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Effect of acute environmental hypoxia on protein metabolism in human skeletal muscle

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Physiological relevance: Hypoxia-induced muscle wasting has been observed in several environmental (such as altitude) and pathological (such as lung diseases) conditions. However, the molecular mechanisms behind this loss of muscle mass are far from elucidated, certainly *in vivo*. To the best of our knowledge, no study has previously reported the effect of hypoxia on protein degradation in human skeletal muscle, and protein synthesis has only been scarcely studied at a molecular level. The present article reports new mechanistic explanations about the regulation of muscle protein metabolism, which is not only important for hikers who lose muscle mass during ascent but also for patients suffering from lung diseases or anaemia who present reduced muscle mass.

Abstract

Hypoxia-induced muscle wasting has been observed in several environmental and pathological conditions. However, the molecular mechanisms behind this loss of muscle mass are far from being completely elucidated, certainly *in vivo*. When studying the regulation of muscle mass by environmental hypoxia, many confounding factors have to be taken into account, such as decreased protein ingestion, sleep deprivation or reduced physical activity, which make difficult to know whether hypoxia *per se* causes a reduction in muscle mass.

Aim: We hypothesized that acute exposure to normobaric hypoxia (11% O_2) would repress the activation of the mTOR pathway usually observed after a meal and would activate the proteolytic pathways in skeletal muscle.

Methods: Fifteen subjects were exposed passively for 4 h to normoxic and hypoxic conditions in a random order after consumption of a light breakfast. A muscle biopsy and a blood sample were taken before, after 1 and 4 h of exposure.

Results: After 4 h, plasma insulin concentration and the phosphorylation state of PKB and S6K1 in skeletal muscle were higher in hypoxia than in normoxia ($P < 0.05$). At the same time, Redd1 mRNA level was upregulated ($P < 0.05$), whilst MAFbx mRNA decreased ($P < 0.05$) in hypoxia compared with normoxia. Proteasome, cathepsin L and calpain activities were not altered by environmental hypoxia.

Conclusion: Contrary to our hypothesis and despite an increase in the mRNA level of Redd1, an inhibitor of the mTORC1 pathway, short-term acute environmental hypoxia induced a higher response of PKB and S6K1 to a meal, which may be due to increased plasma insulin concentration.

Keywords insulin, NIRS, PKB, proteasome, S6K1.

Hypoxia is a state of lowered oxygen tension (PO_2) in tissue that can be created by environmental conditions such as high altitude or by pathological conditions such as chronic obstructive pulmonary disease (Baldi *et al.* 2010), obstructive sleep apnoea (Garvey *et al.* 2009) and anaemia (Grocott *et al.* 2007). During exercise, hypoxia can also be generated but, contrary to the previous situations, oxygen restriction is then limited to skeletal muscle (Ameln *et al.* 2005). Whatever the origin of hypoxia, skeletal muscle cells will

adapt acutely and/or chronically to deal with this reduction in oxygen availability. For example, patients exposed to chronic hypoxaemia due to lung disease have a higher 5-year mortality and an associated muscle wasting (Schols *et al.* 2005). Several studies revealed that highlanders and hikers undergo reductions in muscle fibre cross-sectional area, which is associated with increased capillarization (Hoppeler *et al.* 1990, MacDougall *et al.* 1991, Mizuno *et al.* 2008). Although contrasting results have been reported as well (Lundby *et al.* 2004), long-lasting hypoxia generally leads to a negative regulation of protein metabolism and a loss of muscle mass.

Studies on cell culture and animals have provided some more insight into the mechanisms by which hypoxia negatively regulates protein metabolism. In a general way, hypoxia suppresses ATP- and energy-consuming processes like Na^+, K^+ ATPase activity or protein synthesis (Koumenis & Wouters 2006). At a molecular level, a decrease in PO_2 activates the hypoxia-inducible factor-1 alpha (HIF-1α), a transcription factor regulating the expression of genes involved in a large spectrum of functions, that is, angiogenesis, metabolic transition to anaerobic glycolysis and cell motility and invasion (Koumenis & Wouters 2006). Whether HIF-1α is directly responsible for the decrease in protein synthesis is unknown, but HIF-1α and the mammalian target of rapamycin complex 1 (mTORC1) pathway, a key regulator of the mRNA translation machinery, have been shown to regulate each other (Cam *et al.* 2010, Greer *et al.* 2012, Lee *et al.* 2009). On the other hand, hypoxia has also been shown to inhibit mTORC1 in a HIF-1α-independent way in cell cultures (Arsham *et al.* 2003). Whether these observations from cell cultures, where very low concentrations of oxygen (0–5% O_2) are used, may be extrapolated to living organisms remains an open question.

Hypoxia inhibits mTORC1 through at least two intermediates: a small protein called 'regulated in development and DNA damage responses 1' (Redd1) (McGee & Hargreaves 2010) as well as the critical regulator of energy balance 5' AMP-activated protein kinase (AMPK) (Liu *et al.* 2006). Both Redd1 (McGee & Hargreaves 2010) and AMPK (Liu *et al.* 2006) inhibit mTORC1 through phosphorylation of the tuberous suppressor complex 2 (TSC2), which reinforces the inhibition of this complex on mTORC1. Inhibition of mTORC1 results in down-regulation of 5′-terminal oligopyrimidine tract (TOP) mRNA translation through decreased phosphorylation of p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), thereby inhibiting the formation of the eukaryotic initiation factor 4F (eIF4F) complex (Laplante & Sabatini 2012). Hypoxia seems also to impinge directly on the formation of this complex by altering the availability of eukaryotic initiation factor 4E (eIF4E) (Koritzinsky *et al.* 2006).

Endoplasmic reticulum (ER) stress and its downstream response, the unfolded protein response, are mechanisms that have recently been proposed to participate in the reduction of protein synthesis under hypoxia (Koritzinsky *et al.* 2006). Three main sensors, each of which initiates a branch of the unfolded protein response, contribute separately or coordinately for restoration of ER homoeostasis; activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 alpha (IRE1α) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). These three factors associate with the protein, chaperone binding protein (BiP) in normal physiological conditions. Under ER stress, ATF6, IRE1α and PERK are released from BiP and may become activated (Ron & Walter 2007). Severe hypoxia causes ER stress, as O_2 serves as an electron acceptor during oxidative protein folding, an ER process driving formation of disulphide bonds in proteins (Tagliavacca *et al.* 2012). When O_2 is insufficient, disulphide bonds cannot be formed, misfolded proteins accumulate, and the unfolded protein response is activated. However, the underlying molecular mechanisms are poorly understood (Wouters & Koritzinsky 2008). Hypoxia has been shown to activate PERK, which in turn phosphorylates eukaryotic initiation factor 2α (eIF2α) and inhibits translation initiation (Koumenis *et al.* 2002). The regulation of IRE1α and ATF6 by hypoxia has been less studied and requires further investigation.

Muscle protein degradation is a complex process implicating four systems: the lysosomal proteases (cathepsins), the calcium-dependent proteases (calpains), the caspases and the ubiquitin–proteasome pathway (Jackman & Kandarian 2004). The latter mechanism is mainly regulated by E3 ligases amongst which are muscle atrophy F box (MAFbx) and muscle ring finger protein-1 (MuRF-1) (Bodine *et al.* 2001). The transcriptional regulation of MAFbx and MuRF-1 is controlled by, amongst others, the members of the forkhead FoxO family, themselves regulated by protein kinase B (PKB also called Akt) (Stitt *et al.* 2004). To the best of our knowledge, the effect of hypoxia on protein degradation has not been studied in human skeletal muscle.

When studying the regulation of muscle mass by environmental hypoxia, many confounding factors have to be taken into account. Long-term hypoxia at extreme altitude is known to reduce appetite and energy intake, to disturb the sleep cycle and to reduce physical activity, thereby indirectly favouring a catabolic state. It is thus difficult to determine whether the reduction in muscle mass observed after long-term exposure to

hypoxia is due to decreased dietary protein ingestion, sleep deprivation, cold exposure and reduced physical activity or to other mechanisms of regulation. Most of the studies focusing on the regulation of protein metabolism by acute hypoxia have used an exercise paradigm. However, muscle contractile activity *per se* is known to alter oxygen availability, thereby potentially indirectly affecting protein metabolism and adding one supplemental confounding factor. By simulating high altitude in a hypoxic facility, we were able to standardize the nutritional and the physical activity status of the subjects and to study more directly the acute effect of hypoxia on muscle protein metabolism. The purpose of this study was thus to determine at a molecular level how acute environmental hypoxia regulates protein synthesis and breakdown in resting human skeletal muscle. Based on previous studies reporting a negative effect of hypoxia on muscle protein balance (Hoppeler *et al.* 1990, MacDougall *et al.* 1991, Mizuno *et al.* 2008), we hypothesized that hypoxia would repress the response to a breakfast by inhibiting key regulators of protein synthesis and by stimulating protein breakdown.

Materials and methods

Subjects

Fifteen healthy young men (age 21.3 ± 0.4 years; BMI 21.8 ± 0.45 kg m^{-2}) volunteered to participate in this study, which was approved by the local Ethics Committee (KU Leuven) and was in conformity with the Helsinki Declaration. The subjects were all physically active, but they were not involved in any specific resistance training programme during the period of the study. Subjects were asked to refrain from vigorous physical activity for 2 days, as well as to abstain from alcohol consumption the day before the experiments. Furthermore, they were not exposed for more than 7 days to an altitude above 1500 m within a period of 3 months preceding the start of the study. A medical check-up was carried out to detect any contraindications for exposure to extreme altitude, and written consent was obtained from all subjects after explaining all potential risks of the study.

Study design

All subjects underwent two experimental sessions in a randomized crossover designed order, with a 4-week interval period in between. In the normoxic trial (NOR), participants were exposed to normal atmospheric conditions (20.9% O_2). In the hypoxic trial (HYP), participants inspired hypoxic air (approx. 11 ± 0.1% O_2).

Normoxic trial. The night before the experimental session, subjects received a standardized dinner (58% carbohydrates, 28% fat and 14% protein). After an overnight fast of at least 8 h, participants reported to the laboratory at 6:00–8:30 am where they received a standardized breakfast (65% carbohydrates, 29% fat and 6% protein, total energy intake ranged from 489.0 to 671.5 kcal according to the individual weight of the subjects). We chose to study the effect of environmental hypoxia after a light meal and not in the fasted state because we expected that hypoxia would create a state of anabolic resistance rather than impair basal protein metabolism. After breakfast completion, a first near-infrared spectroscopy (NIRS) measurement (Hamamatsu NIRO 200, Louvain-la-Neuve, Belgium) of 20 min was performed to evaluate muscle oxygenation status via the tissue oxygenation index (TOI), which is a valid parameter to assess the fraction of O_2-saturated tissue haemoglobin and myoglobin content (Ferrari *et al.* 2004). One pair of NIRS probes, consisting of one light emitter and one light detector, was attached on the belly of the right m. vastus lateralis in parallel with the long axis of the muscle. We used the right leg for the NIRS measurements because the left leg was used for muscle biopsies. Before positioning of the probes, the skin was shaved to exclude interaction of hair as a chromophore. Forty minutes after completion of the breakfast (T0), a first biopsy sample, with the needle pointing proximally, was taken from the left m. vastus lateralis under local anaesthesia (1–2 mL Lidocaine) through a 5-mm incision in the skin. Immediately after, a blood sample was taken from an antecubital vein. After the baseline measurements, which were all taken in normoxic conditions, subjects were transferred to the air-conditioned (approx. 21 °C) hypoxic facility (Sporting Edge, Leicestershire, UK), yet maintained at 20.9% O_2, where they rested in the seated position for 4 h whilst reading books or watching a movie. Immediately after entering the room, blood oxygen saturation (SpO_2) was measured with a pulse oximeter (Oximax, Nellcor, UK). One hour after the first biopsy (T60), a second biopsy was taken through the same incision as the first, yet with the needle pointing distally. Furthermore, a venous blood sample was taken. At T220, a second 20-min NIRS measurement was performed. Finally, at T240, the last biopsy and blood sample were taken. The last biopsy was taken with the needle pointing distally and through a new incision in the skin 3 cm distally to the first incision.

Hypoxic trial. All experimental conditions in the hypoxic trial were similar to those in the normoxic trial except that the hypoxic facility was maintained at 11%

ambient O_2 content (approx. 5000 m altitude) instead of 20.9%.

Western blot

Details of the immunoblotting procedures have been described previously (Deldicque *et al.* 2010b). Briefly, frozen muscle tissue (approx. 20 mg) was homogenized 3 × 5 s with a Polytron mixer in ice-cold 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 then centrifuged at 10 000 ***g*** for 10 min at 4 °C. The supernatant was collected and immediately stored at −80 °C. The protein concentration was measured using the DC protein assay kit (Bio-Rad laboratories, Nazareth, Belgium). 30–80 μg of proteins was separated by SDS-PAGE (8–12% gels) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% non-fat milk for 1 h and afterwards incubated overnight (4 °C) with the following antibodies (1 : 1000, Cell Signaling, Leiden, the Netherlands): phospho-Akt/PKB Ser^{473}, Akt/PKB pan, total eEF2, phospho-S6K1 Thr^{389}, total S6K1, phospho-4E-BP1 $Thr^{37/46}$, total 4E-BP1, p-FoxO1 Thr^{24}/FoxO3a Thr^{32}, total FoxO3a, p-eIF2α Ser^{51}, total eIF2α, BiP, C/EBP homologous protein (CHOP) and HIF-1α. Horseradish peroxidase-conjugated anti-mouse (1 : 10 000), anti-rabbit (1 : 5000) or anti-goat (1 : 20 000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap softwares (Syngene, Cambridge, UK) respectively. Then, membranes were stripped and reprobed with the antibody for the total form of the respective protein to ascertain the relative amount of the phosphorylated protein compared with the total form throughout the whole experiment. The results are presented as the ratio protein of interest/eEF2 or as the ratio phosphorylated/total forms of the proteins when the phosphorylation status of the protein was measured. A value of 1.0 was assigned to the mean value of the first sample (T0) in NOR and in HYP to which all other values from the respective condition were reported.

RNA extraction and reverse transcription

The method used for reverse transcription is described in detail elsewhere (Jamart *et al.* 2011, Vincent *et al.* 2010). Briefly, total RNA was extracted using TRIzol (Invitrogen, Vilvoorde, Belgium) from 20–25 mg of frozen muscle tissue. RNA quality and quantity were assessed by spectrophotometry with a Nanodrop (Thermo Scientific, Erembodegem, Belgium). One μg of RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium) according to manufacturer's instructions.

Real-time qPCR analysis

A SYBR Green-based master mix (Applied Biosystems) was used for real-time PCR analyses using the ABI PRISM 7300 (Applied Biosystems). Real-time PCR primers were designed for human MuRF-1, MAFbx, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), activating transcription factor 4 (ATF4), spliced XBP1 (XBP1s), CHOP, HIF-1α and Redd1 (Table 1). Thermal cycling conditions consisted of 40 three-step cycles including denaturation of 30 s at 95 °C, annealing of 30 s at 58 °C and extension of 30 s at 72 °C. All reactions were performed in duplicate. To compensate for variations in input RNA amounts and efficiency of reverse transcription, cyclophilin A (Cyclo A) and beta-2-microglobulin (β-2-MG) mRNA were quantified, and results were normalized to these values. These genes were chosen out of five normalization genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere (Vandesompele *et al.* 2002). A value of 1.0 was assigned to the mean value of the first sample (T0) in NOR and in HYP to which all other values from the respective condition were reported.

Enzymatic activity assays

Enzymatic activities were determined fluorometrically using specific substrates and specific inhibitors. Each sample was assessed in triplicate with one replicate containing a specific inhibitor to the activity studied. 26S *β5* subunit proteasome activity was determined by adding 100 μM Suc-LLVY-AMC (Calbiochem) to 10 μg proteins in a reaction buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT and 100 μM ATP, ±20 μM epoxomicin. Cathepsin L activity was determined by adding 100 μM Z-Phe-Arg-AMC (Peptide Institute, Sandhausen, Germany) to 10 μg proteins in a buffer containing 100 mM sodium acetate, pH 5.5, 1 mM EDTA, 1 mM DTT, ±10 μM cathepsin L inhibitor I (Calbiochem, Overijse, Belgium). Calpain activity was determined by adding 200 μM Suc-LLVY-AMC (Calbiochem) to 10 μg proteins in a buffer containing 25 mM Tris, pH7.5, 0.5 mM

Table 1 Primer sequences

	Forward	Reverse
ATF4	CCA ACA ACA GCA AGG AGG ATG	GTC ATC CAA CGT GGT CAG AAG G
Bnip3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
CHOP	CTG GCT TGG CTG ACT GAG GAG	CGG GCT GGG GAA TGA CC
HIF-1α	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG AAC
Redd1	TGA GGC ACG GAG TGG GAA	CAG CTC GAA GTC GGG CAA
MuRF-1	AAA CAG GAG TGC TCC AGT CGG	CGC CAC CAG CAT GGA GAT ACA
MAFbx	CCC AAG GAA AGA GCA GTA TGG AGA	GGG TGA AAG TGA AAC GGA GCA
XBP1s	CCG CAG CAG GTG CAG G	GAG TCA ATA CCG CCA GAA TCC A
Cyclo A	CTT CAT CCT AAA GCA TAC GGG TC	TGC CAT CCA ACC ACT CAG TCT
β-2-MG	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG

ATF4, activating transcription factor 4; Bnip3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; CHOP, C/EBP homologous protein; HIF-1α, hypoxia-inducible factor-1 alpha; Redd1, regulated in development and DNA damage responses 1; MuRF-1, muscle ring finger protein-1; MAFbx, muscle atrophy F box; XBP1s, spliced X-box binding protein 1; Cyclo A, cyclophilin A; β-2-MG, beta-2-microglobulin.

EDTA, 5 mM $CaCl_2$, 75 mM NaCl, 0.025 mM DTT, ±125 μM calpain inhibitor IV (Calbiochem). Fluorescence was monitored every 5 min for 105 min on a fluorometer (Fluostar Optima, BMG labtech, Sint-Pieters-Leeuw, Belgium) at an excitation and emission wavelengths of 370 and 460 nm respectively. Enzymatic activities were calculated as the difference of the slope of the accumulation of fluorescence as a function of time in the absence of inhibitor and presence of inhibitor.

Analysis of blood samples

Plasma insulin and cortisol were assayed by chemiluminescence using the Siemens DPC kit according to the instructions of the manufacturer. Blood glucose was measured with a Glucocard X-Meter (Arkray, Kyoto, Japan).

Statistical analyses

A repeated-measures ANOVA design was used to assess the statistical significance of differences between mean values over time and between conditions. When appropriate, Holm–Sidak pairwise multiple comparison test was used as *post hoc*. A Pearson correlation coefficient was calculated between ΔSpO_2 and ΔTOI values. The threshold of significance was set at 0.05. Results are expressed as the means ± SEM.

Results

Blood oxygen saturation/tissue oxygenation index

SpO_2. Mean SpO_2 was markedly lower (−25%) in HYP than in NOR during the whole experimental trial ($P < 0.05$, Table 2). Individual decreases in HYP compared with NOR ranged from 12 to 36%.

TOI. There were no differences in TOI between T0 and T240 in the NOR trial. Conversely, during the HYP trial, TOI values were approx. 6% lower at T240 than at T0 ($P < 0.05$, Table 2). A correlation of $r = 0.451$ was found between ΔSpO_2 [($SpO_{2\ NOR\ T240}$-$SpO_{2\ NOR\ T0}$)-($SpO_{2\ HYP\ T240}$-$SpO_{2\ HYP\ T0}$)] and ΔTOI [($TOI_{NOR\ T240}$-$TOI_{NOR\ T0}$)-($TOI_{HYP\ T240}$-$TOI_{HYP\ T0}$)] ($P < 0.05$, data not shown).

Blood biochemistry

Insulin. Plasma insulin levels decreased throughout the experiment in both NOR and HYP ($P < 0.05$,

Table 2 Arterial oxygen saturation and tissue oxygenation index values

		Mean during whole experiment
SpO_2 (%)		
NOR		99.0 ± 0.2
HYP		75.5 ± 2.0*
	T0	T240
TOI (%)		
NOR	69.7 ± 1.5	68.4 ± 1.2
HYP	70.8 ± 2.0	65.7 ± 1.4†

Mean arterial oxygen saturation (SpO_2) during the whole experiment and tissue oxygenation index (TOI) at the start (T0) and at the end (T240) of the experimental session in either normoxia (NOR) or hypoxia (HYP). Values are means ± SEM ($n = 15$).
*$P < 0.05$ vs. NOR;
†$P < 0.05$ vs. T0.

Table 3) with a larger decrease observed in NOR, resulting in approx. 2-fold lower plasma insulin values in NOR compared with HYP at T240 ($P < 0.05$).

Glucose. Blood glucose increased by 15% from T0 to T60 in NOR ($P < 0.05$) but not in HYP, resulting in lower glucose concentrations in HYP compared with NOR at T60 ($P < 0.05$). At 240, glucose values were similar to those at T0 in both NOR and HYP.

Cortisol. Plasma cortisol levels increased by about 30% between T0 and T240 in NOR and in HYP ($P < 0.05$, Table 3). No differences were present between NOR and HYP.

Effect of environmental hypoxia on the regulation of protein synthesis

Phosphorylation of PKB at Ser^{473} decreased from T0 to T240 in NOR ($P < 0.05$), but not in HYP (Fig. 1a), resulting in a approx. 40% lower phosphorylation of PKB in NOR compared with HYP at T240 ($P < 0.05$). Downstream of PKB and mTORC1, phosphorylation of S6K1 at Thr^{389} followed a similar phosphorylation pattern as PKB (Fig. 1b), but the reduction in phosphorylation was more pronounced than for PKB in both NOR and HYP ($P < 0.05$). Like phospho-PKB, phospho-S6K1 was lower in NOR compared with HYP at T240 ($P < 0.05$). Phosphorylation of 4E-BP1 at $Thr^{37/46}$ (Fig. 1c), another downstream target of mTORC1, was not affected by any condition. We also measured the mRNA level of Redd1 as the latter is known to inhibit the mTOR pathway and to be up-regulated during hypoxia (Schwarzer *et al.* 2005). There was a trend to increase in the mRNA level of Redd1 during both experimental trials, but this increase was only significant in HYP (T240, approx. fourfold increase vs. basal and approx. twofold increase vs. NOR, $P < 0.05$, Fig. 1d).

Table 3 Plasma insulin, blood glucose and plasma cortisol concentrations

	T0	T60	T240
Insulin (μU mL^{-1})			
NOR	27.3 ± 3.6	20.9 ± 3.6	5.9 ± 1.2*†
HYP	31.5 ± 4.3	17.9 ± 3.3*	13.4 ± 2.5*‡
Glucose (mg dL^{-1})			
NOR	94.2 ± 2.5	110.1 ± 5.4*	81.6 ± 2.0†
HYP	86.0 ± 4.0	89.5 ± 4.7‡	87.7 ± 2.8
Cortisol (nM)			
NOR	257.8 ± 13.1	305.9 ± 20.5	337.9 ± 16.7*
HYP	234.9 ± 14.9	310.6 ± 24.3*	325.9 ± 29.9*

Plasma insulin, blood glucose and plasma cortisol concentrations at basal (T0), after 1 h (T60) and after 4 h (T240) in normoxia (NOR) or in hypoxia (HYP). Values are means ± SEM ($n = 15$).
*$P < 0.05$ vs. T0;
†$P < 0.05$ vs. T60;
‡$P < 0.05$ vs. NOR.

Effect of environmental hypoxia on the regulation of protein breakdown

Activities of 26S proteasome $\beta 5$, cathepsin L and calpain, which have been previously described as key proteins in muscle protein degradation (Jackman & Kandarian 2004), were assessed by fluorometric assays. Compared with T0, activity of 26S proteasome $\beta 5$ increased by 19% in NOR at T240 ($P < 0.05$, Fig. 2a). No time effect was observed in HYP. Cathepsin L and calpain activities showed the same activation pattern, although the statistical threshold was not reached (time effect for cathepsin L, $P = 0.089$, Fig. 2b; time effect for calpain, $P = 0.056$, Fig. 2c). The activity of the proteasome is regulated by E3 ligases, the two best described of which in skeletal muscle are MAFbx and MuRF-1 (Bodine *et al.* 2001). Their expressions are under the control of a family of transcription factors called FoxO (Stitt *et al.* 2004). Phosphorylation of FoxO1/3a at $Thr^{24/32}$ was higher at T60 and T240 than at basal in NOR ($P < 0.05$), but not in HYP (Fig. 2d). No differences between conditions were present. Neither MAFbx nor MuRF-1 mRNA expression followed the phosphorylation of FoxO1/3a. The mRNA levels of MuRF-1 were up-regulated by approx. 15% at T240 in HYP ($P < 0.05$, Fig. 2e). MAFbx mRNA content decreased throughout the experiment in both NOR and HYP. However, the decrease was larger in HYP compared with NOR ($P < 0.05$, Fig. 2f). Bnip3 mRNA expression, a marker of the autophagy/lysosome pathway (Tracy *et al.* 2007), was not modified by the experimental conditions (Fig. 2g).

Effect of environmental hypoxia on the unfolded protein response

Activation of the unfolded protein response, triggered by endoplasmic reticulum stress, was assessed by measuring several markers previously described (Deldicque *et al.* 2010a). ATF4 and spliced XBP1 mRNA contents increased at T240 compared with T0 but only in HYP (by 30% for ATF4 and by 25% for XBP1s, $P < 0.05$, Fig. 3a,b). Moreover, the mRNA expression of ATF4 was approx. 30% higher in HYP compared with NOR at T240 ($P < 0.05$, Fig. 3a). BiP expression increased throughout the experiment in both conditions, but this increase was only significant in NOR at T240 ($P < 0.05$, Fig. 3e). CHOP protein expression

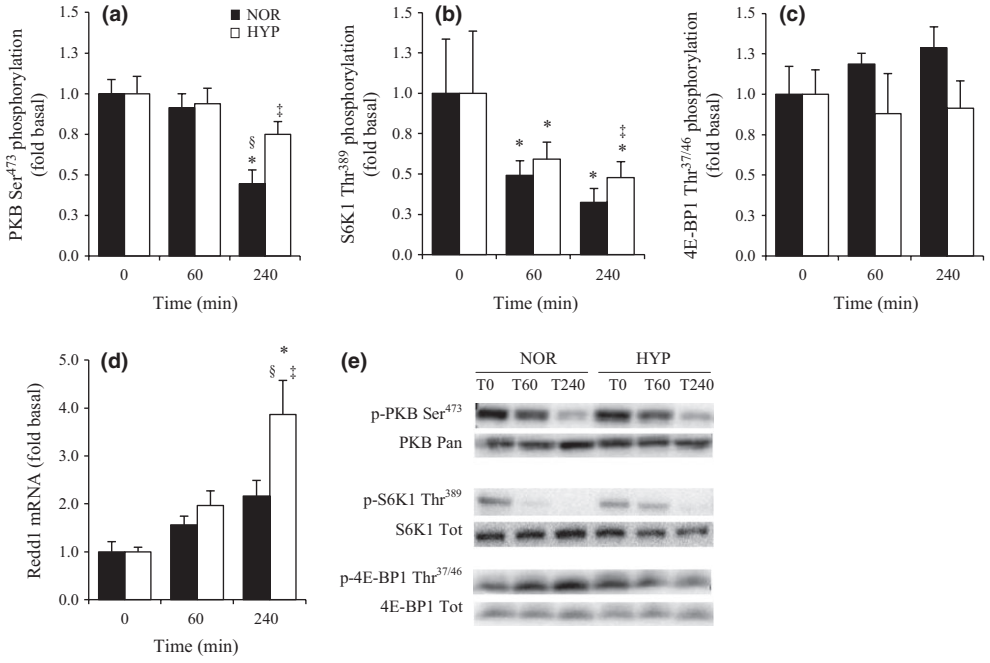

Figure 1 Effect of hypoxia on protein synthesis markers and regulators. (a) PKB, (b) S6K1, (c) 4E-BP1 phosphorylation and (d) Redd1 mRNA level at basal (0), after 1 h (60) and after 4 h (240) in normoxia (NOR) or in hypoxia (HYP). (e) Representative blots. Data shown are expressed as means ± SEM (n = 15). *P < 0.05 vs. T0; §P < 0.05 vs. T60; ‡P < 0.05 vs. NOR.

tended to display a time effect (P = 0.075) with a tendency to higher CHOP expression in HYP compared with NOR at T60 (P = 0.055, Fig. 3d). No effects were found in CHOP mRNA (Fig. 3c) or eIF2α phosphorylation at Ser^{51} (Fig. 3f).

Hypoxia-inducible factor-1 and its downstream targets

The acute bout of hypoxia did not alter HIF-1α either at the mRNA level or at the protein level (Fig. 4), neither did it activate the transcriptional activity of HIF-1α. To test the latter, we measured the expression of several genes known to be regulated by HIF-1α. Neither GLUT-1 nor GAPDH mRNA levels were modified in HYP (data not shown). VEGF-A mRNA was the only one to increase in HYP. At T240, VEGF-A mRNA content was approx. 1.5-fold higher in HYP compared with NOR (P < 0.05, Fig. 4b). We tried to quantify HIF-1α at the protein level. However, the basal expression in normoxia was barely detectable, and hypoxia did not increase its expression (Fig. 4c). To ensure that our antibody was able to detect modifications in the protein levels, we treated human umbilical vein endothelial cells (HUVEC) with 1 mM dimethyloxalylglycine (DMOG), an inhibitor of the prolyl-hydroxylases to provoke accumulation of HIF-1α in the cell, and 10 mM MG132, an inhibitor of the proteasome to block the degradation of HIF-1α. We were able to reproduce the results presented in the datasheet of the antibody (Cell Signaling), that is, no increase in HIF-1α in MG-132-treated cells but a large increase in DMOG-treated cells, confirming thereby that if there had been any increase in muscle HIF-1α expression, we should have detected it in our conditions. Altogether, our results show that the muscle HIF-1α pathway was not activated by acute environmental hypoxia and, thus, that the effects observed in this study are probably HIF-1α-independent. However, we did not separate membrane and cytosolic cell fractions. We can thus not rule out a possible translocation of HIF-1α to the cell nucleus. If this was the case, it did not result in higher transcriptional activity as measured by unchanged levels of mRNA known to be up-regulated by HIF-1α.

Discussion

Hypoxia-induced muscle wasting has been previously described as a consequence of high altitude (Hoppeler *et al.* 1990, MacDougall *et al.* 1991, Mizuno *et al.* 2008) and several diseases (Grocott *et al.* 2007, Wust & Degens 2007), but the molecular mechanisms behind this loss of muscle mass are far to be elucidated. Contrary to our hypothesis and to previous reports on long-term environmental hypoxia, we show in the present study that acute and severe hypoxia alters the mTORC1 pathway in a way that is theoretically favourable for muscle protein accretion.

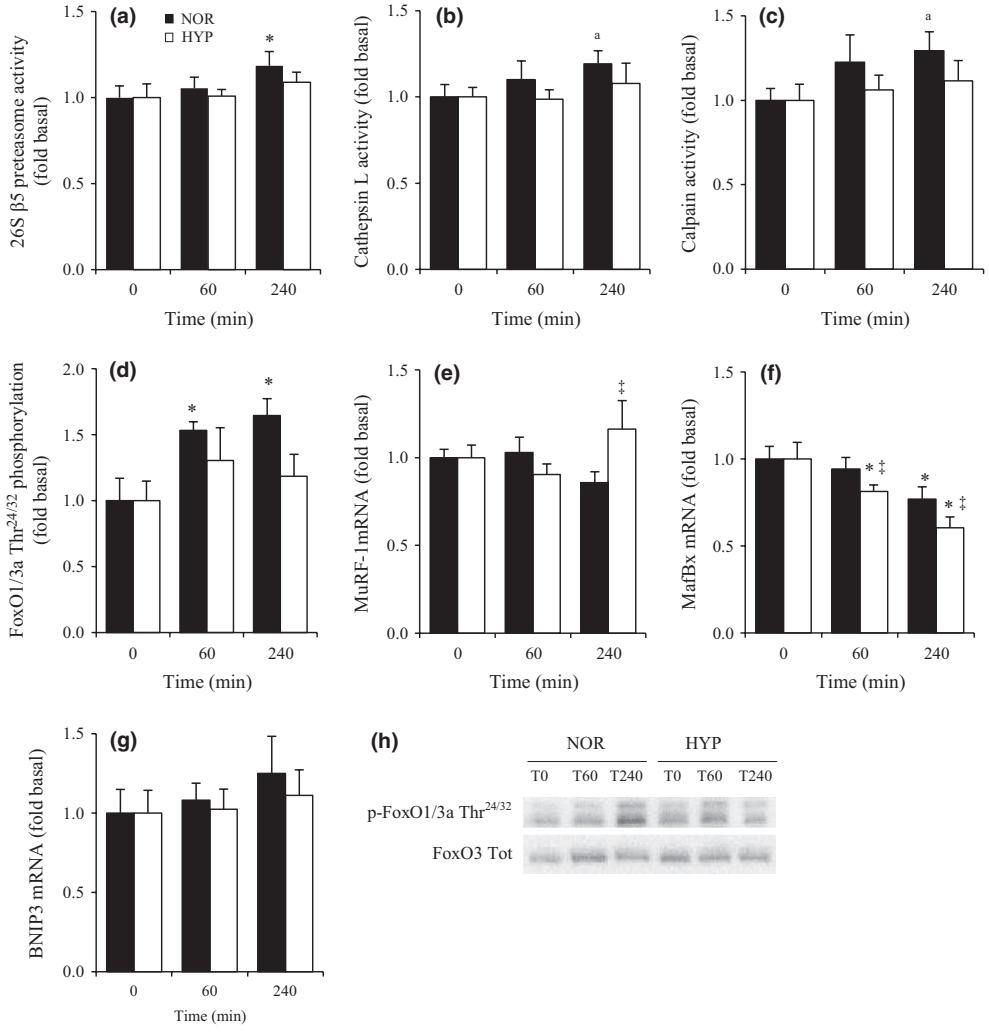

Figure 2 Effect of hypoxia on protein breakdown markers and regulators. (a) 26S *β5* proteasome activity, (b) cathepsin L activity, (c) calpain activity, (d) FoxO1/3a phosphorylation, (e) MuRF-1, (f) MAFbx and (g) Bnip3 mRNA levels at basal (0), after 1 h (60) and after 4 h (240) in normoxia (NOR) or in hypoxia (HYP). (h) Representative blots. Data shown are expressed as means ± SEM ($n = 15$). *$P < 0.05$ vs. T0; $^{a}P < 0.1$ vs. T0; $^{‡}P < 0.05$ vs. NOR.

Up-regulation of the mTORC1 pathway by environmental hypoxia

Whereas it is generally accepted that hypoxia induces muscle wasting, there is clearly a lack of data describing the molecular mechanisms in human. Muscle wasting reflects a decrease in protein synthesis, an increase in protein breakdown or both at the same time. Only very few studies have attempted to clarify the molecular mechanisms by which acute or more prolonged environmental hypoxia regulates muscle mass in human. A slight decrease in total mTORC1 was found after 7- to 9-day sojourn at 4559 m (Vigano *et al.* 2008). In another recent study, subjects showed a blunted exercise-induced increase in muscle protein synthesis when breathing hypoxic air (12% O_2) compared with normoxic air (Etheridge *et al.* 2011). Hypoxia did not modify phosphorylation of S6K1 or expression of Redd1 whether at rest or after exercise. In another study, in rat soleus muscle, Redd1 expression was markedly increased, and the PKB/mTORC1 pathway was concomitantly down-regulated after a 3-week exposure to 6300 m. Furthermore, in hypoxemic patients suffering from chronic obstructive pulmonary disease, a substantial decrease in phosphorylation of several intermediates of the PKB/mTORC1 pathway was reported (Favier *et al.* 2010). In contrast to the aforementioned studies, our data show that PKB and S6K1 displayed a higher phosphorylation state after 4-h exposure to hypoxia following a meal. All biopsies were taken after a light meal explaining why PKB and S6K1

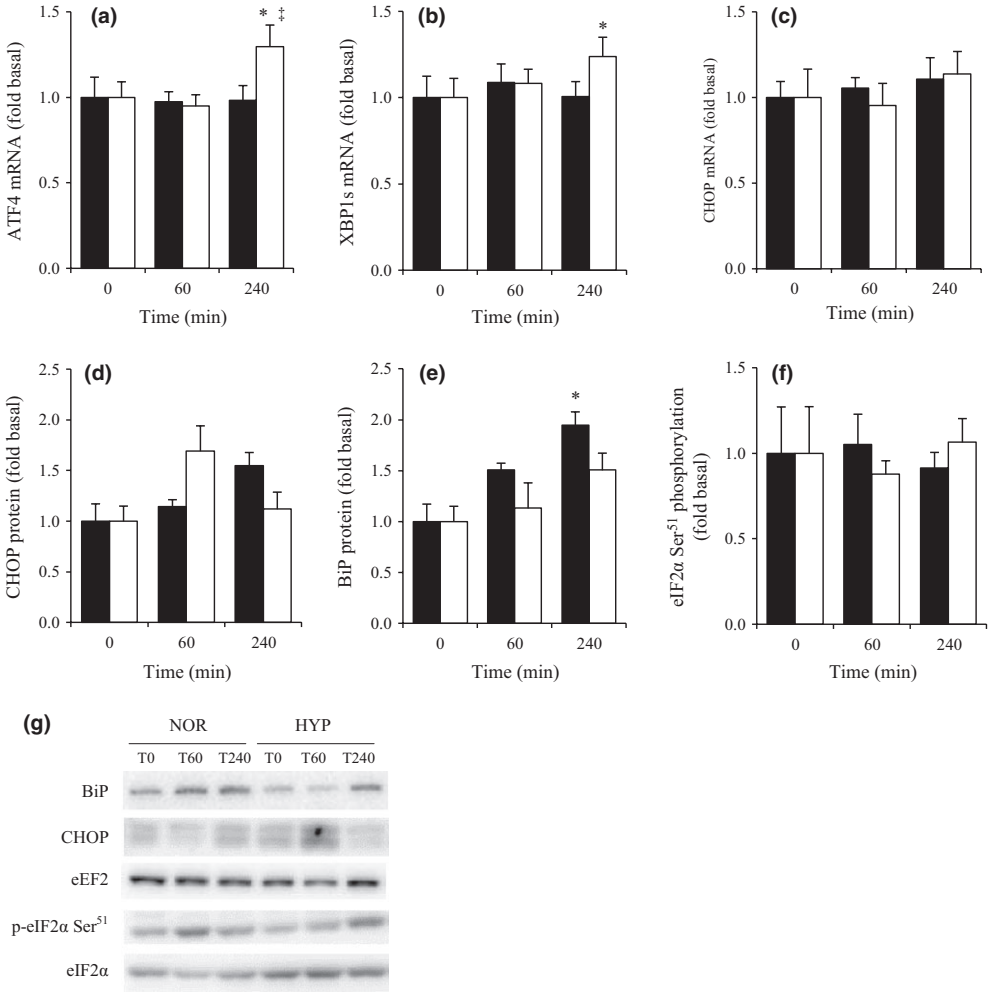

Figure 3 Effect of acute hypoxia on markers of the unfolded protein response. (a) ATF4, (b) XBP1s and (c) CHOP mRNA levels, (d) CHOP and (e) BiP protein expressions and (f) eIF2α phosphorylation at basal (0), after 1 h (60) and after 4 h (240) in normoxia (NOR) or in hypoxia (HYP). (g) Representative blots. Data shown are expressed as means ± SEM (n = 15). *P < 0.05 vs. T0; ‡P < 0.05 vs. NOR.

phosphorylation decreased between the first and the third biopsy in normoxia and hypoxia. In hypoxia, however, the return to basal phosphorylation states was slowed down for both proteins. When subjects remained fasted for 3.5 h in hypoxia, S6K1 phosphorylation was not changed compared with pre-exposure (Etheridge *et al.* 2011), indicating that short-term hypoxia does not modify basal phosphorylation of S6K1. Based on our data, it is however likely that environmental hypoxia alters the response of S6K1 and PKB to a meal. According to previous *in vitro* studies (Koritzinsky *et al.* 2006), we could have expected a down-regulation of 4E-BP1 phosphorylation due to hypoxia. However, such increase did not occur. We choose to use an antibody specific to Thr^{37} and Thr^{46}, the sites phosphorylated by mTORC1 as the aim of the study was to focus on the mTORC1 pathway. However, Thr^{37} and Thr^{46} are also known to be less sensitive to serum and other growth factors than other sites of the protein, for example Thr^{65} and Thr^{70} (Gingras *et al.* 1999). We can thus not rule out that other sites of 4E-BP1, more sensitive to changes in insulin concentrations *in vivo*, reacted the same way as PKB and S6K1. However, it is difficult to screen each of them as six different sites (Thr 37, Thr 46, Ser 65, Thr 70, Ser 83 and Ser 112) have been discovered up to now (Gingras *et al.* 2001).

Redd-1 expression is regulated by HIF-1α-independent mechanisms

Redd-1 was originally discovered in cell cultures as a hypoxia-responsive gene (Shoshani *et al.* 2002), and since then, many studies have confirmed that Redd-1 is a direct target of the transcription factor HIF-1α

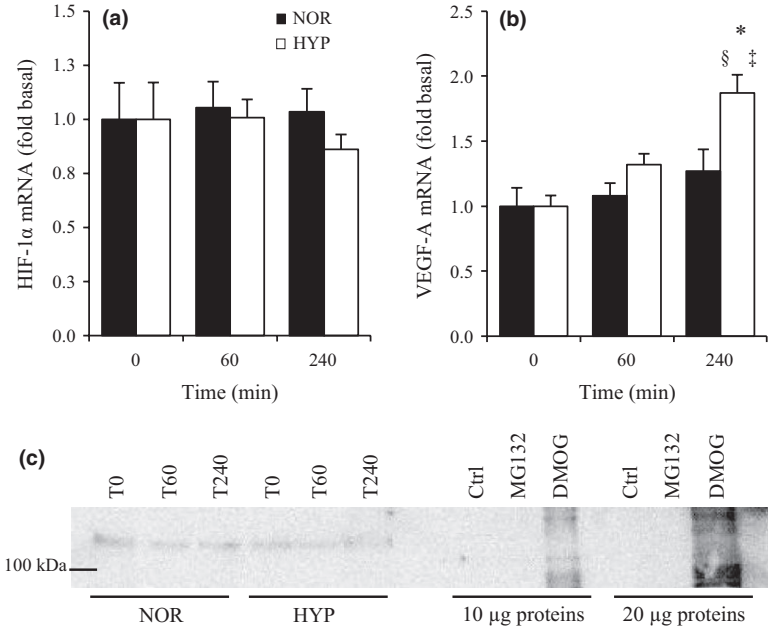

Figure 4 Effect of acute hypoxia on the hypoxia-inducible factor pathway. (a) HIF-1α mRNA level (b) VEGF-A mRNA level at basal (0), after 1 h (60) and after 4 h (240) in normoxia (NOR) or in hypoxia (HYP). (c) Representative western blot for HIF-1α from one subject and the different controls we used on HUVEC cell cultures (see material and methods for further details). Data shown are expressed as means ± SEM (n = 15). *$P < 0.05$ vs. T0; $^{§}P < 0.05$ vs. T60; $^{‡}P < 0.05$ vs. NOR.

(Schwarzer *et al.* 2005). It has also been shown to act as an inhibitor of mTORC1 through regulation of 14-3-3 protein shuttling (DeYoung *et al.* 2008). Here, we show a clear elevation of Redd-1 mRNA after 4 h of exposure to hypoxia against the face of constant HIF-1α. We did not assessed Redd-1 at the protein level, but it has previously been shown that the mRNA and protein expressions follow the same regulation pattern (Frost *et al.* 2009). Endoplasmic reticulum stress and its downstream adaptive response, the unfolded protein response, have been proposed as another HIF-1α-independent mechanism by which Redd-1 can be up-regulated (Whitney *et al.* 2009). To test this hypothesis, we measured ATF4 and the active (spliced) form of XBP1 at the mRNA level, and we found a higher expression of both genes after exposure to hypoxia, indeed. The unfolded protein response could also have contributed to the increase in VEGF-A mRNA, usually attributed to HIF-1α, that we observed at the end of the hypoxic trial. IRE1α and XBP1s have previously been reported to induce VEGF-A mRNA expression (Drogat *et al.* 2007). Altogether, our data show that PKB and S6K1 phosphorylation remained higher in hypoxia potentially due to higher plasma insulin concentrations and despite an increase in Redd-1 mRNA, a well-known inhibitor of the PKB/mTOR pathway. However, it is highly probable that other untested factors also contributed to the regulation of PKB and S6K1.

Environmental hypoxia affects protein breakdown to a minor extent

As mentioned above, protein breakdown has often been understudied in studies dealing with hypoxia. Two studies performed on animal models have directly investigated the effect of hypoxia on the different proteolytic pathways. Favier et al. did not find any effect of chronic hypoxia (3 weeks, 6300 m) on any component of the proteasomal, lysosomal and calcium-dependent systems in rat skeletal muscle (Favier *et al.* 2010). On the contrary, Chaudhary et al. reported that chronic hypoxia (2 weeks, 7620 m) induced muscle atrophy and that the proteasomal and calpain systems were activated at the same time (Chaudhary *et al.* 2012). Based on those 2 studies, a clear-cut conclusion is thus difficult to draw. Both used very similar methodologies except the strain (Wistar (Favier *et al.* 2010) vs. Sprague-Dawley males (Chaudhary *et al.* 2012)), the altitude and the age of the animals. The latter issue could explain why Chaudhary, using young rats, found an increase in protein breakdown after exposure to hypoxia as protein turnover is known to be higher in younger rats than in adult rats. Growing rats are thus more sensitive to factors affecting protein synthesis and/or protein breakdown. Moreover, in the study of Chaudhary, the altitude was 1000 m higher than that in the study of Favier, which has probably favoured the catabolic

state of the animals. In the present study, we did not find any change in either proteasome, or cathepsin, or calpain activities in adult subjects. This is the first time that protease activities are measured in skeletal muscle of subjects exposed to hypoxia. Based on our results, hypoxia seems to affect markers of protein synthesis to a larger extent than those of protein breakdown. Interestingly, we found that independently of the hypoxic response, the activity of the three main proteolytic systems in skeletal muscle was higher a few hours after a light meal in normoxia. This higher activity could not be explained by a decreased phosphorylation of FoxO1/3a, a master regulator of protein breakdown, as contrary to what expected its phosphorylation increased throughout the normoxic trial. Although the activities of the proteasome, cathepsin and calpain systems were not modified, we hypothesized that short-term hypoxia could have altered some key regulators of these pathways. This would prepare the different machineries to become more activated in case of prolonged exposition to hypoxia. Several well-known markers of the ubiquitin –proteasome pathway (MuRF-1 and MAFbx mRNA) (Bodine *et al.* 2001), the autophagy–lysosome (Bnip3 mRNA) (Tracy *et al.* 2007) and the ER stress-associated degradation systems (CHOP mRNA and protein) (Oyadomari & Mori 2004) were quantified. Only MuRF-1 and MAFbx mRNA were modified after 4-h hypoxia and these changes occurred in an opposite way, MuRF-1 level being up-regulated and MAFbx down-regulated. Those 2 muscle-specific ligases have originally been thought to play a pivotal role in the regulation of the ubiquitin–proteasome pathway (Bodine *et al.* 2001). Since then, their role has been revised somewhat downwards, and it is not uncommon to see a specific regulation of each factor (Greenhaff *et al.* 2008, Jamart *et al.* 2012). We could have expected that the higher insulin concentration induced by hypoxia would have affected protein breakdown to a larger extent as insulin is known to affect protein breakdown rather than protein synthesis in skeletal muscle (Greenhaff *et al.* 2008). However, it was not the case in the present study. It is possible that exposure to hypoxia modulates the response of protein breakdown to insulin usually observed in normoxia, but this needs further investigation.

Acute environmental hypoxia does not activate HIF-1α in skeletal muscle

Acute exposure to 11% O_2 resulted in a large decrease in SpO_2 in all subjects. Interestingly, individual SpO_2 drops ranged from 12 to 36%, indicating a high variability in hypoxic adaptation between subjects. Irrespective of the large decrease in SpO_2, TOI only decreased by approx. 6% in hypoxia, which is in line with previous studies (Martin *et al.* 2009). Even though oxygen supply to skeletal muscle is drastically reduced, muscle oxygenation seems to stay rather stable under severe environmental hypoxia, probably because of the low muscle oxygen consumption at rest and the high affinity of myoglobin for oxygen (Ordway & Garry 2004). It is noteworthy that, during exercise, TOI decreases much more severely than the 6% presented here not only in hypoxia but in normoxia as well (Martin *et al.* 2009, Masschelein *et al.* 2012). This large decrease in tissue oxygenation induced by exercise is accompanied by an up-regulation of HIF-1α (Ameln *et al.* 2005), whereas in our resting conditions, we did not measure any change of the latter whether at the protein or at the mRNA level. All the observations we made in the present study are thus independent of HIF-1α, which is not surprising as muscle oxygenation was barely affected. The latter suggests that other factors, that could be systemic, are involved in the activation of intramuscular signalling. Two likely candidates are insulin and cortisol, previously shown to be altered during hypoxic exposure (Larsen *et al.* 1997). Whereas plasma cortisol concentration was not modified by hypoxia, insulin returned more slowly to basal after a standardized breakfast in the hypoxic trial. Insulin concentration was twofold higher at the end of the exposure to environmental hypoxia and could partially explain several observations we made at this time, that is, higher phosphorylation of PKB and S6K1. The higher insulin concentration in the group exposed to hypoxia was not due to higher blood glucose concentrations. Glucose is a well-known stimulator of insulin secretion, but the 4-h exposure to hypoxia did not increase glucose blood level compared with normoxia. On the contrary, hypoxia tended to reduce blood glucose levels. Although we could not measure plasma catecholamine concentrations due to a lack of sample, it is possible that adrenaline contributed to the increase in insulin as adrenaline increases at high altitude (Mazzeo *et al.* 1994), and it regulates insulin secretion by the pancreas (Lacey *et al.* 1993). It is also possible that adrenaline also directly altered protein metabolism as it has been shown to increase protein synthesis (Navegantes *et al.* 2004) and to repress proteolysis in rat skeletal muscle (Navegantes *et al.* 2000).

Limitations of the present study

The major limitation of this study is not having taken a biopsy before the breakfast. The lack of sample at this time point does not allow drawing any conclusion about the effect of breakfast itself. However, this was not the purpose of this study as the effect of feeding on protein synthesis and breakdown is already well

known (Atherton & Smith 2012). We also have to acknowledge that we did not directly measure protein synthesis and protein breakdown, but we quantified several markers of those processes. Finally, the physiological challenges experienced by the human body at high altitude are far more complex than those experienced with the normobaric model used in this study. Therefore, the results should be interpreted with caution when extrapolating to muscle protein synthesis and protein breakdown at high altitude.

Conclusion

In conclusion, contrary to our original hypothesis and despite an increase in the mRNA level of Redd1, an inhibitor of the mTOR pathway, short-term acute hypoxia induces a higher response of PKB and S6K1 4 h after a meal that could at least partially been explained by an increase in plasma insulin concentration. Further investigation will be required to study the repeated response to a standardized meal during chronic hypoxia, which could differ from the one observed in this study. Long-term exposure to hypoxia could create a state of anabolic resistance that we did not observe on a short-term.

Conflict of interest

The authors do not have any conflicts of interest.

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