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Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes

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ABSTRACT

Aim. To determine whether repeated maximal-intensity hypoxic exercise induces larger beneficial adaptations on the hypoxia inducible factor-1α pathway and its target genes than similar normoxic exercise, when combined with chronic hypoxic exposure.

Methods. Lowland elite male team-sport athletes underwent 14 days of passive normobaric hypoxic exposure (≥14 h.day^{-1} at F_iO_2 14.5-14.2%) with the addition of six maximal-intensity exercise sessions either in normobaric hypoxia (F_iO_2 ~14.2%) (LHTLH; n = 9) or in normoxia (F_iO_2 20.9%) (LHTL; n = 11). A group living in normoxia with no additional maximal-intensity exercise (LLTL; n = 10) served as control. Before (Pre), immediately after (Post-1), and 3 weeks after (Post-2) the intervention, muscle biopsies were obtained from the *vastus lateralis*.

Results. Hypoxia inducible factor-1α subunit, vascular endothelial growth factor, myoglobin, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha and mitochondrial transcription factor A mRNA levels increased at Post-1 (all $P \leq 0.05$) in LHTLH, but not in LHTL or LLTL, and returned near baseline levels at Post-2. The protein expression of citrate synthase increased in LHTLH ($P<0.001$ and $P<0.01$ at Post-1 and Post-2, respectively) and

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LLTL (P<0.01 and P<0.05 at Post-1 and Post-2, respectively), whereas it decreased in LHTL at Post-1 and Post-2 (both P<0.001).

Conclusion. Combined with residence in normobaric hypoxia, repeated maximal-intensity hypoxic exercise induces short-term post-intervention beneficial changes in muscle transcriptional factors that are of larger magnitude (or not observed) than with similar normoxic exercise. The decay of molecular adaptations was relatively fast, with most of benefits already absent 3 weeks post-intervention.

Key words. Hypoxia, physical exercise, maximal intensity, gene, protein, oxygen sensor system.

INTRODUCTION

Human skeletal muscle is a greatly specialized and a highly adaptive tissue. Increased oxygen (O_2) consumption and/or a lowered tissue O_2 tension (hypoxia) are known to initiate a cascade of systemic, local and cellular adaptations, all aiming to restore O_2 homeostasis (Semenza, 1999, Semenza, 1998). Whatever the origin of the hypoxic stimulus [*i.e.*, exercise-induced (Ameln *et al.*, 2005) or environmentally- (O_2-deprived environments) (Hoppeler and Vogt, 2001)], the hypoxia inducible factor-1α subunit (HIF-1α), an O_2 sensitive transcriptional activator that stabilizes in the nucleus under hypoxic conditions, is the main factor mediating these responses (Wang and Semenza, 1995). HIF-1α is a key regulator responsible for the induction of hypoxia-induced genes (Ke and Costa, 2006) in turn involved in erythropoiesis/iron metabolism, angiogenesis, glucose metabolism as well as cell proliferation/survival and apoptosis (Lundby et al., 2009, Semenza, 1998, Semenza, 1999).

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The current scientific literature indicates that prolonged hypoxic residence (≥10-12 h.day^{-1} for a minimum of 10 consecutive days) would only induce minimal adaptations in human skeletal muscle tissue. Specifically, down-regulation of HIF-1α (-49%) and its target genes [*e.g.*, vascular endothelial growth factor (VEGF) (-66%)] (D'Hulst *et al.*, 2016), absence of muscle angiogenesis as well as marginal changes in oxidative enzymes [*e.g.*, citrate synthase (CS)] (Lundby *et al.*, 2009) have been reported. Contrastingly, exercising in hypoxia appears appealing to promote structural and functional adaptations in skeletal muscle (Lundby et al., 2009, Hoppeler and Vogt, 2001). While these changes have been reported to occur immediately after (within days post-intervention) the aforementioned hypoxic interventions, the extent to which delayed (after several weeks) positive muscle phenotype adaptations also occur is unknown.

While chronic low-intensity 'aerobic' exercise in hypoxia may evoke cellular adaptations via HIF-1α activation (Vogt et al., 2001, Zoll et al., 2006), the magnitude of these responses likely depends on the hypoxic dose (Lundby *et al.*, 2009), and may not necessarily translate in substantial physical performance benefits in endurance individuals (Lundby et al., 2012, Roels et al., 2007, Truijens et al., 2003). Interestingly, it has been postulated that exercise intensity in hypoxia *per se* modulates muscle molecular mechanisms of O_2 homeostasis with '*adaptations that compensate for the reduced availability of O_2 during exercise*' (Hoppeler and Vogt, 2001). Reportedly, maximal-intensity hypoxic exercise, where short 'all-out' efforts (≤ 10 s) with incomplete recoveries (< 30 s) are repeated (namely repeated sprints in hypoxia or RSH), induces additional molecular adaptations at the skeletal muscle level compared to similar exercise in normoxia (RSN) (Faiss *et al.*, 2013b). Specifically, the mRNA expression of genes involved in O_2 signaling (HIF-1α), O_2 carrying [myoglobin (Mb)] and pH regulation [carbonic anhydrase-3 (CA-3)] were up-regulated after RSH but not after RSN. Nonetheless, the observation of a concomitant down-regulation of genes involved in

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mitochondrial biogenesis [mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α)] after RSH would suggest a shift from aerobic to anaerobic glycolytic activity in the muscle and a more efficient use of fast-twitch (FT) muscle fibers (Faiss et al., 2013a, Faiss et al., 2013b, Puype et al., 2013).

We recently demonstrated that the combination of the 'live high-train low' (LHTL) paradigm and RSH, namely 'live high-train low and high' (LHTLH), produces superior short- (2-3 days post-intervention) and long-term (3 weeks post-intervention) performance benefits (*i.e.*, repeated-sprint ability and high-intensity intermittent exercise capacity) than when LHTL and RSN are combined (Brocherie *et al.*, 2015). While short- and long-term hematological adaptations (*e.g.*, increase in hemoglobin mass) were similar between these two training interventions, twice larger immediate repeated-sprint performance gains associated with LHTLH (which were also maintained after 3 weeks with this training regimen only) advocate that non-hematological factors outside the role played by O_2 carrying-capacity are probably more robust to explain why LHTLH maximizes performance changes. Thus, chronic hypoxic exposure in combination with repeated maximal-intensity hypoxic exercise (LHTLH) appears as a promising intervention to induce concomitant hematological and molecular adaptations. To date, however, changes in skeletal muscle molecular mechanisms of O_2 homeostasis in response to LHTLH remain undetermined.

Therefore, the purpose of this study was to investigate the immediate (*i.e.*, few days post-intervention) and delayed (*i.e.*, several weeks post-intervention) skeletal muscle molecular adaptations associated with 14 days of passive normobaric hypoxic exposure combined with RSH (LHTL+RSH, namely LHTLH) or RSN (LHTL+RSN, namely LHTL) [both compared to a control condition, *i.e.*, 'live low-train low' (LLTL)]. Our hypothesis was that, when combined with passive normobaric hypoxic residence, repeated maximal-intensity hypoxic

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exercise induces immediate post-intervention muscle molecular adaptations – *i.e.*, HIF-1α pathway and its target genes – not observed (or to a lower extent) with similar normoxic exercise. We also expected that the molecular adaptations would be maintained for a longer period when combining passive normobaric hypoxic residence with repeated maximal-intensity hypoxic exercise compared to the other conditions tested. This is the first study to investigate the effects of prolonged passive normobaric hypoxic exposure with superimposed repeated maximal-intensity exercise sessions in hypoxia (LHTLH) *vs*. normoxia (LHTL) on molecular adaptations in human skeletal muscle.

MATERIALS AND METHODS

Subjects

Thirty lowland elite male field hockey players (age 25.1 ± 4.5 years, height 177.8 ± 5.6 cm, body weight 75.2 ± 7.7 kg and estimated VO_2max 52.0 ± 1.9 $mL.min^{-1}.kg^{-1}$) were recruited among Belgium, Spanish and Dutch first division clubs to participate in this study.

The subjects were fully informed of the possible risks involved in the study before providing written consent. The study was approved by the Anti-Doping Lab Qatar institutional review board (Agreement SCH-ADL-070) and was conducted according to the Helsinki Declaration. Exclusion criteria for participation were acclimatization or exposure to hypoxia of more than 2000 m for more than 48 h during a period of 6 months before the study, and any history of altitude-related sickness and health risk that could compromise the subject's safety during exercise and/or hypoxia exposure. During the study, two subjects (control group: n = 1; experimental groups: n = 1) were excluded due to injury.

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Procedures

The experimental design and physical performance results have been described in details elsewhere (Brocherie *et al.*, 2015). Briefly, it consisted in three testing sessions conducted before (Pre-), 2-3 days (Post-1) and three weeks (Post-2) following the 14-days intervention period. After the completion of Pre-, subjects were randomly assigned to one of the three following groups: 14 days of residence in normobaric hypoxia (≥14 $h.day^{-1}$ at 2800-3000 m, inspired oxygen fraction (F_iO_2) 14.5-14.2%) during which subjects exercised (*i.e.*, regular field hockey practices) at sea level with the addition of six repeated maximal-intensity exercise sessions either in normobaric hypoxia simulating an altitude of 3000 m (LHTL+RSH, namely LHTLH; n = 8) or in normoxia (LHTL+RSN, namely LHTL; n = 11) and control (LLTL; n= 9) where subjects did not performed any additional specific exercise. Importantly, this research was successfully run in a double-blinded, controlled manner (Brocherie *et al.*, 2015).

Living hypoxic exposure. The sleeping and recreational hypoxic facilities were fully furnished normobaric hypoxic rooms with O_2-filtration membrane that reduces the molecular concentration of O_2 in ambient air (CAT system, Colorado Altitude Training, Louisville, Colorado, USA). The two intervention groups (LHTLH and LHTL) were exposed (*i.e.*, from 22:00 to 07:00, from 08:00 to 10:00 and again from 13:00 to 16:00; and were encouraged to spend more time in if desired) to a normobaric hypoxia equivalent to 2500 m (F_iO_2 15.1%, BP 768.0 mmHg, P_iO_2 108.3 mmHg) for the first 24 h of the intervention period (day 1). Thereafter, the O_2 fraction was further decreased to the equivalent of 2800 m (F_iO_2 14.5 ± 0.1%, BP 766.8 ± 1.1 mmHg, P_iO_2 104.5 ± 0.6 mmHg; days 2-5) and 3000 m (F_iO_2 14.2 ± 0.1%, BP 765.3 ± 1.5 mmHg, P_iO_2 101.7 ± 0.8 mmHg; days 6-14). Concentrations of ferritin

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(155.2 ± 78.7 μg.L^{-1}, range: 45-279 μg.L^{-1}) and soluble transferrin receptor (256.6 ± 33.7 mg.dL^{-1}, range: 202-330 mg.dL^{-1}) measured during the 2-weeks lead-in period at sea level indicated that none of the subjects was iron deficient at the commencement of the study.

Supervised exercise protocol. In addition to their usual field hockey practices and matches [carefully monitored and reported in (Brocherie *et al.*, 2015)], subjects of the two intervention groups (LHTLH and LHTL) completed six specific repeated maximal-intensity exercise sessions (with at least 36 h in between) on a synthetic grass ground, inside a 45-m long mobile inflatable simulated hypoxic equipment (Altitude Technology Solutions Pty Ltd, Brisbane, Queensland, Australia), as described elsewhere (Girard *et al.*, 2013). For RSH, ambient air was mixed with nitrogen (from pressurized tanks) to reduce F_iO_2 to ~14.2% in order to simulate an altitude of 3000 m. In order to blind subjects to altitude, the system was also run for RSN with normoxic airflow (F_iO_2 21.0%) into the tunnel.

Each session lasted ~50 min including a 15-min warm-up, the repeated maximal-intensity exercise and a 10-min recovery phase (*i.e.*, a total of 300 min for the 6 sessions among the 14-days intervention). Specifically, the repeated maximal-intensity exercise included 4 sets of 5 × 5-s maximal sprints interspersed with 25 s of passive recovery with 5 min of standing rest between sets.

Muscle Biopsy Samples

Biopsy samples were all taken by the same experienced medical doctor with a 5-mm Bergström type needle (in conjunction with a suction device to create a negative pressure) in the mid portion of the *vastus lateralis* muscle after local anesthesia (1% xylocaine,

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subcutaneously). Biopsies were preceded by 48 h without any exercise activity and were taken randomly in opposite legs (*i.e.*, left-right-left or vice versa) during subsequent test sessions. For mRNA analysis, the muscle tissue portion was immediately frozen in liquid nitrogen and stored at -80°C until required for analyses.

Western blotting

Frozen muscle tissue (~20 mg) was homogenized 3 × 5 s with a Tissuelyser (Qiagen, Hilden, Germany) in an ice-cold buffer (1:10, w/v) [50 mM Tris-HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 1 % Triton-X 100 and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium)]. After centrifugation of homogenates at 10 000 g for 10 min at 4°C, the supernatants were stored at -80°C. Protein concentration was measured using the DC protein assay kit (Bio-Rad laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Proteins (30-50 µg) were separated by SDS-PAGE (7.5 – 12.5%) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% non-fat milk for 1 h and incubated overnight (4°C) with the following antibodies: glucose transporter 4 (GLUT-4, #PA1-1065, Thermo Scientific, Erembodegem, Belgium), phosphofructo kinase (PFK, #166722, Santa Cruz, Huissen, The Netherlands), monocarboxylate transporter-1 (MCT-1, #AB3538P, Millipore, Overijse, Belgium), monocarboxylate transporter-4 (MCT-4, #AB3316P, Millipore), CA-3 (#135995, Abcam, Cambridge, UK), AMP-activated protein kinase alpha (AMPKα, #2532, Cell Signaling, Leiden, The Netherlands), phospho-AMPKα Thr172 (#2535, Cell Signaling), CS (#14309, Cell Signaling), eukaryotic elongation factor 2 (eEF2, #2332, Cell Signaling). Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Bornem, Belgium)

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were used for chemiluminescent detection of the proteins of interest. Membranes were scanned and quantified with Genesnap and Genetools softwares (Syngene, Cambridge, UK), respectively. The results are presented as the ratio protein of interest/eEF2 or as the ratio phosphorylated/total form for AMPK.

Real-time quantitative Polymerase Chain Reaction

RNA was extracted using TRIzol (Invitrogen, Vilvoorde, Belgium) from 20–25 mg of frozen muscle tissue. RNA quality and quantity were assessed by spectrophotometry with a Nanodrop (Thermo Scientific, Erembodegem, Belgium). One µg of RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium) according to manufacturer's instructions. A SybrGreen-based master mix (Applied Biosystems, Erembodegem, Belgium) was used for real-time PCR analyses using the ABIPRISM 7300 (Applied Biosystems). Real-time PCR primers were designed for human HIF-1α, PHD-2 (prolyl hydroxylase domain protein 2), VEGF, Mb, cytochrome c oxidase subunit 4 isoform 1 (COX-4_1) and isoform 2 (COX-4_2), PGC-1α, TFAM, endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), pyruvate dehydrogenase kinase 1 (PDK-1), glucose transporter 1 (GLUT-1) and, phosphofructokinase (PFK) (Table 1). Thermal cycling conditions consisted of 40 three-step cycles including denaturation of 30 s at 95°C, annealing of 30 s at 58°C and extension of 30 s at 72°C. All reactions were performed in triplicate. To compensate for variations in input RNA amounts and efficiency of reverse transcription ribosomal protein L19 (RPL19) and beta-2-microglobulin (B2M) mRNA were quantified, and results were normalized to these values. These genes were chosen out of three normalization genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere (Vandesompele *et al.*, 2002).

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Citrate synthase activity

Five µg of protein were added to the reaction buffer containing 100 mM Tris base, 2 mM EDTA, 1.25 mM malate, 0.25 mM NAD and 6 $U.ml^{-1}$ L-malate dehydrogenase (10127256001, Roche, Brussels, Belgium). The temperature was set at 37˚C and the reaction started by the addition of acetyl-CoA. NADH production was measured by fluorescence (340 nm excitation, 460 nm emission).

Data and statistical analysis

Because of large inter-individual differences in absolute mRNA levels and protein expressions, post-intervention values are expressed as percent changes in means (Pre- *vs*. Post-1 and Post-2, respectively), while the mean of the Pre- values was assigned to the arbitrarily value of 1.00±0.00. Two-way ANOVA with repeated measures [Time (Pre- *vs*. Post-1 *vs*. Post-2) × Group (LHTLH *vs*. LHTL *vs*. LLTL)] was used to compare each measured variable. When significant modifications were found, Holm-Sidak post-hoc test was performed to localize the effect. All analyses were made using Sigmaplot 11.0 software (Systat Software, CA, USA). Null hypothesis was rejected at $P < 0.05$.

RESULTS

Skeletal muscle mRNA expression analysis and enzyme activity

Figures 1, 2 and 3 and Table 2 display changes from Pre- to Post-1 and Post-2 in mRNA and protein expression/activity levels in the three groups.

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O_2 signaling. Compared to Pre-, HIF-1α mRNA levels increased at Post-1 ($P < 0.01$) in LHTLH only, before returning to near Pre- values at Post-2 (Fig. 1). At Post-1, higher HIF-1α mRNA levels were observed in LHTLH compared to LHTL and LLTL (both $P < 0.05$).

O_2 carrier. Compared to Pre-, higher mRNA levels of VEGF ($P = 0.05$) and Mb ($P < 0.05$) occurred at Post-1 in LHTLH (Fig. 1). At Post-1, VEGF and Mb values were also higher in LHTLH than in LLTL (both $P < 0.05$).

Mitochondrial biogenesis and metabolism. Transcript levels of regulators of mitochondrial biogenesis PGC-1α ($P < 0.05$ at Post-1) and TFAM ($P < 0.05$ at Post-1 and Post-2) were increased in LHTLH only. No significant changes were detected in COX-4_1 and COX-4_2 mRNA levels. The protein expression of CS increased in LHTLH ($P < 0.001$ at Post-1 and $P < 0.01$ at Post-2) and LLTL ($P < 0.01$ at Post-1, and $P < 0.05$ at Post-2), whereas it decreased in LHTL ($P < 0.001$ at Post-1 and Post-2) (Fig. 2). Similar results were obtained for CS activity (Table 2).

Nitric oxide synthase pathway. Compared to Pre-, eNOS and nNOS mRNA levels tended to increase at Post-1 in LHTLH (P = 0.30 and P = 0.09, respectively) (Fig. 1). In LHTL, only eNOS mRNA levels increased significantly from Pre- to Post-1 ($P < 0.01$), while values of both eNOS and nNOS were higher at Post-2 *vs*. Pre (both $P < 0.05$). The mRNA levels of eNOS at Post-1 were higher in LHTLH and LHTL compared to LLTL (both $P < 0.05$).

pH regulation. The protein expression of MCT-1, but not of MCT-4 and CA-3, was increased in LHTLH ($P < 0.05$) and LHTL ($P < 0.05$) at Post-1 in reference to Pre- (Figs. 2 and 3). Whereas MCT-1 at Post-2 returned to near Pre- values in LHTLH, lower values were observed from Pre- to Post-2 in LHTL ($P < 0.05$).

Glucose metabolism. At Post-1, the mRNA levels of GLUT-1 were higher in LHTLH compared to Pre- (Fig. 1, $P < 0.05$) while the protein expression of PFK was lower in LHTLH

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compared to LLTL (Figs. 2 and 3, $P < 0.05$). No changes were measured in the protein expression levels of GLUT-4 (Figs. 2 and 3).

AMPK total protein content and phosphorylation status. Compared to Pre-, AMPK total protein content ($P < 0.01$ for LHTLH and LHTL) and pAMPK-tot ($P < 0.001$ for LHTLH and LHTL) increased significantly at Post-1, whereas lower pAMPK-tot values were noted at Post-2 ($P < 0.01$ and < 0.001 for LHTLH and LHTL, respectively) (Figs. 2 and 3).

DISCUSSION

This is the first study to investigate the effects of prolonged passive exposure to normobaric hypoxia with superimposed repeated maximal-intensity exercise sessions in hypoxia (LHTLH) *vs*. normoxia (LHTL) on molecular regulations in human skeletal muscle. The novel findings are that LHTLH elicits higher short-term (first few days post-intervention) molecular responses of factors implicated in the regulation of O_2 signaling and carrying, mitochondrial biogenesis, as well as of enzymes implicated in mitochondrial metabolism compared to LHTL [with also no change in control (LLTL)]. We further indicate that the majority of these positive molecular responses disappeared already three weeks post-intervention. We confirm our hypothesis of larger specific transcriptional responses when passive normobaric hypoxic exposure and repeated maximal-intensity hypoxic exercise are combined, yet with normalization of molecular adaptations three weeks after the intervention. These adaptations in O_2 signaling and transport, mitochondrial biogenesis, as well as in enzymes implicated in mitochondrial metabolism, may contribute to the sport-specific performance gains (Post-1) reported elsewhere (Brocherie et al., 2015). The functional benefits (*e.g.*, performance gains) were maintained up to 3 weeks post-LHTLH (Brocherie et

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al., 2015) and were likely caused by the molecular adaptations which may have promoted changes in muscle biochemistry and vascularity.

O_2 signaling and gene regulation

In the present study, the level of HIF-1α mRNA increased immediately after the intervention in LHTLH but, unexpectedly, it was not the case for LHTL. First, the absence of HIF-1α increase after 14 days of LHTL supports the hypothesis that HIF-1α response to hypoxia is time-dependent and activated shortly and briefly after hypoxic stimulus exposure (Lundby *et al.*, 2009). Accordingly, it has been previously demonstrated that HIF-1α protein levels peak within the first hours of hypoxic exposure then progressively decline toward basal levels (Stroka et al., 2001, Vigano et al., 2008), suggesting a possible local or systemic 'acclimatization' after several days (Lundby *et al.*, 2009). As the protein level of HIF-1α is only briefly increased after a hypoxic stimulus and our intention was to determine stable molecular changes after repeated expositions to hypoxia, we solely quantified the mRNA level of this transcription factor, which reflects the long-term activation of the HIF-1α pathway (Galban and Gorospe, 2009). With this in mind, the enhanced mRNA level of HIF-1α after LHTLH clearly suggests that the addition of repeated maximal-intensity exercise in hypoxia (but not in normoxia) plays an important role for up-regulating the activation of the HIF-1α pathway and some of its downstream genes (Zoll et al., 2006, Vogt et al., 2001, Faiss et al., 2013b). This is further supported by the return to near HIF-1α mRNA Pre- values at 3 weeks post-intervention when no additional repeated maximal-intensity exercises and/or hypoxic stimulation were performed.

Activation of HIF-1α is known to lead to cellular adaptations [*i.e.*, O_2 carrying-capacity (Wenger and Gassmann, 1997), neovascularization (Forsythe *et al.*, 1996), glucose oxidation

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(Wenger and Gassmann, 1997)], which in turn would positively influence exercise capacity in humans (Vogt et al., 2001, Zoll et al., 2006, Faiss et al., 2013b). Accordingly, the mRNA levels for the capillary growth factor VEGF, *i.e.*, an HIF-1-regulated gene (Semenza, 1999, Semenza et al., 1999, Wenger and Gassmann, 1997) and Mb mRNA, significantly increased after LHTLH, whereas no changes were observed in both LHTL and LLTL. This corroborates previous works, which demonstrated Mb mRNA or protein levels enhancement after hypoxic endurance exercise [*i.e.*, 3-5 sessions of intermittent hypobaric (~2300 m) or normobaric hypoxic (~3850 m) training per week for 4-6 weeks (Vogt et al., 2001, Terrados et al., 1990)]. Overall, it seems that these RSH-related molecular adaptations were not blunted by the passive normobaric residence and may participate in the twice-larger repeated-sprint performance improvements observed in LHTLH *vs*. LHTL (Brocherie et al., 2015).

Metabolic Phenotype

After hypoxic endurance exercise, it has been demonstrated that mRNA levels for PGC-1α (Zoll *et al.*, 2006), COX-1, COX-4 and CS (Zoll et al., 2006, Vogt et al., 2001, Terrados et al., 1990) increase to a greater extent compared to similar intervention in normoxia. In the present study, LHTLH induced larger mitochondrial adaptations (*i.e.*, increased mRNA levels for PGC-1α and TFAM) compared to LHTL and LLTL, thereby suggesting a preponderant role of the superimposed RSH for muscle phenotypic adaptations. Whereas the protein expression and protein activity of CS decreased after LHTL, it increased after both LHTLH and LLTL. Conversely, when used in isolation (*i.e.*, with normoxic residence), Faiss et al. (Faiss *et al.*, 2013b) indicated that RSH induced a down-regulation in mitochondrial biogenesis (PGC-1α and TFAM), despite unchanged oxidative capacity (CS). Some methodological differences (*i.e.*, exercise mode, frequency and duration) between studies might be responsible for these

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discrepant findings. In summary, the increase in PGC-1α and TFAM measured after intervention in LHTLH, for which mRNA expression predicts protein levels quite well (Collu-Marchese et al., 2015, Handschin et al., 2003), appears also as a plausible molecular explanation for the beneficial adaptations observed in LHTLH *vs*. LHTL *vs*. LLTL.

Compensatory vasodilation

Among the HIF-1α target genes, VEGF is a critical signal in vascular remodeling, which maintains vascular integrity and stimulates the production of the vasodilatory mediator nitric oxide. Hence, eNOS plays a key role in blood flow regulation and vascular tone (Gielen *et al.*, 2011). Concomitantly, nNOS expression exerts a functionally significant effect in hypoxic tissue, thereby influencing tissue O_2 delivery (Fish et al., 2007, Tsui et al., 2014), ventilatory regulation and metabolic adaptations to hypoxia (Gardiner *et al.*, 2011). Although we did not measure the protein expression, an increase in eNOS and nNOS may promote changes in the blood flow and vascular tone (Gielen *et al.*, 2011), participate in angiogenesis (Viboolvorakul and Patumraj, 2014) and enhance O_2 delivery.

pH-regulating System

In line with previous sprint interval intervention studies (Puype et al., 2013, Burgomaster et al., 2007), LHTLH and LHTL increased muscle MCT-1 protein content, whereas MCT-4 and CA-3 contents did not change. This potential up-regulation of lactate metabolism may reflect the similar sport-specific aerobic performance gains observed in LHTLH and LHTL (Brocherie et al., 2015).

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Delayed effects on skeletal muscle transcriptional regulation

We recently demonstrated that LHTLH and LHTL resulted in similar short-term (2-3 days post-intervention) hematological adaptations (*i.e.*, increase in hemoglobin mass) that were maintained for at least 3 weeks post-intervention (Brocherie *et al.*, 2015). Contrastingly, our novel findings indicate that most of the transcriptional adaptations were back to near Pre-values 3 weeks post-intervention. The present study is the first to show the time course of HIF-1α and related genes transcription in human skeletal muscle adaptations following residence in normobaric hypoxia superimposed with repeated maximal-intensity exercise in hypoxia or normoxia. With the post-intervention carefully supervised and controlled (*i.e.*, similar between groups), the fact that this period did not include any environmental hypoxic stress – be it during residence or exercise in normobaric hypoxia – indicates that the lack of external hypoxic 'stimulus' could be responsible for the rapid reversal of skeletal muscle transcriptional regulation. Meanwhile, it may have already promoted some beneficial muscle function adjustments and therefore functional benefits without affecting hemoglobin mass improvement (Brocherie *et al.*, 2015). Additionally, as the post-intervention period (3 weeks) did not include any repeated maximal-intensity exercise session (as performed six times during the 14-days intervention), the influence of such exercise modality and/or frequency on normalization of molecular responses should not be overlooked. One aspect that deserves more research attention is whether additional hypoxic stress and/or intense exercise when individuals return to sea level after an hypoxic intervention induces a better maintenance of molecular adaptations. The decay of molecular responses over time after return to sea level, that occur more rapidly than the hematological adaptations, would suggest that mechanisms (not measured here) such as those involved in the factor-inhibiting HIF regulation (Lindholm and Rundqvist, 2016) may be at play.

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Strengths and limitations

One potential strength of this study is that elite field hockey players volunteered to participate, while mechanistic studies are generally carried out with recreational participants. In these studies, results are often extrapolated to elite athletes while exercise-related adaptations are probably less prominent as the level of practice/performance increases. Here, we found that LHTLH was the most efficient strategy to activate in the short-term the transcription of specific genes involved in key physiological processes for adaptations such as angiogenesis or O_2 transport. It is therefore tempting to associate the molecular regulations we measured at Post-1 and the enhanced performance that we previously reported at both Post-1 and Post-2 in LHTLH (Brocherie *et al.*, 2015).

Methodologically, it is known that most gene expressions generally peak within 2-8 h after a single exercise stimulus (Pilegaard *et al.*, 2003). Although we acknowledge that short-term (hours) molecular responses are the basis for long term structural, enzymatic and functional adaptations, we are confident that, with muscle biopsies obtained 48 h following the last exercise session in our subjects, the augmented expression of several genes actually is the result of the proposed intervention *per se*, and not a side effect of the final exercise session. Despite careful control of biopsy sampling (*i.e.*, no physical activity allowed between the last exercise session, similar sampling timing between 08:00 and 10:00 a.m. during the three test sessions and corresponding samples ran in the same assay) (Fluck *et al.*, 2005), a large inter-subject gene and protein expression variation occurred here. Potentially, this may be explained by: (i) DNA sequence variations resulting from HIF-1α gene polymorphism in the promoter region of HIF-1α gene (Prior *et al.*, 2006), (ii) fiber type differences in the sampling from *vastus lateralis* muscle, which has an unequal typology and metabolic properties (Pette, 1985), (iii) pulsative nature of gene expression in muscular fiber (Newlands *et al.*, 1998), and (iv) involvement of reactive oxygen species in the regulation of HIF-1α mRNA and HIF-1α

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target genes (Pialoux *et al.*, 2009). That said, it is worth mentioning that biopsies obtained alternatively from the left and right *vastus lateralis* muscle actually give similar mRNA expression profiles when the two legs are compared (Lundby *et al.*, 2005).

In conclusion, combined with normobaric hypoxic residence, repeated maximal-intensity hypoxic exercise elicited higher short-term (first few days post-intervention) skeletal muscle molecular beneficial adaptations not observed (or with smaller magnitudes) after similar normoxic exercise. The large and specific adaptations in mRNA levels of factors involved in O_2 signaling and transport, mitochondrial biogenesis, as well as in enzymes implicated in mitochondrial metabolism, are seen to highlight the prominence of superimposed repeated maximal-intensity hypoxic exercise, yet with a rapid decay and normalization of molecular adaptations after cessation of the intervention.

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Conflict of interest

No conflict of interest, financial or otherwise, is declared by the authors.

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Table 1. PCR Primers sequences.

Gene	Forward Primer	Reverse Primer
HIF-1α	GCCCCAGATTCAGGATCAGA	TGGGACTATTAGGCTCAGGTGAAC
PHD-2	AGGTGAAGCCAGCCTAT	CCTGATGCTAGCTGATACTTG
VEGF	TTTCTGCTGTCTTGGGTGCATTGG	ACCACTTCGTGATGATTCTGCCCT
Mb	GCCACCAAGCACAAGATC	GGCATCAGCACCAAAGT
COX-4_1	GAGAGCTTTGCTGAGATGAA	CCGTACACATAGTGCTTCTG
COX-4_2	CCTTCTGCACAGAACTCAAC	CGGTACAAGGCCACCTTT
PGC-1α	GGGATGATGGAGACAGCTATGG	CTCTTGGTGGAAGCAGGGTC
TFAM	AGCGTTGGAGGGAACTTCCTGATT	TTCTTTATATACCTGCCACTCCGCCC
eNOS	CAGTTACCAGCTAGCCAAAGT	CTCATTCTCCAGGTGCTTCAT
nNOS	CAGAACTCACACAAGGTCTATC	GTTGACCGACTGGATTTAGG
PDK-1	TGCCCATATCACGTCTTTAC	GTCTGTTGACAGAGCCTTAAT
GLUT-1	CCTGCAGTTTGGCTACAACA	GTGGACCCATGTCTGGTTG
PFK	ATTTGACGAAGCCCTGAAG	GTGCGAACCACTCTTAGATAC
RPL19	CGCTGTGGCAAGAAGAAGGTC	GGAATGGACCGTCACAGGC
B2M	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG

Table 2. Citrate synthase protein activity.

	LHTLH	LHTL	LLTL
Pre-	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Post-1	1.19 ± 0.27*,†	0.82 ± 0.25	1.22 ± 0.25*,†
Post-2	1.20 ± 0.31†	0.79 ± 0.32*	1.35 ± 0.22***,†

* $P < 0.05$ *** $P < 0.001$ significantly different from Pre-; $P < 0.05$ † significantly different from LHTL.

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Figures Legend

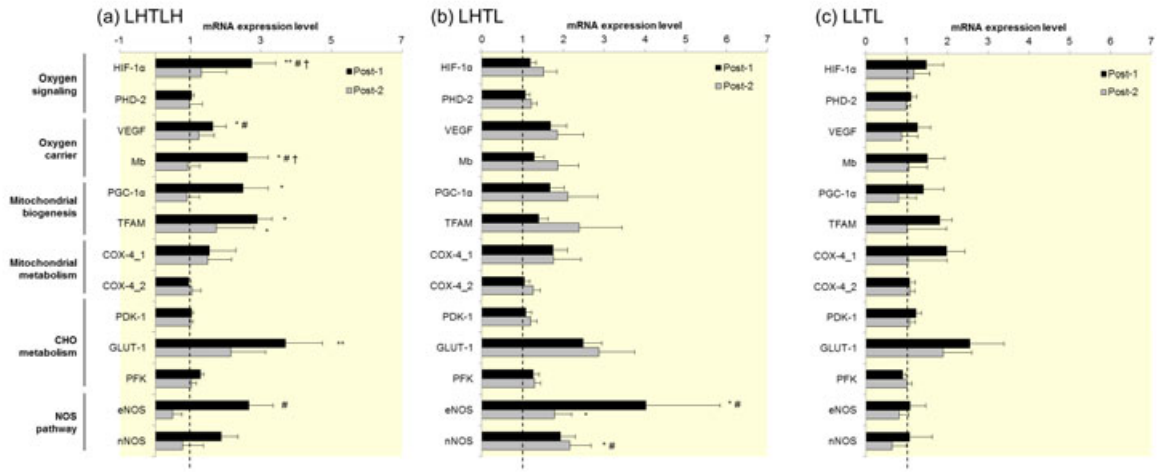
Figure 1. Relative changes in selected mRNA expression markers from before (Pre-) to immediately (Post-1) and 3 weeks (Post-2) after the intervention. The intervention consisted in 14-days of passive normobaric hypoxic exposure combined with (a) repeated maximal-intensity hypoxic exercise in hypoxia (LHTLH) or (b) normoxia (LHTL). (c) A control group followed a 'live low-train low' (LLTL) protocol. Black and grey bars represent Post-1 and Post-2 values of mRNA concentrations in *vastus lateralis* muscle, respectively. These values were normalized to baseline values (Pre-), which were set to 1.00±0.00 (dashed line). Values are means ± SD. * $P < 0.05$, ** $P < 0.01$ *vs*. Pre-intervention; # $P < 0.05$ *vs*. LLTL and † $P < 0.05$ *vs*. LHTL. HIF-1α, hypoxia inducible factor-1a; PHD-2, prolyl hydroxylase domain protein 2; VEGF, vascular endothelial growth factor; Mb, myoglobin; PGC1-α, proliferator-activated receptor gamma coactivator-1α; TFAM, mitochondrial transcription factor A; COX-4_1, cytochrome oxidase 4 isoform 1; COX-4_2, cytochrome oxidase 4 isoform 2; PDK-1, pyruvate dehydrogenase kinase 1; GLUT-1, glucose transporter 1; PFK, phosphofructokinase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

Figure 2. Relative protein expression of selected markers from baseline (Pre-) to the end of the intervention (Post-1) and after 3 weeks (Post-2). The intervention consisted in 14-days of passive normobaric hypoxic exposure combined with (a) repeated maximal-intensity hypoxic exercise in hypoxia (LHTLH) or (b) normoxia (LHTL). (c) A control group followed a 'live low-train low' (LLTL) protocol. Black and grey bars represent Post-1 and Post-2 values of protein concentrations in *vastus lateralis* muscle, respectively, and were normalized to baseline values (Pre-), which were set to 1.00±0.00 (dashed line). Values are means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *vs*. Pre- and # $P < 0.05$, ## $P < 0.01$ *vs*. LLTL. CS,

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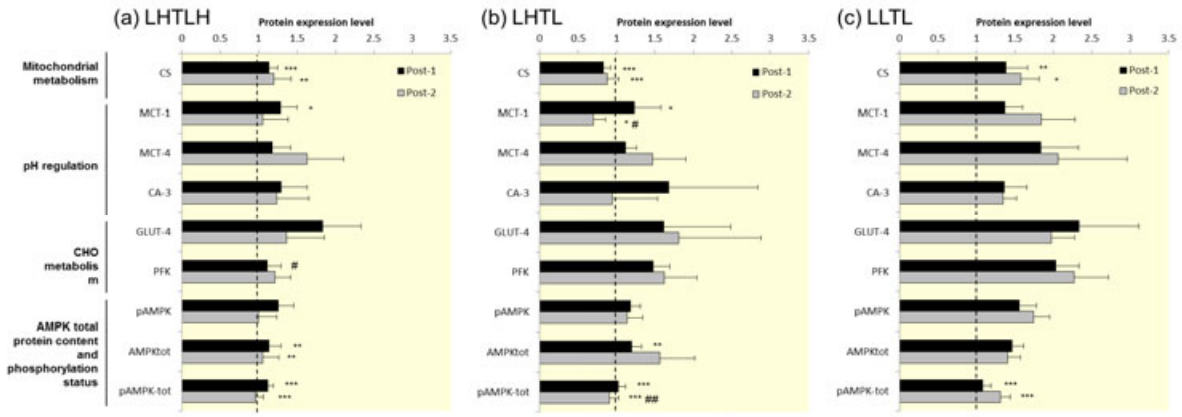
citrate synthase; MCT-1, monocarboxylate transporter 1; MCT-4, monocarboxylate transporter 4; CA-3, carbonic anhydrase III; GLUT-4, glucose transporter 4; PFK, phosphofructokinase; AMPK, AMP-activated protein kinase.

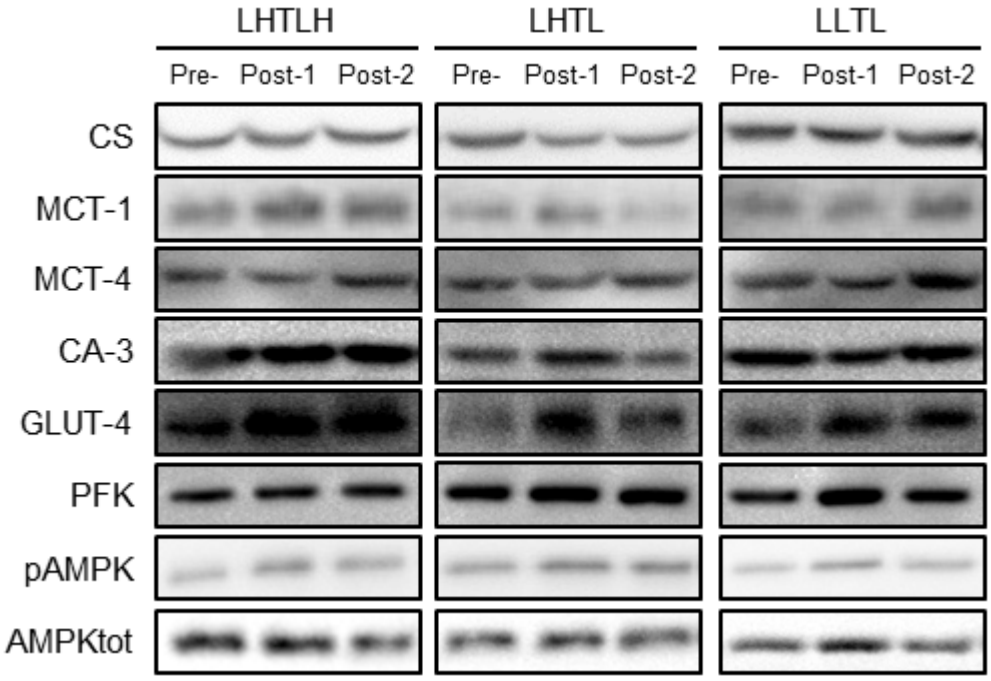
Figure 3. Representative Western blots showing the content of selected proteins from baseline (Pre-) to the end of the intervention (Post-1) and after 3 weeks (Post-2). The intervention consisted in 14-days of passive normobaric hypoxic exposure combined with (a) repeated maximal-intensity hypoxic exercise in hypoxia (LHTLH) or (b) normoxia (LHTL). (c) A control group followed a 'live low-train low' (LLTL) protocol. CS, citrate synthase; MCT-1, monocarboxylate transporter 1; MCT-4, monocarboxylate transporter 4; CA-3, carbonic anhydrase III; GLUT-4, glucose transporter 4; PFK, phosphofructokinase; AMPK, AMP-activated protein kinase.



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