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Higher strength gain after hypoxic vs normoxic resistance training despite no changes in muscle thickness and fractional protein synthetic rate

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Abstract

Acute hypoxia has previously been suggested to potentiate resistance training-induced hypertrophy by activating satellite cell-dependent myogenesis rather than an improvement in protein balance in human. Here, we tested this hypothesis after a 4-week hypoxic vs normoxic resistance training protocol. For that purpose, 19 physically active male subjects were recruited to perform 6 sets of 10 repetitions of a one-leg knee extension exercise at 80% 1-RM 3 times/week for 4 weeks in normoxia (FiO_2: 0.21; n = 9) or in hypoxia (FiO_2: 0.135, n = 10). Blood and skeletal muscle samples were taken before and after the training period. Muscle fractional protein synthetic rate was measured over the whole period by deuterium incorporation into the protein pool and muscle thickness by ultrasound. At the end of the training protocol, the strength gain was higher in the hypoxic vs the normoxic group despite no changes in muscle thickness and in the fractional protein synthetic rate. Only early myogenesis, as assessed by higher MyoD and Myf5 mRNA levels, appeared to be enhanced by hypoxia compared to normoxia. No effects were found on myosin heavy chain expression, markers of oxidative metabolism and lactate transport in the skeletal muscle. Though the present study failed to unravel clearly the mechanisms by which hypoxic resistance training is particularly potent to increase muscle strength,

Abbreviations: B2M, beta-2-microglobulin; BSA, bovine serum albumin; CS, citrate synthase; CSA, cross-sectional area; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HPRT-1, hypoxanthine phosphoribosyltransferase 1; MCT, monocarboxylate transporter; MRF, myogenic regulatory factors; Myf5, myogenic factor 5; MyHC, myosin heavy chain; MyoD, myoblast determination protein; NIRS, near-infrared spectroscopy; OCT, optimal cutting temperature; Pax7, paired box 7; PBS, phosphate-buffered saline; RPL19, ribosomal protein L19; TSI, tissue saturation index.

it is important message to keep in mind that this training strategy could be effective for all athletes looking at developing and optimizing their maximal muscle strength.

KEYWORDS

deuterium, hypoxia, muscle thickness, myogenesis, protein synthesis

1 | INTRODUCTION

Environmental hypoxia is a condition characterized by low partial oxygen pressure (pO_2) levels, either by decreased atmospheric pressure at altitude, or by reduction of inspired oxygen fraction at sea level. Lower oxygen availability leads to adaptations within many tissues, including skeletal muscle.[1] Those adaptations depend on the duration of the exposure and the severity of hypoxia, the so-called hypoxic dose. At a macroscopic level, prolonged exposure to hypoxia is known to induce skeletal muscle atrophy, but maintaining physical activity and a proper diet during a long stay in hypoxic conditions may limit skeletal muscle mass loss.[2] In contrast, repeated resistance exercise in acute hypoxia may result in larger skeletal muscle hypertrophy and maximal strength gains than in normoxia.[3,4] Athletes willing to maximize the effects of resistance training on muscle mass do not hesitate to perform some of their sessions in hypoxic conditions, despite a limited amount of scientific evidence and a lack of comprehension. Only about ten articles dealing with resistance training in hypoxic conditions have been published so far and the mechanisms are largely unknown. Two recent reviews on the topic have concluded that there is still insufficient data to determine if and how hypoxia potentiates the effects of resistance training.[5,6]

Skeletal muscle mass is controlled by the balance between protein synthesis and degradation as well as the inclusion and myogenesis of satellite cells.[7] Looking at the anabolic effects of repeated resistance training in acute hypoxia, no study investigated how protein balance is regulated after several sessions. After one single exercise session, the protein synthesis rate seems to be blunted in environmental hypoxia compared to normoxia.[8,9] But what occurs acutely does not necessarily reflect long-term adaptations.[10] Therefore, how protein synthesis is regulated after repeated resistance exercise in environmental hypoxia still remains to be investigated.

Beyond the regulation of protein synthetic rate, environmental hypoxia is expected to modify the expression of specific proteins in response to exercise training. Particularly of interest in the present context are the different myosin heavy chain (MyHC) isoforms as well as markers for the oxidative and the glycolytic metabolism.[8,11] A previous microarray analysis from our laboratory highlighted an up-regulation of the expression of genes related to fiber typing and muscle contraction after a single-leg resistance exercise bout as well as the expression of genes related to glucose metabolism and glycolysis in the rested leg exposed to hypoxia vs normoxia.[8] Another study reported that despite similar gains in muscle cross-sectional area (CSA) after resistance training, hypoxic training induced higher capillary-to-fiber ratio, circulating vascular endothelial growth factor (VEGF) levels as well as muscular endurance compared to normoxic training, indicating that hypoxic resistance training not only promotes classical adaptations to resistance training but also some adaptations associated to endurance training. However, it should also be mentioned that a lack of effect of hypoxic resistance training on muscle CSA, hypoxia markers, glycolytic enzymes and myosin heavy chain isoforms has been reported as well.[12] Further investigation is thus necessary to better understand the mechanisms induced by hypoxic resistance training in skeletal muscle.

Finally, in addition to changes in protein balance and the expression of specific proteins as mentioned above, environmental hypoxia has been shown to enhance satellite cell myogenesis.[13-15] Myogenesis is defined as the process whereby satellite cells are activated, proliferated, differentiated into myocytes and fuse with existing myofibers, which may contribute to muscle growth and repair.[16] Each step is characterized by a sequential activation of paired box 7 (Pax7) and the myogenic regulators factors (MRF), namely myogenic regulatory factor 4 (Mrf4), myogenic factor 5 (Myf5), myoblast determination protein (MyoD), and myogenin.[17] We previously found that in human skeletal muscle, the mRNA levels of myogenin and Mrf4, both markers of satellite cell differentiation, were higher after a single resistance exercise in hypoxia vs normoxia [8] and that the inflammatory pathway could contribute to this regulation.[13] Altogether, our previous results suggested that acute hypoxia would potentiate resistance training-induced hypertrophy by activating satellite cell-dependent myogenesis rather than an improvement in protein balance.[8] As a follow-up study, we tested this hypothesis after a 4-week hypoxic vs normoxic resistance training protocol. Secondarily, we expected hypoxic resistance training to induce a larger strength gain compared to normoxic training via an increased expression of proteins involved in muscle contraction and substrate handling.

2 | MATERIALS AND METHODS

2.1 | Experimental design

2.1.1 | Participants

Nineteen physically active male subjects gave their written consent to voluntary participate in the experiment (Table 1).

The experiment was approved by the ethical committee of the UCLouvain and conducted in accordance with the declaration of Helsinki. The exclusion criteria were: cardiovascular or pulmonary disease, chronic medication, any contraindication for exertional physical activity doing resistance training or having been exposed to an altitude above 1500 m during the month before the experiment.

2.1.2 | Protocol

The subjects were randomly assigned to two groups, hypoxia (n = 10) or normoxia (n = 9). In total, 17 visits were planned: 3 pre-training visits, 12 training sessions, and 2 post-training visits.

2.1.3 | First visit

A week before the experiment, height and weight were determined and body mass index was calculated. The one-repetition maximum (1RM) of the right leg was determined on a knee extension device (Pro-Dual, Body Solid, Forest Park, IL, USA). Subcutaneous fat thickness of the vastus lateralis was measured using a skin fold caliper (Harpenden, Baty, Burgess Hill, West Sussex, UK) according to the procedure described by Müller et al[18], fat thickness determination was needed for correcting near-infrared spectroscopy (NIRS) data (see below) and was therefore measured exactly at the same place as muscle thickness. Vastus lateralis muscle thickness was measured by B-mode ultrasound (Acuson Sequoia 512, Siemens Healthineers, Beersel, Belgium) according to the procedure described by Yasuda et al.[19]

Second visit

The day before the experiment, saliva and blood samples were collected before ingestion of deuterium (2.5 mL/kg body weight spread over 2 hours; #613428, Sigma-Aldrich, Saint-Louis, MO, USA). A second sample of saliva was collected 1hour 30 minutes after ingestion of the last dose of deuterium. Thereafter, a standardized dinner was provided around 8.00 PM and the subjects were asked to fast until the next morning for their third visit.

TABLE 1 Pre-training characteristics of the participants

	Normoxia	Hypoxia
n	9	10
Age (years)	20.7 ± 0.8	21.2 ± 0.5
Size (m)	1.81 ± 0.06	1.82 ± 0.06
Weight (kg)	71.1 ± 3.1	73.3 ± 3.0
BMI (kg·m^2)	21.6 ± 0.8	21.9 ± 0.6
VLT (cm)	2.2 ± 0.1	2.1 ± 0.1
1RM (kg)	54.5 ± 4.4	54.3 ± 3.9

Note: Results are presented as the mean ± SEM.

Abbreviations: BMI, body mass index; VL, vastus lateralis; VLT, vastus lateralis thickness.

Third visit

The morning (8.00-10.00 AM) after the second visit, the subjects came to the laboratory in the fasted state. After collection of blood and saliva samples, a muscle sample was taken from the vastus lateralis.

Training sessions

The 12 training sessions were spread over 4 weeks (3×/week). Each session consisted in 6 sets of 10 repetitions of one leg knee extension at 80% 1RM in normoxic or hypoxic (FiO_2: 0.135, which corresponds to an altitude of 3500 m) conditions. At the beginning and at the end (week 4) of the training protocol, peripheral blood oxygen saturation (SpO_2, %) was monitored by pulse oximetry (TSM Tosca $tcpCO_2$-SpO_2 monitor, Medical ApS, Hadsund, Denmark) and muscle oxygenation was monitored by NIRS (Artinis Medical Systems, Elst, The Netherlands) to determine whether the adaptations to hypoxic training detected in skeletal muscle were dependent on changes in blood and/or muscle tissue oxygenation. Blood and muscle oxygenation were monitored in half of the subjects (n = 5 in normoxia and n = 5 in hypoxia) due to material and technical limitation. The results of SpO_2 and tissue saturation index (TSI, %) at baseline represent the average during 30 seconds preceding the start of the first set and the results during each set represent the average during the set. Deuterium top-up doses (0.9 mL/kg body weight) were given at the beginning of each week and saliva samples were taken before and 1 hour 30 minutes after each top-up doses.

Final visits

Two days after the last training session, the subjects received a standardized dinner and the day after, they reported to the laboratory in the fasted state. Body weight, subcutaneous fat thickness, and vastus lateralis muscle thickness were determined and saliva, blood, and muscle samples were collected as previously described. The 1RM of the right leg was determined on the same knee extension device as for the pre-training measures.

2.1.4 | Near-infrared spectroscopy

Changes in muscle oxygenation were measured by NIRS using the difference in absorption characteristics of light between 760 and 850 nm (Portamon, Artinis Medical Systems, Elst, The Netherlands). Skinfold was measured with Harpenden caliper in order to confirm that the distance between the NIRS apparatus and the muscle was not higher than 16 mm. As described in Brocherie et al,[20] 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.

2.1.5 | B-mode ultrasound

Vastus lateralis muscle thickness was measured by B-mode ultrasound (Acuson Sequoia 512, Siemens) according to the procedure described by Yasuda et al.[19] Briefly, a 10.0 MHz scanning head (5.5 cm length probe) was placed on the skin perpendicular to the tissue interface. The scanning head was coated with a transmission gel to provide acoustic contact without depressing the dermal surface. Vastus lateralis thickness was measured at the lower third of the distance between the greater trochanter and the lateral condyle. The perpendicular distance from the adipose tissue-muscle interface to the vastus lateralis-vastus intermedius interface was considered as the vastus lateralis thickness.

2.1.6 | Sample collection and storage

Blood

Samples were taken in an antecubital vein in two 5 mL EDTA tubes and immediately put on ice until centrifugation. After centrifugation at 4°C for 10 minutes at 3000 *g*, plasma was transferred to microtubes and stored at −80°C.

Saliva

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

Muscle biopsies

Vastus lateralis muscle biopsies were taken according to the modified Bergström technique with suction.[21] After local anesthesia (xylocaine 2% without adrenaline, Astra Zeneca, Cambridge, UK), a 5 mm incision was made with a scalpel and pressure was applied for 10 minutes 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 150-200 mg per sample. The samples were immediately frozen in liquid nitrogen and stored at −80°C. About 15-20 mg of each sample was embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, VWR, Radnor, PA, USA), frozen in liquid nitrogen-cooled isopropanol and stored at −80°C for histochemical analyses.

2.2 | Protein extraction

Muscle samples were homogenized with a Polytron mixer in ice-cold lysis buffer (20 mM Tris–HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM B-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1mM DTT, 1% Triton-X 100 and a complete protease inhibitor tablet), centrifuged at 10 000 *g* for 10 minutes at 4°C and the supernatant was aliquoted and stored at −80°C. The DC protein assay kit was used to determine the protein concentration of each sample using bovine serum albumin (BSA, Bio-Rad, Hercules, CA, USA) as a standard.

2.3 | Western blotting

Twenty to fifty μg proteins were separated by SDS-PAGE (8%-10% gels) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1h and incubated overnight at 4°C with the following antibodies: citrate synthase (CS, 1:1000, BSA 5%, 14309, Cell signaling,), eEF2 (1:1000, BSA 5%, 2332, Cell signaling), monocarboxylate transporter 1 (MCT1, 1:1000, BSA 5%, AB3538P, Millipore), MCT4 (1:1000, BSA 5%, AB3548P, Millipore), MyHCI (BA-D5, 1:2000, milk 5%, 2235587, DSHB), MyHCIIa (SC-71, 1:1000, milk 5%, 2147165, DSHB), MyoD (1:500, milk 5%, sc-377460, Santa Cruz), myogenin (1:1000, milk 5%, sc-576, Santa Cruz), Pax7 (supernatant, 1:100, milk 5%, 528428, DSHB), succinate dehydrogenase A (SDH-A; 1:10 000, milk 5%, sc-59687, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse (1:10 000, A9044, Sigma) or anti-rabbit (1:5000, A6154 Sigma) secondary antibodies were used for detection of proteins. Membranes were thereafter scanned, and proteins were quantified with GeneSnap softwares and tools (Syngene, Cambridge, UK). The results are presented as the ratio protein of interest/eEF2.

CS activity was measured through the reaction of CoA-SH, a product of citrate synthase, with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Protein lysates (2.5-10 μg) were incubated with acetyl-CoA, DTNB, and oxaloacetic acid at 37°C. Absorbance of 5-thio-2-nitrobenzoic acid was measured at 412 nm every minute for 60 minutes. CS activity is expressed in μmol/min/g protein.

2.4 | Fractional synthetic rate

Following the procedure described by Wilkinson et al based on oral administration of stable isotope deuterium oxide, the muscle protein synthetic rate was measured over the 4 week period.[22]

2.5 | RNA extraction and real-time PCR

About 20 mg of muscle biopsy sample was homogenized in 1 mL 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 1 µg RNA using the iScript cDNA Synthesis Kit from Bio-Rad Laboratories (Nazareth, Belgium), following the manufacturer's instructions. The primers used are presented in Table 2. PCR was run using the following conditions: 3 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C. All samples were run in duplicate, and each reaction was processed in a 10 µL volume containing 4.8 µL SsoAdvanced Universal SYBR Green SuperMix (Bio-Rad Laboratories), 0.1 µL of each primer (100 nM final), and 5 µL 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, the gene expression of ribosomal protein L19 (RPL19), hypoxanthine phosphoribosyltransferase 1 (HPRT-1), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified. The results of each gene of interest were normalized to the geometric mean of the four normalization genes,[23] the expression of each not being influenced by the experimental conditions.

2.6 | Immunofluorescence

Sections of 6 µm from OCT-embedded tissue were cut at −20°C using a cryostat microtome (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA, USA) and put on histological blades. The blades were dried at room temperature for 1 hour and stored at −80°C upon immunofluorescence analysis. Tissues sections were washed 2 × 5 minutes in phosphate-buffered saline (PBS) and blocked for 30 minutes at room temperature in PBS with 10% BSA, 10% normal goat serum, and 5% Triton. After blocking and washing, sections were incubated overnight at 4°C with the primary antibodies MyoD (1:250, IgG2b anti-mouse, sc-377460, Santa-Cruz, Dallas, TX, USA) and Pax7 (1:5, supernatant, IgG1 anti-mouse, 528428, DSHB, Iowa City, IA, USA) for satellite cell identification and BA-D5 (MyHCI, concentrate, 1:200, 2235587, DSHB), SC-71 (MyHCIIa, concentrate, 1:500, 2147165, DSHB) and laminin (1:1000, NB300-144, Novus/Interchim, Montluçon, France) for muscle fiber typing. Of note, unstained muscle fibers were considered as containing MyHCIIx. Sections were washed 2 × 5 minutes and incubated for 1h at room temperature with the secondary antibody Alexa Fluor 488 anti-mouse IgG2b (1:250, A-21141, Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 568 anti-mouse IgG1 (1:250, A21124, Invitrogen) for satellite cell identification and Alexa Fluor 488 anti-rabbit IgG (1:500, A-11008, Invitrogen), Alexa Fluor 350 anti-mouse IgG2b (1:500, A-21140, Invitrogen), and Alexa Fluor 568 anti-mouse IgG1 (1:500, A21124, Invitrogen) for muscle fiber typing. Finally, tissue sections were washed 2 × 5 minutes and covered with an antifade mounting medium containing DAPI (Vectashield, Labconsult, Brussels, Belgium). Blades were stored for 24 hours at 4°C before visualization with the microscope Axio Vert A1 (Zeiss, Oberkochen, Deutschland) equipped with an AxioCam ICm1 camera and ZEN lite 2011 (Zeiss) software. Image processing and analysis were realized with ImageJ.

TABLE 2 Primers sequences

Gene	Forward	Reverse
B2M	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG
GADPH	CAT GTT CGT CAT GGG TGT GAA CCA	AGT GAT GGC ATG GAC TGT GGT CAT
HPRT-1	TCC TTG GTC AGG CAG TAT	ATC CAA CAA AGT CTG GCT TAT
Mrf4	AAT CTT GAG GGT GCG GAT TTC CTG	TGC TCC TCC TTC CTT AGC CGT TAT
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
Myh2	TGT TGA AGA GTG CAG AAA CTG AG	TGG CAA GTT CGT CTT TAA TTT TC
Myh7	ACA CCC TGA CTA AGG CCA AA	GTC CAT GCG CAC CTT CTT
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
PAX7	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
RPL19	CGC TGT GGC AAG AAG AAG GTC	GGA ATG GAC CGT CAC AGG C

Abbreviations: B2M, beta-2-microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT-1, hypoxanthine phosphoribosyltransferase 1; Mrf4, muscle regulatory factor 4; Myf5, myogenic factor 5; Myh, Myosin heavy chain; MyoD, myogenic differentiation; PAX7, paired box 7; RPL19, ribosomal protein L19.

2.7 | Statistics

All values are expressed as the mean ± SEM. Except for SpO_2 and TSI, for which n = 5 in normoxia and n = 5 in hypoxia, for all other variables, n = 9 in normoxia and n = 10 in hypoxia. Mixed linear models were applied on the data, with groups (normoxia or hypoxia), condition (rest and exercise), and time (pre-training and post-training) as fixed effects and the subjects as random effect. Statistical significance was fixed at $P < .05$. Statistical analyses were performed with lme4 and lmerTest library in R. Graphs were realized with ggplot2 library in R. Training effect sizes were calculated according to Cohen's d and interpreted using the following classification[24]: d < 0.01 = very small, 0.01 < d < 0.2 = small, 0.2 < d < 0.5 = medium, 0.5 < d < 0.8 = large, 0.8 < d < 1.2 = very large, and 1.2 < d < 2.0 = huge. All main effects and effect sizes (Cohen's d values) are reported in Supporting Information file 1.

3 | RESULTS

3.1 | Environmental hypoxia exacerbates the drop in tissue oxygenation during resistance exercise

At baseline and during each set, SpO_2 was about 98% in normoxia and about 94% in hypoxia ($P < .001$ vs normoxia), with no difference between the first and the last training (Table 3). At baseline, the TSI was about 10% lower in hypoxia compared to normoxia during the last training only ($P = .025$). While no drop was measured during the first training, during the last training, the TSI decreased from 66% at baseline to 44%-47% during resistance exercise in normoxia ($P < .001$) and from 56% to 35%-41% in hypoxia ($P < .01$). During the last training, the TSI in the hypoxic group was lower than in the normoxic group during S1 ($P = .015$), S3 ($P = .043$), and S4 ($P = .032$) and lower than during the first training during S3 ($P = .008$), S4 ($P = .032$), and S5 ($P = .032$).

3.2 | Additional effect of environmental hypoxia on resistance training-induced increase in maximal strength

The maximal strength gain after 4-week resistance training was higher in hypoxia (+18.5%) compared to normoxia (+12.8%) ($P = .010$, Figure 1A). The latter observation could not be explained by a higher vastus lateralis thickness in hypoxia vs normoxia as no change in muscle thickness was observed in any condition (Figure 1B). Neither did contribute a higher protein accretion as the fractional synthetic rate was similarly higher in the exercised vs the rested leg post-training in normoxia (+0.19%/day, $P < .001$) as in hypoxia (+0.17%/day, $P = .016$), with no difference between both groups (Figure 1C).

3.3 | Resistance training regulates myosin heavy chain expression at the mRNA but not at the protein levels

While environmental hypoxia up-regulated the gene expression of Myh1, coding for MyHCIIx, after a single resistance exercise,[8] we did not confirm this result after a 4-week training period (Figure 2A). Oppositely, a decrease in Myh1 mRNA levels post-training compared to pre-training was found in the hypoxic group ($P = .018$). In addition, a tendency toward lower Myh1 mRNA levels was found in the exercised compared to the rested leg in both normoxia ($P = .097$) and hypoxia ($P = .068$). From pre- to post-training, Myh2 mRNA levels, coding for MyHCIIa, tended to increase in both normoxia ($P = .070$) and hypoxia ($P = .070$) (Figure 2B). In the normoxic group, Myh2 mRNA levels tended to be higher in the exercised vs the rested leg post-training ($P = .092$). In the hypoxic group, Myh7 mRNA levels, coding for MyHCI, was higher post-training in the rested leg compared to normoxia ($P = .020$, Figure 2C). In the normoxic group, Myh7 mRNA levels were higher in the exercised vs the rested leg post-training ($P = .023$). The tendency toward an up-regulation of the oxidative phenotype (Myh2/7) and a down-regulation of the glycolytic phenotype (Myh1) in both groups was not confirmed at the protein levels as none of the conditions did modify the expression of MyHCI (Figure 2D,F) and MyHCIIa (Figure 2E,F), as quantified by western blotting. Of note, MyHCIIx was not detectable by western blotting in our conditions.

3.4 | Minor modulation of fiber type distribution by environmental hypoxia

No difference in the area of type I, IIa, and IIx was found between pre- to post-training nor between the normoxic and the hypoxic group (Table 4). Fiber type distribution was slightly modulated at 3 levels: (1) in the rested leg in normoxia, the percentage of type I fibers decreased ($P = .026$) while the percentage of type IIa fibers increased ($P = .031$) after 4 weeks; (2) in normoxia, the percentage of type IIx fibers was lower post-training in the exercised vs the rested leg ($P = .036$); (3) in the rested leg post-training, the percentage of type I was higher ($P = .038$) and the percentage of type IIa fiber was lower ($P = .011$) in the hypoxic vs the normoxic group. In summary and focusing on the effects of environmental hypoxia, hypoxic exposure resulted in a slightly higher proportion of type I and lower proportion of type IIa compared to normoxic exposure.

TABLE 3 Blood and skeletal muscle oxygenation

	SpO_2 (%)				TSI (%)			
	First training		Last training		First training		Last training	
Set	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
BL	98.8 ± 0.2	94.2 ± 0.7$^{###}$	97.9 ± 0.3	93.0 ± 1.8$^{###}$	57.7 ± 6.6	49.3 ± 4.2	66.3 ± 2.5	56.0 ± 2.7$^{\#}$
			d = −1.490	d = −0.411			d = 0.779	d = 0.845
S1	99.0 ± 0.2	94.2 ± 0.4$^{###}$	98.3 ± 0.2	92.7 ± 2.0$^{###}$	44.6 ± 2.9	40.0 ± 3.2	46.5 ± 1.7***	39.2 ± 1.6$^{**,\#}$
			d = −1.570	d = −0.476			d = 0.355	d = −0.134
S2	98.6 ± 0.2	94.5 ± 0.5$^{###}$	98.4 ± 0.3	93.8 ± 1.2$^{###}$	49.7 ± 2.9	41.5 ± 1.8$^{\#}$	45.2 ± 2.7***	38.6 ± 2.0**
			d = −0.392	d = −0.340			d = −0.729	d = −0.685
S3	98.4 ± 0.2	94.6 ± 0.5$^{###}$	98.5 ± 0.3	93.1 ± 1.4$^{###}$	52.4 ± 2.7	50.5 ± 1.2	46.5 ± 2.6***	36.8 ± 3.1$^{**,\#,\$\$}$
			d = 0.014	d = −0.674			d = −0.994	d = −2.590
S4	98.3 ± 0.3	94.2 ± 0.3$^{###}$	98.5 ± 0.2	94.2 ± 0.9$^{###}$	50.7 ± 3.9	46.6 ± 3.1	44.3 ± 1.4***	35.8 ± 2.7$^{***,\#,\$}$
			d = 3.341	d = 0.052			d = −0.989	d = −1.679
S5	98.5 ± 0.2	94.0 ± 0.5$^{###}$	98.3 ± 0.2	93.1 ± 0.9$^{###}$	49.9 ± 3.1	51.2 ± 2.6	47.2 ± 2.4***	41.4 ± 2.1
			d = −0.375	d = −0.498			d = −0.442	d = −1.823
S6	98.5 ± 0.2	93.6 ± 0.5$^{###}$	98.4 ± 0.2	93.6 ± 1.2$^{###}$	53.1 ± 2.6	48.1 ± 1.8	45.0 ± 1.9***	40.8 ± 2.8**
			d = −0.138	d = 0.005			d = −1.569	d = −1.398

Note: Data are presented as the mean ± SEM.

Abbreviations: BL, baseline; S, set; SpO_2, blood oxygenation; TSI, tissue saturation index.

$^{**}P < .01$; $^{***}P < .001$ vs baseline;

$^{\#}P < .05$;

$^{###}P < .001$ vs Normoxia;

$^{\$}P < .05$;

$^{\$\$}P < .01$ vs First training.

3.5 | Markers for oxidative metabolism and lactate transport are unchanged by resistance exercise, no matter the environmental conditions

No effect was found on CS expression (Figure 3A,F), CS activity (Figure 3B), MCT1 (Figure 3C,F), MCT4 (Figure 3D,F), and SDH-A (Figure 3E,F) protein expression, suggesting that our hypoxic training protocol had probably no major impact, if any, on oxidative metabolism and lactate transport.

3.6 | Additional effects of environmental hypoxia on resistance exercise-induced regulation of satellite cells during early myogenesis

Post-training, the mRNA levels of Pax7 in the normoxic group (P =.066, Figure 4A) and the protein expression (P =.074, Figure 4B,I) in the hypoxic group tended to be higher in the exercised compared to the rested leg. Post-training, MyoD (Figure 4C) and Myf5 (Figure 4D) mRNA levels were higher in the hypoxic vs the normoxic group in the rested leg (P = .074 for MyoD and P = .011 for Myf5), and in the exercised leg for MyoD (P = .049). Compared to the rested leg, Myf5 mRNA levels were higher post-training in the normoxic group (P = .007). Resistance training increased Myf5 mRNA levels in the normoxic (P = .019) and hypoxic group (P = .026). The mRNA levels of myogenin (Figure 4E) and MRF4 (Figure 4F) tended to be higher pre-training (P = .088 and P = .063, respectively) and were higher post-training (P = .032 and P = .022, respectively) in the exercised vs the rested leg in the normoxic group. Compared to pre-training, MRF4 mRNA levels decreased in the rested leg in the normoxic group (P = .013) and increased in the exercised leg in the hypoxic group (P = .023). The specificity of the signals for Pax7, MyoD, and myogenin protein expression were confirmed with human cell protein extracts (Figure 4I). MyoD protein expression increased in the rested (P = .011) and the exercised (P = .005) leg in the normoxic group and tended to increase in the exercised leg in the hypoxic group (P = .060) after training (Figure 4G,I). In addition, MyoD expression was higher in the rested leg post-training in the hypoxic vs normoxic group (P = .038). Finally, myogenin protein expression remained unchanged in our conditions (Figure 4H,I). Altogether and specifically looking at the main effect of hypoxia, the preceding results

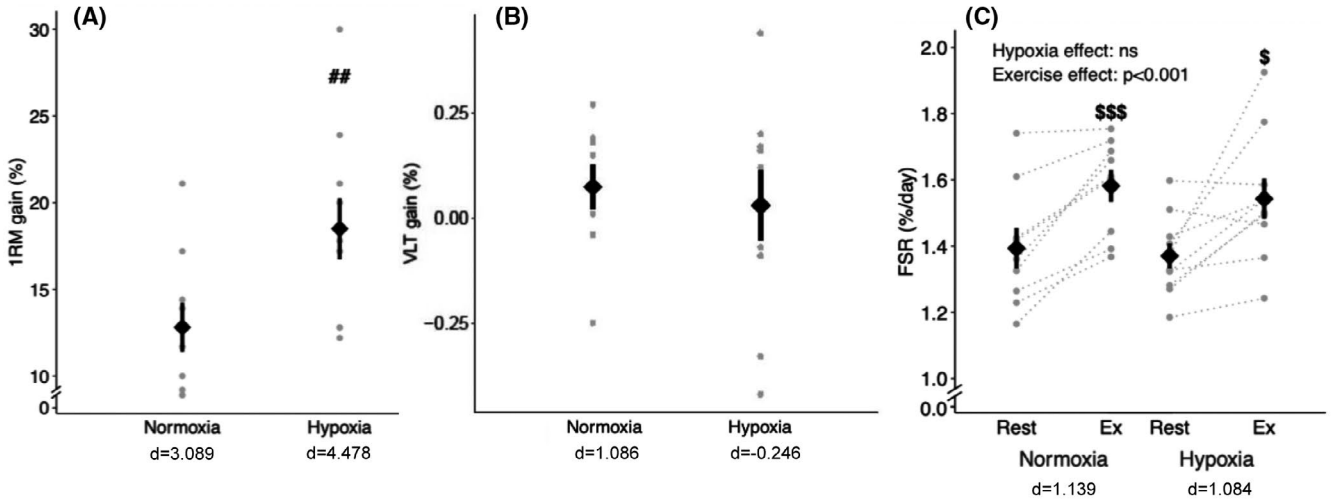

FIGURE 1 Changes in muscle strength, muscle thickness, and protein synthesis by hypoxia and exercise. A, One-repetition maximum (1RM, %) and (B) vastus lateralis thickness (VLT, %) gain; (C) fractional synthetic rate (FSR) (%/d) in the rested and exercised (Ex) legs after 4 weeks of resistance training in normoxia and in hypoxia. $^{\#\#}P < .01$ vs Normoxia; $^{\$}P < .05$, $^{\$\$\$}P < .001$ vs rest, d = training size effect

may be summarized as follows: MyoD ($P = .006$) and Myf5 ($P = .042$) mRNA levels were higher while Pax7 ($P = .070$) and MyoD ($P = .059$) protein expression tended to be lower in hypoxia than in normoxia. However, we did not confirm the latter result using immunofluorescence to detect the presence ($Pax7^+/MyoD^-$) and activation ($Pax7^+/MyoD^+$) of satellite cells in myofibers by co-staining Pax7, MyoD, and myonuclei (Figure 5). [25]

4 | DISCUSSION

Two recent reviews have concluded that there is still insufficient data to determine if and how hypoxia potentiates the effects of resistance training.[5,6] The present study aimed at extending previous results from our lab suggesting that acute hypoxia would potentiate resistance training-induced hypertrophy by activating satellite cell-dependent myogenesis rather than an improvement in protein balance.[8] As a follow-up study, we tested this hypothesis after a 4-week hypoxic vs normoxic resistance training protocol. Secondarily, we expected hypoxic resistance training to induce a larger strength gain compared to normoxic training via an increased expression of proteins involved in muscle contraction and substrate handling.

The main finding of the present study is the higher muscle strength gain after hypoxic compared to normoxic resistance training, which could neither be explained by a greater muscle thickness nor a faster accretion of muscle protein. It could be argued that the duration of the training protocol was not long enough to detect changes in muscle mass. We purposely chose a duration of 4 weeks as it was previously shown that 3-week resistance training induced significant gains in muscle thickness and protein accretion with diminished anabolic signaling responses from the third to the sixth week of training.[26] In addition, in a 6 week-long study investigating arm muscle hypertrophy in environmental hypoxia, a larger increase in arm flexor and arm extensor cross-sectional area has been reported as soon as after 3 weeks resistance training in hypoxia vs normoxia.[3] This said, differences in arm and leg muscle fiber type distribution may affect adaptations to resistance exercise. In healthy adults, the vastus lateralis contains about 50% of type I and 50% of type II muscle fibers, whereas the proportion of upper body muscles usually reaches 30% of type I and 70% of type II fibers.[27] Thereby, resistance training in arms seems more prone to induce muscle hypertrophy, at least more rapidly, than in legs.[28,29] It should also be highlighted that two studies[11,12] of four[3,11,12,30] did not find any difference in muscle cross-sectional area after hypoxic vs normoxic resistance training. An additional factor that could have contributed to the lack of effect on muscle mass in both normoxic and hypoxic conditions is the duration of the inter-set recovery period. Ideally, 30-60 seconds inter-set periods should be observed to promote muscle hypertrophy.[31] Studies using inter-set periods between 60 and 90 seconds[3,11,32] found an increase in muscle/lean mass but not with 120 seconds.[33] Here as well, 120 seconds between each set were used as this lap duration was necessary to stabilize blood and tissue oxygenation values. The latter measurements witness the severity of hypoxia, which is also an important factor to take into consideration in the present context. At rest and during exercise, blood oxygenation was lower in hypoxia than in normoxia, reaching a desaturation of 93%-94%, which is consistent with previous studies using similar hypoxic conditions.[3] Of note, exercise by itself did not lower blood but well muscle oxygenation. The tissue

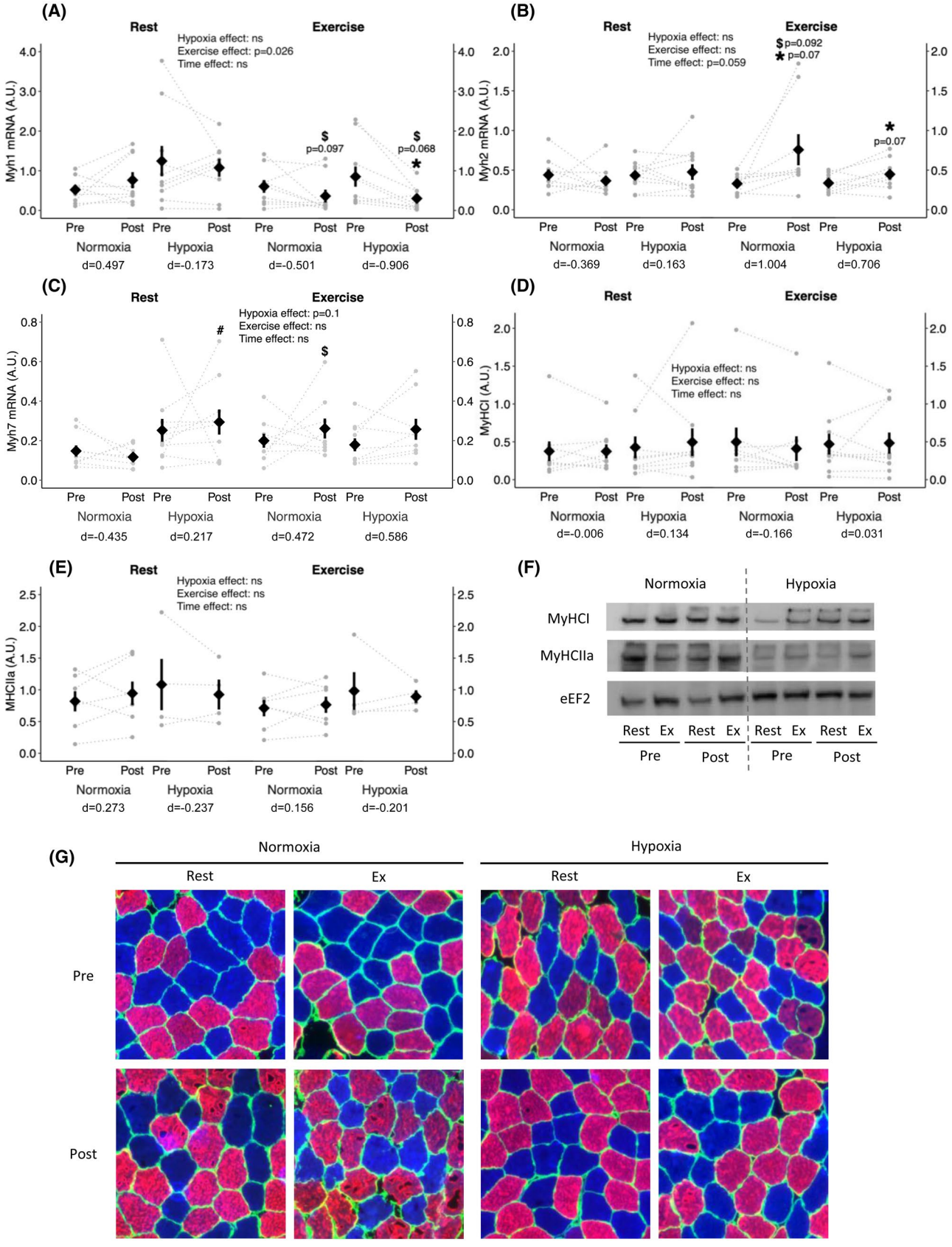

FIGURE 2 Regulation of myosin heavy chain by hypoxia and exercise. mRNA levels of (A) Myh1, (B) Myh2, and (C) Myh7 and protein expression of (D) myosin heavy chain I (MyHCI) and (E) MyHCIIa in the rested and exercised legs before (Pre) and after (Post) training in normoxia and in hypoxia. F, Representative Western blots. G, Representative immunofluorescence with laminin (green), MyHCI (blue), MyHCIIa (red), and MyHCIIx (black). AU, arbitrary units. $^{\#}P < .05$ vs Normoxia; $^{\$}P < .05$ vs rest; $^{*}P < .05$ vs Pre-training, d = training size effect

TABLE 4 Fiber type area and distribution

	Rest				Exercise			
	Normoxia		Hypoxia		Normoxia		Hypoxia	
Area (μm^2)	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Type I	4309 ± 475	4484 ± 430	3803 ± 302	4250 ± 395	4953 ± 519	4018 ± 476	4186 ± 432	4120 ± 321
		d = 0.138		d = 0.450		d = −0.710		d = −0.055
Type IIa	4990 ± 635	5180 ± 560	3952 ± 287	4301 ± 267	4717 ± 558	4538 ± 432	4158 ± 446	3964 ± 309
		d = 0.113		d = 0.445		d = −0.135		d = −0.159
Type IIx	3828 ± 595	4248 ± 601	3186 ± 465	3554 ± 344	2984 ± 618	3852 ± 1206	2873 ± 316	2963 ± 405
	d = 0.299		d = 0.357		d = 0.505		d = 0.100	
Distribution (%)								
Type I	40.8 ± 3.1	36.2 ± 3.0*	45.4 ± 4.0	46.9 ± 3.7#	45.0 ± 4.1	41.5 ± 4.5	44.4 ± 3.3	48.6 ± 5.1
		d = −0.517		d = 0.145		d = −0.277		d = 0.314
Type IIa	53.8 ± 2.4	57.9 ± 2.8*	46.5 ± 4.1	46.1 ± 3.0#	52.4 ± 3.4	56.1 ± 4.0	51.1 ± 3.2	48.9 ± 4.8
		d = 0.541		d = −0.041		d = 0.346		d = −0.171
Type IIx	5.5 ± 2.9	5.9 ± 1.9	8.0±2.7	7.0 ± 1.7	2.5 ± 1.0	2.4 ± 1.2$	4.5 ± 1.4	2.5 ± 1.3
		d = 0.065		d = −0.159		d = −0.009		d = −0.469

Note: Data are presented as the mean ± SEM.

#$P < .05$ vs Normoxia;

$$P < .05$ vs Rest;

*$P < .05$ vs Pre.

saturation index dropped at the beginning of the exercise in both normoxia and hypoxia. This is in line with a drop in tissue oxygenation reported at the onset of resistance exercise beyond 60% of maximal working rate.[34] In addition, during the last training session, muscle oxygenation was lower in the hypoxic vs the normoxic group. Those results confirm that hypoxia occurs in blood and muscle and that hypoxic training may induce additional effects to those of normoxic training in the skeletal muscle. Despite a lack of effect on muscle thickness and the fractional synthetic rate, hypoxic training could have induced a larger gain in muscle strength compared to normoxic training by favoring molecular adaptations related to muscle contraction, cell differentiation and/or energy metabolism.

Amongst those molecular adaptations, a switch in muscle fiber type from slower to faster fibers could contribute to a larger strength gain after resistance training in hypoxia, certainly as hypoxic exposure has previously been shown to induce a fiber switch toward the rapid phenotype in rodents.[35] In human, after an acute resistance exercise in hypoxia, the expression of genes encoding for the slow phenotype was decreased, whereas those encoding for the fast phenotype were up-regulated.[8] When analyzing the mRNA levels of Myh1, Myh2, and Myh7, which encode for MyHCIIx, MyHCIIa, and MyHCI, respectively, a trend toward up-regulation of the oxidative phenotype and down-regulation of the glycolytic phenotype was observed in both environmental conditions. Those tendencies were not translated into changes in the protein expression and cross-sectional area of the MyHC isoforms as no differences were found between the conditions and only minor modifications were found in fiber type distribution. Similarly, the increase in strength endurance capacity observed after a 4-week low-resistance/high repetition training in both normoxia and hypoxia was not matched by changes in MyHC mRNA levels, fiber type distribution or fiber cross-sectional area.[12] It is likely that 4 weeks is not long enough to induce a switch in muscle fiber type, as studies that observed an increase in type IIa together with a decrease in type I muscle fibers usually last from 6 to 12 weeks.[36] Even though our study was probably a bit too short to observe changes in muscle fiber types and distribution, the changes observed at the mRNA levels were opposite to those expected to explain the higher gain in muscle strength after hypoxic resistance training.

Based on the results from our previous microarray analysis after one single hypoxic resistance exercise[8] and the literature in the field,[11,37] we were interested in measuring the expression and activation of key markers of oxidative metabolism and lactate transport. Despite MyHC mRNA levels being regulated toward the oxidative phenotype, markers of oxidative metabolism remained unchanged after resistance training in both normoxia and hypoxia. To the best of our knowledge, no study investigated markers of the oxidative metabolism after hypoxic resistance training. Our results

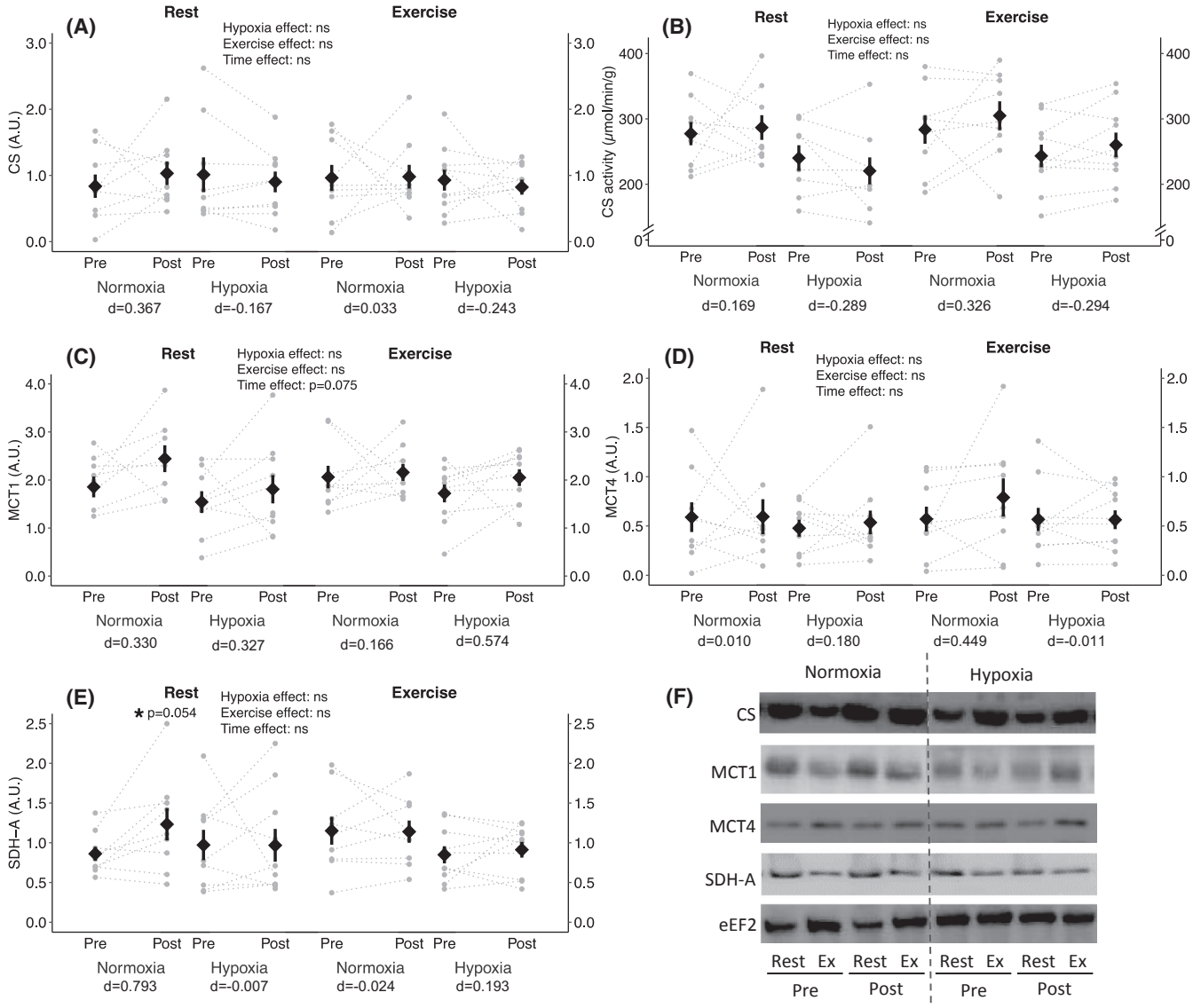

FIGURE 3 Regulation of markers of the oxidative metabolism and lactate transport by hypoxia and exercise. Protein expression of (A) citrate synthase (CS), (C) monocarboxylate transporter (MCT) 1, (D) MCT4 and (E) succinate dehydrogenase (SDH)-A and (B) citrate synthase activity in the rested and exercised legs before (Pre) and after (Post) training in normoxia and in hypoxia. (F) Representative Western blots. AU, arbitrary units. *$P < .05$ vs Pre-training, d = training size effect

tend to suggest that it is unaffected as it is the case for lactate transport, though we only indirectly assessed those processes via well-known markers. We hereby extend previous results showing that markers of the glycolytic metabolism, ie, the mRNA levels of phosphofructokinase and lactate dehydrogenase, were not modified after 4 weeks of resistance training in either hypoxia or normoxia.[12]

Finally, as the larger increase in muscle strength after hypoxic vs normoxic resistance training could not be explained by changes in muscle fiber phenotype or energy metabolism, we focused on activation, proliferation, and differentiation of satellite cells, which could create a favorable anabolic environment for further and delayed accretion of muscle mass. In other words, we hypothesized that a favorable anabolic environment could translate into muscle hypertrophy in a timeframe beyond the one of the present study. In addition, current evidences show that satellite cell activation and differentiation are necessary to induce adaptations to exercise training in skeletal muscles[38] and that hypoxia can regulate myogenesis.[13,39] Despite the latter evidence, few studies have investigated whether and how hypoxic exercise regulates myogenesis.[8,13,40] To the best of our knowledge, this study is the first to analyze the mRNA and protein expression of the MRF after hypoxic resistance training in humans. Here, we neither find any regulation of the protein level by hypoxia nor determined by western blotting or by immunofluorescence. However, after 4 weeks of resistance training, MyoD and Myf5 mRNA levels were higher in the hypoxic than in the normoxic group. MyoD and Myf5 characterize the proliferation of satellite cells.[17] Looking at markers for

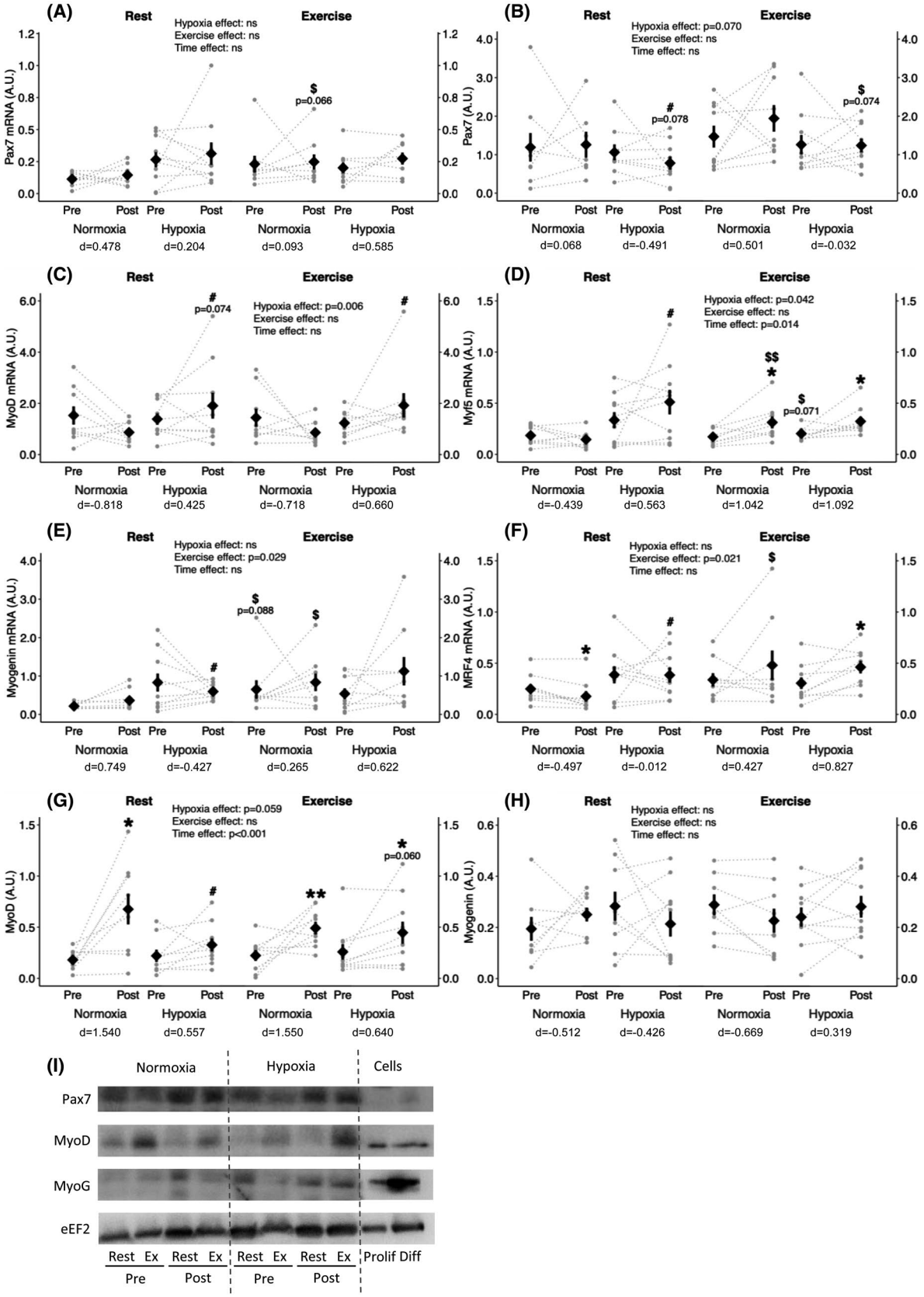

FIGURE 4 Regulation of the myogenic regulatory factors by hypoxia and exercise. mRNA levels of (A) Pax7, (C) MyoD, (D) Myf5, (D) myogenin and (F) MRF4 and protein expression of (B) Pax7, (G) MyoD and (H) myogenin in the rested and exercised legs before (Pre) and after (Post) training in normoxia and in hypoxia. I, Representative Western blots with proliferating (Prolif) and differentiating cells as control. $^{\#}P < .05$, $^{\#\#}P < .01$ vs Normoxia; $^{\$}P < .05$ vs rest; $^{*}P < .05$, $^{**}P < .01$ vs Pre-training, d = training size effect

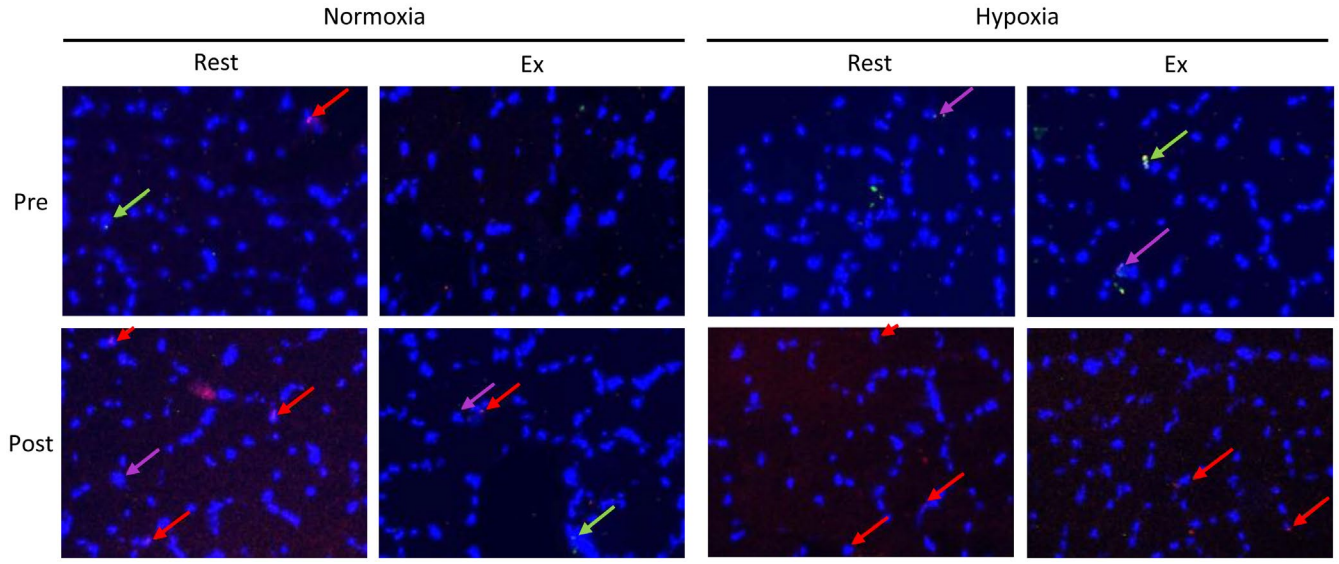

FIGURE 5 Localization and activation of satellite cells. Representative immunofluorescence pictures with myonuclei (blue), Pax7 (green arrow), and MyoD (red arrow)

cell differentiation, no changes were observed in myogenin in either hypoxia or normoxia, and MRF4 was regulated by exercise only, not by environmental conditions. Altogether, our results indicate that repeated hypoxic exposure and training rather regulates satellite cell proliferation and not differentiation. We previously found the opposite regulation after one acute hypoxic resistance exercise, which appeared to regulate satellite cell differentiation more than proliferation.[8] In vitro, the percentage of oxygen and therefore the severity of hypoxia is known to differently regulate proliferation and differentiation, with low oxygen levels between 3% and 6% seeming to favor proliferation and very low levels below 1% tending to alter differentiation and myotube formation.[41] Our present and previous results seem to suggest that the duration of hypoxia might differently regulate satellite cell proliferation and differentiation as well. Without any difference in muscle thickness, it is difficult to interpret the present results. It is possible that the higher expression of markers for satellite proliferation witnessed the initiation of myogenesis and a further inclusion of satellite cells beyond the duration of the present study. Although, after resistance training, satellite cells have been shown to participate in muscle hypertrophy as soon as after 4 weeks.[42] On the other hand, satellite cells may also contribute to muscle fiber adaptation and remodeling in the absence of hypertrophy. Six-week sprint interval training decreased the number of type II muscle fibers and increased the number of hybrid type I/II concomitantly to the number of active satellite cells, without any change in fiber cross-sectional area.[43] Based on the minor transcriptional regulation of the proliferation phase of satellite cells and the lack of changes at the protein levels, it is unlikely that satellite cells played a key role in the adaptations to 4 weeks hypoxic training.

Finally, as we could not explain the higher gain in muscle strength in hypoxia by changes in muscle thickness, protein synthesis or molecular adaptations related to muscle contraction, cell differentiation and/or energy metabolism, we hypothesized that hypoxic training induced neuromuscular adaptations beyond those induced by normoxic training. Indeed, especially in the first weeks of resistance training, muscle strength can be improved by a higher recruitment of muscle fibers.[44] In addition, recruitment of type II muscle fibers can be increased in response to lower oxygen availability.[45] Thereby, the higher muscle strength gain after hypoxic vs normoxic resistance training observed here could be due to an increased recruitment of type II fibers. Although plausible, this hypothesis was not tested as it was beyond the scope of the present study looking at changes in muscle mass and molecular adaptations. This certainly constitutes a limitation of the present study. Another limitation is that there was a large interindividual variability in the levels of the different markers at baseline and in response to resistance training in both normoxia and hypoxia, which probably contributed to the lack of effects.

In conclusion, though the present study failed to unravel the mechanisms by which hypoxic resistance training is particularly potent to increase muscle strength, the important message to keep in mind is that this training strategy could be interesting for all athletes looking at developing and optimizing their maximal muscle strength.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

P.J. Atherton, M. Francaux, and L. Deldicque designed the research and had primary responsibility for the final content; O. Gnimassou, G. Warnier, M. R. Belhaj., N. Benoit, D. Naslain, H. Nielens, and L. Deldicque conducted the research; S. van Doorslaer de ten Ryen, G. Warnier, O. Gnimassou, M. R. Belhaj, N. Benoit, D. Naslain, M. S. Brook, K. Smith, D.J. Wilkinson, and L. Deldicque analyzed and interpreted the data; S. van Doorslaer de ten Ryen and L. Deldicque wrote the manuscript; and all authors critically revised, contributed to, and approved the final version of the manuscript.

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