Title

Environmental hypoxia favors myoblast differentiation and fast phenotype but blunts activation of protein synthesis after resistance exercise in human skeletal muscle

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Short title: Muscle adaptations to hypoxic resistance exercise

Abbreviations:

1-RM: one repetition maximum

ACC: acetyl CoA carboxylase

Akt/PKB: protein kinase B

AMPK: adenosine monophosphate-activated protein kinase

Atg: autophagy related-gene

Bnip3: Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3

Bnip3L: Bnip3 like

$CoCl_2$: cobalt chloride

Ctrl: control

D_2O: deuterium

D-3MH: 3D-methyl-histidine

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

Ex: exercise

HIF-1α: hypoxia-inducible factor-1α

HR: heart rate

HYP: hypoxia

LC3b: microtubule-associated protein 1 light chain 3

Lox: lipoxygenase

Mafbx: muscle atrophy F box

mTOR: mammalian target of rapamycin

MuRF-1: muscle ring finger protein-1

Mrf4: myogenic regulatory factor 4

Myf-5: myogenic factor 5

Myh: myosin heavy chain

NIRS: near-infrared spectroscopy

NOR: normoxia

Pcna: proliferating cell nuclear antigen

Pdk4: pyruvate dehydrogenase kinase isoform 4

Pfk: phosphofructokinase

Plin2: perilipin 2

RPL: ribosomal protein L

S6: ribosomal S6 protein

S6K1: ribosomal S6 kinase 1

SpO_2: peripheral blood oxygen saturation

Redd1: regulated in development and DNA damage response 1

Tnni1: troponin I1, slow skeletal type

Tnnt1: troponin T1, slow skeletal type

TSI: tissue saturation index

ULK1: Unc51-like kinase 1

Vegf: vascular endothelial growth factor

Abstract

We hypothesized that a single session of resistance exercise performed in moderate environmental hypoxic conditions would potentiate the anabolic response during the recovery period spent in normoxia. Twenty subjects performed a one-leg knee extension session in normoxic or hypoxic (FiO_2: 14%) conditions. Muscle biopsies were taken 15min and 4h after exercise in the vastus lateralis of the exercised and the non-exercised legs. Blood and saliva samples were taken at regular intervals before, during and after the exercise session. The muscle fractional protein synthetic rate was determined using deuterium incorporation into proteins and the protein degradation rate by methylhistidine release from skeletal muscle. We found that: 1) hypoxia blunted the activation of protein synthesis after resistance exercise; 2) hypoxia down-regulated the transcriptional program of autophagy; 3) hypoxia regulated the expression of genes involved in glucose metabolism at rest, and genes involved in myoblast differentiation and fusion, and in muscle contraction machinery after exercise; 4) the hypoxia-inducible factor-1α pathway was not activated at the time points studied. Contrary to our hypothesis, environmental hypoxia did not potentiate the short-term anabolic response after resistance exercise, but it initiated transcriptional regulations that could potentially translate into satellite cells incorporation and higher force production on the long-term.

Keywords: hypoxia-inducible factor, deuterium, autophagy, redd1, tissue oxygenation index

Introduction

Hypoxia is a state of lowered oxygen tension in tissue that can be created by environmental conditions such as high altitude, or by pathological conditions such as chronic obstructive pulmonary disease (1) and obstructive sleep apnea (2). During exercise, hypoxia can also be generated but, contrary to the previous situations, oxygen restriction is then limited to skeletal muscle (3). Whatever the origin of hypoxia, different tissues will adapt acutely and/or chronically to deal with this reduction in oxygen availability. Interestingly, hypoxia has recently emerged as a particularly efficient stimulus to stimulate muscle cell proliferation and accretion of muscle mass (4). While long lasting hypoxia generally leads to a negative regulation of protein metabolism and a loss of muscle mass (5-7), repeated intermittent hypoxia seems to rather exert a positive effect on protein balance (4). Thus, hypoxic resistance training has become popular amongst athletes as it is thought to favor muscle accretion. Indeed, previous observations suggest that muscle hypertrophy can be optimized when resistance training is performed in a hypoxic environment (8, 9). However, the molecular mechanisms are largely unknown.

Skeletal muscle mass reflects a dynamic turnover between net protein synthesis and degradation. In addition, satellite cell inclusion may contribute to increase muscle mass, while fiber loss results in a reduction of muscle mass. While controversial (10), protein synthesis is regularly assessed by measuring the activation of the protein kinase B (Akt/PKB)/mammalian target of rapamycin (mTOR) pathway and its downstream targets ribosomal S6 kinase 1 (S6K1) and ribosomal S6 protein (S6). Muscle protein degradation is mainly regulated by the autophagy-lysosomal and the ubiquitin–proteasome pathways. The latter mechanism is largely regulated by E3 ligases such as muscle atrophy F box (Mafbx) and muscle ring finger protein-1 (MuRF-1) (11). The transcriptional regulation of those E3 ligases is partly controlled by the members of the forkhead FoxO family, themselves regulated by Akt (12). Unc51-like kinase 1 (ULK1) plays an essential role in the initiation of autophagy and more specifically in the autophagosome membrane formation (13). The subsequent elongation of the membrane is under the control of several autophagy related-gene (Atg) proteins, including microtubule-associated protein 1 light chain 3 (LC3b). The mature autophagosome, whose membrane includes the lipidated form of LC3 (LC3bII), fuses with lysosomes containing hydrolases. As mentioned earlier, satellite cells may contribute to muscle hypertrophy as well (14). Their activation, proliferation and differentiation are tightly controlled by the so-called myogenic regulatory factors, namely myogenin, MyoD, myf-5 and myogenic regulatory

factor 4 (Mrf4) (14). At a molecular level, a decrease in oxygen tension activates the hypoxia-inducible factor-1α (HIF-1α), which is responsible for many responses to hypoxic conditions such as angiogenesis or increased glucose metabolism, via increased gene expression of vascular endothelial growth factor (Vegf) and phosphofructokinase (Pfk), respectively (15). Whether HIF-1α is directly responsible for the regulation of protein metabolism under hypoxic conditions is unknown.

Due to the lack of data, the purpose of the present study was to determine the rates of protein synthesis and protein degradation as well as the molecular adaptations after resistance exercise in hypoxia in human skeletal muscle. We hypothesized that one session of resistance exercise performed in moderate environmental hypoxic compared to normoxic conditions would potentiate the anabolic response during the recovery period spent in normoxia.

Materials and methods

Subjects

Twenty young, physically active, men gave their written informed consent to participate in the study (BMI 22.2 ± 0.3kg/m^2, age 22.2 ± 0.4y). The experiment was approved by the ethical committee of the Université catholique de Louvain and conducted in accordance with the declaration of Helsinki. The exclusion criteria were: cardiovascular or pulmonary disease, chronic medication, any contraindication to resistance training or having been exposed to an altitude above 1500m during the month before the experiment.

Protocol

The subjects were randomly distributed into two groups, hypoxia (HYP, n=10) or normoxia (NOR, n=10) (Fig. 1). Each subject came 3 times to the laboratory.

First visit: A week before the experiment, height and weight were determined. The one-repetition maximum (1-RM) of the right leg was determined on a knee extension device (Pro-Dual Body Solid).

Second visit: The day before the experiment, saliva and blood samples were collected prior to ingestion of deuterium oxide (400ml spread over 2h, Sigma-Aldrich #613428, Overijse, Belgium) and 3D-methyl-histidine (10mg diluted in 100ml water, Cambridge Isotope Laboratories DLM-2949-MPT-PK, Apeldoorn, The Netherlands). A second sample of saliva

was collected 90min after ingestion of the last dose of deuterium. Thereafter, a standardized low meat spaghetti bolognese was provided.

Third visit: The day after the second visit, the subjects came to the laboratory in the fasted state. After collection of blood, saliva and urine samples (Fasted), they received a standardized meat-free breakfast complemented with 10g essential amino acids. One hour after meal consumption, the one-leg knee extension exercise session began (8 sets of 8 repetitions at 80% 1-RM, 2-min rest between sets) either in normoxic or hypoxic (FiO_2: 14%) conditions. Each set lasted about 25s to be completed. In total, the whole session, and thereby the exposition to hypoxia or normoxia, lasted for 30min. During exercise, vastus lateralis muscle oxygenation was monitored by near-infrared spectroscopy (NIRS) (Artinis, Elst, The Netherlands), and peripheral blood oxygen saturation (SpO_2) and heart rate (HR) were monitored by pulse oximetry (TCM Tosca, Radiometer, Zoetermeer, The Netherlands). Blood, saliva and urine samples were collected immediately (Post), 2h (2h Post) and 4h after exercise (4h Post). Muscle biopsies were taken from the vastus lateralis of the exercised and non-exercised (control) legs within 15min after exercise (Post) and 4h after exercise (4h Post).

Near-infrared spectroscopy

Changes in muscle oxygenation were measured by NIRS using the difference in absorption characteristics of light between 760nm and 850nm (Portamon, Artinis). Tissue saturation index (TSI, %) was monitored at baseline (30-s averaging before the start of the first set) and during each set. As described in Brocherie et al. (16), the apparatus was fixed with a large bandage to avoid interference by extraneous light and loss of transmitted light out of the field of investigation.

Samples collection and storage

Blood: Samples were taken in an antecubital vein in two 5-ml tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately put on ice until centrifugation. After centrifugation at 4°C for 10min at 3000g, plasma was transferred to microtubes and stored at -80°C.

Saliva: Samples were collected with a cotton wool, gently chewed during 1 to 2min. The swab was then placed in the suspended insert and the salivette was firmly closed using the stopper. The samples were immediately placed on ice until centrifugation. The salivettes were then centrifuged at 3°C for 10min at 3000g. The supernatant was aliquoted and stored at -20°C.

Urine: Samples were collected in 2-l bottles, transferred and aliquoted in 50-ml tubes and immediately stored at -20°C.

Muscle biopsies: Vastus lateralis muscle biopsies were taken according to the modified Bergström technique with suction. After local anesthesia (xylocaine 2% without adrenaline, Astra Zeneca, Uccle, Belgium), a 5-mm incision was made with a scalpel and a pressure applied for 10min to reduce blood flow. The Bergström needle was then gently inserted into the incision, at least 1cm beyond the fascia, to reach the muscle and three successive cuts were performed to obtain 150mg per sample. The samples were immediately frozen in liquid nitrogen and stored at -80°C.

Plasma insulin and cortisol concentrations

Plasma concentration of insulin and cortisol were determined by ELISA using the ultrasensitive insulin kit from Mercodia (Uppsala, Sweden) and the human cortisol kit from Abcam (Cambridge, UK), respectively.

Protein extraction

Muscles samples were homogenized with a Polytron mixer in ice-cold lysis buffer (20mM Tris–HCl pH 7.0, 270mM sucrose, 5mM ethylene glycol tetraacetic acid (EGTA), 1mM EDTA, 1mM sodium orthovanadate, 50mM B-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM DTT, 1% Triton-X 100 and a complete protease inhibitor tablet), centrifuged at 10.000g for 10min at 4°C and the supernatant aliquoted and stored at -80°C. The pellet, containing non-soluble proteins, was stored at -80°C for the determination of the protein synthetic rate. The myofibrillar fraction was isolated from the pellet by the addition of 0.3M NaOH and placed at 37^{o}C for 30min. The non-soluble collagen proteins were removed by centrifugation and the myofibrillar proteins precipitated with 1M PCA. Protein bound amino acids were released using acid hydrolysis in 0.1M HCl and Dowex H+ resin slurry overnight before being eluted from the resin with 2M NH_4OH and evaporated to dryness. The DC protein assay kit was used to determine the protein concentration of each sample using bovine serum albumin as a standard (Bio-Rad, Nazareth, Belgium).

Western Blotting

Thirty to seventy μg proteins were separated by SDS-PAGE (8%-15% gels) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1h and incubated overnight at 4°C with one of the following antibodies: phospho-AMP activated protein kinase

(AMPK, Thr^{172}), total-AMPK, phospho-S6K1 (Thr^{389}), total-S6K1, phospho-Akt (Ser^{473}), total-Akt, phospho-FoxO1 (Thr^{24})/FoxO3a (Thr^{32}), total-FoxO1/FoxO3a, phospho-S6 ($Ser^{235/236}$), total S6, phospho-acetyl CoA carboxylase (ACC, Ser^{79}), total-ACC, p62/SQSTM1, HIF-1α (all from Cell Signaling, Leiden, The Netherlands, 1:1000, BSA 1%), regulated in development and DNA damage response 1 (Redd1, ProteinTech, 1:750, BSA 1%), LC3b (Sigma-Aldrich, 1:1000, milk 5%). Secondary anti-mouse (1:10000) or anti-rabbit (1:5000) antibodies conjugated to horseradish peroxidase were used for detection of proteins of interest. Membranes were thereafter scanned and proteins quantified with GeneSnap software and tools (Syngene, Cambridge, UK). For detection of total protein forms, membranes were stripped and re-probed 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 phosphorylated/total form of the protein and are expressed in arbitrary units.

Protein synthesis and protein degradation

The muscle fractional synthetic rate was determined using the stable isotope deuterium following the same procedure as in Wilkinson et al (17). The muscle protein degradation rate was determined using the stable isotope tau-methyl-l-histidine based on the procedure described in Sheffield-Moore et al (18). Both rates were determined between 15min and 4h post-exercise.

Immunofluorescence

Cryosections of 6μm thickness were fixed with 3.7% formaldehyde for 10min and incubated in a permeabilization buffer (20mM Tris-HCl pH 8.0, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose, 0.5% Triton X-100) at 37°C for 20min. After blocking nonspecific staining with 1% normal goat serum (NGS) in phosphate buffered saline (PBS) for 30min, sections were incubated with an anti-HIF1α (1:25 in 1% NGS diluted in PBS, Cell Signaling) overnight at 4°C, followed by incubation with a goat anti-rabbit IgG AF 488 (1:200 in 1% NGS diluted in PBS, Invitrogen, Merelbeke, Belgium) for 1h at room temperature. Nuclei were identified with 4',6-diaminido-2-phenylindole (DAPI) provided in the mounting medium (Vectashield, Vector Laboratories Inc, Brussels, Belgium). Specimens were examined with a Zeiss Axio Vert.A1 fluorescence microscope. HeLa cells and human myotubes treated with 400μM $CoCl_2$ for 24h were used as a positive control.

RNA extraction and real-time PCR

About 20-25mg of muscle biopsy sample was homogenized in 1ml Trizol reagent (Invitrogen, Merelbeke, Belgium) using a Polytron mixer. RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop spectrophotometry. Reverse transcription was performed from 1µg RNA using the iScriptcDNA Synthesis Kit from Bio-Rad, following the manufacturer's instructions. The primers used are presented in Table 1. PCR was run using the following conditions: 3min at 95°C, followed by 35 cycles of 30s at 95°C, 30s at 60°C, and 30s at 72°C. All samples were run in duplicate and each reaction was processed in a 10µl volume containing 4.8µl IQ SybrGreen SuperMix (Bio-Rad), 0.1µl of each primer (100nM final), and 5µl cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. To compensate for variations in input RNA amounts and efficiency of reverse transcription, ribosomal protein L4 (RPL4) and RPL19 mRNA were quantified, and results were normalized to these values. These genes were chosen out of four normalization genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere (19). The results are expressed in arbitrary units.

Microarray

Microarray analyses were performed on biopsies obtained 4h after exercise in the exercised and non-exercised control legs in NOR and in HYP. Bioanalyzer nano 6000 chips (Agilent Technologies, Santa Clara, CA, USA) were used to assess RNA quality. Only samples with an RNA integrity number > 7.7 were used. We thus selected subjects in normoxia (n=8) and hypoxia (n=7), and then pooled their RNA for each condition, i.e. NOR.Rest, NOR.Exercise, HYP.Rest, and HYP.Exercise. GeneChip Human Transcriptome Array 2.0 and GeneChip WT PLUS Reagent Kit were used following manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Affymetrix Transcriptome Analysis Console Software was used to compute gene signals. Functional annotation and pathway analysis for comparisons at rest (HYP.Rest vs. NOR.Rest) and after exercise (HYP.Exercise vs. NOR.Exercise) were performed with DAVID web tool (20). In the DAVID analyses, Fischer exact p-values < 0.05, which are equivalent to $-\log_{10}$(p-value) > 1.3, were considered statistically significant.

Statistics

All values are expressed as means ± SEM. Repeated measures ANOVA was performed considering groups (HYP or NOR) as inter-group factor, and time (15min post-exercise and

4h post-exercise) and condition (rest and exercise) as intra-group factors. Corrected Bonferroni post-hoc analyses were performed when indicated, and statistical significance was fixed at $p<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc version 24.0).

Results

Blood but not muscle oxygenation was decreased in hypoxia

SpO_2 was about 98% at baseline and during each set in normoxia (Table 2). In hypoxia, SpO_2 decreased to ~93% at baseline, and was lower compared to normoxia during the whole experiment ($p<0.001$). From the 2nd set in hypoxia, SpO_2 increased compared to baseline reaching 94.6 to 95.1% ($p<0.05$). TSI decreased by about 10 to 15% during exercise ($p<0.001$) but was not different between normoxia and hypoxia. Heart rate progressively increased during exercise ($p<0.001$), with no difference between normoxia and hypoxia (+ 30 beats/min in normoxia and + 25 beats/min in hypoxia). Plasma insulin levels were higher at Post (after breakfast) compared to Fasted in both normoxia and hypoxia ($p<0.001$, Table 3), as expected. No difference in plasma insulin concentrations was observed between normoxia and hypoxia, although a trend to higher values in hypoxia was measured at 2h Post ($p=0.061$). Compared to Fasted, plasma cortisol concentrations were lower at Post, 2h Post and 4h Post ($p<0.05$), with no difference between normoxia and hypoxia.

Hypoxia blunts the activation of protein synthesis after resistance exercise

The fractional synthetic rate was increased by about 40% after resistance exercise in normoxia ($p=0.023$, Fig. 2a), but not in hypoxia. The fractional synthetic rate was not different between normoxia and hypoxia in the control rested leg.

No effect of hypoxia on the mTOR pathway despite decreased AMPK phosphorylation

We next analyzed the phosphorylation state of key mediators of the mTOR pathway. Compared to Post, the phosphorylation of Akt, S6K1 and S6 were lower at 4h Post in the exercised leg in normoxia and hypoxia ($p<0.001$, Fig. 2b-d). Compared to Post, phospho-Akt ($p=0.004$) and phospho-S6 ($p=0.036$) were lower at 4h Post in the control leg only in hypoxia, while phospho-S6K1 was lower in both normoxia ($p=0.012$) and hypoxia ($p=0.004$). In addition, phospho-S6K1 was higher in the exercised leg compared to the control leg at Post in normoxia ($p=0.016$), and at 4h Post in hypoxia ($p=0.012$). Compared to the control leg,

phospho-S6 was higher in the exercised leg at Post in normoxia (p=0.012) and hypoxia (p=0.012). The expression of the mTOR inhibitor Redd1 was lower at 4h Post in the exercised leg compared to the control leg in both normoxia (p=0.020) and hypoxia (p=0.050) (Fig. 2e). Hypoxia lowered Redd1 protein expression at 4h Post in the control (p=0.047) and in the exercised leg (p=0.041). Hypoxia decreased phospho-AMPK at Post in the control leg (p=0.022) and at 4h Post in the exercised leg (p=0.001) (Fig. 2f). As a readout of AMPK activity, we also tested the phosphorylation of its substrate ACC. Compared to Post in the exercised leg, phospho-ACC was lower at 4h Post in normoxia (p=0.040) and in hypoxia (p=0.004) (Fig. 2g). Exercise increased phospho-ACC in hypoxia (p=0.004) and tended to increase it in normoxia (p=0.084).

Hypoxia down-regulated autophagy transcriptional regulation

The muscle protein degradation rate, assessed by the rate of 3-D-methyl-histidine disappearance from the plasma, was similar between normoxia and hypoxia (Fig. 3a). LC3bII/I ratio increased in both legs at 4h Post in normoxia and hypoxia ($p<0.01$, Fig. 3b). This increase was partially repressed by exercise in normoxia (p=0.012) as well as in hypoxia (p=0.004). A trend to a main exercise effect was observed for p62 (p=0.096, Fig. 3c). Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3, Fig. 3d) and Bnip 3 like (Bnip3L, Fig. 3e) mRNA levels were lower at all time points studied in hypoxia compared to normoxia ($p<0.05$). Compared to Post, Bnip3 mRNA was lower in the control leg in hypoxia (p=0.036) and tended to be lower in the exercised leg in normoxia at 4h Post (p=0.072). Compared to Post, Bnip3L mRNA was lower in the exercised leg in hypoxia at 4h Post (p=0.024). Similarly, Lc3b mRNA was lower in hypoxia compared to normoxia ($p<0.05$) except at Post in the control leg (Fig. 3f). Compared to Post, Lc3b mRNA was lower in the control leg in hypoxia at 4h Post (p=0.016). Atg12 mRNA levels were not modified by any condition (Fig. 3g). Compared to normoxia, pFoxO was higher in hypoxia at 4h Post in the exercised leg (p=0.047) and tended to be higher at the same time point in the control leg (p=0.072) (Fig. 3h). Murf-1 mRNA levels were higher at 4h Post in the exercised leg compared to the control leg (p=0.024) (Fig. 3i). Compared to Post in hypoxia, Mafbx mRNA levels were lower at 4h Post in both the control (p=0.004) and exercised leg (p=0.016) (Fig. 3j).

Hypoxia differentially regulated gene expression at rest and after exercise

Microarray analyses showed that hypoxia up- or down-regulated by at least 1.5-fold a total of 392 genes at rest and 480 genes 4h after exercise (see Fig. 4a for selected genes, and Supplementary file 1 for the full list). Analyses of functional annotation and signaling pathways revealed that, at rest, hypoxia influenced glucose metabolism and oxygen transport (Fig. 4b and Supplementary file 1). Similar analyses after exercise revealed that hypoxia influenced myoblast differentiation and fusion, and several components of the muscle contraction machinery (Fig. 4c and Supplementary file 1). Focusing on hypoxia-induced changes, qPCR analyses confirmed that Myh1 mRNA levels were increased while Tnnt1 mRNA levels were decreased in hypoxia compared to normoxia ($p<0.05$, Supplementary file 2).

Hypoxia regulated markers for cell differentiation but not proliferation

The mRNA levels of Fos were lower at 4h Post compared to Post in all conditions ($p<0.05$, Fig. 5a). At Post, Fos mRNA levels were higher in the exercised leg compared to the control leg in both normoxia and hypoxia ($p<0.01$). In addition, at Post, Fos mRNA levels were higher in hypoxia in the control leg ($p=0.012$) and tended to be higher in the exercised leg ($p=0.098$). The mRNA levels of Cyclin D1 were higher in the exercised leg compared to the control leg at 4h Post in hypoxia ($p=0.028$, Fig. 5c) The mRNA levels of Pcna were lower in the exercised leg compared to the control leg at 4h Post in normoxia ($p=0.020$, Fig. 5c). The mRNA levels of p21 were similarly increased by exercise at 4h Post in normoxia and in hypoxia ($p<0.05$, Fig. 5d). Myf5 mRNA levels were not modified by any condition (Fig. 5e). A time ($p=0.006$) and an exercise ($p=0.046$) effects were found for MyoD (Fig. 5f). The mRNA levels of myogenin were higher in hypoxia compared to normoxia in the control leg at Post ($p=0.009$) and tended to be higher at 4h Post compared to Post in the exercised leg in hypoxia ($p=0.072$). Mrf4 mRNA levels were decreased 4h Post compared to Post in both normoxia and hypoxia in the control leg as well as in the exercised leg ($p<0.05$, Fig. 5h). In addition, exercise increased the mRNA levels of Mrf4 at 4h Post in both normoxia ($p=0.016$) and hypoxia ($p=0.008$) and at Post in hypoxia only ($p=0.044$).

Hypoxia-inducible factor pathway was not modified by any condition

Neither the localization (Fig. 6a, Supplementary file 3), the protein expression (Fig. 6b), nor the mRNA levels (Fig. 6c) of HIF-1α was modified by any condition. Similar results were found for Hif-2α (Fig. 6d) and Plin2 (Fig. 6g) mRNA levels. Vegf mRNA levels were higher

4h Post in normoxia (p=0.004) and tended to be higher in hypoxia (p=0.064) compared to Post (Fig. 6e). In addition Vegf mRNA was higher in the exercised leg 4h Post compared to the control leg in both normoxia (p<0.001) and hypoxia (p=0.024). A main time effect was observed for Lox mRNA levels (p=0.032, Fig. 6f), with lower values at 4h Post compared to Post.

Discussion

Skeletal muscle adapts to hypoxia and resistance training. Combining both stimuli intermittently seems an effective strategy to favor muscle hypertrophy (21-23), even if the molecular bases remain unclear. To the best of our knowledge, only one study focused on the regulation of protein metabolism after an acute hypoxic resistance exercise in human (24). Muscle protein synthesis was blunted by hypoxia after resistance training, but no amino acids were provided to favor muscle anabolism and the hypoxic conditions were harsher (12% for 3.5h) than the one typically used by sportsmen (14-15% O_2 for 30min to 1h). As the hypoxic dose is crucial for the regulation of muscle mass (25), we hypothesized that different doses could induce different responses and a resistance exercise session performed in a moderate environmental hypoxia could induce a positive protein balance and favor muscle mass accretion when repeated (26). Contrary to our hypothesis, a lower dose of hypoxia blunted protein synthesis as well. This is surprising because an inverse correlation has been previously found between SpO_2 and the fractional synthetic rate after resistance exercise, with the lowest rates being measured in subjects displaying SpO_2 below 85% (24). Here, SpO_2 averaged 93-94% in the hypoxic group and still protein synthesis did not increase after exercise. As we found no relationship between SpO_2 or TSI and the protein synthetic rate (data not shown), we looked for other possible molecular mechanisms that could have led to the lack of activation of protein synthesis after resistance exercise in hypoxia. We have previously found that the Akt/mTOR pathway at rest (27) and the autophagy pathway after endurance exercise (28) were modulated by hypoxia. Here, despite a reduced phosphorylation of AMPK and a reduced expression of Redd1, the activation of the Akt/mTOR pathway after exercise was not modified in hypoxia. These results indicate that the activation of AMPK (29) and Redd1 (30) was not sufficient to inhibit the Akt/mTOR pathway or that this inhibitory role was overruled by another undetermined signal. One of those signals could have been insulin, as it has been previously related to an activation of the Akt/mTOR pathway under hypoxic conditions (27). Insulin increased after breakfast in our study, but this increase was not modulated by the environmental conditions in which exercise was performed. Those results suggest that

hypoxia did not blunt protein synthesis through a decrease in insulin concentrations and down-regulation of the Akt/mTOR pathway.

For the first time, the muscle protein degradation rate was measured after hypoxic training. We found that the protein degradation rate was similar after normoxic and hypoxic training. Unfortunately, the technique used to determine the muscle degradation rate, i.e. the rate of disappearance of 3-D-methyl-histidine from the plasma (18), only allows comparison between normoxia and hypoxia, not between the rested and the exercised leg. The only study having determined the rate of muscle protein degradation in hypoxic conditions was made in rats exposed to severe hypobaric hypoxia (7620m) for 14 days (31). The degradation rate was higher after 3 days of exposure and increased further after 14 days. As those conditions profoundly differ from ours (species, duration of exposure, hypoxic dose, exercise), no comparison is possible. Though, we ensure the values we obtained were in the range of those reported previously for both protein synthesis (17) and protein degradation (18) with the same techniques. While calculation of the net protein balance was not possible due to the use of two different isotopes, we can speculate that the exercise session in normoxia induced a more favorable balance than in hypoxia, contrary to our hypothesis.

A similar global protein degradation rate did not exclude that specific proteolytic processes were regulated by hypoxia or that protein degradation was differently regulated in the exercised and in the control leg. Therefore, as we have previously found that hypoxia could activate autophagy after endurance exercise (28), we investigated whether the same occurred after resistance exercise. Initiation of autophagy through LC3b lipidation was highly activated by the nutritional state, inversely following insulin levels, and partially reversed by resistance exercise. However, hypoxia had no influence on those regulations on LC3bII/I, nor on p62 expression, which remained unaltered by any condition. Those results contrast with our previous study (28), probably because the hypoxic conditions were less severe here. The level of oxygen was higher here (14% vs.11%) and the duration much shorter (30min vs. 8h), indicating that moderate hypoxia does not influence the post-translational regulation of autophagy. Unexpectedly after such short exposure duration, hypoxia down-regulated the expression of a series of genes that compose the so-called transcriptional response of autophagy, amongst which Lc3b and Bnip3. Usually, the transcriptional response is activated when autophagy lasts for hours or days to replenish the components of the machinery (32). The lower mRNA levels of Lc3b and Bnip3 in hypoxia are therefore surprising and opposite to the regulation of FoxO. The latter activates autophagy at both the post-translational and

transcriptional levels (32). Here, FoxO phosphorylation was higher in hypoxia but did not translate into an activation of autophagy at any level. It still remains to determine why Lc3b and Bnip3 mRNA levels were lower, and except a regulation of their stability by hypoxia, no other explanation can be put forward. In summary, moderate hypoxia down-regulated the expression of a series of genes involved in the transcriptional response of autophagy without impacting the initiation of the process as measured by the unchanged LC3bII/I ratio in hypoxic compared to normoxic conditions.

The micorarray analyses revealed that hypoxia regulated the expression of genes involved in glucose metabolism at rest, and genes involved in myoblast differentiation and fusion, and in muscle contraction machinery after exercise. This is the first microarray analysis reporting the transcriptional regulation induced by a single exposure to environmental hypoxia in human skeletal muscle. The higher expression of genes involved in glucose metabolism at rest is not surprising, as a better glucose handling (33) and higher dependence on blood glucose (34, 35) have been shown in people living at moderate to high altitude. Here, we show that a single exposure of 30min is enough to initiate those adaptations at a transcriptional level, confirming previous results from our lab showing a higher abundance of GLUT4 at the sarcolemmal membrane after 4h of hypoxic exposure (36). After exercise, genes involved in myoblast differentiation and fusion, and in muscle contraction machinery were differently regulated in hypoxia compared to normoxia. Globally, genes related to the slow phenotype were down-regulated, such as troponin I1 and troponin T1, whereas those related to the fast phenotype were-upregulated, such as troponin T3 and MYH1. Studies having looked at fiber typing before and after hypoxic exposure in human are scarce and data are not straightforward. In addition, the role of physical activity in those adaptations is unclear. One study found no change in myosin heavy chain type I and type IIx mRNA levels after 6 weeks of endurance training (37), while another study found an increase in type I and a decrease in type IIx protein expression after a 43-day Himalayan expedition (38). However, the higher mRNA level we observed for MYH1, which encodes for myosin heavy chain type IIx, is a positive molecular response to resistance exercise, which requires explosive and powerful muscle contractions. Whether this will translate into higher protein content and better performance needs to be investigated with a training study.

Environmental hypoxia regulates myogenesis during embryonic developement, myoblasts and satellite cell proliferation and differentiation (39). Here, we were particularly interested in the effect of hypoxia on the proliferation of satellite cells and their differentiation into myoblasts.

Both processes are tightly regulated by the myogenic regulatory factors. Myf5 and MyoD are expressed upon activation and proliferation of satellite cells (40). MyoD remains expressed at early stages of differentiation and myogenin, followed by Mrf4, at a later stage (40). In vitro, low oxygen levels (3-6%) seem to favor myoblast proliferation, whereas very low oxygen levels (<1%) tend to alter myogenic differentiation and myotube formation (41). Murine myoblasts cultured in a severe hypoxic environment (1% O_2) have been shown to up-regulate the expression of genes related to cell cycle and metabolic processes and to down-regulate the expression of genes associated with catabolic processes and muscle development (42). Our results contrast with the aforementioned studies as no effect of hypoxia was found on specific markers for cell proliferation such as Cyclin D1, Pcna or p21 whereas hypoxia increased the expression of some markers for cell differentiation. Both the microarray and the qPCR analyses showed that the mRNA levels of myogenin were higher in hypoxia compared to normoxia. In addition, a trend to an increase was observed for MyoD and Mrf4 mRNA. If this translates at the protein level, a well-controlled dose of hypoxia would favor satellite cell incorporation and differentiation into existing myofibres, which could be a mechanism contributing to the increased muscle mass after intermittent and moderate hypoxia previously reported in human (8).

Whether HIF-1α is responsible for the regulation of protein metabolism and satellite cells under hypoxic conditions is currently unknown. In vitro, HIF-1α and the mTORC1 pathway have been shown to regulate each other (43-45), but this has not been confirmed in vivo. Data on intramuscular pO_2 are sparse, but a critical threshold of 7-8 mmHg has been suggested below which intramuscular HIF-1α accumulates (46). When humans are passively exposed to environmental hypoxia, the drop in intramuscular pO_2 does not surpass this threshold (47) and HIF-1α does not stabilize as we previously showed (27). Contrarily, HIF-1α can be stabilized during repeated muscle contractions in humans, which are known to largely decrease intramuscular pO_2 (47), coupled or not to blood flow restriction (3). Here, the tissue saturation index decreased by about 10% during resistance exercise, but no additional effect of hypoxia was observed. It seems therefore that repeated contractions or endurance exercise is more potent to stabilize HIF-1α than resistance exercise. Of note, no decrease in tissue oxygenation index was detected under hypoxia, probably because 14% O_2 was not severe enough as skeletal muscle is a potent tissue to face a lack of oxygen. Therefore, the molecular regulations observed in the present study after resistance exercise and/or hypoxia were probably independent of the HIF-1α pathway as HIF-1α was not stabilized and the expression

of downstream target genes (Vegf, Redd1, Lox and Plin2 mRNA) was not increased at the two time points analyzed here.

The strength of the present study is the direct measurement of protein synthesis and degradation together with the quantification of commonly used markers to assess these processes. The microarray data provide new perspectives in terms of further investigation and more particularly at the level of satellite cells. The main limitation of the present study is the absence of muscle biopsy before the exercise session, as the protocol already comprised four muscle biopsies within a period of four hours. Thus, we cannot exclude a systemic effect from the exercised to the rested leg since both biopsies were taken at the same time. This said, we were mainly interested in the effect of hypoxia, which could be properly studied by comparison to the normoxic condition.

All together, we conclude that, contrary to our hypothesis, environmental hypoxia did not potentiate the short-term anabolic response after resistance exercise, but it initiated transcriptional regulations that could potentially translate into satellite cells incorporation and higher force production on the long-term, which remains to be investigated.

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Author contributions

L.D., M.F., K.S. and P.J.A. designed the research; O.G., H.N., D.N. and L.D. conducted the research; A. D. and J-B. D participated to the development of analytical techniques; O.G., R.F-V., M.B., E.B., D.N., M.S. and J.C. analyzed and interpreted data; O.G. and L.D. wrote the manuscript; all authors critically revised and contributed to the manuscript; all authors approved the final version of the manuscript; L.D. has primary responsibility for final content. Data collection took place at Université catholique de Louvain. Data analyses took place at Université catholique de Louvain and the University of Nottingham.

Additional information

The authors declare no competing financial interests.

Tables

Table 1. Primer sequences

	Forward	Reverse
Atg12	AGT AGA GCG AAC ACG AAC CAT C	CCA TCA CTG CCA AAA CAC TCA T
Bnip3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
Bnip3L	CCA AGG AGT TCC ACT TCA GAC	AGT AGG TGC TGG CAG AGG GTG T
Cyclin D1	GAA CAA GCT CAA GTG GAA CC	CAC AGA GGG CAA CGA AGG
Fos	GGC AAG GTG GAA CAG TTA TC	TCT CCG CTT GGA GTG TAT C
Hif-1α	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG
Hif-2α	AAG CTG AAG CGA CAG CTG GAG TAT	GTA CAT TTG CGC TCA GTG GCT TGT
Lc3b	AAT CCC GGT GAT AAT AGA ACG A	GGA GAC GCT GAC CAT GCT GT
Lox	GCA CAG TTG TCA TCA ACA TTA C	GAT GTC CTG TGT AGC GAA TG
Mafbx	CGA CCT CAG CAG TTA CTG CAA	TTT GCT ATC AGC TCC AAC AG
Mrf4	AAT CTT GAG GGT GCG GAT TTC CTG	TGC TCC TCC TTC CTT AGC CGT TAT
Murf-1	AAA CAG GAG TGC TCC AGT CGG	CGC CAC CAG CAT GGA GAT ACA
Myf5	TGA GAG AGC AGG TGG AGA ACT ACT	AGA CAG GAC TGT TAC ATT CGG GCA
Myh1	CAC ACT AGT TTC ACA GCT CTC	GGC ACT CTT GGC CTT TAT C
Myh11	GGA GGA GCA GCT ATC CAT ATT	GCC TGG TCT GTG TTT CTT TC
MyoD	TGC CAC AAC GGA CGA CTT CTA TGA	AAG TGC GAG TGC TCT TCG GGT TT
Myogenin	AAA CTA CCT GCC TGT CCA CCT C	ACA CCG ACT TCC TCT TAC ACA CCT
p21	GCA GAC CAG CAT GAC AGA TTT	GGA TTA GGG CTT CCT CTT GGA
Pcna	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
Pdk4	CAG CTA CTG GAC TTT GGT TC	CTA ATT GGG TCG GGA GGA TA
Pfk	ATT TGA CGA AGC CCT GAA G	GTG CGA ACC ACT CTT AGA TAC
Plin2	CTC ATG TCC TCA GCC TAT CT	TAG GCA GTC TCT CCT CAA TC
Rpl19	CGC TGT GGC AAG AAG AAG GTC	GGA ATG GAC CGT CAC AGG C
Rpl4	ATA CGC CAT CTG TTC TGC CCT	GCT TCC TTG GTC TTC TTG TAG CCT
Tnni1	CAA CAC CAG GGA GAT TAA GG	ATG GAC ACC TTG TGC TTG
Tnnt1	GAG CTG TCG GAC TGG AT	ACA GCA CGT TGA TCT CAT ATT
Vegf	TTT CTG CTG TCT TGG GTG CAT TGG	ACC ACT TCG TGA TGA TTC TGC CCT

Atg12, autophagy-related protein 12; Atg4b, autophagy-related protein 4b; Bnip3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; Bnip3L, Bnip3 like; Hif-1α, hypoxia-inducible factor 1 alpha; Hif-2α, hypoxia-inducible factor 2 alpha; Lc3b, microtubule-associated proteins 1A/1B light chain 3B; Lox, lipoxygenase; Mafbx, muscle atrophy F box; Mrf4, muscle regulatory factor 4; Murf-1, muscle ring finger protein-1; Myf5, myogenic factor 5; Myh1, myosin heavy chain IIx/D; Myh11, myosin heavy chain polypeptide 11, smooth muscle; Pcna, proliferating cell nuclear antigen; Pdk4, pyruvate dehydrogenase kinase isoform 4; Pfk, phosphofructokinase; Plin2, perilipin 2; Redd1, protein regulated in development and DNA damage response 1; Rpl19, ribosomal protein L19; Rpl4, ribosomal

protein L4; Tnni1, troponin I1, slow skeletal type; Tnnt1, troponin T1, slow skeletal type; Vegf, vascular endothelial growth factor.

Table 2. Tissue oxygenation and heart rate during exercise

	SpO_2 (%)		TSI (%)		HR (beats/min)	
	NOR	**HYP**	**NOR**	**HYP**	**NOR**	**HYP**
Baseline	98.2 ± 0.1	93.3 ± 0.4***	61.3 ± 0.9	61.4 ± 0.8	73.2 ± 3.1	85.1 ± 4.2
Set 1	98.2 ± 0.2	93.1 ± 0.4***	48.8 ± 1.1$^{###}$	50.4 ± 1.5$^{###}$	92.6 ± 2.3$^{###}$	101.6 ± 3.6$^{###}$
Set 2	98.0 ± 0.1	94.6 ± 0.3$^{\#,***}$	51.1 ± 1.1$^{###}$	49.4 ± 1.9$^{###}$	93.3 ± 3.5$^{###}$	100.5 ± 4.6$^{###}$
Set 3	98.0 ± 0.1	95.1 ± 0.4$^{###,***}$	50.3 ± 1.3$^{###}$	50.1 ± 1.8$^{###}$	92.5 ± 4.1$^{###}$	101.2 ± 5.1$^{###}$
Set 4	98.0 ± 0.1	94.9 ± 0.5$^{\#,***}$	51.8 ± 1.4$^{###}$	50.5 ± 1.7$^{###}$	95.5 ± 3.3$^{###}$	101.7 ± 4.7$^{###}$
Set 5	98.3 ± 0.1	94.7 ± 0.3$^{\#,***}$	50.9 ± 1.1$^{###}$	50.6 ± 1.8$^{###}$	98.8 ± 3.9$^{###}$	104.6 ± 5.3$^{###}$
Set 6	98.3 ± 0.1	95.1 ± 0.4$^{###,***}$	52.4 ± 0.9$^{###}$	51.4 ± 1.5$^{###}$	97.4 ± 4.5$^{###}$	106.7 ± 5.1$^{###}$
Set 7	98.4 ± 0.1	94.9 ± 0.4$^{\#,***}$	52.5 ± 0.9$^{###}$	52.4 ± 1.7$^{###}$	102.6 ± 4.8$^{###}$	106.7 ± 4.6$^{###}$
Set 8	98.4 ± 0.1	94.6 ± 0.5$^{\#,***}$	54.0 ± 0.7$^{###}$	52.5 ±1.6$^{###}$	106.2 ± 6.1$^{###}$	109.3 ± 5.1$^{###}$

SpO_2, blood oxygenation; TSI, tissue saturation index; HR, heart rate; NOR, normoxia; HYP, hypoxia. $^{\#}p<0.05$, $^{###}p<0.001$ vs Baseline; $^{*}p<0.05$, $^{***}p<0.001$ vs NOR.

Table 3. Plasma insulin and cortisol concentrations

		Fasted	Post	2h Post	4h Post
Insulin	**NOR**	2.7 ± 0.5	12.3 ± 2.2$^{###}$	2.5 ± 0.5	1.9 ± 0.3
(mIU/ml)	**HYP**	2.8 ± 0.4	15.6 ± 1.9$^{###}$	4.9 ± 1.2	2.4 ±0.4
Cortisol	**NOR**	273 ± 33	167 ± 25$^{###}$	120 ± 17$^{##}$	128 ± 26$^{###}$
(ng/ml)	**HYP**	245 ± 29	160 ± 22$^{#}$	138 ± 19$^{###}$	139 ± 19$^{###}$

NOR, normoxia; HYP, hypoxia. $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ vs Fasted

Figure legends

Figure 1. Study protocol

NIRS, near infrared spectroscopy; TSI, tissue saturation index; SpO_2, peripheral blood oxygen saturation; HR, heart rate; Ex, exercise; R, right; L, left.

Figure 2. Regulation of the fractional protein synthetic rate and protein synthesis markers by hypoxia and exercise

(a) Fractional protein synthetic rate between 15min and 4h post-exercise in the control and the exercised leg in normoxia and hypoxia. Phosphorylation of (b) Akt Ser^{473}, (c) S6K1 Thr^{389}, (d) S6 $Ser^{235/236}$, (f) AMPK Thr^{172} and (g) ACC Ser^{79} and (e) protein expression of Redd1 in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. (h) Representative western blots. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg; FSR, fractional synthetic rate. *p<0.05, ***p<001 HYP vs NOR; $^{\$}$p<0.05, $^{\$\$}$p<0.01 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 4h Post vs Post. n = 10/group.

Figure 3. Regulation of the protein degradation rate and protein degradation markers by hypoxia and exercise

(a) Protein degradation rate between 15min and 4h post-exercise in normoxia and hypoxia. (b) LC3bII/I ratio, (c) p62 protein expression, (d) Bnip3 mRNA levels, (e) Bnip3L mRNA levels, (f) Lc3b mRNA levels, (g) Atg12 mRNA levels, (h) FoxO1 Thr^{24}/FoxO3a Thr^{32} phosphorylation, (i) Murf-1 mRNA levels and (j) Mafbx mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. (k) Representative western blots. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg; D-3MH: 3D-methyl-histidine. *p<0.05, **p<0.01 HYP vs NOR; $^{\$}$p<0.05 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01 4h Post vs Post. n = 10/group.

Figure 4. Regulation of gene expression by hypoxia and exercise

(a) Heat map with selected genes >1.5-fold up- or down-regulated in hypoxia vs. normoxia in the control (Re) and the exercised (Ex) legs. Gene ontology by DAVID web tool using the

genes >1.5-fold up- or down-regulated in hypoxia vs. normoxia in the control (b) and in the exercised (c) legs; only significantly modified terms are presented, as −log10(p-value) > 1.3 (Fischer exact p-value). Percentage of intersection represents the percentage of genes regulated in hypoxia vs. normoxia in relation to the genes in the DAVID database. n = 8 in normoxia and n = 7 in hypoxia. BP, biological process; CC, cellular component; GO, gene ontology; MF, molecular function.

Figure 5. Regulation of the myogenic regulatory factors by hypoxia and exercise

(a) Fos, (b) Cyclin D1, (c) Pcna, (d) p21, (e) Myf5, (f) MyoD, (g) Myogenin and (h) Mrf4 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg. *p<0.05, **p<0.01 HYP vs NOR; $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 4h Post vs Post. n = 10/group.

Figure 6. Regulation of the hypoxia-inducible factor 1 alpha pathway by hypoxia and exercise

(a) HIF-1α localization and representative western blots of HIF-1α and eEF2, (b) HIF-1α protein expression, (c) Hif-1α, (d) Hif-2α, (e) Vegf, (f) Lox and (g) Plin2 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg. $^{\$}$p<0.05, $^{\$\$\$}$p<0.001 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01 4h Post vs Post. n = 10/group.

Supplementary file 1

Detailed report of the microarray analyses.

Supplementary file 2

(a) Myh1, (b) Myh11, (c) Tnni1, (d) Tnnt1, (e) Pfk and (f) Pdk4 mRNA levels in the control and in the exercised leg at Post and at 4h Post in normoxia and hypoxia. NOR, normoxia; HYP, hypoxia; Ctrl, control leg; Ex, exercised leg. *p<0.05 HYP vs NOR; $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001 Ex vs Ctrl; $^{\#}$p<0.05, $^{\#\#\#}$p<0.001 4h Post vs Post. n = 10/group.

Supplementary file 3

Cobald cloride ($CoCl_2$) 400 μM for 24h was used as a positive control to validate the antibody used to localize HIF-1α as well as the technique for immunofluorescence in (a) HeLa cells and (b) human myotubes.

Figure 1

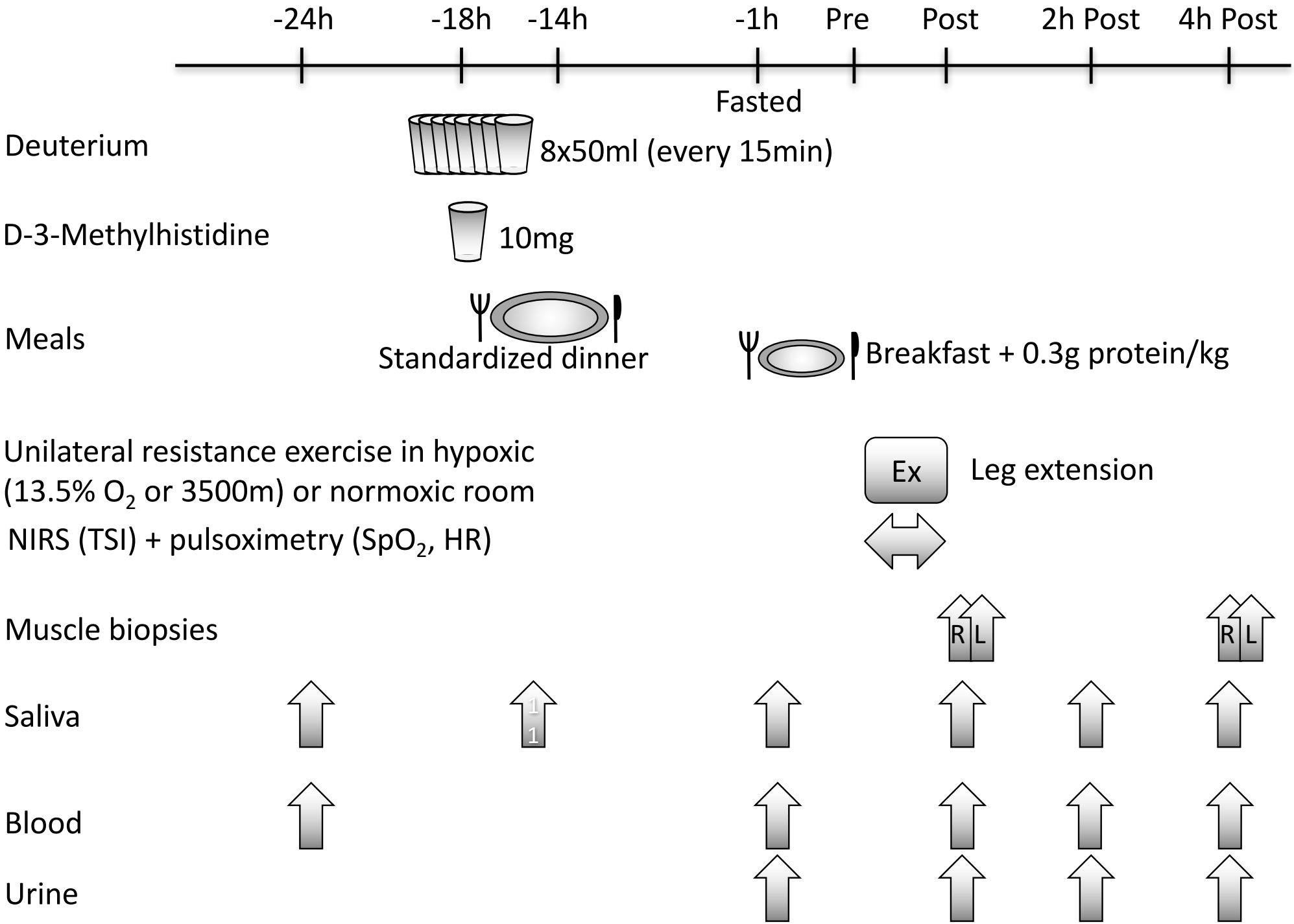

-24h
-18h
-14h
-1h
Pre
Post
2h Post
4h Post
Fasted
Deuterium
8x50ml (every 15min)
D-3-Methylhistidine
10mg
Meals
Standardized dinner
Breakfast + 0.3g protein/kg
Unilateral resistance exercise in hypoxic
(13.5% O_2 or 3500m) or normoxic room
NIRS (TSI) + pulsoximetry (SpO_2, HR)
Ex
Leg extension
Muscle biopsies
R L
R L
Saliva
Blood
Urine

Figure 2

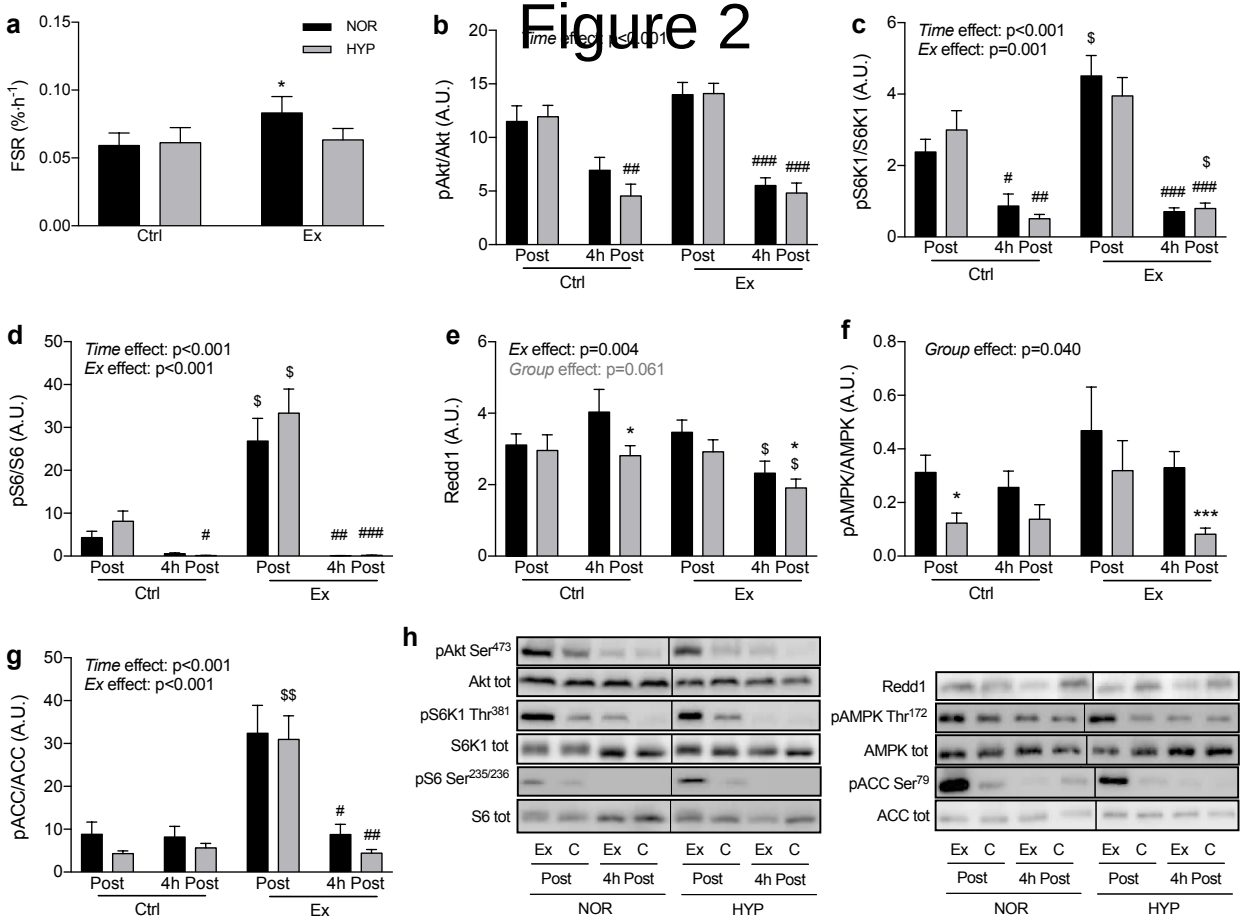

a
FSR (%·h-1)
NOR
HYP
Ctrl
Ex
b
pAkt/Akt (A.U.)
Time effect: p<0.001
Post
4h Post
Ctrl
Ex
c
pS6K1/S6K1 (A.U.)
Time effect: p<0.001
Ex effect: p=0.001
d
pS6/S6 (A.U.)
Time effect: p<0.001
Ex effect: p<0.001
e
Redd1 (A.U.)
Ex effect: p=0.004
Group effect: p=0.061
f
pAMPK/AMPK (A.U.)
Group effect: p=0.040
g
pACC/ACC (A.U.)
Time effect: p<0.001
Ex effect: p<0.001
h
pAkt Ser473
Akt tot
pS6K1 Thr381
S6K1 tot
pS6 Ser235/236
S6 tot
Redd1
pAMPK Thr172
AMPK tot
pACC Ser79
ACC tot
Ex C
NOR
HYP

Figure 3

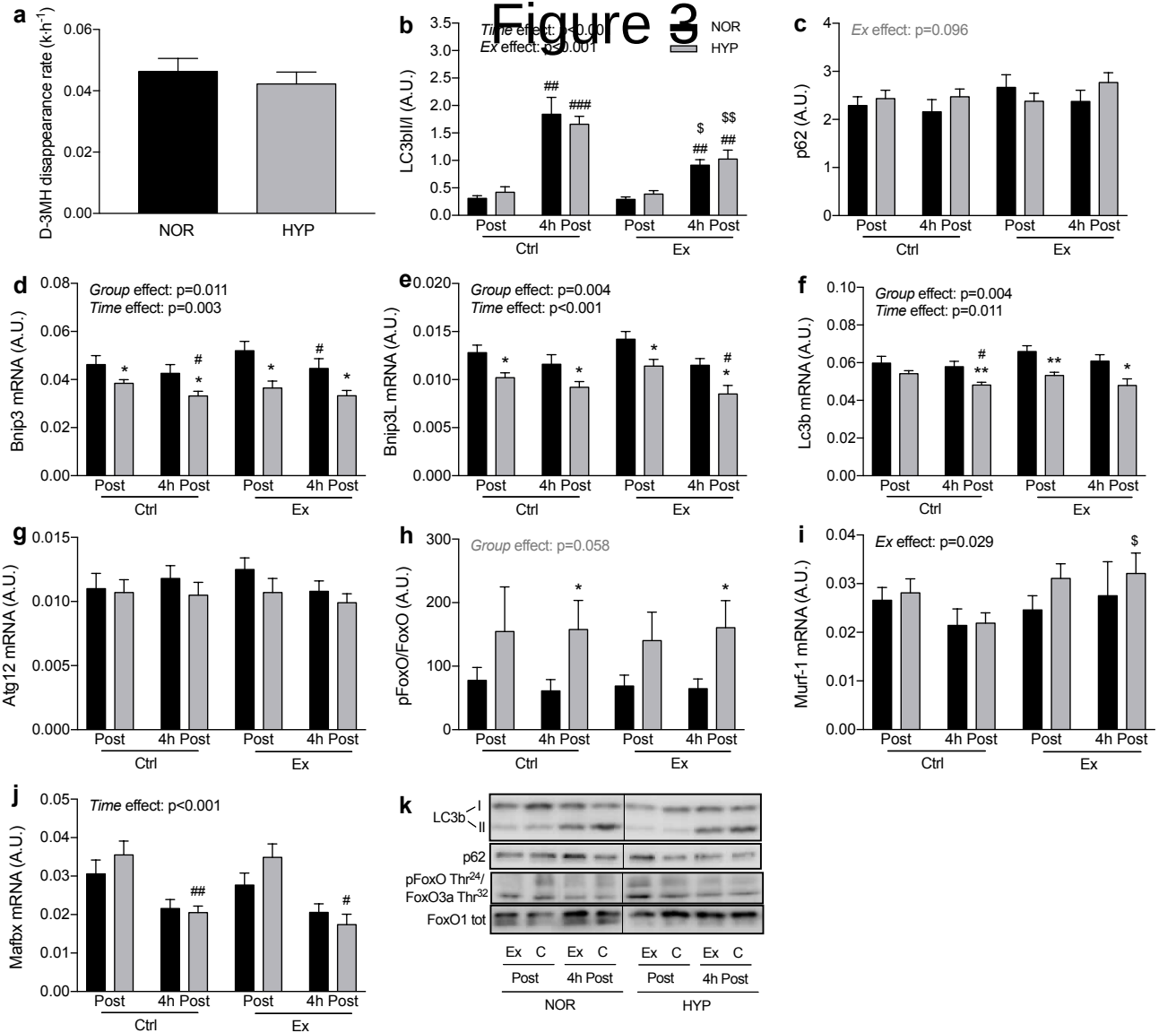
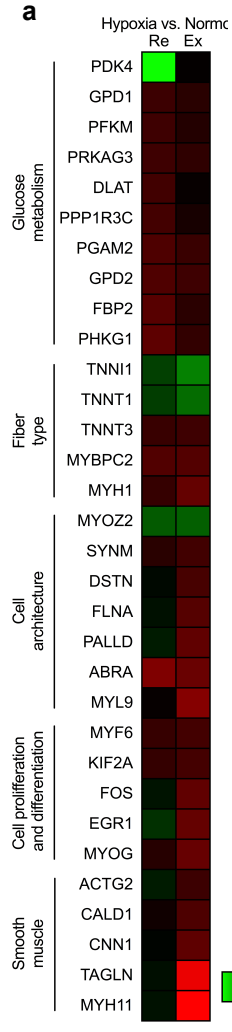
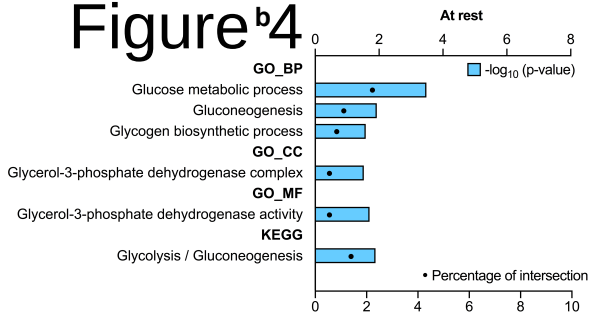

a
D-3MH disappearance rate (k·h-1)
NOR
HYP
b
Time effect: p<0.001
Ex effect: p<0.001
NOR
HYP
LC3bII/I (A.U.)
Post
4h Post
Ctrl
Ex
c
Ex effect: p=0.096
p62 (A.U.)
d
Group effect: p=0.011
Time effect: p=0.003
Bnip3 mRNA (A.U.)
e
Group effect: p=0.004
Time effect: p<0.001
Bnip3L mRNA (A.U.)
f
Group effect: p=0.004
Time effect: p=0.011
Lc3b mRNA (A.U.)
g
Atg12 mRNA (A.U.)
h
Group effect: p=0.058
pFoxO/FoxO (A.U.)
i
Ex effect: p=0.029
Murf-1 mRNA (A.U.)
j
Time effect: p<0.001
Mafbx mRNA (A.U.)
k
LC3b I
LC3b II
p62
pFoxO Thr24/
FoxO3a Thr32
FoxO1 tot
Ex C Ex C Ex C Ex C
Post
4h Post
NOR
HYP

Figure 4


a
Hypoxia vs. Normo
Re
Ex
PDK4
GPD1
PFKM
PRKAG3
DLAT
PPP1R3C
PGAM2
GPD2
FBP2
PHKG1
TNNI1
TNNT1
TNNT3
MYBPC2
MYH1
MYOZ2
SYNM
DSTN
FLNA
PALLD
ABRA
MYL9
MYF6
KIF2A
FOS
EGR1
MYOG
ACTG2
CALD1
CNN1
TAGLN
MYH11
Glucose metabolism
Fiber type
Cell architecture
Cell proliferation and differentiation
Smooth muscle
Log2 ratio
-1
0
1
2


b
At rest
0
2
4
6
8
-log10 (p-value)
GO_BP
Glucose metabolic process
Gluconeogenesis
Glycogen biosynthetic process
GO_CC
Glycerol-3-phosphate dehydrogenase complex
GO_MF
Glycerol-3-phosphate dehydrogenase activity
KEGG
Glycolysis / Gluconeogenesis
• Percentage of intersection
0
2
4
6
8
10

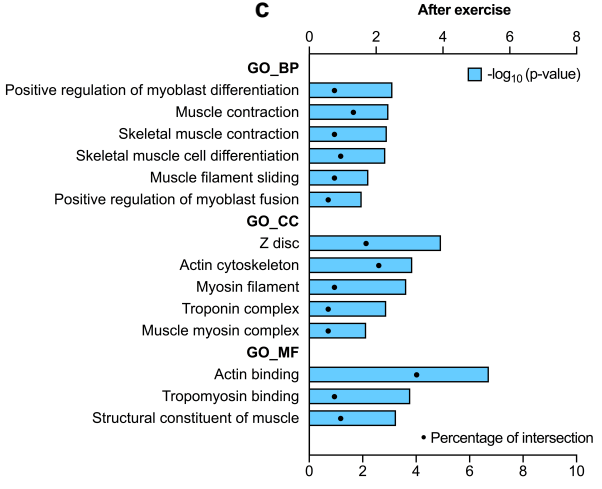

c
After exercise
0
2
4
6
8
-log10 (p-value)
GO_BP
Positive regulation of myoblast differentiation
Muscle contraction
Skeletal muscle contraction
Skeletal muscle cell differentiation
Muscle filament sliding
Positive regulation of myoblast fusion
GO_CC
Z disc
Actin cytoskeleton
Myosin filament
Troponin complex
Muscle myosin complex
GO_MF
Actin binding
Tropomyosin binding
Structural constituent of muscle
• Percentage of intersection
0
2
4
6
8
10

Figure 5

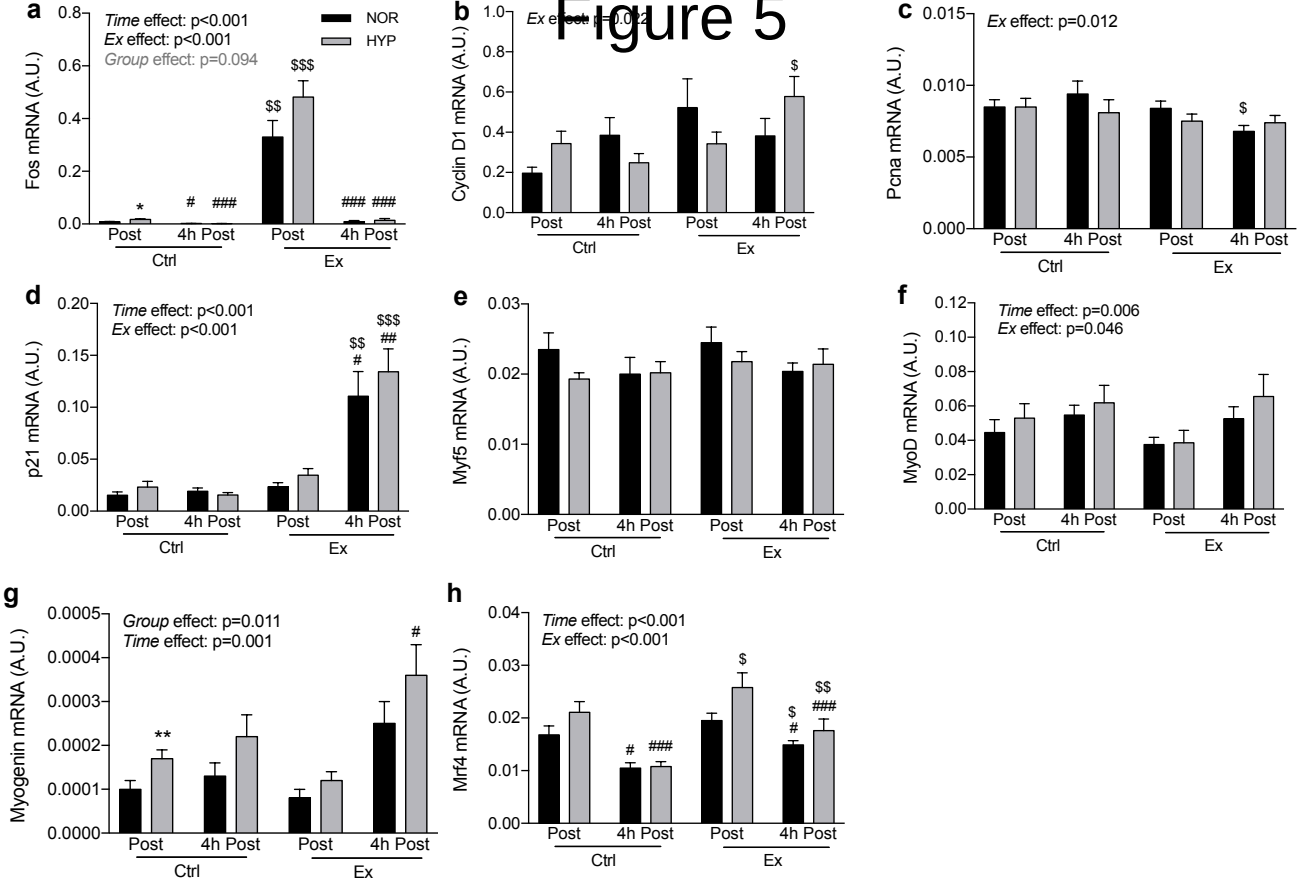
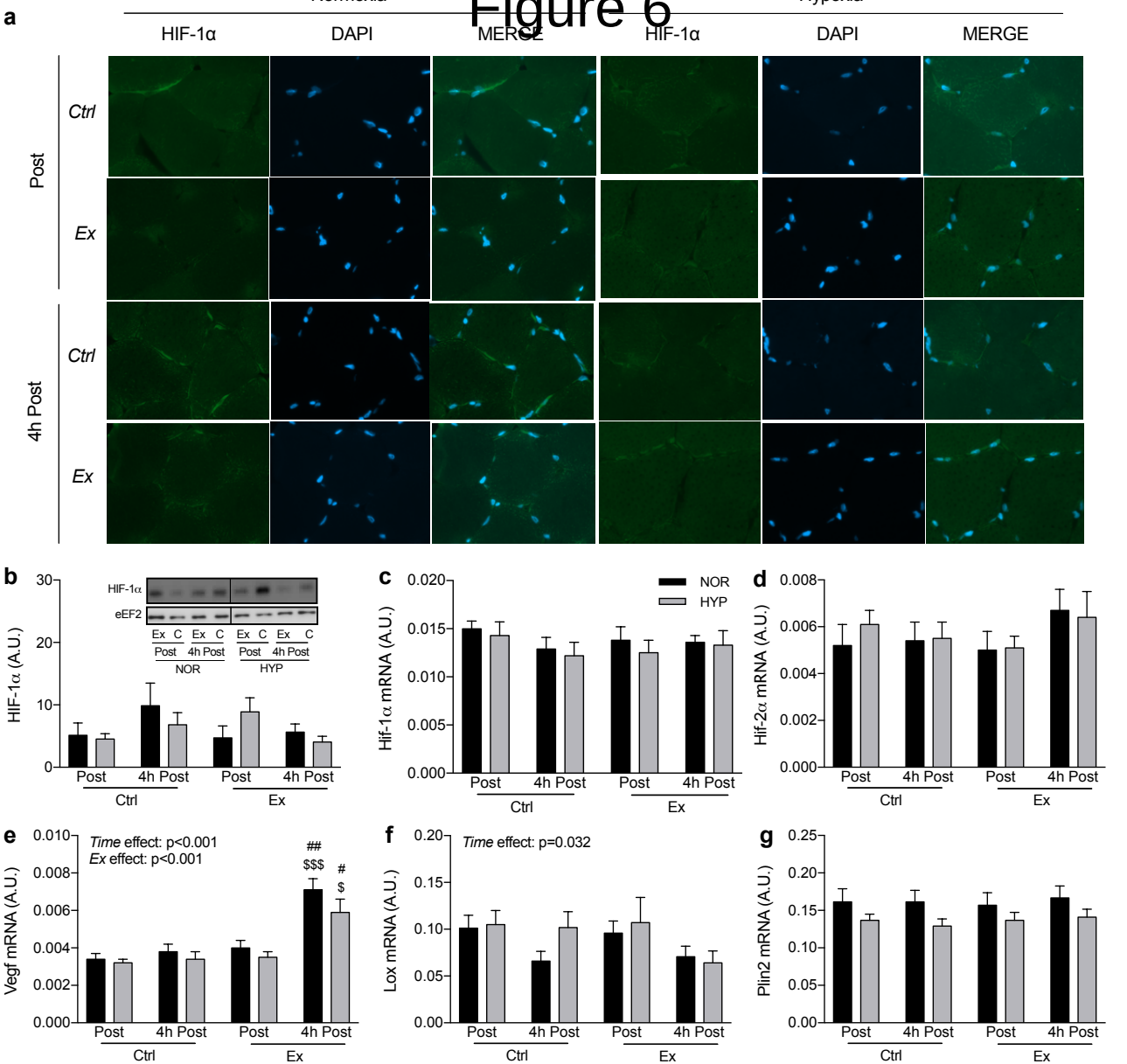
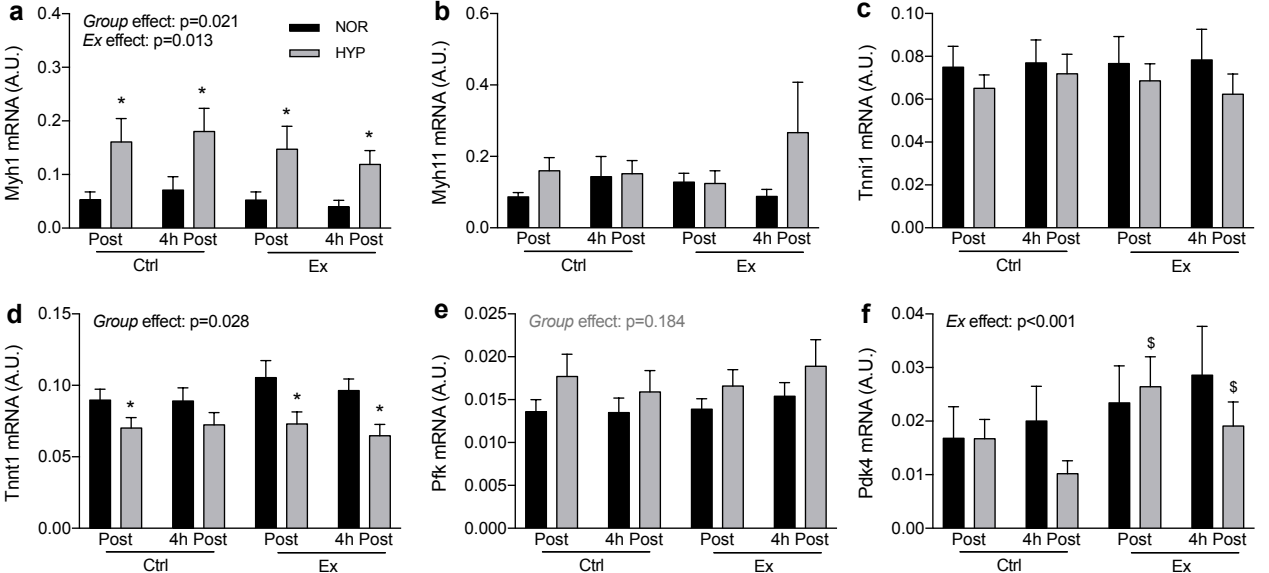

a
Fos mRNA (A.U.)
Time effect: p<0.001
Ex effect: p<0.001
Group effect: p=0.094
NOR
HYP
Post
4h Post
Ctrl
Ex
b
Cyclin D1 mRNA (A.U.)
Ex effect: p=0.021
c
Pcna mRNA (A.U.)
Ex effect: p=0.012
d
p21 mRNA (A.U.)
Time effect: p<0.001
Ex effect: p<0.001
e
Myf5 mRNA (A.U.)
f
MyoD mRNA (A.U.)
Time effect: p=0.006
Ex effect: p=0.046
g
Myogenin mRNA (A.U.)
Group effect: p=0.011
Time effect: p=0.001
h
Mrf4 mRNA (A.U.)
Time effect: p<0.001
Ex effect: p<0.001

Figure 6


a
Normoxia
Hypoxia
HIF-1α
DAPI
MERGE
HIF-1α
DAPI
MERGE
Post
4h Post
Ctrl
Ex
Ctrl
Ex
b
HIF-1α (A.U.)
HIF-1α
eEF2
Ex C Ex C Ex C Ex C
Post 4h Post Post 4h Post
NOR
HYP
Post 4h Post Post 4h Post
Ctrl
Ex
c
Hif-1α mRNA (A.U.)
NOR
HYP
Post 4h Post Post 4h Post
Ctrl
Ex
d
Hif-2α mRNA (A.U.)
Post 4h Post Post 4h Post
Ctrl
Ex
e
Vegf mRNA (A.U.)
Time effect: p<0.001
Ex effect: p<0.001
##
$$$
#
$
Post 4h Post Post 4h Post
Ctrl
Ex
f
Lox mRNA (A.U.)
Time effect: p=0.032
Post 4h Post Post 4h Post
Ctrl
Ex
g
Plin2 mRNA (A.U.)
Post 4h Post Post 4h Post
Ctrl
Ex


a
Group effect: p=0.021
Ex effect: p=0.013
NOR
HYP
Myh1 mRNA (A.U.)
0.4
0.3
0.2
0.1
0.0
Post
4h Post
Post
4h Post
Ctrl
Ex
b
Myh11 mRNA (A.U.)
0.6
0.4
0.2
0.0
Post
4h Post
Post
4h Post
Ctrl
Ex
c
Tnni1 mRNA (A.U.)
0.10
0.08
0.06
0.04
0.02
0.00
Post
4h Post
Post
4h Post
Ctrl
Ex
d
Group effect: p=0.028
Tnnt1 mRNA (A.U.)
0.15
0.10
0.05
0.00
Post
4h Post
Post
4h Post
Ctrl
Ex
e
Group effect: p=0.184
Pfk mRNA (A.U.)
0.025
0.020
0.015
0.010
0.005
0.000
Post
4h Post
Post
4h Post
Ctrl
Ex
f
Ex effect: p<0.001
Pdk4 mRNA (A.U.)
0.04
0.03
0.02
0.01
0.00
$
$
Post
4h Post
Post
4h Post
Ctrl
Ex

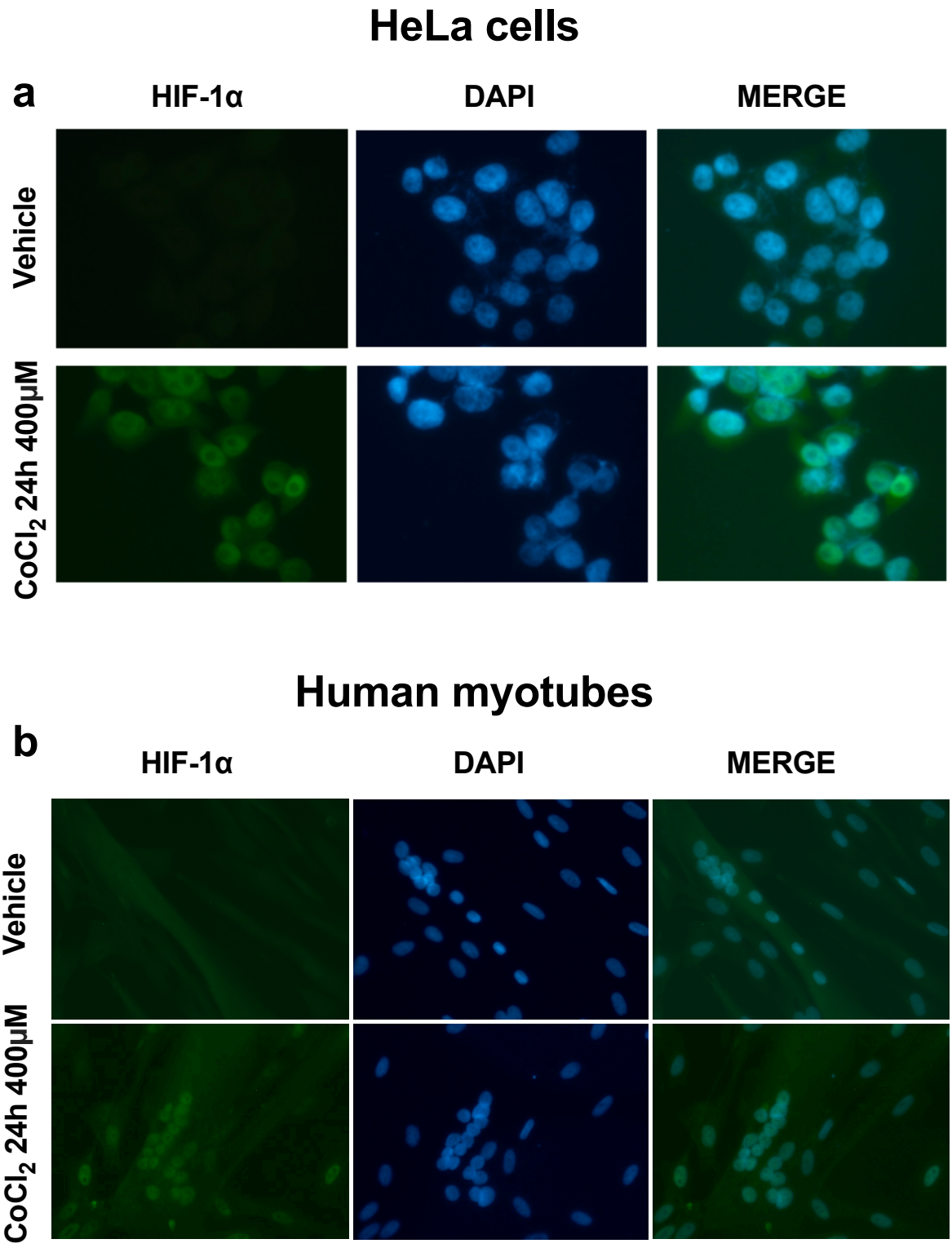

HeLa cells
a
HIF-1α
DAPI
MERGE
Vehicle
CoCl2 24h 400μM
Human myotubes
b
HIF-1α
DAPI
MERGE
Vehicle
CoCl2 24h 400μM

Table 1. Primer sequences

	Forward	Reverse
Atg12	AGT AGA GCG AAC ACG AAC CAT C	CCA TCA CTG CCA AAA CAC TCA T
Bnip3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
Bnip3L	CCA AGG AGT TCC ACT TCA GAC	AGT AGG TGC TGG CAG AGG GTG T
Cyclin D1	GAA CAA GCT CAA GTG GAA CC	CAC AGA GGG CAA CGA AGG
Fos	GGC AAG GTG GAA CAG TTA TC	TCT CCG CTT GGA GTG TAT C
Hif-1α	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG
Hif-2α	AAG CTG AAG CGA CAG CTG GAG TAT	GTA CAT TTG CGC TCA GTG GCT TGT
Lc3b	AAT CCC GGT GAT AAT AGA ACG A	GGA GAC GCT GAC CAT GCT GT
Lox	GCA CAG TTG TCA TCA ACA TTA C	GAT GTC CTG TGT AGC GAA TG
Mafbx	CGA CCT CAG CAG TTA CTG CAA	TTT GCT ATC AGC TCC AAC AG
Mrf4	AAT CTT GAG GGT GCG GAT TTC CTG	TGC TCC TCC TTC CTT AGC CGT TAT
Murf-1	AAA CAG GAG TGC TCC AGT CGG	CGC CAC CAG CAT GGA GAT ACA
Myf5	TGA GAG AGC AGG TGG AGA ACT ACT	AGA CAG GAC TGT TAC ATT CGG GCA
Myh1	CAC ACT AGT TTC ACA GCT CTC	GGC ACT CTT GGC CTT TAT C
Myh11	GGA GGA GCA GCT ATC CAT ATT	GCC TGG TCT GTG TTT CTT TC
MyoD	TGC CAC AAC GGA CGA CTT CTA TGA	AAG TGC GAG TGC TCT TCG GGT TT
Myogenin	AAA CTA CCT GCC TGT CCA CCT C	ACA CCG ACT TCC TCT TAC ACA CCT
p21	GCA GAC CAG CAT GAC AGA TTT	GGA TTA GGG CTT CCT CTT GGA
Pcna	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
Pdk4	CAG CTA CTG GAC TTT GGT TC	CTA ATT GGG TCG GGA GGA TA
Pfk	ATT TGA CGA AGC CCT GAA G	GTG CGA ACC ACT CTT AGA TAC
Plin2	CTC ATG TCC TCA GCC TAT CT	TAG GCA GTC TCT CCT CAA TC
Rpl19	CGC TGT GGC AAG AAG AAG GTC	GGA ATG GAC CGT CAC AGG C
Rpl4	ATA CGC CAT CTG TTC TGC CCT	GCT TCC TTG GTC TTC TTG TAG CCT
Tnni1	CAA CAC CAG GGA GAT TAA GG	ATG GAC ACC TTG TGC TTG
Tnnt1	GAG CTG TCG GAC TGG AT	ACA GCA CGT TGA TCT CAT ATT
Vegf	TTT CTG CTG TCT TGG GTG CAT TGG	ACC ACT TCG TGA TGA TTC TGC CCT

Atg12, autophagy-related protein 12; Atg4b, autophagy-related protein 4b; Bnip3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; Bnip3L, Bnip3 like; Hif-1α, hypoxia-inducible factor 1 alpha; Hif-2α, hypoxia-inducible factor 2 alpha; Lc3b, microtubule-associated proteins 1A/1B light chain 3B; Lox, lipoxygenase; Mafbx, muscle atrophy F box; Mrf4, muscle regulatory factor 4; Murf-1, muscle ring finger protein-1; Myf5, myogenic factor 5; Myh1, myosin heavy chain IIx/D; Myh11, myosin heavy chain polypeptide 11, smooth muscle; Pcna, proliferating cell nuclear antigen; Pdk4, pyruvate dehydrogenase kinase isoform 4; Pfk, phosphofructokinase; Plin2, perilipin 2; Redd1, protein regulated in development and DNA damage response 1; Rpl19, ribosomal protein L19; Rpl4, ribosomal protein L4; Tnni1, troponin I1, slow skeletal type; Tnnt1, troponin T1, slow skeletal type; Vegf, vascular endothelial growth factor.

Table 2. Tissue oxygenation and heart rate during exercise

	SpO_2 (%)		TSI (%)		HR (beats/min)	
	NOR	**HYP**	**NOR**	**HYP**	**NOR**	**HYP**
Baseline	98.2 ± 0.1	93.3 ± 0.4***	61.3 ± 0.9	61.4 ± 0.8	73.2 ± 3.1	85.1 ± 4.2
Set 1	98.2 ± 0.2	93.1 ± 0.4***	48.8 ± 1.1$^{###}$	50.4 ± 1.5$^{###}$	92.6 ± 2.3$^{###}$	101.6 ± 3.6$^{###}$
Set 2	98.0 ± 0.1	94.6 ± 0.3$^{\#,***}$	51.1 ± 1.1$^{###}$	49.4 ± 1.9$^{###}$	93.3 ± 3.5$^{###}$	100.5 ± 4.6$^{###}$
Set 3	98.0 ± 0.1	95.1 ± 0.4$^{###,***}$	50.3 ± 1.3$^{###}$	50.1 ± 1.8$^{###}$	92.5 ± 4.1$^{###}$	101.2 ± 5.1$^{###}$
Set 4	98.0 ± 0.1	94.9 ± 0.5$^{\#,***}$	51.8 ± 1.4$^{###}$	50.5 ± 1.7$^{###}$	95.5 ± 3.3$^{###}$	101.7 ± 4.7$^{###}$
Set 5	98.3 ± 0.1	94.7 ± 0.3$^{\#,***}$	50.9 ± 1.1$^{###}$	50.6 ± 1.8$^{###}$	98.8 ± 3.9$^{###}$	104.6 ± 5.3$^{###}$
Set 6	98.3 ± 0.1	95.1 ± 0.4$^{###,***}$	52.4 ± 0.9$^{###}$	51.4 ± 1.5$^{###}$	97.4 ± 4.5$^{###}$	106.7 ± 5.1$^{###}$
Set 7	98.4 ± 0.1	94.9 ± 0.4$^{\#,***}$	52.5 ± 0.9$^{###}$	52.4 ± 1.7$^{###}$	102.6 ± 4.8$^{###}$	106.7 ± 4.6$^{###}$
Set 8	98.4 ± 0.1	94.6 ± 0.5$^{\#,***}$	54.0 ± 0.7$^{###}$	52.5 ±1.6$^{###}$	106.2 ± 6.1$^{###}$	109.3 ± 5.1$^{###}$

SpO_2, blood oxygenation; TSI, tissue saturation index; HR, heart rate; NOR, normoxia; HYP, hypoxia. $^{\#}p<0.05$, $^{###}p<0.001$ vs Baseline; $^{*}p<0.05$, $^{***}p<0.001$ vs NOR.

Table 3. Plasma insulin and cortisol concentrations

		Fasted	Post	2h Post	4h Post
Insulin	**NOR**	2.7 ± 0.5	12.3 ± 2.2$^{###}$	2.5 ± 0.5	1.9 ± 0.3
(mIU/ml)	**HYP**	2.8 ± 0.4	15.6 ± 1.9$^{###}$	4.9 ± 1.2	2.4 ±0.4
Cortisol	**NOR**	273 ± 33	167 ± 25$^{###}$	120 ± 17$^{##}$	128 ± 26$^{###}$
(ng/ml)	**HYP**	245 ± 29	160 ± 22$^{#}$	138 ± 19$^{###}$	139 ± 19$^{###}$

NOR, normoxia; HYP, hypoxia. $^{#}p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ vs Fasted