The FASEB Journal

RESEARCH ARTICLE

Regulation of Satellite Cells and Myogenesis in Response to Eccentric Resistance Exercise in Hypoxic Conditions in Healthy Young Men

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Received: 24 January 2026 | **Revised:** 20 April 2026 | **Accepted:** 8 May 2026

Keywords: blood flow restriction | exercise | hypoxia | myogenesis | regeneration | resistance | satellite cell | skeletal muscle

ABSTRACT

Satellite cells participate in myogenesis and contribute to skeletal muscle regeneration and hypertrophy. Amongst other stimuli, satellite cells can be activated by exercise and hypoxia. However, the cumulative effect of exercise on hypoxia on myogenesis is not well understood, certainly in humans. Furthermore, whether satellite cell activation and myogenesis differ between environmental hypoxia and blood flow restriction is not known. The purpose of this study was to analyze satellite cell and myogenic markers in response to acute eccentric resistance exercise in normoxia, normobaric environmental hypoxia, and with blood flow restriction (local hypoxia). Thirty-eight healthy young men were allocated to one of the three experimental conditions: normoxia ($n = 13$), normobaric environmental hypoxia ($n = 12$), and blood flow restriction ($n = 13$). They all performed 5 series of 15 repetitions at 60°/s for the knee extension and 30°/s for the knee flexion on an isokinetic dynamometer. Vastus lateralis muscle biopsies and blood samples were taken before, 1, 24, and 72 h after exercise. Myogenic regulatory factor expression was upregulated after exercise similarly in the normoxic and hypoxic groups and attenuated in the blood flow restriction group. Despite differential regulation of myogenic regulatory factor expression and circulating creatine kinase levels after eccentric resistance exercise, none of the investigated hypoxia markers and immediate early genes, inflammatory markers, growth factors, except insulin-like growth factor-1, and mitogen-activated protein kinase members were differently regulated between the groups. Contrary to our hypothesis, satellite cell activation and myogenesis were not potentiated by the combination of eccentric resistance exercise and hypoxic conditions.

1 | Introduction

Exercising in hypoxia at equal absolute loads induces a greater metabolic stress than in normoxia and is used by many athletes to foster systemic and intramuscular adaptations [1]. Over the years, various strategies to train intermittently in hypoxia while staying at sea level have emerged [2]. Amongst the different strategies that may be used to create hypoxic conditions at sea level, hypoxic rooms and blood flow restriction (BFR) are intensively used by athletes. Both have been shown to be potentially beneficial for muscle mass accretion and strength gain following resistance training, though results are sometimes equivocal [3, 4]. In the search of deciphering the mechanisms beyond the adaptations to hypoxic resistance training, we previously found that an acute hypoxic resistance exercise could regulate myogenesis [5], possibly via the inflammatory pathway [6]. However, we could not confirm those results when resistance exercise in hypoxia was repeated over time, excluding any contribution of satellite cells to the higher gain in muscle strength after 4 weeks when compared to normoxia [3]. However, the protocol was based on concentric contractions, which are not as potent as eccentric contractions to activate satellite cells [7].

Satellite cells are muscle stem cells localized between the sarcolemma and the basal lamina of skeletal muscle fibers [8], which, once activated by mechanical stress such as exercise, may undergo myogenesis and contribute to skeletal muscle regeneration and hypertrophy [9]. Whole myogenesis is tightly controlled by the up- and down-regulation of Paired box protein 7 (Pax7) and the myogenic regulatory factors (MRF), namely myogenic differentiation (MyoD), myogenic factor 5 (Myf5), MRF4, and myogenin. The expression of myosin heavy chains (MyHC) marks the end of the process [10]. Myogenesis can last up to 8 days [9], although the proportion of activated satellite cells is increased as soon as 1 h after resistance exercise [11]. Satellite cell number has been shown to be at its highest levels 72 h after resistance exercise [9]. The regulation of myogenesis is dependent on oxygen levels. In normoxia, satellite cells reside in a so-called niche with a partial oxygen pressure of ~25–45 Torr, corresponding to 3%–6% O_2 in vitro, a range in which they exhibit robust proliferative capacities [12]. In those physiological conditions, differentiation and fusion of myocytes are promoted, and myonuclear accretion of myofibers is increased [13]. When oxygen tension is reduced below 20 Torr, or 2%–3% O_2 in vitro, self-renewal of the satellite cell pool is upregulated at the cost of lower differentiation and fusion [13]. Finally, severe hypoxia (< 1 Torr or 1% O_2 in vitro) blocks myogenic proliferation and differentiation, and satellite cells are maintained in an undifferentiated state [12, 14]. How myogenesis can be regulated by hypoxic levels has been mainly observed in vitro, but how these observations translate in vivo remains poorly understood, especially after exercise in humans.

Several molecular pathways are involved in the regulation of myogenesis, amongst which the mitogen-activated protein kinases (MAPK) and the phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathways. Their activation is also dependent on O_2 availability. In normoxia, activation of p38 MAPK, Akt, and mTOR by insulin-like growth factor (IGF)-1 tends to promote satellite cell differentiation and fusion, whereas activation of the Ras/Raf/mitogen-activated kinase (MEK)/extracellular signal regulated kinase (Erk) pathway by IGF-1 will rather enhance self-renewal and proliferation [13]. Lower levels of O_2 and associated stabilization of hypoxia-inducible factor (HIF)-1α inhibit IGF-1-induced p38 MAPK phosphorylation and activation of the PI3K/Akt–mTOR pathway. On the contrary, IGF-1-induced Erk activation will be promoted by hypoxic conditions, thereby enhancing satellite cell proliferation [15]. MAPK activity is also sensitive to mechanical tension and muscle damage. In response to those changes, the higher phosphorylation of Jun amino-terminal kinase (JNK) will block the transcription of MyoD, which is responsible for the switch from proliferation to differentiation of satellite cells [16]. Additionally to the previous mechanisms, inflammation also plays an important role in myogenesis, as it is the case after muscle cell degeneration induced by repeated contractions [17]. Degeneration is the first phase of the regenerative process, rapidly followed by the recruitment of immune cells, and especially pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β [18]. A pro-inflammatory stage will promote satellite cell proliferation, but a switch to an anti-inflammatory state is necessary to enable satellite cell differentiation [17]. The inflammatory phase is followed by regeneration, maturation, and functional recovery of the muscle fibers [19].

As both eccentric [20] and hypoxic exercise [21] induce a large inflammatory response and inflammation is a key regulator of myogenesis, we hypothesized that an acute eccentric resistance exercise in hypoxia would induce a larger activation of satellite cells than the same exercise in normoxia. In addition, we were interested in determining whether systemic hypoxia induced by exercising in a normobaric hypoxic room would trigger a different pattern of responses to local hypoxia induced by BFR. Indeed, when both methods promote metabolic stress, angiogenesis, and glycolytic metabolism, systemic hypoxia induces metabolic vasodilation whereas blood flow restriction induces vascular resistance [1]. How this latter difference affects myogenesis is not known yet.

2 | Materials and Methods

2.1 | Experimental Design

Thirty-eight healthy young men (24 ± 1 years, 23 ± 1 kg/m^2) were recruited and gave their written consent to voluntarily participate in the experiment. All participants were physically active and did not engage in resistance training on a regular basis. The experiment was approved by the ethical committee of the UCLouvain and conducted in accordance with the declaration of Helsinki. The exclusion criteria were: consumption of tobacco and drugs, inflammatory disease or status, contraindication for resistance exercise and for the use of blood flow restriction or having been exposed to an altitude above 1500 m during the month before the experiment.

2.1.1 | Pre-Visit (D-7)

On the first visit, height and body mass were determined, and body mass index was calculated. Afterwards, a familiarization and quadriceps femoris maximal force test was realized on an isokinetic dynamometer (Biodex System 4 Pro, Biodex Medical Systems, USA). All participants started with a 5 min warm-up on a cyclo-ergometer at 1.5 W/kg (60–80 rpm) and a warm-up on the isokinetic dynamometer (10 submaximal repetitions at 90°/s) [22]. Thereafter, they performed a maximal isometric force test at 60° of knee flexion, as well as an isokinetic test at 60°/s, 90°/s, 120°/s, and 150°/s, with 2 min rest between each speed test [22, 23]. Based on their maximal isometric strength, participants were assigned to one of the three homogenous groups: normoxia ($n = 13$), normobaric environmental hypoxia ($n = 12$), and BFR ($n = 13$) (Table 1).

2.1.2 | Experiment Week (D0, D + 1, D + 3)

At least 7 days after the first visit, participants came to the lab in the morning, after an overnight fast. Blood samples were collected from the antecubital vein in tubes (all from Vacuette, Greiner Bio-One, Austria) containing EDTA (KG454023) and heparin lithium (KG454084) for plasma and in dry tubes for serum (KG455071). After blood sampling, a muscle biopsy was taken from the vastus lateralis according to the modified Bergström technique with suction. After local anesthesia (Linisol 2%, B. Braun Medical NV/SA, Germany), a 5-mm

TABLE 1 | Participant characteristics.

	NOR	BFR	HYP	*p*
Age (years)	24 ± 1	25 ± 1	24 ± 1	0.163
Height (cm)	182 ± 2	179 ± 1	180 ± 2	0.554
Weight (kg)	76.3 ± 3.6	76.0 ± 2.9	72.9 ± 3.4	0.736
BMI (kg/m^2)	22.9 ± 0.9	23.6 ± 0.8	22.6 ± 1.0	0.732
Maximal isometric torque (N m)	209 ± 14	203 ± 8	197 ± 12	0.741
Relative isometric torque (N m/kg)	2.8 ± 0.2	2.7 ± 0.1	2.7 ± 0.2	0.959

Note: Results are presented as mean ± SEM. One-way ANOVAs were used for statistical analyses.
Abbreviations: BFR, blood flow restriction; HYP, hypoxia; NOR, normoxia.

incision was made with a scalpel, and pressure was applied for 10 min to reduce blood flow. The Bergström needle was then gently inserted into the incision, ≥1 cm beyond the fascia, to reach the muscle, and three successive cuts were performed to obtain about 80–120 mg tissue/sample. The samples were immediately frozen in liquid nitrogen and stored at −80°C.

After the pre-exercise measures and sampling, the participants started the resistance exercise session in one of the three experimental conditions in a normoxic/hypoxic room (High altitude system B-Cat, Tiel, The Netherlands): normoxia (fraction of inspired oxygen (FiO_2) = 0.209), environmental normobaric hypoxia (FiO_2 = 0.140) or BFR (FiO_2 = 0.209, 60% of arterial occlusion pressure (AOP) [24]). Environmental hypoxia was achieved following nitrogen injection into the confined room to dilute oxygen content in the air. AOP was calculated with the formula proposed by Tuncali et al. [25], requiring systolic blood pressure (SBP) and thigh circumference measured at mid-thigh, halfway between the greater trochanter and lateral condyle. Thigh circumference was associated with a tissue padding coefficient (KtP). AOP was calculated in the following way: AOP = (SBP + 10)/KtP. BFR was induced by a pneumatic tourniquet (GCCM106, DDMedical, France) and an automatic pump (DDM Easy Pump Dual, DDMedical), managing the pressure at each time during exercise. The cuff was inflated before the start of the first set and deflated directly after the end of the last set.

The exercise session started with 5 min warm-up on a cycloergometer (1.5 W/kg) followed by a warm-up on the isokinetic dynamometer (2 × 20 submaximal reps at 60°/s). The resistance exercise targeted knee flexion-extension consisted of 5 sets of 15 reps at 60°/s for the concentric contraction (knee extension), and 30°/s for the eccentric contraction (knee flexion), with 30 s of rest between each set [26]. All participants got the same instruction to push as much as they could during knee extension, and to refrain from knee flexion as much as possible. They were encouraged by the staff during the whole exercise session. Repetitions in reserve (RIR) and rate of perceived pain (RPP) in the quadriceps were evaluated by the participant at the end of each set. During exercise, changes in vastus lateralis oxygenation were monitored by near-infrared spectroscopy (NIRS; Portamon, Artinis Medical Systems, The Nederland's), using the difference in absorption characteristics of light between 760 and 850 nm. Skinfold was measured with Harpenden caliper to confirm that the distance between the NIRS apparatus and the muscle was not higher than 20 mm [27]. As described in Brocherie et al. [28], 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. Tissue saturation index (TSI, %) was monitored at baseline (2 min averaging at rest, sitting with knees at 90° of flexion) and during the whole exercise session. Average values for each set and for each recovery were analyzed separately. Peripheral blood oxygen saturation (SpO_2) and heart rate were monitored by pulse oximetry (TCM Tosca; Radiometer, Denmark), at baseline and during the whole exercise session. Average values for each set were also analyzed separately. Blood lactate concentrations (Lactate Pro 2, LT-1730, Arkray, Japan) were measured by taking a blood droplet from the ear lobe before warm-up, directly after the end of exercise and every 2 min upon a peak was observed. Additional blood and muscle samples were collected 1, 24, and 72 h post-exercise, all in the fasted state. Muscle biopsies were taken 3 cm apart from each other, starting from the most distal part of the vastus lateralis and going more proximally, to avoid biopsy-induced inflammation [29].

2.2 | Blood Analysis

Blood samples were immediately put on ice until centrifugation except for serum, which stayed 30 min at room temperature before centrifugation. Tubes were centrifuged at 3000 g for 10 min at 4°C. Plasma and serum were transferred to microtubes and stored at −80°C upon analysis. Creatine kinase (CK) and C-Reactive protein (CRP) concentrations were measured on plasma-heparin with an automated analyzer (Pentra C 200, Horiba medical, Axonlab, Switzerland). Fibroblast growth factor (FGF), IL-1β, IL-6, IL-10, and TNF-α were analyzed on plasma-EDTA with U-PLEX assay (MESO QuickPlex SQ 120MM, Meso Scale Discovery, USA), following the manufacturer's instructions. All blood analyses were performed in single.

2.3 | RNA Isolation and Real-Time PCR

About 20 mg of muscle biopsy sample was homogenized in 1 mL Trizol reagent (Invitrogen) using a Polytron mixer (Kinematica, Switzerland). 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 (Bio-Rad Laboratories, Belgium), following the manufacturer's instructions. The primers used are presented in Table 2. All primer sets were designed with at least one primer overlapping 2 exons to avoid amplification of contaminating genomic DNA. PCR was run using the following conditions: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s 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.

TABLE 2 | Primers sequences.

Gene	Forward	Reverse
B2M	ATG AGT ATG CCT GCC GTG TGA	GGC ATC TTC AAA CCT CCA TG
BNIP3	CTG AAA CAG ATA CCC ATA GCA TT	CCG ACT TGA CCA ATC CCA
CD68	TGG GCA AAG TTT CTC CTG	GGA TGA TGC AGA AAG CAA TAA G
CyclinD1	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-1	GCC CCA GAT TCA GGA TCA GA	TGG GAC TAT TAG GCT CAG GTG
IGF-1	TAT TTC AAC AAG CCC ACA GG	CAT CTC CAG CCT CCT TAG AT
Jun	AAG ATG GAA ACG ACC TTC TAT G	CAG GGT CAT GCT CTG TTT C
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
PCNA	ATC CTC AAG AAG GTG TTG GAG GCA	ACG AGT CCA TGC TCT GCA GGT TTA
PDK1	TGC CCA TAT CAC GTC TTT AC	GTC TGT TGA CAG AGC CTT AAT
Redd1/DDIT4	CTT TGG GAC CGC TTC TC	CAG GTA AGC CGT GTC TTC
RPL19	CGC TGT GGC AAG AAG AAG GTC	GGA ATG GAC CGT CAC AGG C
VEGFa	TTT CTG CTG TCT TGG GTG CAT TGG	ACC ACT TCG TGA TGA TTC TGC CCT

Abbreviations: B2M, beta-2-microglobulin; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; CD68, cluster of differentiation 68; HIF, Hypoxia inducible factor; IGF-1, insulin-like growth factor 1; Mrf4, muscle regulatory factor 4; Myf5, myogenic factor 5; Myh, myosin heavy chain; MyoD, myogenic differentiation; PAX7, paired box 7; PCNA, proliferating cell nuclear antigen; PDK1, pyruvate dehydrogenase kinase 1; Redd1/DDIT4, regulated in development and DNA damage response 1/DNA-damage-inducible transcript 4; RPL19, ribosomal protein L19; VEGF, vascular endothelial growth factor.

To compensate for variations in input RNA amounts and efficiency of reverse transcription, the mRNA levels of ribosomal protein L19 (RPL19) and beta-2-microglobulin (B2M) were quantified. The results of each gene of interest were normalized to the geometric mean of the two normalization genes [30], the mRNA levels of each not being influenced by the experimental conditions.

2.4 | Protein Isolation and Western Blotting

Approximately 20–25 mg of muscle samples were homogenized with a Polytron mixer in an ice-cold lysis buffer [1 mM 1,4-dithiothreitol, 20 mM Tris–HCl (pH 7.0), 50 mM beta-glycerophosphate, 1 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 270 mM sucrose, 1% Triton X-100, and 10% protease inhibitor], centrifuged at 10000 g for 10 min at 4°C. The supernatant was then transferred to microtubes and stored at −80°C upon analysis. Twenty to 60 μg proteins were separated by SDS-PAGE (8%–10%) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4°C with the following antibodies (all from Cell Signaling Technology, USA, 1:1000 excepted specified): eukaryotic translation elongation factor 2 (eEF2) (#2332), HIF-1α (1:500, #14179), MyoD (1:100, sc-377 460, Santa Cruz, USA), myogenin (1:200, sc-52 903, Santa Cruz), phospho-nuclear factor-kappa B (NFκB) p65 Ser536 (#5536), NFκB p65 (#8242), phospho-p38 MAPK Thr180/Tyr182 (#9211), p38 MAPK (#9212), phospho-p44/42 MAPK Thr202/Tyr204 (#4370), and p44/42 MAPK (Erk1/2) (#4696), Pax7 (supernatant, 1:100, AB_528 428, Developmental Studies Hybridoma Bank (DSHB), USA), phospho-p70S6K Thr389 (#9234), p70S6K (#2708), phospho-stress-activated protein kinases (SAPK)/JNK Thr183/Tyr185 (#9251), SAPK/JNK (#9252), phospho-signal transducer and activator of transcription 3 (STAT3) Tyr705 (#9131) and STAT3 (#12640). Horseradish peroxidase-conjugated anti-mouse (1:10000, A9044, Sigma-Aldrich, USA) or anti-rabbit (1:5000, A6154, Sigma-Aldrich) secondary antibodies were used for detection of proteins. Membranes were thereafter scanned, and proteins were quantified with GeneSnap and GeneTools softwares (Syngene, UK). The results are presented as the ratio protein of interest/eEF2 or as the ratio phosphorylated protein/total protein. All results are expressed in arbitrary units.

2.5 | Immunostaining

Sections of 6 μm from OCT-embedded tissue were cut at −20°C using a cryostat microtome (CryoStar NX70, Thermo Fisher Scientific, USA) and put on histological blades. The blades were dried at room temperature for 1 h and stored at −80°C upon immunofluorescence analysis. Tissue sections were washed 2 × 5 min in phosphate-buffered saline (PBS) and blocked for 30 min 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 laminin (1:1000, IgG, rabbit, NB300-144, Novus/Interchim), MyoD (1:200, IgG2b, mouse, sc-377 460) and Pax7 (1:5, supernatant, IgG1, mouse, 528 428, DSHB) or myogenin (1:200, IgG1, mouse, sc-52 903) for satellite cell identification. Sections were washed 2 × 5 min and incubated for 1 h at room temperature with the secondary antibody Alexa Fluor 488 anti-mouse IgG2b (1:250, A-21141, Invitrogen, USA), Alexa Fluor 568 anti-mouse IgG1 (1:250, A21124, Invitrogen) and Alexa Fluor 660 anti-rabbit IgG (1:500, A21074, Thermofisher). Finally, tissue sections were washed 2 × 5 min and covered with an antifade mounting medium containing DAPI (Vectashield, Labconsult, Belgium). Blades were stored for 4 h at 4°C before visualization with the microscope Axio Vert A1 (Zeiss, Deutschland) equipped with an AxioCam ICm1 camera and ZEN lite 2011 (Zeiss) software. Image processing and analysis were realized with ImageJ.

2.6 | Cell Cultures and Electrical Pulse Stimulation

Myoblasts were purified from a human muscle biopsy (young healthy man, 24 years old, BMI = 25 kg/m^2) and were cultured in standard conditions (21% O_2, 5% CO_2, 37°C) in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, stable glutamine and sodium pyruvate (L0103-500, VWR), 1% penicillin/streptomycin and 0.1% amphotericin B (L0009-020, Biowest, VWR) = DMEM*. Proliferation medium was composed of 79.8% DMEM*, 20% fetal bovine serum (FBS) and 0.2% Ultroser G. For differentiation, cells were cultured in 6-well dishes (10 469 282, Fisherscientific) and the medium was composed of 98% DMEM* and 2% FBS.

On D5 of differentiation, 1 h before electrical pulse stimulation (EPS), differentiation medium was replaced by DMEM* (medium without serum) after rinsing with PBS. Myotubes were subjected to EPS (1 s contraction, 4 s rest, 50 Hz pacing frequency, 15 V, 2 ms pulse duration) in a resistance exercise-like manner (5 series of 15 repetitions, 2 min rest between series) using the C-Pace EP 200 system (IonOptix) in normoxia (21% O_2, 37°C), physiological hypoxia (5% O_2, 37°C), and severe hypoxia (1% O_2, 37°C). Hypoxic conditions were induced in a hypoxic gloves box (GP Campus, Jacomex). Directly following stimulation, the medium was removed, cells were rinsed with cold PBS and scrapped with ice cold lysis buffer for protein isolation or scrapped with Trizol (15 596 018, Thermofischer Scientific) for RNA isolation. Samples were frozen upon further analysis. The experience was repeated four times.

2.7 | Statistics

All values are expressed as means ± SEM. Linear mixed models were applied on the data, with groups (normoxia vs. hypoxia vs. BFR) and time (pre, 1, 24, and 72 h post-exercise) as fixed effects and subjects as random effects for the human experiment. One-way ANOVAs were applied to confirm that groups were homogenous before the experimental trial (Table 1), and *T*-tests were applied for the preliminary tests of the in vitro experiment to validate the resistance-like EPS protocol (Figure 6A). For the main in vitro experiment, linear mixed models were applied with groups (non-stimulated and stimulated) and conditions (21%, 5%, and 1% O_2) set as fixed effects and the date of the experiment (cells coming from the same plate) as the random effect. Statistical significance was fixed at $p < 0.05$. Linear mixed models were performed with lme4 and lmerTest libraries in R. Normality of the residuals was assessed with qqPlot. Post hoc analyses were performed with emmeans Package in R, using Bonferroni as the adjustment method of *p*-values. Graphs were realized with ggpplot2 library in R.

3 | Results

3.1 | Average Peak Torque and Blood Lactate Concentrations Indicate That Exercise Was Maximal in All Groups

Average peak torque decreased over sets for both concentric and eccentric contractions in all conditions (Table 3). Compared to Set 1, the decrease was larger and earlier in BFR, starting at Set 2 ($p < 0.001$) compared to Normoxia and Hypoxia, starting at Set 4 ($p < 0.001$), for both concentric and eccentric contractions. The capacity of each participant to perform an extra contraction at the end of each set was assessed with the RIR scale (Table S1). RIR were ≤ 3 in the 1st set and at 0 in all groups at the 4th set ($p < 0.001$). At the end of the exercise, blood lactate increased in all groups ($p < 0.001$) but was higher in Hypoxia (7.8 ± 0.3 mmol/L) than in Normoxia (6.5 ± 0.5 mmol/L, $p = 0.021$ vs. Hypoxia) and BFR (6.4 ± 0.4 mmol/L, $p = 0.010$ vs. Hypoxia) ($p < 0.001$, Table S2). In summary, despite all participants giving a maximal effort, we observed a larger decrease of the average peak torque in the BFR group and higher post-exercise lactate concentrations in the hypoxic group.

3.2 | Blood and Muscle Oxygenation Confirms Environmental and Local Hypoxia

SpO_2 remained constant over the whole exercise session but was globally lower in Hypoxia compared to Normoxia and BFR ($p < 0.001$, Table 4). TSI decreased from baseline to Set 1 in both Normoxia (61% ± 2% to 54% ± 3%, $p < 0.001$) and BFR (63% ± 1% to 52% ± 2%, $p < 0.001$) and remained lower during all sets. In Normoxia and BFR, TSI was lower than baseline through the sets ($p < 0.001$) but no changes between baseline and sets were observed in Hypoxia. In summary, lower SpO_2 values in the hypoxic group confirm the environmental hypoxic exposure in that specific group.

TABLE 3 | Average peak torque.

	Concentric contraction (N m)			Eccentric contraction (N m)		
Set	**NOR**	**BFR**	**HYP**	**NOR**	**BFR**	**HYP**
1	156 ± 11	128 ± 9	133 ± 9	154 ± 16	152 ± 10	171 ± 17
2	124 ± 9	60 ± 6***, $$, #	108 ± 11	138 ± 11	84 ± 12***, $$, ###	164 ± 16
3	98 ± 9	32 ± 6***, $$$, ###	87 ± 11	118 ± 10	45 ± 8***, $$$, ###	143 ± 17
4	83 ± 8***	26 ± 5***, $$$, ###	70 ± 11***	104 ± 9**	39 ± 7***, $$$, ###	117 ± 15**
5	80 ± 9***	23 ± 5***, $$$, ###	64 ± 11***	95 ± 10***	30 ± 6***, $$$, ###	103 ± 13***

Note: Results are presented as mean ± SEM. Linear mixed models were used for statistical analyses.
Abbreviations: BFR, blood flow restriction; HYP, hypoxia; NOR, normoxia.
**$p < 0.01$.
***$p < 0.001$ vs. Set 1.
$$$p < 0.01$.
$$$$p < 0.001$ vs. Normoxia.
#$p < 0.05$.
###$p < 0.001$ vs. Hypoxia.

TABLE 4 | Blood and tissue oxygenation during exercise.

	SpO_2 (%)			TSI (%)		
Set	**NOR**	**BFR**	**HYP**	**NOR**	**BFR**	**HYP**
Bsl	97.79 ± 0.19###	97.80 ± 0.16###	90.36 ± 0.78	61 ± 2	63 ± 1	58 ± 2
1	97.92 ± 0.21###	97.80 ± 0.19###	90.70 ± 0.90	54 ± 3***	52 ± 2***	56 ± 2
2	98.22 ± 0.21###	98.09 ± 0.24###	92.62 ± 0.65***	54 ± 3***	54 ± 2***	56 ± 2
3	98.24 ± 0.22###	97.92 ± 0.26###	93.16 ± 0.58***	55 ± 3***	54 ± 2***	57 ± 2
4	98.23 ± 0.23###	98.07 ± 0.25###	93.03 ± 0.57***	55 ± 2**	55 ± 2***	57 ± 2
5	98.25 ± 0.22###	97.98 ± 0.24###	92.52 ± 0.64***	58 ± 2	55 ± 2***	57 ± 2

Note: Results are presented as mean ± SEM. Linear mixed models were used for statistical analyses.
Abbreviations: BFR, blood flow restriction; HYP, hypoxia; NOR, normoxia; SpO_2, peripheral blood oxygen saturation; TSI, tissue saturation index.
**$p < 0.01$.
***$p < 0.001$ vs. Baseline (Bsl).
###$p < 0.001$ vs. Hypoxia.

3.3 | Myogenic Regulatory Factor Expression Changes Over Time to a Larger Extent in Normoxia and Hypoxia Versus Blood Flow Restriction

In skeletal muscle, proliferating cell nuclear antigen (PCNA) mRNA levels, a marker of satellite cell quiescence and activation and thus early myogenesis [8], were globally lower at 24 h ($p = 0.002$) and 72 h ($p = 0.010$) post-exercise compared to Pre (Figure 1A). Post hoc analyses revealed that PCNA mRNA levels were lower at 24 h ($p = 0.011$) and 72 h ($p = 0.049$) in Normoxia and at 24 h ($p = 0.027$) in Hypoxia compared to Pre. Pax7 protein expression, which characterizes satellite cell activation [8], was increased at 1 h ($p < 0.001$), 24 h ($p < 0.001$) and 72 h ($p = 0.004$) compared to Pre (Figure 1B,I). Myf5 mRNA levels, another marker of satellite cell proliferation [8], was globally lower at 1 h post-exercise compared to Pre ($p < 0.001$), and returned to basal values at the following timings (Figure 1C). MRF4 mRNA levels, a marker of early differentiation [8], were globally higher than pre-exercise values at 1 h ($p < 0.001$), 24 h ($p < 0.001$) and 72 h ($p = 0.022$) post-exercise (Figure 1D). Post hoc analyses showed that those increases compared to Pre were specifically found at 1 h in Normoxia ($p < 0.001$), BFR ($p = 0.002$) and Hypoxia ($p < 0.001$) and at 24 h in Normoxia ($p < 0.001$) and Hypoxia ($p = 0.002$). Additionally, MRF4 mRNA levels at 24 h were lower in BFR compared to Normoxia ($p = 0.031$). As it was technically possible to determine the protein expression of MyoD, a marker for satellite cell proliferation and differentiation [8], and myogenin, a marker for differentiation [8], both mRNA and protein expression were measured to get a better picture of transcriptional and post-translational regulation of those 2 markers of myogenesis. MyoD mRNA levels were globally higher 1 h post-exercise ($p = 0.006$) and lower 72 h post-exercise ($p = 0.016$) compared to Pre (Figure 1E). Similarly to its mRNA levels, MyoD protein expression was globally higher at 1 h post-exercise compared to Pre ($p < 0.001$), with post hoc analyses showing a higher expression in Normoxia ($p < 0.001$) and Hypoxia ($p = 0.003$) (Figure 1F,I). Additionally, MyoD protein expression was still higher in Normoxia at 24 h compared to Pre ($p = 0.009$) and was lower in BFR at 72 h compared to Normoxia ($p = 0.007$). Finally, myogenin mRNA levels ($p < 0.001$, Figure 1G) and protein expression ($p = 0.004$, Figure 1H,I) were globally higher at 24 h compared to Pre. Post hoc analyses revealed that compared to Pre, at 24 h, myogenin mRNA levels were higher in Normoxia ($p < 0.001$) and Hypoxia ($p < 0.001$). At 24 h, myogenin mRNA levels in BFR were lower than in Normoxia ($p = 0.004$) and Hypoxia ($p = 0.020$). Costaining of nuclei, MyoD and Pax7 and nuclei, MyoD and

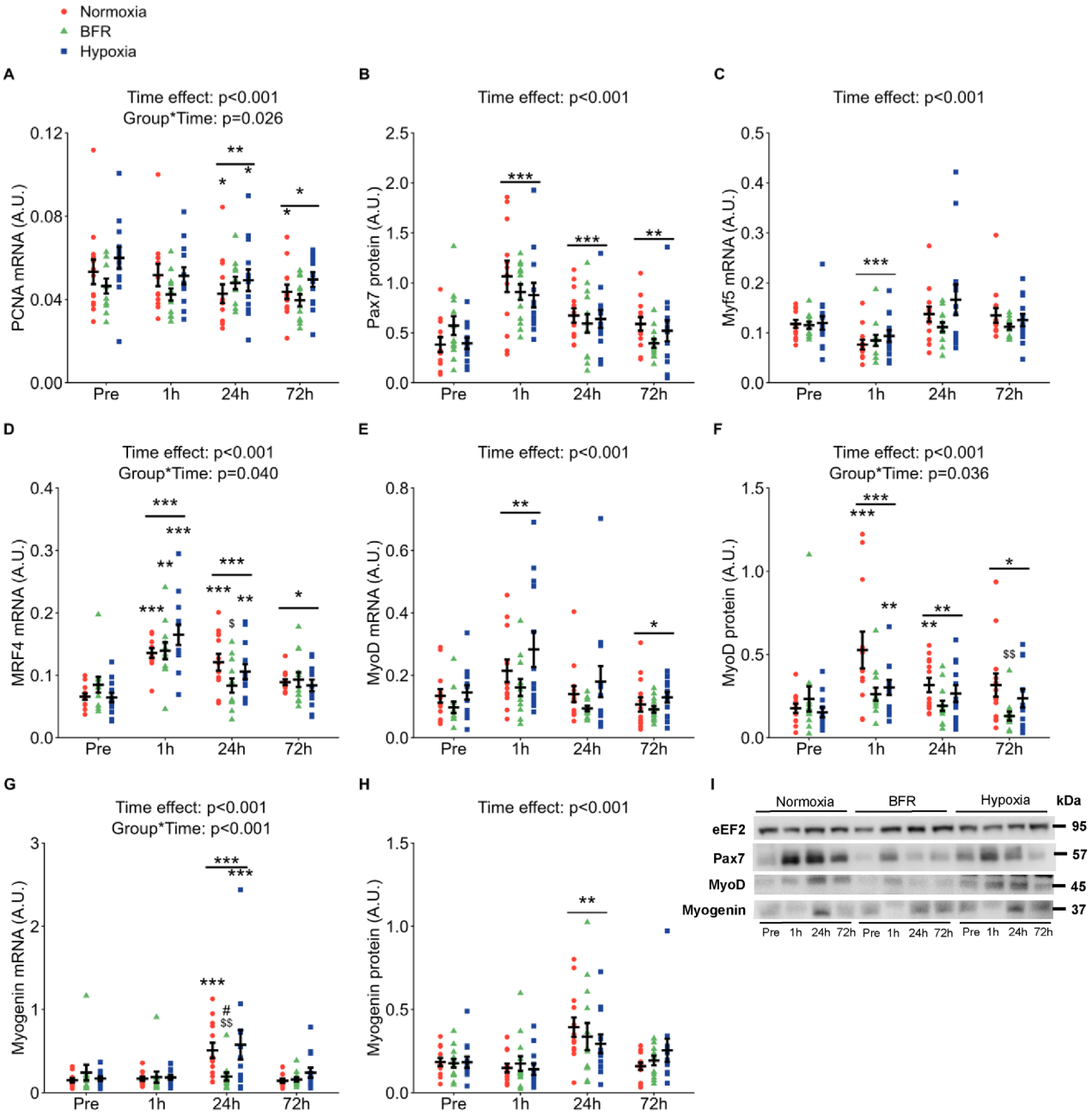

FIGURE 1 | Regulation of the myogenic regulatory factors by eccentric resistance exercise in Normoxia, BFR, and Hypoxia. (A) PCNA mRNA levels; (B) Pax7 protein expression; mRNA levels of (C) Myf5, (D) MRF4, and (E) MyoD; (F) MyoD protein expression; (G) myogenin mRNA levels; and (H) myogenin protein expression before and 1, 24, and 72 h after eccentric resistance exercise in Normoxia (○, $n=13$), with BFR (Δ, $n=13$), or in Hypoxia (□, $n=12$); (I) Representative Western blots. Mean ± SEM. Linear mixed models were used for statistical analyses. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. Pre-exercise; $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs. Normoxia same time; $^{\#}p<0.05$ vs. Hypoxia same time. BFR, blood flow restriction.

myogenin are presented in Figure 2 and Figure S1, respectively. In summary, exercise induced larger changes in MRF mRNA levels and protein expression in the normoxic and hypoxic groups in comparison to the BFR group.

3.4 | Few Differences in Hypoxic Responsive and Immediate Early Gene Regulation

Several changes were observed in the expression of hypoxic responsive genes, that is, Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (Bnip3), pyruvate dehydrogenase kinase 1 (PDK1), regulated in development and DNA damage response 1 (Redd1), vascular endothelial growth factor (VEGF)a [31–33] and immediate early genes involved in the regulation of myogenesis, that is, cyclin D1 and Fos [34, 35]. Compared to Pre, Bnip3 mRNA levels were lower at 24 h post-exercise ($p<0.001$, Figure 3A). At 24 h, post hoc analyses showed that Bnip3 mRNA levels in Normoxia were lower than in Pre ($p<0.001$) and in BFR at the same timepoint ($p=0.004$). Globally, at 1 h post-exercise, PDK1 mRNA levels were lower ($p=0.039$, Figure 3B) and Redd1 mRNA levels were higher ($p=0.012$, Figure 3C) than

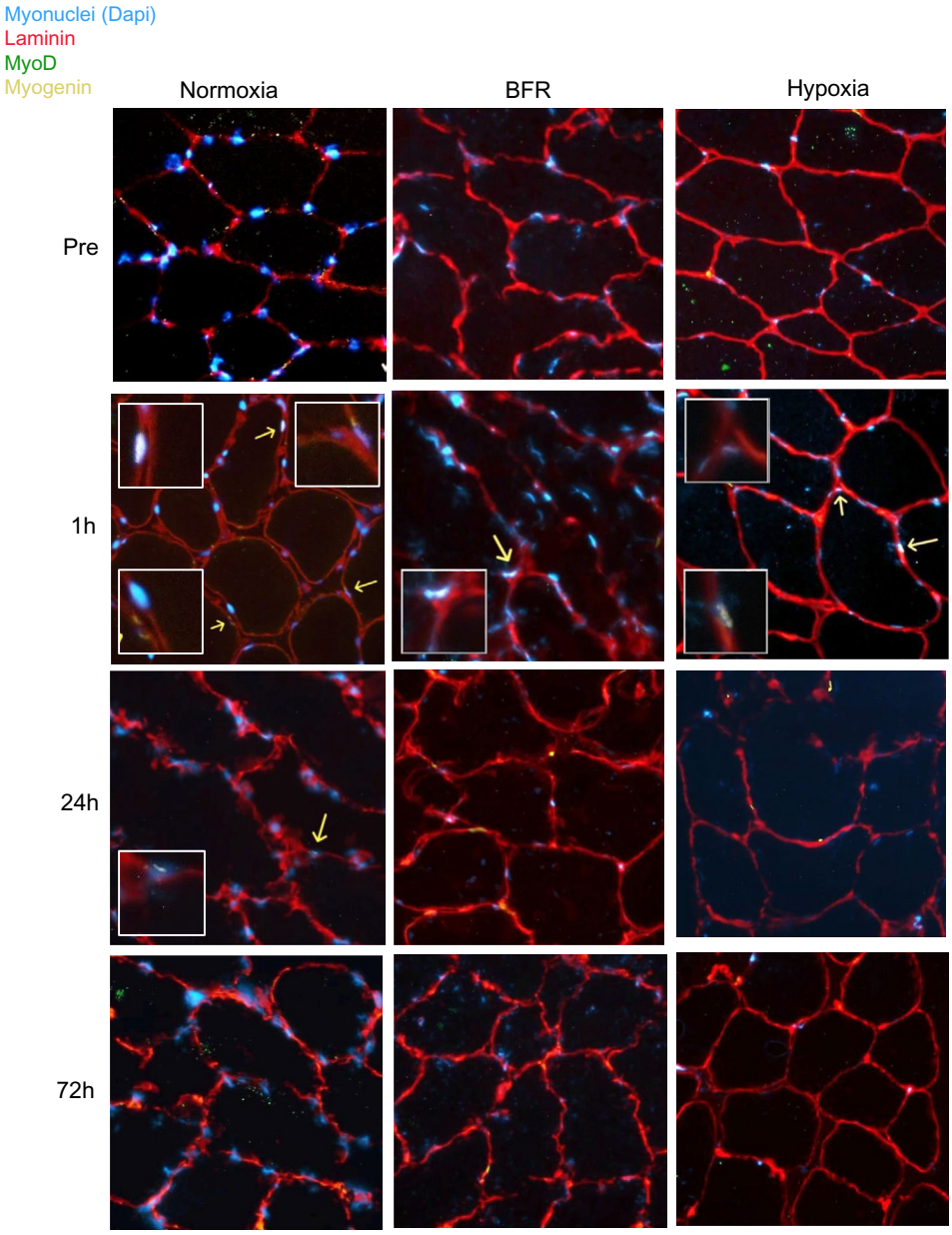

FIGURE 2 | Representative immunofluorescence pictures with myonuclei (Dapi blue), Pax7 (yellow), MyoD (green), and laminin (red). Yellow arrow: Costaining of Pax7 and Dapi. BFR, blood flow restriction.

pre-exercise values, but no difference between groups was observed. Compared to Pre, VEGFa mRNA levels were globally higher at 1 h ($p < 0.001$) and 24 h post-exercise ($p < 0.001$, Figure 3D). Compared to Pre, Cyclin D1 mRNA levels were lower at 72 h ($p < 0.001$, Figure 3E). Compared to Pre, Fos mRNA levels were higher at 1 h ($p < 0.001$) and lower at 24 h ($p < 0.001$) and 72 h ($p = 0.050$) post-exercise (Figure 3F). Compared to Pre, at 1 h, all three groups had higher Fos mRNA levels ($p < 0.01$) but at 24 h, only Normoxia displayed lower values ($p < 0.001$). Finally, compared to Pre, HIF-1α protein expression was higher at 1 and 24 h post-exercise ($p < 0.001$, Figure 3G). In summary, the main effects were observed at 1 and/or 24 h post-exercise, with higher expression for VEGFa, Fos and HIF-1α and lower expression for Bnip3, but no further differences were observed between groups in the expression of hypoxic responsive and immediate early genes.

3.5 | Hypoxic Exercise Elicits High CK Levels

As the inflammatory state has previously been shown to regulate myogenesis [6], we analyzed several inflammatory markers in skeletal muscle and blood. In skeletal muscle, phospho-NFκB p65 at Ser536 was unchanged by exercise in all groups (Figure 4A,C). Compared to Pre, phospho-STAT3 at Tyr705 was higher at 1 h post-exercise ($p < 0.001$, Figure 4B,C). Compared to pre, the mRNA levels of the macrophage/neutrophil marker CD68 [36] were higher at 24 h ($p < 0.001$) and 72 h ($p = 0.001$) post-exercise (Figure 4D).

In blood, no differences in CRP levels were observed between groups and over time (Figure S2D), but some changes were observed in the circulating levels of some ILs. A global time effect ($p = 0.015$) was observed for IL-1β levels, a pro-inflammatory

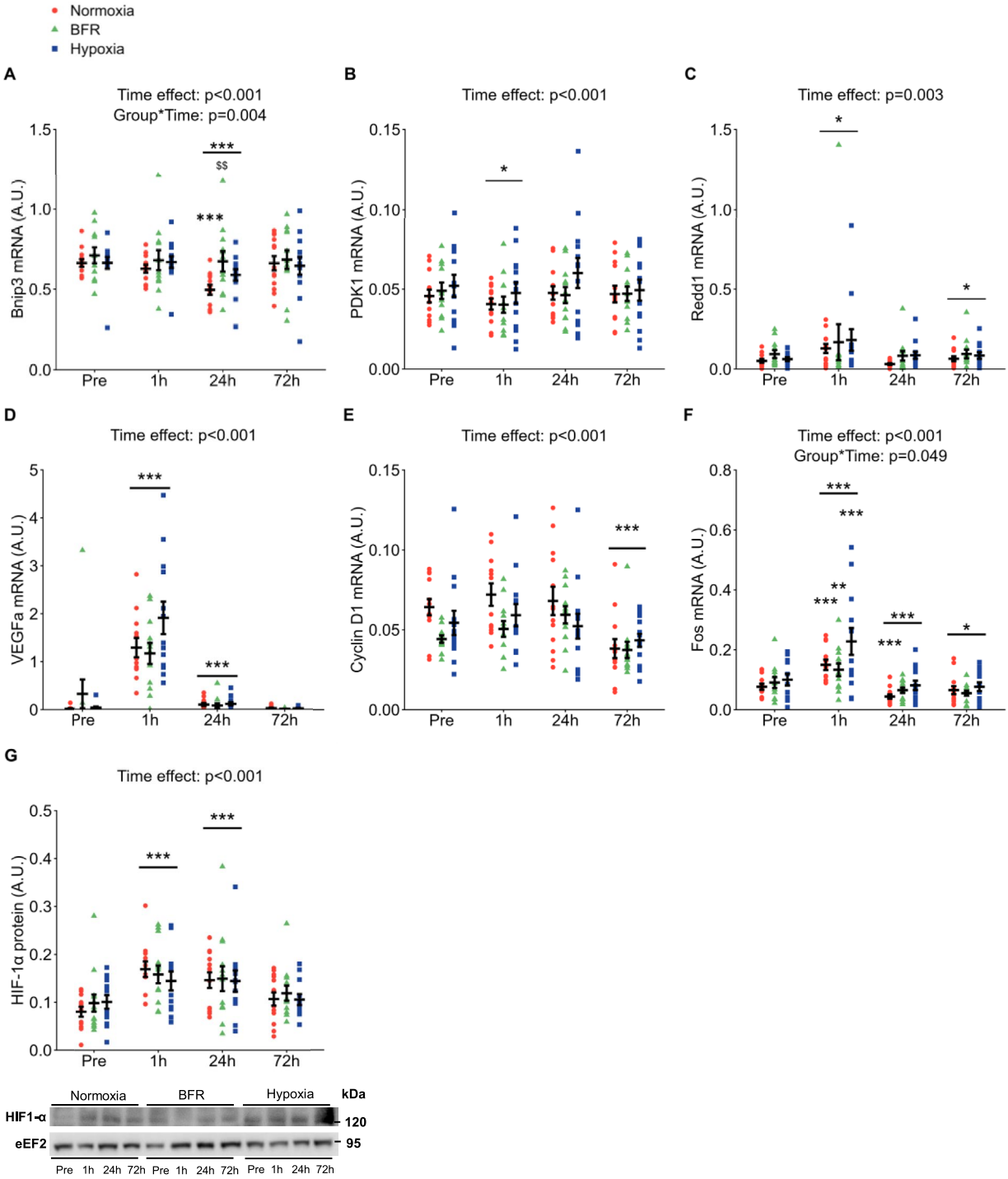

FIGURE 3 | Regulation of the hypoxic responsive and immediate early genes by eccentric resistance exercise in Normoxia, BFR, and Hypoxia. mRNA levels of (A) Bnip3, (B) PDK1, (C) Redd1, (D) VEGFa, (E) Cyclin D1, and (F) Fos; (G) HIF-1α protein expression and representative Western blots before and 1, 24, and 72 h after eccentric resistance exercise in Normoxia (○, $n = 13$), with BFR (Δ, $n = 13$), or in Hypoxia (□, $n = 12$). Mean ± SEM. Linear mixed models were used for statistical analyses. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Pre-exercise; $^{\$\$}p < 0.01$ vs. Normoxia same time. BFR, blood flow restriction.

cytokine [36], without any further differences being detected (Figure 4E). Compared to Pre, IL-6 levels, which have pro- and anti-inflammatory roles [37], were lower at 1, 24, and 72 h post-exercise ($p = 0.017$, $p < 0.001$ and $p = 0.007$, respectively, Figure 4F). Post hoc analyses showed that compared to Pre, IL-6 levels were lower in Normoxia at 24 h ($p = 0.005$) and 72 h ($p = 0.037$). Moreover, IL-6 levels in Hypoxia were higher than in Normoxia at 1 h ($p = 0.029$) and lower than Pre at 72 h ($p = 0.027$). Compared to Pre, blood levels of the anti-inflammatory cytokine IL-10 were globally lower at 24 h post-exercise ($p = 0.037$, Figure 4G). Compared to Pre, higher levels of TNF-α, which is known to stimulate satellite cell differentiation [17], were globally observed at 24 h ($p = 0.004$) and 72 h post-exercise ($p = 0.020$, Figure 4H). Finally, CK levels, which are an indirect marker of muscle damage [36], were globally increased at 72 h post-exercise compared to

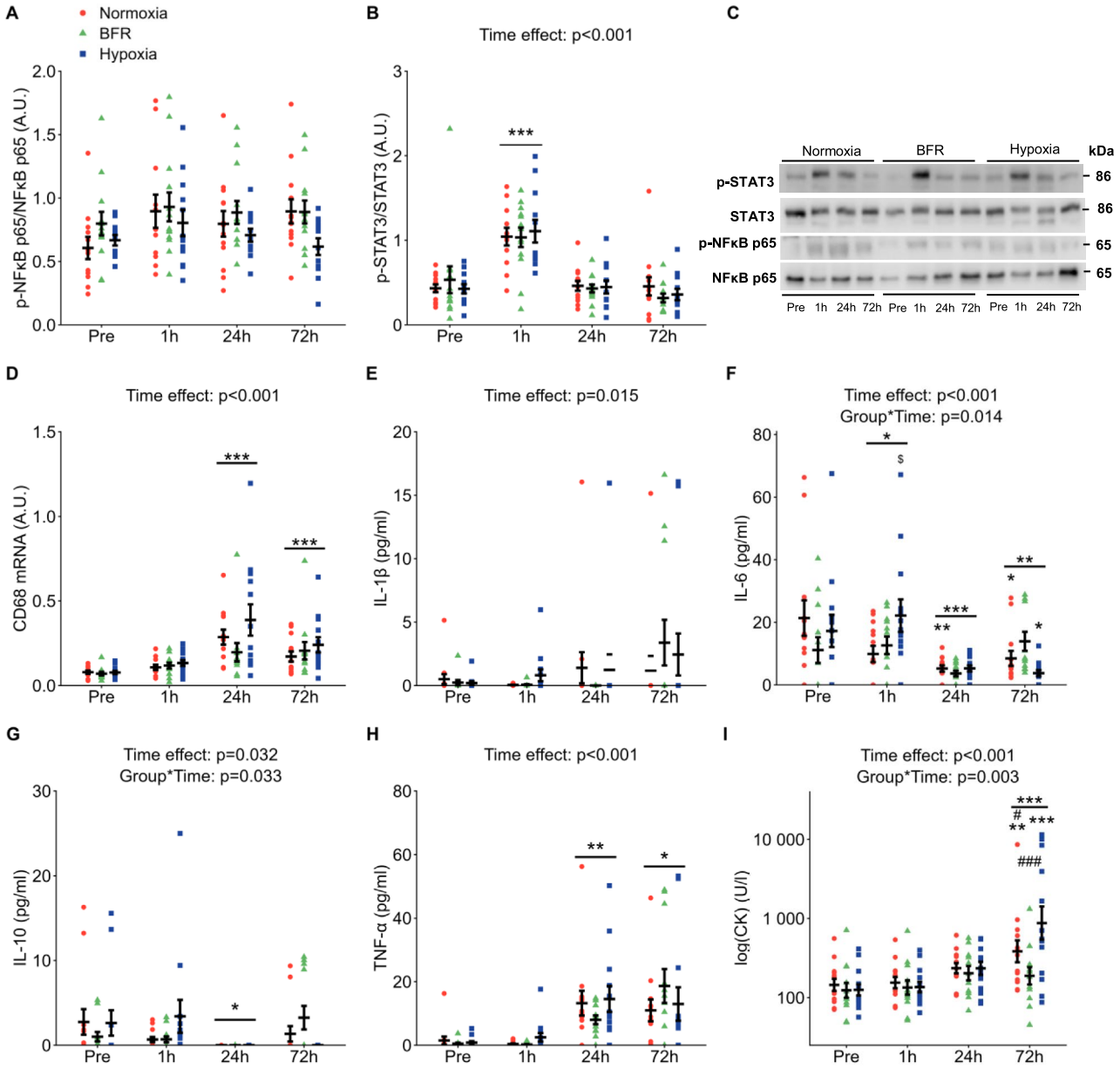

FIGURE 4 | Regulation of the inflammatory markers by eccentric resistance exercise in Normoxia, BFR, and Hypoxia. Protein expression of (A) p-NFκB p65, (B) p-STAT3, and (C) representative Western blots, (D) mRNA levels of CD68; blood levels of (E) IL-1β and (F) IL-6, (G) IL-10, (H) TNF-α, and (I) CK (results displayed in log10) before and 1, 24, and 72 h after resistance exercise in Normoxia (○, $n=13$), with BFR (Δ, $n=13$), or in Hypoxia (□, $n=12$). Mean ± SEM. Linear mixed models were used for statistical analyses. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. Pre-exercise; $^{\$}p<0.05$ vs. Normoxia same time; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. Hypoxia same time. BFR, blood flow restriction.

pre-exercise ($p<0.001$, Figure 4I). Post hoc analyses revealed that CK levels were higher at 72 h post-exercise compared to Pre in Normoxia ($p=0.004$) and Hypoxia ($p<0.001$) and were lower in Normoxia ($p=0.044$) and BFR ($p<0.001$) compared to Hypoxia at the same time point.

In summary, exercise increased the inflammatory status level as measured by a global increase in circulating IL-1β and TNF-α levels and in STAT3 phosphorylation and CD68 mRNA levels in skeletal muscle together with a global decrease in IL-6 and IL-10 over time. TNF-α blood levels were higher at 24 and 72 h post-exercise. No major difference was observed between the groups, except for circulating CK levels, which increased at 72 h post-exercise by about 5-fold in Normoxia and by about 20-fold in Hypoxia compared to pre-exercise but did not change in BFR.

3.6 | Growth Factors and MAPK Expression Varies Over Time, With Generally no Differences Between Groups

In the context of eccentric exercise, growth factors are particularly interesting to investigate as they regulate myogenesis, through amongst others, the MAPK and mTOR pathways [38]. We observed a global increase of circulating FGF levels at 24 h ($p=0.002$) and 72 h ($p<0.001$) (Figure 5A). In skeletal muscle,

an interaction effect was found between groups and time for IGF-1 mRNA levels ($p = 0.044$, Figure 5B). Post hoc analyses revealed that IGF-1 mRNA levels were lower in Hypoxia at 1 h compared to Normoxia ($p = 0.043$) and at 24 h compared to BFR ($p = 0.019$) and to Pre ($p = 0.035$). Compared to Pre, phospho-p38 MAPK at Thr180/Tyr182 was globally lower at 1 h post-exercise ($p = 0.0013$, Figure 5C,G). A global time effect was found for phospho-Erk1/2 at Thr202/Tyr204 ($p = 0.017$), with no further differences observed (Figure 5D,G). Compared to Pre, phospho-JNK at Thr183/Tyr185 (Figure 5E,G) and phospho-p70S6K at Thr389 (Figure 5F,G) were globally higher at 1 h ($p < 0.001$) and phospho-JNK at 72 h ($p = 0.045$). Finally, a group ($p = 0.004$) and time effect ($p = 0.037$) was found for Myh1 mRNA levels, a group*time interaction for Myh2 levels ($p = 0.010$) and no main effect for Myh7 mRNA levels (File S2A–C). In summary, most changes were observed at 1 h post-exercise, where p38 phosphorylation was lower while Erk1/2, JNK and p70S6K phosphorylation was higher than pre-exercise values. No differences between groups were observed, except for IGF-1 mRNA levels.

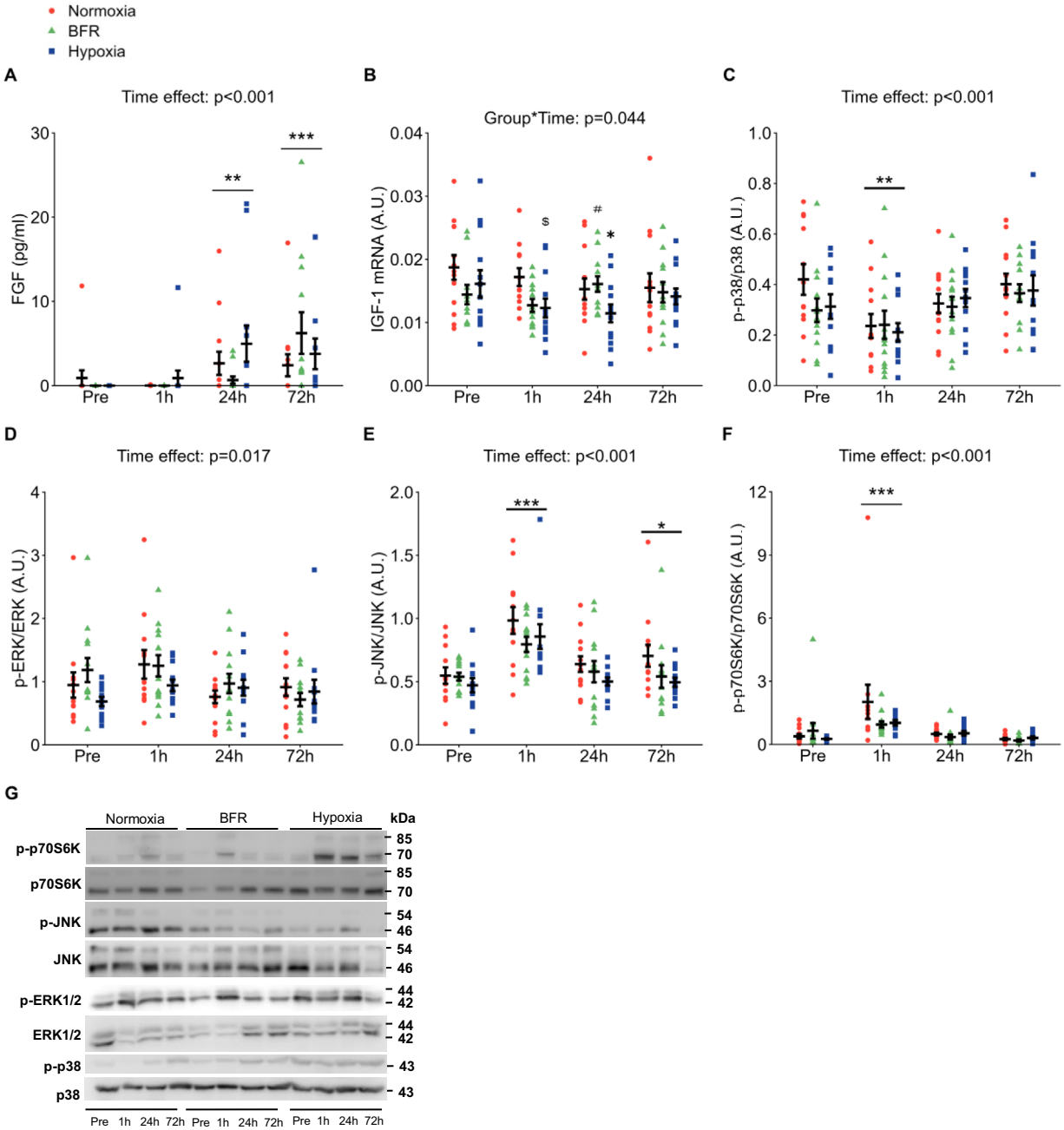

FIGURE 5 | Regulation of growth factors and MAPK by eccentric resistance exercise in Normoxia, BFR, and Hypoxia. (A) Blood levels of FGF; (B) mRNA levels of IGF-1; protein expression of (C) p-p38 MAPK, (D) p-Erk1/2, (E) p-JNK, and (F) p-p70S6K before and 1, 24, and 72 h after resistance exercise in Normoxia (○, $n = 13$), with BFR (Δ, $n = 13$), or in Hypoxia (□, $n = 12$). (G) Representative Western blots. Mean ± SEM. Linear mixed models were used for statistical analyses. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Pre-exercise; $^{\$}p < 0.05$ vs. Normoxia same time; $^{\#}p < 0.05$ vs. Hypoxia same time. BFR, blood flow restriction.

3.7 | Oxygen Levels Modify the Molecular Response to Resistance Exercise-Like EPS, but Not the mRNA Levels of the Myogenic Regulatory Factors

We conducted in vitro experiments with human myotubes to better understand the pathways activated in response to muscle contraction and hypoxia. We first validated the resistance exercise-like protocol by observing differences between stimulated and non-stimulated myotubes (Figure 6A,B). Typical markers of resistance exercise, namely phospho-S6 ($p=0.041$), phospho-p38 ($p=0.089$), and the mRNA levels of the early-responsive gene Fos ($p=0.004$), were higher in the stimulated versus non-stimulated myotubes. Conversely, phospho-AMPK, which characterizes the response to endurance exercise, was not modified by EPS ($p=0.483$).

Myotubes were then stimulated in three different conditions of oxygen availability. For phospho-S6, an interaction effect ($p=0.009$) and an oxygen condition effect ($p<0.001$) were observed, with higher phospho-S6 at 1% compared to 21% ($p=0.0011$) and 5% ($p=0.008$, Figure 6C,H). Post hoc analyses revealed that phospho-S6 at 1% O_2 was higher than at 21% O_2 in unstimulated cells ($p<0.001$) and higher than at 5% in stimulated cells ($p=0.016$). Phospho-S6 tended to be higher overall in the stimulated versus non-stimulated myotubes (group effect: $p=0.084$), especially at 21% of oxygen ($p=0.017$). A stimulation effect ($p=0.011$) and an oxygen condition effect ($p=0.007$) were observed for phospho-p38, with a higher phosphorylation at 5% versus 21% ($p=0.037$, Figure 6D,H). A group*condition interaction ($p=0.018$) and an oxygen condition effect ($p=0.025$) were found for phospho-AMPK (Figure 6E,H). Post hoc analyses revealed that phospho-AMPK was higher in stimulated myotubes at 1% versus 21% ($p=0.023$) and versus 5% ($p=0.034$). The mRNA levels of Jun ($p=0.021$, Figure 6F) were influenced and the mRNA levels of VEGFa tended to be influenced by the oxygen levels ($p=0.097$, Figure 6G). Finally, MyoD, Myf5, MRF4, myogenin, Fos, and HIF-1α mRNA levels were unchanged by EPS and oxygen conditions (Figure S3). In summary, the combination of EPS and hypoxia regulated AMPK and S6 phosphorylation whereas the mRNA levels of the MRFs were unchanged in those conditions.

4 | Discussion

As both eccentric [20] and hypoxic exercise [21] induce a large inflammatory response and inflammation is a key regulator of myogenesis, we hypothesized that an acute eccentric resistance exercise performed in hypoxia would induce a larger activation of satellite cells and myogenesis than in normoxia. Our results showed that MRF expression was upregulated after exercise similarly in the normoxic and hypoxic groups and attenuated in the BFR group. Those changes were particularly visible at 1 h and/or 24 h for MRF4 and myogenin mRNA levels and MyoD protein expression. Additionally, blood CK levels increased to a larger extent in hypoxia compared to normoxia at 72 h post-exercise, while those levels did not change over time in the BFR group. Finally, although changes over time occurred, no differences between groups were observed in the regulation of hypoxia markers and immediate early genes, inflammatory markers, growth factors, except for IGF-1 mRNA levels, and MAPK.

Previous studies looking at the effects of resistance exercise in hypoxia found an effect of exercise on MRF regulation without any further effect of hypoxia. Horwath et al. observed an increase of MyoD, MRF4 and myogenin mRNA levels 3 h after 6×8^{-10} repetitions of unilateral leg extension at 70% 1RM in both the normoxic ($FiO_2=0.21$) and the hypoxic group ($FiO_2=0.12$, face mask) [39]. Drummond et al. also observed an increase in MyoD, but not myogenin, mRNA levels 3 h after 4 sets of 30–15–15–15 repetitions of knee extension at 20% 1RM both with and without blood flow restriction (200 mmHg) [40]. The lack of effect on myogenin could likely be explained by the low intensity used in both groups. In the present study, we analyzed both the mRNA and the protein levels of MyoD and myogenin. MRF are particularly challenging to measure at the protein levels in human skeletal muscle due to the poor quality of antibodies commercially available. We found a global effect of eccentric resistance exercise on PCNA mRNA levels and Pax7 protein expression as well as for the 4 MRFs at the mRNA levels and MyoD and myogenin at the protein levels. Looking at the effects of the conditions in which the exercise was performed, we found the more pronounced responses on MRF4 and myogenin mRNA levels and MyoD protein expression in the normoxic and the hypoxic group at 1 h and/or 24 h, with an attenuated response in the BFR group. Altogether, those results infirm our hypothesis of a higher adaptive response in hypoxia and with BFR compared to normoxia.

To better understand the regulation of myogenesis in our study, we investigated additional mechanisms, such as the immediate early genes, inflammation, MAPK, growth factors and lactate. When looking at immediate early gene expression, Fos mRNA levels, a negative regulator of MyoD expression [41], were higher in all conditions at 1 h post-exercise but lower at 24 h, especially in the normoxic group. Higher Fos mRNA levels were also observed directly after 8×8 repetitions at 80% 1RM (2 min rest) in normoxia and hypoxia ($FiO_2=0.14$) [5]. Four hours after exercise, Fos mRNA levels were returned to basal levels. In another study, Fos mRNA levels were found to be higher only at 2 h but not 4 h and 24 h post-exercise (3×12 repetitions at 60°/s, 2 min rest) [42]. Globally, the rapid increase in Fos mRNA levels after both concentric and eccentric resistance exercise suggests that Fos primarily prevents early differentiation immediately after satellite cell activation, but enables satellite cell differentiation in a second time, when its expression is reduced. In addition, Fos mRNA levels were reduced at 24 h only in the normoxic group, re-enforcing the higher response observed in that group specifically on the MRF expression.

Inflammation plays a major role in the regulation of myogenesis, with the switch from pro- to anti-inflammatory markers enabling the transition from proliferation to differentiation of satellite cells [43]. Here we found that, compared to pre-exercise, circulating IL-6 levels were lower from 1 to 72 h post-exercise, except at 1 h post-exercise in the hypoxic group, in which IL-6 levels were not decreased. This decrease in circulating IL-6 levels is quite surprising looking at other studies having found no changes in its levels up to 48 h following eccentric and concentric resistance exercise of low- and high-volume and intensity

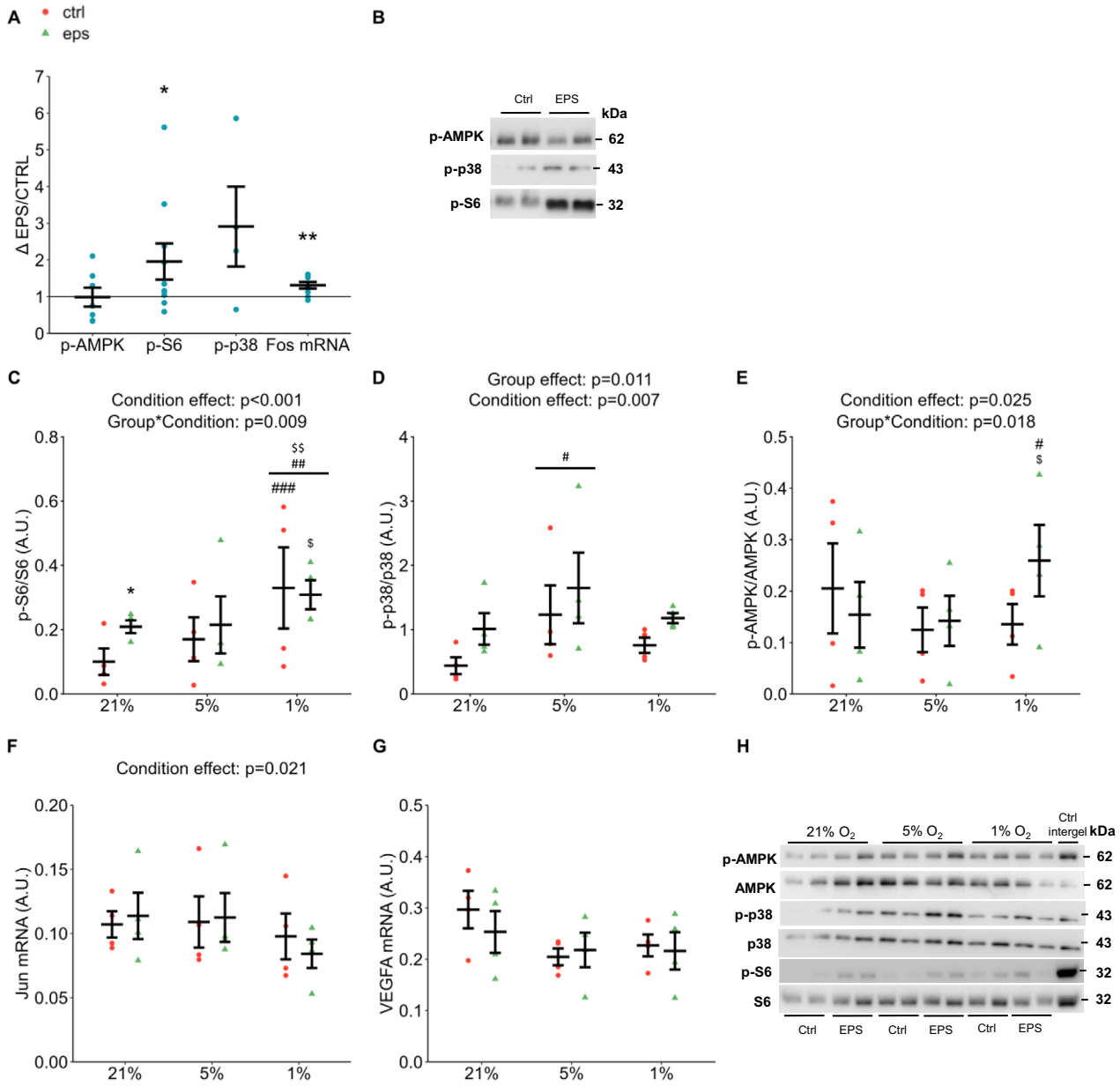

FIGURE 6 | Resistance-exercise like electrical pulse stimulation in human myotubes at 21%, 5%, and 1% oxygen. (A) Protocol validation: Differences in p-S6, p-p38, and p-AMPK protein expression and in Fos mRNA levels between stimulated and non-stimulated cells at 21% O_2. Mean ± SEM. Student's *T*-Tests were used for statistical analyses. (B) Representative Western blots for the EPS protocol validation. Protein expression of (C) p-S6, (D) p-p38, and (E) p-AMPK and mRNA levels of (F) Jun and (G) VEGFa in non-stimulated (○, $n = 4$ per condition) and stimulated (Δ, $n = 4$ per condition) human myotubes at 21%, 5%, and 1% O_2. (H) Representative Western blots. Mean ± SEM. Linear mixed models were used for statistical analyses from (C) to (G). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control unstimulated myotubes, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. 21% O_2 and $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. 5% O_2.

[6, 20, 44]. IL-6 secretion is associated with satellite cell proliferation through the phosphorylation of STAT3 [45]. Higher STAT3 phosphorylation was observed by Trenerry et al. [42] in skeletal muscle 2 h after 3 × 12 repetitions of knee extension at 60°/s, whereas those levels were returned to resting values no later than 4 h post-exercise. Our results corroborate those observations and indicate that STAT3 phosphorylation may occur earlier, at 1 h after eccentric resistance exercise. Of the few studies that investigated the levels of the anti-inflammatory cytokine IL-10 after resistance exercise in hypoxia, Perez-Regalodo et al. observed that IL-10 blood levels were higher in hypoxia (2320 m) versus normoxia at 5 min and 30 min after 3 sets of 10 repetitions of 6 different exercises at 70% 1RM [46]. A trend toward a decrease from 5 to 30 min post-exercise suggests that IL-10 blood levels could have returned to basal values as soon as 1 h post-exercise in hypoxia. Of note, in that study, IL-10 blood levels were unchanged in normoxia, while we found a similar downregulation in both normoxia and hypoxia. Interestingly, IL-10 is known to inhibit the pro-inflammatory pathway of TNF-α [47]. Here we found a concomitant decrease in circulating IL-10 levels and increase in TNF-α at 24 h and 72 h after all 3 exercise conditions. Whereas Britto et al. observed no changes in blood TNF-α levels

up to 4 h after 8 × 8 repetitions at 80% 1RM in normoxia and hypoxia [6], blood TNF-α levels were higher in young men 24 h and 48 h, but not earlier, after 8 × 10 repetitions of knee extension at 60°/s in normoxia [48]. Similarly to TNF-α, CD68 mRNA levels were also higher at 24 and 72 h post-exercise in our study, which is not surprising as CD68 secretes pro-inflammatory cytokines such as TNF-α and IL-1β [36]. Confirming the interrelationship between CD68, TNF-α and IL-1β, we found an increase over time as well for IL-1β. Altogether, those inflammatory markers might be linked in the response to resistance exercise, but the lack of differences in their regulation between groups fails to explain the difference we found in MRF expression between normoxia, hypoxia and BFR.

MAPK are also involved in the regulation of myogenesis, and their activity relies on oxygen availability [13]. In normoxia, p38 and mTOR phosphorylation are upregulated, promoting satellite cell differentiation and fusion. In hypoxia, both pathways are inhibited and the MEK–Erk pathway is upregulated, which promotes satellite cell proliferation and self-renewal [13]. We observed a decrease in p38 phosphorylation in skeletal muscle in all groups at 1 h post-exercise, with no further difference between groups. The downregulation of p38 phosphorylation coincided with the upregulation of the proliferating markers Pax7 and MyoD, whereas p38 phosphorylation returned to basal values at 24 h and 72 h post-exercise, likely to promote satellite cell differentiation and fusion. A decrease in p38 phosphorylation was also observed 1 h after a high-intensity resistance (1 × 20 repetitions of leg extension at maximal force at 40°/s, 70 s time under tension) [49]. In that study as well, p38 phosphorylation returned to baseline values at 24 h post-exercise. This suggests that an acute resistance exercise, whatever the environmental condition, can reduce p38 phosphorylation, likely to enable satellite cell proliferation and self-renewal.

Finally, we specifically developed and validated an EPS protocol mimicking resistance exercise in primary human myotubes. Typical markers of resistance exercise, namely S6 and p38 phosphorylation as well as the mRNA levels of the early-responsive gene Fos, were higher in the stimulated versus non-stimulated myotubes. Conversely, phospho-AMPK, which characterizes the response to endurance exercise, was not modified by EPS. Having validated the protocol, we were interested in investigating the effects of different oxygen levels on the regulation of the MRFs. We used the classical 21% O_2 levels, which do not reflect physiological conditions as myogenic cells are surrounded by a corresponding 5% O_2 [50]. We therefore opted for a third O_2 condition to create a real hypoxic environment, using 1% O_2. Unfortunately, we did not find any difference in any MRF or HIF-1α mRNA levels in stimulated versus unstimulated human myotubes, nor did we find any difference between O_2 levels. Our results contrast with previous results in C2C12 cells showing that MyoD and myogenin mRNA levels were higher 3 h after 1 h EPS at 10 Hz, 2 ms, 11.5 V, which is closer to an endurance exercise [51]. Future in vitro studies mimicking resistance exercise should consider rest time before harvesting to examine MRF as we collected the cells directly at the end of the EPS protocol. Another parameter to consider is the phase of myogenesis as proliferating myogenic cells seem more sensitive to O_2 levels than differentiating cells. Indeed, higher Pax7, Myf5, and MyoD mRNA levels and a higher proliferation rate were observed in 2% versus 21% O_2 proliferating cells, while no difference was found after 5 days of differentiation [52], neither after 4 days between 20% and 5% O_2 in another study [53].

The strengths of our study rely on the high intensity of the resistance exercise that induced muscle damage and enabled satellite cell activation. Our study is one of the sole studies with a very high eccentric component. Furthermore, the protocol conducted to failure ensured that the exercise intensity was similar between conditions when most of the studies compared BFR resistance exercise with a low load free-flow resistance exercise. This latter does not result in similar adaptations as the exercise intensity is lower than recommendations to develop hypertrophy or maximal force through resistance training. Our exercise protocol is therefore closer to the practice of usual resistance training.

The lack of differences observed between conditions can be attributed to several factors. Expected hypoxia-induced adaptations could have been prevented by the relatively short exposure to hypoxic conditions (40 min) or blunted by the stress induced by the eccentric contractions. In addition, we were limited in the number of muscle biopsies per participant and may have missed changes that could have occurred at other times, such as directly after exercise or 48 h or up to 7 days post-exercise. We also chose to recover in normoxia for all groups, following the live low-train high method, but recovery in hypoxia could affect myogenesis and upstream factors in another manner. We also focused on the main regulators of myogenesis such as inflammatory markers, growth factors, and MAPK, but angiogenic factors could be affected by our experimental conditions as well [54]. Finally, when participants conducted concentric and eccentric contractions in vivo, skeletal muscle myotubes in vitro underwent an isometric contraction, due to the EPS mechanism.

In conclusion, contrary to our hypothesis that an acute eccentric resistance exercise performed in hypoxia would induce a larger activation of satellite cells and myogenesis than in normoxia, we found that MRF expression was upregulated after exercise similarly in the normoxic and hypoxic groups and attenuated in the BFR group. Despite exercise being conducted to failure in the three groups, the BFR group had a lower total exercise work and peak torque. Thus, the stimulus might not have been high enough to reach the same intramuscular adaptations as in normoxia or environmental hypoxia.

Author Contributions

S.v.D.d.t.R. and L.D. designed the protocol. S.v.D.d.t.R., G.W., N.A., E.B., S.C., and L.D. acquired the data. S.v.D.d.t.R., P.K.-C., M.F., and L.D. analyzed and interpreted the data. S.v.D.d.t.R. and L.D. drafted the manuscript and all authors contributed to, and approved the final version of the manuscript.

Acknowledgments

This work was supported by a grant from the FNRS (J.0048.21). The authors would like to thank T. Bacq, A. de Wergifosse, F. Lwango, P. Kinart, and I. Ziedins for technical assistance during experiments, D. C. Navarro for the development of the resistance exercise-like EPS protocol and C. Bugli for assistance in statistical analysis.

Funding

This work was supported by a grant from FNRS (J.0048.21).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are not publicly available. Data are however available from the authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Representative immunofluorescence pictures with myonuclei (Dapi blue), myogenin (yellow), MyoD (green) and laminin (red). Yellow arrow: costaining of myogenin and Dapi. **Figure S2:** (A) Myh1, (B) Myh2, and (C) Myh7 mRNA levels in skeletal muscle and (D) Blood levels of C-reactive protein (CRP) before and 1, 24, and 72 h after eccentric resistance exercise in Normoxia (○, $n = 13$), with BFR (Δ, $n = 13$), or in Hypoxia (□, $n = 12$). Mean ± SEM. Linear mixed models were used for statistical analyses. BFR, blood flow restriction. **Figure S3:** mRNA levels of (A) MyoD, (B) Myf5, (C) MRF4, (D) myogenin, (E) Fos, and (F) HIF-1α in non-stimulated (○, $n = 4$ per condition) and stimulated (Δ, $n = 4$ per condition) human myotubes at 21%, 5%, and 1% O_2. Mean ± SEM. Linear mixed models were used for statistical analyses. **Table S1:** Repetitions in reserve. **Table S2:** Blood lactate.