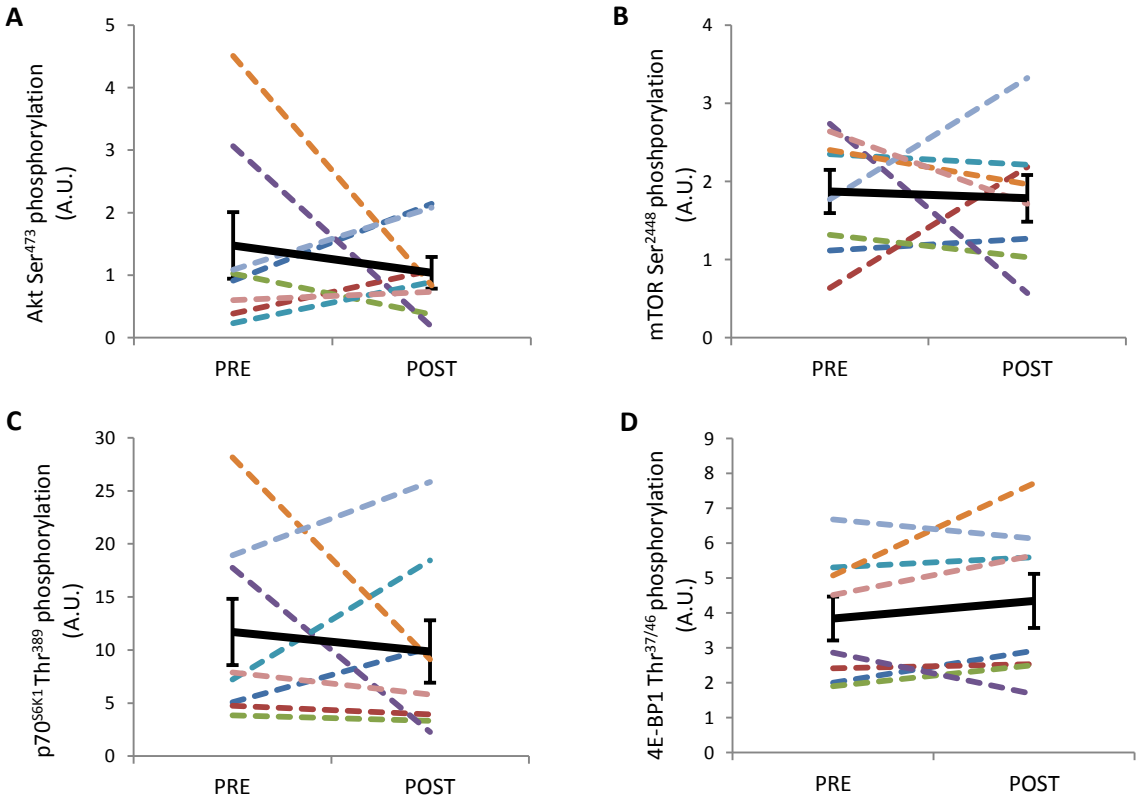


Fig. 2. Effect of chronic environmental hypoxia (15 days) on markers of protein synthesis: (A) Akt, (B) mTOR, (C) $p70^{S6K1}$ and (D) 4E-BP1 phosphorylation. Individual (n=8, dotted line) and mean±SEM (solid line) values before (PRE) and after (POST) exposure to hypoxia are presented. Phosphorylated proteins are normalized to their total forms. Data are expressed as arbitrary units (A.U.). *significantly different from PRE, $p<0.05$.

Effect of chronic hypoxia on markers of hypoxia

Hypoxia can inhibit mTORC1 and thus protein synthesis via two distinct mechanisms. First, hypoxia can phosphorylate and activate AMPK, a sensor of energetic status of the cell (39). However, phosphorylation of AMPK at Thr^{172} (Fig. 3A) was unchanged by exposure to chronic hypoxia. In line with this, phosphorylation of Acetyl CoA Carboxylase (ACC) at Ser^{79} (Fig. 3B), an indicator for AMPK activity (50), was unaffected.

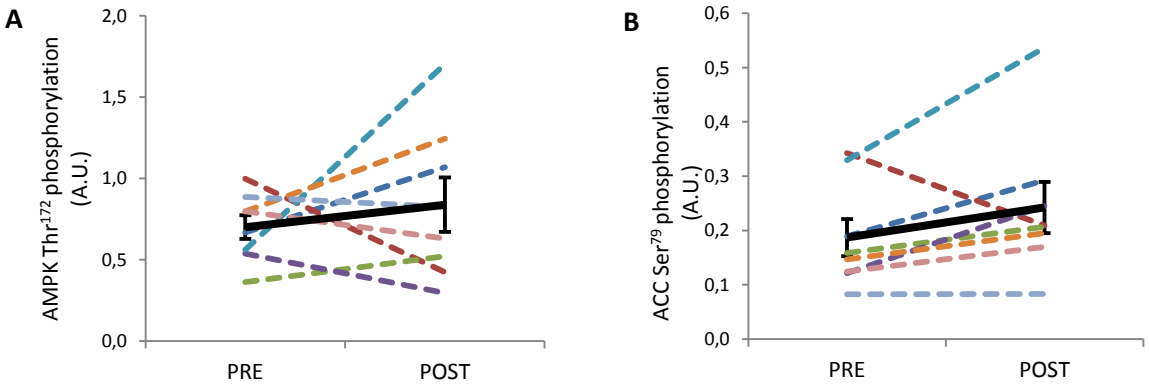
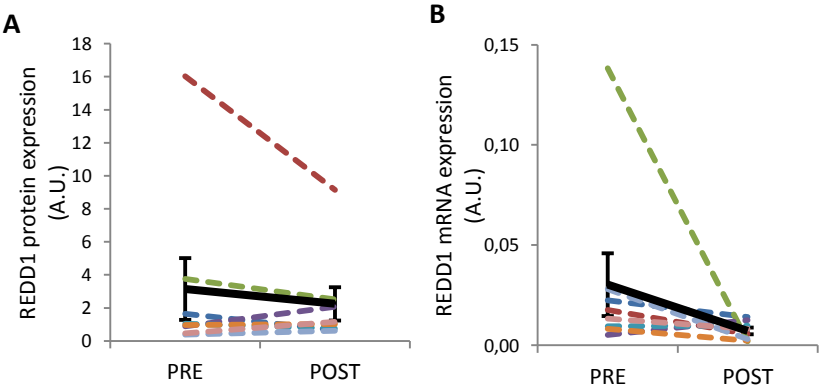


Fig. 3. Effect of chronic environmental hypoxia (15 days) on (A) AMPK and (B) ACC phosphorylation. Individual (n=8, dotted line) and mean±SEM (solid line) values before (PRE) and after (POST) exposure to hypoxia are presented. Phosphorylated proteins are normalized to their total forms. Data are expressed as arbitrary units (A.U.). *significantly different from PRE, $p<0.05$.

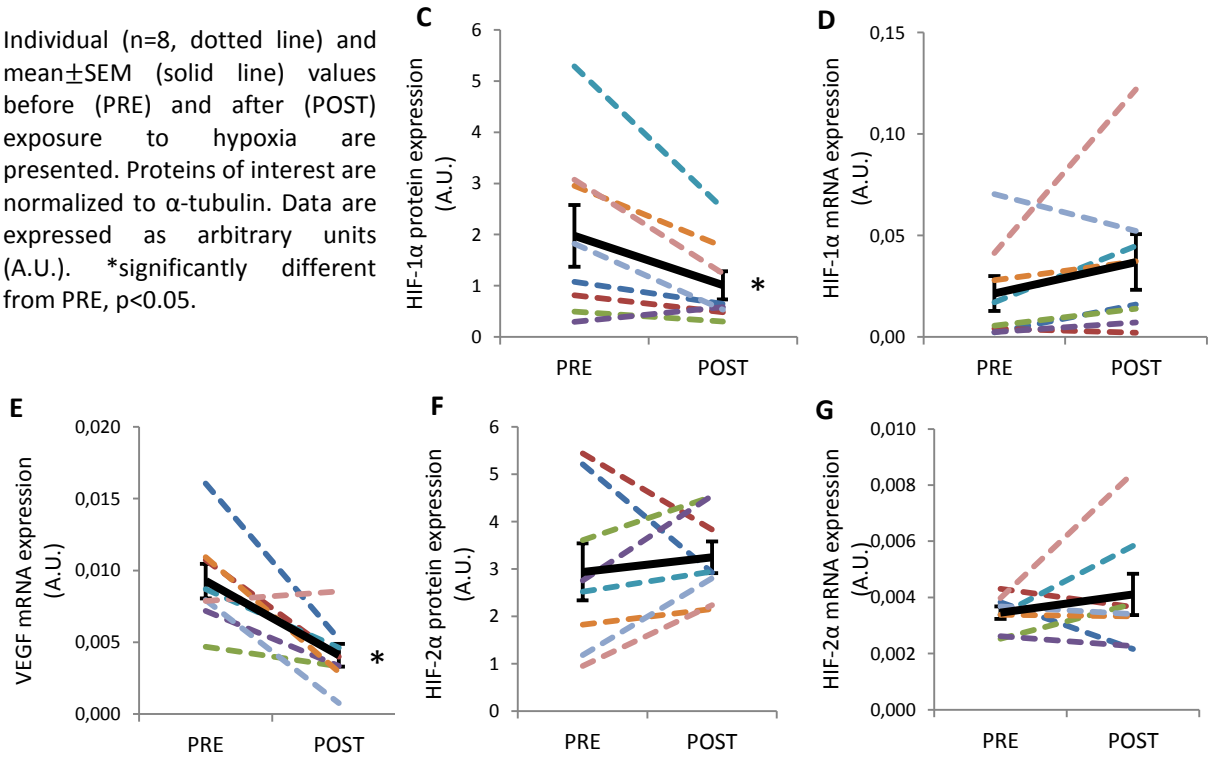
Secondly, hypoxia can inhibit mTORC1 via the expression of REDD1 (8,16), which is induced by HIF-1α (9). However, there was no difference in REDD1 protein (Fig. 4A) and mRNA (Fig. 4B) expression between PRE and POST. In contrast, HIF-1α protein expression (Fig. 4C) was decreased ($p<0.05$) after exposure to hypoxia compared with baseline (PRE). In line with this, mRNA expression of vascular endothelial growth factor (VEGF) (Fig. 4E), a target gene of HIF-1α, was decreased ($p<0.05$) by chronic hypoxia. However, mRNA expression of HIF-1α (Fig. 4D) was not modified.

Another member of the HIF family is HIF-2α. Protein (Fig. 4F) and mRNA (Fig. 4G) expression of HIF-2α remained unchanged by chronic hypoxia.

Fig. 4. Effect of chronic environmental hypoxia (15 days) on markers of the HIF pathway: (A) REDD1 protein expression, (B) REDD1 mRNA expression, (C) HIF-1α protein expression, (D) HIF-1α mRNA expression, (E) VEGF mRNA expression, (F) HIF-2α protein expression and (G) HIF-2α mRNA expression.



Individual (n=8, dotted line) and mean±SEM (solid line) values before (PRE) and after (POST) exposure to hypoxia are presented. Proteins of interest are normalized to α-tubulin. Data are expressed as arbitrary units (A.U.). *significantly different from PRE, p<0.05.



Effect of chronic hypoxia on markers of satellite cells

Besides protein turnover, cell turnover can also participate in muscle mass regulation (49). MyoD belongs to the family of myogenic regulatory factors (MRFs) and plays a key role in satellite cell differentiation into muscle cells. Therefore, MyoD is a marker of myogenic commitment of satellite cells. MyoD mRNA expression (Fig. 5A) was not different between PRE and POST. However, mRNA expression of PCNA (Fig. 5B), a marker of DNA synthesis and satellite cell activation, decreased following 15 days of hypoxia ($p<0.05$).

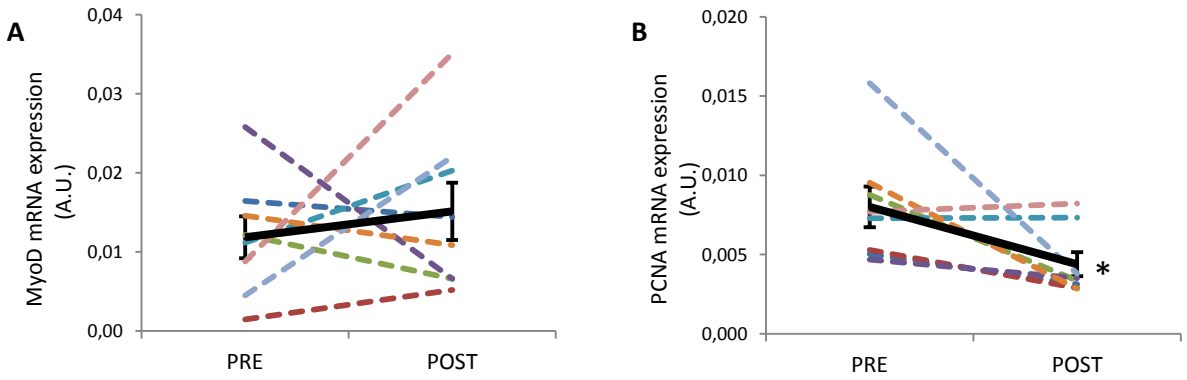


Fig. 5. Effect of chronic environmental hypoxia (15 days) on markers of satellite cells: (A) MyoD and (B) PCNA mRNA level. Individual (n=8, dotted line) and mean±SEM (solid line) values before (PRE) and after (POST) exposure to hypoxia are presented. Data are expressed as arbitrary units (A.U.). *significantly different from PRE, p<0.05.

DISCUSSION

Loss of skeletal muscle mass is a well-known phenomenon during exposure to chronic hypoxia, induced by environmental (29,41,47) or pathological (20,32) conditions. However, the underlying molecular mechanisms remain poorly understood. Besides, only a limited number of studies have examined the effect of hypoxia on protein turnover in human. In addition, the purpose of the present study was to investigate the effect of chronic environmental hypoxia (14.1% O_2, 15 days) on protein synthesis signaling pathway in resting human skeletal muscle. We hypothesized that chronic hypoxia would up-regulate the HIF pathway and subsequently reduce markers of protein synthesis, resulting in muscle atrophy. In contrast to our hypothesis, we showed presently that chronic normobaric hypoxia of 15 days does not alter phosphorylation status of markers of the mTORC1 pathway, which mainly regulates protein synthesis in skeletal muscle. We also found no alteration in AMPK phosphorylation and REDD1 protein and mRNA expression, two markers that are known to be up-regulated in hypoxia and to inhibit the mTORC1 pathway (8,16,39). However, we did find a decrease in HIF-1α protein expression following chronic exposure, parallel with a decrease in mRNA expression of VEGF, a target gene of HIF-1α.

Chronic hypoxia does not inhibit the mTORC1 pathway

It has been suggested that decreased protein synthesis, in order to spare oxygen, is the key underlying mechanism for hypoxia-induced muscle atrophy. *In vitro* studies (2,35,39) and studies in COPD patients (20,32) reported reduced phosphorylation of markers of the mTORC1 pathway in response to respectively acute and chronic hypoxia. Also *in vivo* studies using rats found a decreased protein synthesis due to chronic hypoxia (11,20). In one study, male rats showed muscle atrophy, independently of hypophagia, that was associated with a down-regulation of the Akt/mTORC1 pathway after 3 weeks exposure to hypobaric hypoxia (6300m) (20). In another recent study, hypobaric hypoxia (5500m) for 12 days blunted the overload-induced activation of mTORC1/$p70^{S6K1}$ in female rats (11). Furthermore, male subjects showed a small reduction in total mTORC1 protein content after staying 7-9 days at 4559m (57). However, in the present study, we found that chronic normobaric hypoxia had no effect on markers of protein synthesis: phosphorylation status of Akt, mTOR, $p70^{S6K1}$ and 4E-BP1 remained unchanged following 15 days of hypoxia (Fig. 6). In agreement, phosphorylation status of y4E-BP1, the most phosphorylated form of 4E-BP1, was not modified by chronic hypoxia (data not shown). Our results indicate that a decreased protein synthesis is probably not the responsible mechanism for hypoxia-induced muscle wasting, although we did not assess protein synthesis directly. In line with our data, another recent study of our laboratory found that acute normobaric hypoxia (11% O_2) did not alter markers of protein synthesis in resting human

skeletal muscle (44). However, a similar study of our laboratory examined the effect of acute (4h) normobaric hypoxia (11% O_2) in resting human skeletal muscle after eating a light meal and found, surprisingly, an increased phosphorylation of Akt and $p70^{S6K1}$. These results can be explained by a higher plasma insulin level in hypoxia, caused by the light meal (17).

Contrasting results can be due to different factors: duration and degree of hypoxia, normobaric versus hypobaric hypoxia, physical activity levels and food intake. These factors have an influence on the adaptation of skeletal muscle to hypoxia. Moreover, a muscle biopsy was only taken after 15 days of hypoxia exposure. In this way, short-term effects (several hours or days) of hypoxia on markers of protein synthesis were not determined. Thus, we cannot rule out a transient decrease in protein synthesis signaling earlier during hypoxia whereas this transitory change returns back to basal values after 15 days.

Muscle atrophy is due to an imbalance between protein synthesis and protein degradation. Since the present study showed an unaltered protein synthesis in response to chronic hypoxia, we suggest that the hypoxia-induced muscle loss is attributable to an increased protein degradation. Two main proteolytic systems in skeletal muscle are the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP). In the UPS, target proteins are marked with multiple ubiquitin monomers and then recognized and degraded by the 26S proteasome (6,21,31,48). In the ALP, double membrane vesicles are formed around protein aggregates and fuse with lysosomes, resulting in degradation of the content by lysosomal hydrolases (30,54). Although not confirmed by everyone (20), there are indications in the literature that markers of UPS, including the E3 ligases MuRF-1 and MAFbx (24,54) or level of ubiquitinated proteins (12), are up-regulated in COPD patients (18,51) and in rats, exposed to chronic hypoxia (11,12). One study also reported an increased autophagic flux in response to acute hypoxia in resting human skeletal muscle (44).

Based on our data and the aforementioned studies, we suggest, although we did not measure markers of protein degradation, that hypoxia-induced muscle wasting is possibly caused by an increased protein degradation instead of a reduced protein synthesis. However, also other pathways, besides the mTORC1 pathway, and other mechanisms could have regulated protein synthesis. Another pathway that can influence protein synthesis is the Ras-Raf-MEK-ERK1/2 pathway (45). Activated ERK1/2 has many targets, including MAPK-interacting kinase 1 (MNK1). Active MNK1 phosphorylates and upregulates eIF-4E, resulting in enhanced translation initiation and efficiency. Another ERK1/2 target is p90 ribosomal protein S6 kinase ($p90^{RSK}$) which, when activated, stimulates eukaryotic elongation factor 2 (eEF2) and thus translation elongation. However, the role of this pathway in regulating protein synthesis during hypoxia is unknown. Another mechanism that can

contribute to reduced protein synthesis under hypoxic conditions is endoplasmic reticulum (ER) stress, i.e. an accumulation of misfolded proteins within the ER lumen, and the subsequent unfolded protein response (UPR) (35,39). As part of the UPR, the protein kinase RNA-like endoplasmic reticulum kinase (PERK) is released from Bip, becomes activated and, in turn, phosphorylates eukaryotic initiation factor 2α (eIF2α). This leads to inhibition of the mRNA translation initiation at earlier time points during hypoxia (36) in order to prevent further accumulation of misfolded proteins and to restore ER homeostasis. However, in this study, we did not evaluate markers of the ERK1/2 pathway and UPR since this is beyond the scope of this paper.

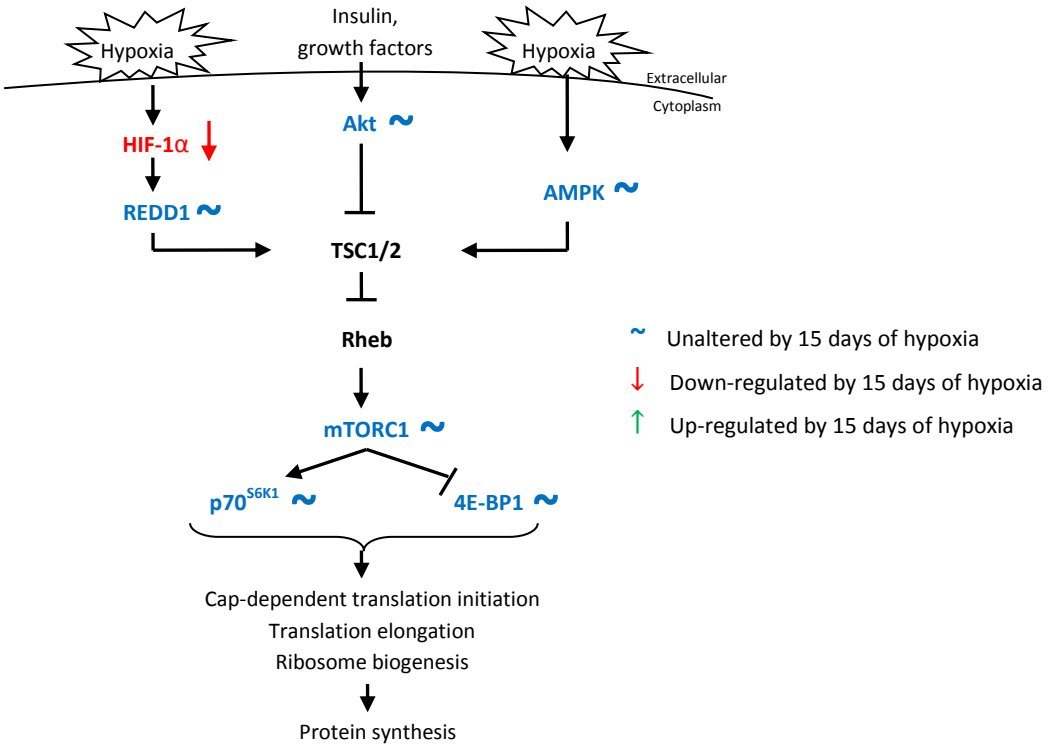


Fig. 6. Effect of chronic normobaric hypoxia (15 days) on markers of mTORC1 and HIF pathway. In the present study, we found a down-regulation of HIF-1α protein expression, an unchanged REDD1 protein expression and an unchanged phosphorylation status of AMPK, Akt, mTOR, $p70^{S6K1}$ and 4E-BP1 after 15 days of hypoxia (POST) compared with baseline (PRE).

Chronic hypoxia does not up-regulate AMPK and REDD1

Hypoxia can inhibit the mTORC1 pathway via two independent mechanisms: 1) AMPK activation (39) and 2) REDD1 expression (8,16). Exposure to chronic hypoxia can lead to energy starvation and subsequently AMPK activation, resulting in inhibition of the mTORC1 pathway. (39). Surprisingly, the present study found no alteration in phosphorylation of AMPK, a sensor of energetic status of the

cell, in response to chronic hypoxia. To evaluate the activity of AMPK, we measured the phosphorylation status of ACC, a substrate of AMPK (50). In agreement, phosphorylation of ACC was not affected by chronic hypoxia, indicating that AMPK was not activated. In line with our results, another recent study showed that chronic hypoxia (5500m for 12 days) does not alter AMPK phosphorylation in female rats (11). Thus, our results do not support the idea that chronic hypoxia leads to energy starvation and subsequently AMPK activation.

Another mechanism that can inhibit the mTORC1 pathway is the expression of REDD1 (8,16). A recent study reported that muscle wasting in male rats, exposed to chronic hypoxia (6300m for 3 weeks), is associated with a marked increase in REDD1 protein expression and its association with 14-3-3 partners, resulting in down-regulation of Akt/mTORC1 pathway (20). In contrast, our results showed no alteration in REDD1 expression as a result of chronic hypoxia.

Taken together, the present study reported that chronic hypoxia does not up-regulate REDD1 protein expression and AMPK activation, two markers that can inhibit the mTORC1 pathway (Fig. 6). This is consistent with no alterations in the mTORC1 pathway.

HIF-1α protein expression and transcriptional activity decrease in response to chronic hypoxia

HIF-1α is a transcription factor that is stabilized and up-regulated in hypoxia and thereby regulates adaptive responses to cope with reduced oxygen tension (27,33,40). A recent study found a five-fold increase in HIF-1α protein expression in rats after 14 days of hypobaric hypoxia (7620m) (12). Contrary, we found presently a decrease in HIF-1α protein expression after 15 days of hypoxia (Fig. 6). This can be the result of a time-dependent HIF-1α response to hypoxia. It is possible that HIF-1α protein levels peak within the first hours or days of hypoxic exposure in order to cope with the low oxygen tension and then, after adaptation to hypoxic conditions, progressively decline towards or even under basal levels. This decline in HIF-1α expression after prolonged hypoxia exposure can possibly be explained by earlier adaptive responses that attenuate, restore or even overcompensate for the low oxygen tension in the muscles. Because only one muscle biopsy was taken in hypoxia, a possible time-dependent response of HIF-1α cannot be established with certainty.

Differently to a decrease in HIF-1α protein level, the mRNA level was unaffected in response to hypoxia, indicating that HIF-1α is modified at the protein level by post-transcriptional mechanisms. To test the transcriptional activity of HIF-1α, we measured the mRNA expression of VEGF, a target gene of HIF-1α and a main regulator of angiogenesis. Our data showed a decrease in VEGF mRNA level in response to 15 days hypoxia. In addition to the decrease in HIF-1α protein expression, also the transcription of the target gene VEGF decreased, possibly due to earlier acclimatization to the hypoxic conditions.

Chronic hypoxia reduces satellite cell activity

Changes in muscle mass are not only determined by protein turnover but also by cell turnover, the balance between myonuclear accretion and myonuclear loss (49). Satellite cells are quiescent, myogenic progenitor cells located between the basal lamina and sarcolemma of muscle fibers and are important for muscle development and regeneration. In the case of myonuclear accretion, e.g. some forms of muscle hypertrophy (55), the normally quiescent satellite cells become activated, proliferate and fuse with myofibers, resulting in an increased number of myonuclei within the muscle fibers (49). Contrary, in muscle atrophy, satellite cells and myonuclei may be subjected to apoptosis (49), although this is still debated. The effect of hypoxia on the regulation and (in)activation of satellite cells and the underlying signaling pathways are far from being elucidated. An *in vivo* study in rats reported that chronic hypobaric hypoxia (5500m for 7 days) inhibits satellite cells activity and protein synthesis signaling, resulting in impaired muscle regeneration following injury (10). However, the effect of chronic hypoxia on satellite cells (in)activation in human is barely investigated. One study investigated the muscle regenerative capacity in human skeletal muscle after a 6-week stay above 5000m and found that satellite cells had a decreased ability to regenerate skeletal muscle, partly due to reduced satellite cell activity (43). In the present study, we measured the mRNA expression of MyoD, a marker of satellite cell differentiation into myoblast, and PCNA, a marker of satellite cell activation (4). MyoD mRNA expression was not different before and after exposure to hypoxia, suggesting that satellite cell differentiation was not affected by chronic hypoxia. However, PCNA mRNA expression was decreased in response to chronic hypoxia, indicating that satellite cell activity was reduced. Taken together, our results suggest that chronic hypoxia affects and reduces satellite cell activation but not differentiation. This may impair the regenerative capacity of skeletal muscle and potentially contribute to hypoxia-induced muscle loss. Further research must focus on the regulation of satellite cells by hypoxia, the underlying signaling pathways and the relative contribution of cell turnover in the hypoxia-induced muscle wasting.

Limitations of the study

A first limitation is the absence of a control group, who would undergo the same protocol but live in normoxia for 15 days. The total number of subjects in the study is low whereby the study is probably underpowered. Another limitation is that we only quantified markers of protein synthesis without a direct measurement of this process. This is important because indirect markers may not always represent an accurate reflection of how protein synthesis is really modified (26). We also have to acknowledge that we did not measure the actual changes and losses in skeletal muscle mass in response to chronic hypoxia. However, it is well established in previous studies that chronic hypoxia results in muscle wasting (20,29,32,41,47). Another limiting factor is that we could not control the

procedure of muscle biopsy collection ourselves because the study was conducted by others at Victoria University, Australia. A marked finding in the present study is the large inter-individual variability in baseline values. Also the responses to chronic hypoxia vary widely among the eight subjects. Because of the low number of subjects and the large inter-individual variability, it becomes difficult to draw general conclusions. Another already mentioned limitation is the collection of only one muscle biopsy in hypoxia, namely after 15 days of exposure. In this way, acute, transient and time-dependent changes in response to hypoxia cannot be determined. Finally, an important note is that we examined the effect of chronic normobaric hypoxia (F_iO_2<20.9%, Pb=760 mm Hg), created in a hypoxic hotel, which is not exactly the same as hypobaric hypoxia (F_iO_2=20.9%, Pb<760 mm Hg) at high altitude. Although there is some inconsistency, several studies reported different physiological responses, i.e. parameters of ventilation, fluid retention, nitric oxide (NO) metabolism and acute mountain sickness, between exposure to normobaric and hypobaric hypoxia whereby hypobaric hypoxia seems to be a more severe environmental condition (13,46). Therefore, caution must be taken when extrapolating these normobaric results to high altitude. However, to date, no study compared the regulation of protein synthesis and muscle mass under both hypoxic conditions and it remains unknown if hypobaric hypoxia affects these factors differently than normobaric hypoxia.

CONCLUSION

In contrast to our hypothesis, the present study shows that chronic normobaric hypoxia (14.1% O_2 for 15 days) does not alter markers of the mTORC1 pathway, a key regulator of protein synthesis, in human skeletal muscle. Besides, AMPK phosphorylation and REDD1 expression, two factors known to be up-regulated by hypoxia and inhibit the mTORC1 pathway, are unaffected. However, the protein expression of HIF-1α, the main regulator of hypoxic responses, decreases in response to chronic hypoxia parallel with a decrease in VEGF mRNA expression, a target gene of HIF-1α. These findings indicate that hypoxia-induced muscle atrophy is not caused by a reduced protein synthesis in resting human skeletal muscle. Therefore, other mechanisms, like elevated protein degradation possibly in combination with reduced satellite cell activity, may be responsible for muscle wasting in response to hypoxic conditions. Future research must examine the effect of chronic hypoxia on both protein turnover, i.e. protein synthesis and degradation, and cell turnover in human to establish the exact molecular mechanisms responsible for hypoxia-induced muscle wasting. These findings can help us better understand the phenomenon of muscle atrophy in exposure to chronic hypoxic conditions, which is relevant for athletes, training at high altitude, and mountaineers. This knowledge is also important to better understand pathological conditions, associated with hypoxia, and design therapeutic interventions to counteract muscle loss in patients with hypoxia-related diseases, including COPD.

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