Effects of hypoxia on muscle protein synthesis and anabolic signaling at rest and in response to acute resistance exercise

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Hypoxia is the result of a decrease in arterial O2 availability and may be induced by exposure to high altitude or in pathological conditions such as chronic obstructive pulmonary disease (COPD) (20), obstructive sleep apnea (OSA) (9), anemia, and chronic heart failure (1). Tolerance to hypoxia differs in health and disease. Acclimatized healthy highlanders living above 4,000 m with a resting PO2 of 6.5–8 kPa (17) have a relatively normal life expectancy and suffer few medical problems (although they do have reduced lean mass) (11). Furthermore, patients experiencing chronic hypoxemia (i.e., lung disease PO2 <8 kPa) have a 46% 5-yr mortality and associated muscle wasting (26). Thus, chronic reductions in tissue O2 supply, whether under environmental or pathological conditions, are associated with reduced muscle mass (21). Although the mechanisms for this are unclear, because reductions in muscle protein synthesis (MPS) underlie muscle atrophy in a number of other wasting conditions [e.g., immobilization (10), aging, slow cancer], it is likely that reduced MPS and/or increases in protein degradation also contribute to hypoxia-induced muscle atrophy.

Increases in MPS after RE underpin chronic adaptations (i.e., hypertrophy) to RE training. Indeed, under normoxic conditions, we have shown recently that there is a sigmoidal dose-response relationship between MPS at 1–2 h postexercise and exercise intensity after unilateral resistance-type exercise in the fasted condition (15). Accordingly, we and others have shown that increases in MPS are associated with a stimulation of phosphorylation(s) events on numerous kinases known to regulate mRNA translation, such as Akt (protein kinase B)/mTORC1/p70S6K signaling (15, 29, 31). Although human MPS has not been measured directly during hypoxia either at rest or after exercise, Rennie et al. (22) showed nearly 30 years ago that forearm leucine nonoxidative disposal was reduced in subjects breathing 12% O2 under hypobaric conditions, suggesting that MPS was reduced in response to hypoxia. Similar findings have been obtained in cell culture and whole animal studies, where reductions in anabolic signaling during hypoxia (1–5% O2) via increased activity of inhibitory hypoxia-responsive signaling proteins [e.g., AMPK, regulated in development and DNA damage-1 (REDD1) responses] (14) have been suggested as a mechanism to blunt MPS. As such, reductions in MPS may explain blunted hypertrophic responses to RE training at altitude (19) and in pathological hypoxia (7).

Simulation of altitude in healthy humans under artificial conditions of hypoxia (i.e., breathing O2 at reduced concentrations) not only contribute to understanding the molecular and systemic mechanisms involved in O2 sensing and the ensuing adaptive responses but also help in the understanding of pathological conditions associated with hypoxia. Because of the catabolic effects of hypoxia on muscle mass, our aim was to conduct preliminary investigations to determine the effects of exposing healthy young men to physiologically and clinically important hypoxia [12% O2 to achieve 80–90% blood oxygen saturation (SpO2)] on myofibrillar protein synthesis and associated anabolic signaling. Moreover, we chose to determine these responses not only under resting conditions but also under conditions of anabolic challenge [i.e., a single bout of resistance exercise (RE)] since there have been reports of poorer adaptive capacity to resistance exercise training in hypoxic conditions (19). We hypothesized that hypoxia would
blunt MPS both at rest and in the period after resistance exercise, and this would be associated with increases in REDD1 protein concentrations and blunted p70 S6K1 Thr389 phosphorylation.

**MATERIALS AND METHODS**

**Subjects**

Seven healthy males (age 21.4 ± 0.7 yr, BMI 24.9 ± 1.5 kg/m²) were recruited for the study. Volunteers were recreationally active (≤3 days/wk), had no respiratory ailments, and had not been exposed to any form of hypoxic atmosphere for >6 mo. All subjects were instructed to refrain from heavy exercise for 72 h and caffeine and alcohol for 48 h before the study day. Prior to testing, subjects provided written informed consent. Ethical approval was obtained from the University of Brighton Research Ethics and Governance Committee, and studies complied with standards set by the Declaration of Helsinki.

**Study Design**

At least 2 wk before the study, subjects reported to the laboratory for familiarization of all procedures. Subjects were tested under two experimental conditions in a crossover fashion: normoxia (normal atmospheric conditions, 20.9% inspired O₂ concentration) and normobaric hypoxia (12% inspired O₂ concentration). Trials were ordered and separated by a minimum of 3 mo, with the normoxic condition always being performed first to avoid any long-lasting effect of the hypoxic bout.

**Normoxic trial.** Participants arrived at the laboratories at ~0800 in the postabsorptive state (~12 h overnight fast) for insertion of 18-g catheters into antecubital veins of both arms for blood sampling and tracer infusion. Subjects were then placed in custom-built isometric force apparatuses for performance of unilateral RE. Maximum isometric voluntary contraction (MVC) of the quadriceps was determined by fixing subjects’ knee joint angles at 90° (1.57 rad). A strap was attached to the ankle of the dominant leg, connected via chain to a strain gauge (Teleda, Huntleigh, UK) that measured the amount of isometric force developed in the contracting quadriceps performing the knee extension. The strain gauge signal (sample rate = 0.1 kHz) was amplified, filtered, and converted from analog to digital (model Micro 1401, CED). Results were recorded and analyzed using Spike 2 software. Subjects performed three maximal 5-s efforts, with the peak value taken as the highest value achieved. Following 2 min of recovery, RE consisted of eight sets of six repetitions at 70% MVC, with 2 min of recovery between sets. Immediately after RE a baseline muscle biopsy was taken from the nonexercised leg, and a primed, constant, intravenous infusion (priming dose 7.8 μmol/kg body wt, infusion rate 0.13 μmol/kg body wt·min⁻¹) of 1,2-[¹³C₂]leucine tracer (99 atom%; Cambridge Isotopes, Cambridge, MA) was started and maintained for 2.5 h. Blood samples were collected at 20-min intervals throughout the study to determine plasma α-ketoglutaric acid (KIC) enrichment. Further muscle biopsies were collected from both legs at 2.5 h postexercise. Fingertip measurements of SpO₂ were recorded using pulse oximetry (9500 Onyx, Nonin) every 10 min for the duration of the study, except during exercise. The O₂/CO₂ concentration of inhaled air was also monitored every 10 min using a Servomex Xenta 4100 Gas Purity Analyzer (Servomex, Crowborough, UK). A schematic representation of the study protocol can be viewed in Fig. 1.

**Hypoxic trial.** The experimental conditions for the hypoxic trial were similar to that of the normoxic trial, the difference being that volunteers breathed 12% O₂ for 1 h before RE, during RE, and for the remainder of the study to total 3.5 h of hypoxic exposure. Hypoxic conditions were delivered using a custom-made face mask, which was adapted to allow gas sampling of the internal mask environment via sealed capillary tubing, and connected to a nitrogen generator set to reduce the O₂ concentration of inspired air to 12%.

**Evaluation of Myofibrillar Protein Synthesis**

**Muscle preparation for leucine tracer analysis.** Briefly, muscle tissue (~25 mg) was homogenized with scissors in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.1% Triton X-100, 1 mM EDTA, 10 mM β-glycerophosphate, 50 mM NaF, 0.5 mM activated sodium orthovanadate (all from Sigma-Aldrich, Poole, UK), and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK). The resulting homogenate was centrifuged at 13,000 g for 20 min to precipitate the myofibrillar fraction. The myofibrillar pellet was solubilized with 0.3 M NaOH and centrifuged at 3,000 g for 20 min to separate it from the insoluble collagen fraction. The solubilized myofibrillar protein was precipitated using ice-cold 1 M perchloric acid, and the resulting pellet was washed twice with 70% ethanol and collected by centrifugation. Protein-bound amino acids were released by acid hydrolysis in a Dowex H⁺ resin slurry (0.05M HCl) at 110°C overnight. The amino acids were purified by ion exchange chromatography on Dowex H⁺ resin. The amino acids were derivatized as their N-acetyl-n-propyl esters, as described previously (12). Incorporation of [1,2-¹³C₂]leucine into protein was determined by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XP; ThermoFisher Scientific, Hemel Hempstead, UK) using our standard techniques (16).

**Calculation of myofibrillar protein synthetic rates.** The fractional synthetic rate (FSR) of myofibrillar proteins was calculated based on the incorporation rate of [1,2-¹³C₂]leucine into muscle proteins using a standard precursor-product model as follows: FSR = ΔEₚ/ΔE₀ (1/h) × 100, where ΔEₚ = the change in enrichment (APE) of protein-bound leucine in two subsequent biopsies, Eₚ = the mean enrichment over time of the precursor for protein synthesis [plasma KIC was chosen to represent the immediate precursor for muscle protein synthesis, i.e., leucyl-RNA (8, 28)], and t is the time between biopsies. Values for FSR are expressed as percent per hour (%/h).

**Immunoblotting**

Immunoblotting was performed similarly to previously described methods (4, 5, 15). Briefly, frozen muscle tissue (~20 mg) was rapidly homogenized with scissors in ice-cold buffer [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM glycerophosphate, 50 mM NaF, 0.1% Triton-X, 0.1% 2-mercaptoethanol, 1 complete protease inhibitor tablet (Roche Diagnostics, Burgess Hill, UK)] at 6 μl/mg tissue. Proteins were extracted by shaking for 15 min at 4°C, and samples were then centrifuged at 13,000 g for 10 min at 4°C, and the supernatant containing the proteins was collected. Bradford assays were used to determine sarcoplasmic protein concentration (B6916; Sigma-Aldrich, St. Louis, MO) and adjusted to 1 mg/ml in 3X Laemmli buffer to measure relative phosphorylated protein.
concentrations of P70S6K Thr389, PKB Ser473, mTOR Ser2448, eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) Thr70, eIF4E Ser209, eukaryotic elongation factor 2 (eEF2) Thr56, ERK1/2 Tyr202/204, and acetyl-CoA carboxylase (ACC)/H9252 Ser79 and total protein concentration of REDD1. After heating at 95°C for 7 min, 15 g of protein from each sample were loaded onto 12% NuPAGE Novex Bis-Tris gels (Invitrogen, Paisley, UK), separated by SDS PAGE, and transferred on ice at 100 V for 45 min to methanol-prewetted, 0.2-μm PVDF membranes. Blots were then blocked in 5% (wt/vol) BSA in TBS-T (Tris-buffered saline and 0.1% Tween-20; both Sigma-Aldrich) for 1 h and then incubated in primary antibodies (1:2,000) overnight, rotating at 4°C. Membranes were then washed (3 × 5 min) with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2,000; New England Biolabs) before further washing (3 × 5 min) with TBS-T. Membranes were developed by incubation with enhanced chemiluminescence reagents for 5 min (enhanced chemiluminescence kit, Immunstar; Bio-Rad Laboratories, Richmond, CA), ensuring no pixel saturation. Data were expressed in relation to β-actin loading controls.

Statistical Analysis

Paired sample t-tests were performed on MVC and SpO2 data to determine any differences in these measurements between the normoxic and hypoxic conditions. Data were tested for normality, and, if normally distributed, two-way repeated-measures analysis of variance was used to evaluate possible differences between normoxia and hypoxia in MPS and intracellular signaling elements between unilateral exercised leg and contralateral control leg with post hoc comparisons using GraphPad software (La Jolla, San Diego, CA). If the data failed the normality test, then data were log transformed prior to analyses. Pearson’s product moment correlation was performed to determine relationships between FSR and SpO2. A P value of <0.05 was considered statistically significant. Data are presented as means ± SE.

RESULTS

Mean MVC and SpO2 Responses

Peak MVC, and thus subsequent exercise intensity, was not different between the normoxic and hypoxic conditions (629 ± 63 vs. 613 ± 60 N for normoxic and hypoxic MVC, respectively, P > 0.05; Fig. 2A). Throughout the normoxic trial, SpO2 remained unchanged from baseline (98.2 ± 0.3 vs. 97.4 ± 0.5% for SpO2 at baseline and the average of all subsequent time points, respectively, P > 0.05). In hypoxia, SpO2 decreased significantly from baseline within 10 min to 86.4 ± 1.7% (P < 0.01), and the average of all subsequent time points remained depressed throughout hypoxia for all subjects (82.7 ± 0.5%, P < 0.001, range 75–89%; Fig. 2B), as did all individual 10-min time points (P < 0.01; Fig. 2C).

MPS at Rest and After RE in Normoxia and Hypoxia

After 3.5 h of hypoxia, MPS was not different from that in normoxia in the rested leg (0.033 ± 0.016 vs. 0.043 ± 0.016%/h, P > 0.05; Fig. 3A). In normoxia, MPS was increased significantly from baseline 2.5 h after RE (0.033 ± 0.016 vs. 0.104 ± 0.038%/h, P < 0.01) but not in hypoxia (0.033 ± 0.016 vs. 0.060 ± 0.063%/h, P > 0.05, Fig. 3A).
significant relationship existed between MPS 2.5 h after RE and mean hypoxic SpO2 ($r^2 = 0.49$, $P < 0.05$; Fig. 3B).

**Anabolic Signaling at Rest and After RE in Normoxia and Hypoxia**

Protein concentrations of REDD1 were not altered 2.5 h after RE in normoxia (0.3 ± 0.2-fold increase, $P > 0.05$). After 3.5 h of hypoxic exposure, REDD1 concentrations were also unchanged at rest (0.2 ± 0.2-fold increase, $P > 0.05$) and 2.5 h after RE in hypoxia (0.1 ± 0.2-fold increase, $P > 0.05$; Fig. 4A). In normoxia, the phosphorylation of p70S6K Thr389 increased from baseline values 2.6 ± 1.2-fold 2.5 h after RE ($P < 0.05$). After 3.5 h of exposure to hypoxia, phosphorylation of p70S6K Thr389 increased from normoxic basal values 2.3 ± 1.0-fold in the rest leg and 3.4 ± 1.1-fold 2.5 h after RE (both $P < 0.05$). There were no differences in the level of p70S6K Thr389 phosphorylation between conditions ($P > 0.05$; Fig. 4B). No correlation was present between p70S6K Thr389 phosphorylation and MPS ($r^2 = 0.23$, $P > 0.05$) or SpO2 ($r^2 = 0.18$, $P > 0.05$) 2.5 h after RE in hypoxia. Phosphorylation of PKB Ser473, mTOR Ser2448, 4E-BP1 Thr37/46, eIF4E Ser209, eEF2 Thr56, ERK1/2 Tyr202/204, and ACC-β Ser79 was unchanged from normoxic baseline values in all conditions (all $P > 0.05$; data not shown) and did not correlate with the level of SpO2 2.5 h after RE in hypoxia ($P > 0.05$).

**DISCUSSION**

Falls in SpO2 to between 80 and 90% are experienced frequently, even at moderate altitudes (~4,300 m, ~12% O2, SpO2 81 ± 1%) (32) and in various clinical situations where tissue hypoxia is a feature (26). The primary cause of atrophy in other clinical scenarios is reductions in resting rates of MPS (i.e., disuse atrophy, cancer) (23), and as such we hypothesized that MPS would be suppressed during acute hypoxia exposure. In contrast, we show for the first time that 3.5 h of exposure to hypoxia is insufficient to blunt resting MPS or induce expression of hypoxic-responsive signaling proteins that suppress MPS, such as REDD1. Although these findings contrast to early reports of reduced MPS during acute hypoxia (22), this may be explained by a number of key differences between the studies. First, the techniques utilized to measure MPS were different. Whereas the previous study used leucine uptake into muscle, which provides only an indirect index of MPS, in the present study we used accurate methods to precisely determine MPS. Second, the duration of the hypoxic exposure was almost twice as long in the previous study (6.5 h of exposure vs. 3.5 h presently). Finally, in the earlier study, hypobaric hypoxia was used vs. normobaric hypoxia in the present study. This is a key difference since at equivalent PO2 hypobaric hypoxia induces greater reductions in SpO2 compared with normobaric hypoxia (25). Following
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MPS while in the rested state. Nevertheless, falls in SpO2 to As such, a greater hypoxemic stress with either hypobaric hypoxia or more prolonged exposure may be required to inhibit MPS while in the rested state. Nevertheless, falls in SpO2 to 65% as in Rennie et al. (23) are uncommon, requiring rapid ascent to altitudes of 5,000 m and above (3, 13), and have not been reported in any hypoxic pathology. Finally, we acknowledge interpretative limitations of these ground-based studies in relation to chronic exposure to altitude, i.e., short-term exposure to low O2 and discriminating the effects of normobaric vs. hypobaric hypoxia.

This study is also the first to show that the normal two- to threefold increase in MPS after RE is blunted during hypoxia. Many scenarios exist whereby the adaptive response to RE training is blunted during both environmental (19) and pathological (7, 18, 30) hypoxia. The present findings provide evidence that this effect may be a direct consequence of blunted increases in MPS after individual RE bouts, which over the course of a training program may attenuate increased accretion of muscle proteins and thus hypertrophy. Moreover, we speculate that blunted responses to resistance exercise may perhaps also be translated to much lower levels of activity that normally stimulate muscle protein synthesis (27) and are important for muscle maintenance; i.e., we contend that it is possible that muscles mimic a “mild state of disuse” in low O2 conditions. However, the mechanisms regulating this blunted MPS response to RE in hypoxia remain elusive. As observed at rest in hypoxia, total protein content of REDD1 (or HIF-1/REDD1/2 mRNA; data not shown) and the phosphorylation status of ACC-β Ser79 (a proxy for AMPK activity) were unaffected after hypoxic RE, indicating that other mechanisms must exist to signal low O2 levels to the muscle after RE to attenuate increases in MPS. We also observed dissociation between p70S6K1 and protein synthesis, where increases in p70S6K1 under hypoxic conditions were not translated into elevated MPS. Such dissociation between p70S6K1 phosphorylation and MPS suggests that some other signal is essentially overriding mTORC1 signaling in hypoxia, and although speculative, it is possible that this could be due to induction of an unfolded protein response (which attenuates protein synthesis), a common feature of hypoxic tissue (2). Nonetheless, we have previously reported dissociation between mTOR signaling and muscle protein synthesis (4, 12). Although the reasons for these discrepancies are currently unknown, we are strong advocates that mTOR signaling should not be used as a proxy for muscle protein synthesis, measurements of which we have provided.

We also report that there is a substantive interindividual variation in drops in SpO2 after breathing 12% O2. Although we are able only to speculate at present, this may indicate the existence of a responder and nonresponder phenotype. Indeed, such a “responder/nonresponder” phenomenon relating to falls in SpO2 has been postulated to exist among mountaineers to explain why some experience symptoms of acute mountain sickness whereas others do not (6). Clearly, further work is required to establish the mechanisms underlying this variation, such as investigating individual differences in O2 carrying capacity of the blood (i.e., hemoglobin concentration and/or hematocrit), genetics, or hyperventilatory responses. Importantly, MPS after RE in hypoxia was significantly correlated with extant SpO2; i.e., subjects with a lesser degree of hypoxemia had a greater capacity to maintain post-RE increase in MPS. At present, we cannot establish whether this is a true linear relationship or whether there exists a specific SpO2 threshold below which increases in MPS are blunted, which would suggest a “reserve” in the metabolic capacity to sustain anabolic responses during mild systemic hypoxemia. Nevertheless, individual variation aside, it is likely that the degree of systemic hypoxemia inversely relates to adaptive responses to exercise training. Moreover, our findings support observations that the efficacy (i.e., hypertrophic responses) of exercise prescription varies greatly between individuals with clinical hypoxia of different severities (7, 18).

We report for the first time that a short period of normobaric hypoxia is not sufficient to induce a blunting in MPS, showing that humans have a robust metabolic reserve against acute falls in SpO2. We also report the novel finding that increases in MPS after RE are blunted under conditions of normobaric hypoxia to a level depending upon extant SpO2. In conclusion, human muscles are seemingly able to maintain protein synthesis in hypoxia (at least during acute exposure). However, when faced with the challenge of an anabolic stimulus such as RE, the capacity to increase protein synthesis is limited by extant SpO2 and perhaps the ability to resist declines in SpO2.

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DISCLOSURES

The authors declare no conflicts of interest.

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