

RESEARCH ARTICLE

Repeated sprint training in hypoxia induces specific skeletal muscle adaptations through S100A protein signaling

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Abstract

Athletes increasingly engage in repeated sprint training consisting in repeated short all-out efforts interspersed by short recoveries. When performed in hypoxia (RSH), it may lead to greater training effects than in normoxia (RSN); however, the underlying molecular mechanisms remain unclear. This study aimed at elucidating the effects of RSH on skeletal muscle metabolic adaptations as compared to RSN. Sixteen healthy young men performed nine repeated sprint training sessions in either normoxia ($F_{I}O_2=0.209$, RSN, $n=7$) or normobaric hypoxia ($F_{I}O_2=0.136$, RSH, $n=9$). Before and after the training period, exercise performance was assessed by using repeated sprint ability (RSA) and Wingate tests. *Vastus lateralis* muscle biopsies were performed to investigate muscle metabolic adaptations using proteomics combined with western blot analysis. Similar improvements were observed in RSA and Wingate tests in both RSN and RSH groups. At the muscle level, RSN and RSH reduced oxidative phosphorylation protein content but triggered an increase in mitochondrial biogenesis proteins. Proteomics showed an increase in several S100A family proteins in the RSH group, among which S100A13 most strongly. We confirmed a significant increase

Abbreviations: Akt, protein kinase B; bRP, basic reversed-phase; CamKII, calcium/calmodulin-dependent protein kinase II; DDA, Data-dependent acquisition; DIA, Data-independent acquisition; EMG, electromyography; FIO₂, fraction of inspired oxygen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, glucose transporter 4; GoBP, Gene Ontology Biological Process; HIF-1 α , hypoxia inducible factor 1 α ; HIIT, High-intensity interval training; KEGG, Kyoto Encyclopedia Genes and Genomes; LC, Liquid Chromatography; LDHa, lactate dehydrogenase A; MCT, monocarboxylate transporter; MS, mass spectrometry; MVC, maximal voluntary contraction; M wave, muscle compound action potential; OXPHOS, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase 1; PGC-1 α , peroxisome proliferator-activated receptor coactivator 1 α ; PI3K, phosphatidylinositol 3 kinase; RAGE, receptor for advanced-glycation endproducts; RSA, repeated sprint ability; RSH, repeated sprint in hypoxia; RSN, repeated sprint in normoxia; Sdec, sprint decrement; TIMS PASEF, trapped ion mobility spectrometry parallel accumulation-serial fragmentation; TOM20, mitochondrial import receptor subunit TOM20 homolog; VAL, Voluntary activation level; VEGFa, vascular endothelial growth factor A; VL, vastus lateralis.

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in S100A13 protein by western blot in RSH, which was associated with increased Akt phosphorylation and its downstream targets regulating protein synthesis. Altogether our data indicate that RSH may activate an S100A/Akt pathway to trigger specific adaptations as compared to RSN.

KEYWORDS

exercise, glycolysis, HIF-1 α , OXPHOS, RSH, S100A13

1 | INTRODUCTION

High-intensity interval training (HIIT), characterized by short intense bouts of exercise interspersed by periods of rest, elicits adaptations that improve oxygen transport, glycolytic capacity, and neuromuscular function,^{1,2} according to the training modalities (e.g., work and recovery intervals' intensity and duration, number of repetitions¹). To elicit specific adaptations, various forms of HIIT are employed, including all-out sprint interval training and repeated sprint training in normoxia (RSN). These modalities not only enhance exercise performance but also induce notable glycolytic adaptations in muscle tissue.^{3,4}

Athletes are increasingly using repeated sprint in hypoxia (RSH) as a training modality. The rationale for combining acute hypoxia with exercise is based on the hypothesis that passive exposure to hypoxia would induce not only central⁵ but also peripheral adaptations. Accordingly, cell exposure to hypoxia blunts oxidative phosphorylation (OXPHOS) through pyruvate dehydrogenase kinase 1 (PDK1) activation^{6,7} and increases glycolysis through stimulation of glucose uptake,⁸ and upregulation of glycolytic enzymes and lactate transporters.^{9,10} Although in humans such passive hypoxia exposure-induced peripheral adaptations are not supported by the literature,^{11,12} the combination of acute exercise with hypoxia exposure was found to induce superior performance improvement as compared to RSN.¹³ However, little is known about the specific muscle metabolic response to RSH and whether it might support the beneficial effects of RSH. Two studies reported heterogeneous results: one found increases in glycolytic enzymes and lactate transporters together with reduced mitochondrial biogenesis markers¹⁴; the other did not report any changes in glycolytic or lactate metabolism enzymes but observed an increase in mitochondrial biogenesis markers in response to RSH.¹⁵ Both studies used a transcriptional approach, limiting the interpretation of the results since changes in RNA with training had little overlap with corresponding protein abundance.¹⁶ Clarifying the molecular pathways of muscle adaptations to RSH may thus provide new insights into the role of hypoxia-induced skeletal muscle adaptations in response to training.¹⁷

In this study, we used neuromuscular assessments and muscle biopsies, followed by proteomics and western blot

approaches to investigate the specific molecular pathways induced by RSH, in comparison with RSN. The focus was to compare the glycolytic versus oxidative responses between the two training modalities and to clarify the underlying mechanisms.

2 | MATERIALS AND METHODS

2.1 | Participants

Sixteen physically active men volunteered to participate in this study after being informed of the potential risks and discomfort related to the different experimental procedures. Participants were required to be accustomed to high-intensity training and engage in a minimum of 5 h of training per week. They were instructed to maintain their training activities but at a lower intensity and were asked to not participate in any competition during the study period. The study protocol was approved by the Ethical Commission for Human Research (CER-VD, protocol 2018-02298), and each participant gave his written consent prior to starting the experimentation. The study was conducted according to the Declaration of Helsinki. After meeting the requirements, participants were matched into two groups based on the mean power developed during the Wingate test and RSA test measured during the initial testing; each group performed an identical training protocol but under different conditions. One group trained in normoxia (RSN, $n=7$, age = 27 ± 7 years, height = 179 ± 6 cm, body mass = 71 ± 4 kg, fraction of inspired oxygen [$F_{I}O_2$] of ~ 0.209), while the second group trained in normobaric hypoxia (RSH, $n=9$, age = 25 ± 5 years, height = 183 ± 6 cm, body mass = 77 ± 8 kg, $F_{I}O_2$ of ~ 0.136). The unbalanced number of participants in the groups resulted from the withdrawing of one RSN participant during the study.

2.2 | General procedures

Before starting data collection, each participant underwent familiarization sessions for neuromuscular assessments and repeated sprinting. Then, two test sessions

were conducted (pre). This procedure was repeated two to four days after the training period (post) (see Figure 1). For the first testing session at pre and post, participants were instructed to arrive in an overnight fasting state and to complete a food questionnaire listing everything consumed the previous day. The first test session consisted of four phases: micro biopsies of the *vastus lateralis* (VL), evaluation of knee extensor neuromuscular function with voluntary and electrically evoked contractions coupled with force and electromyography (EMG) recordings, and a RSA test. The second test session consisted of two phases: a Wingate test and a 10 km time trial. All testing was performed in normoxia. The training period lasted three weeks with three training sessions per week for a total of nine training sessions.

2.2.1 | Muscle biopsies

Needle biopsies were taken from the right VL muscle. Briefly, after skin sterilization and local anesthesia, a

1–2-mm-long skin cut was made with the tip of a scalpel. Biopsies were collected using an automatic biopsy device (Bard Biopsy Instrument, Bard Radiology, Covington, GA, USA). A 14-gauge disposable trocar mounted in the device was inserted through the cut, perpendicular to the muscle fibers, until the fascia was pierced. Three or four samples (~15 mg each) were collected at different angles from one puncture site. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

2.2.2 | Torque and EMG recordings

Voluntary and evoked knee extension forces were recorded using an isometric ergometer consisting of a custom-built chair equipped with a strain gauge (STS 250 kg, sensitivity 2.0 mV/V and 1.7 mV/N, SWJ, China), sampled at a rate of 1 kHz using an AD conversion system (MP150, BIOPAC, Goleta, CA, USA), and stored for off-line analysis with commercial software (AcqKnowledge, BIOPAC, Goleta, CA). The knee joint was positioned at an

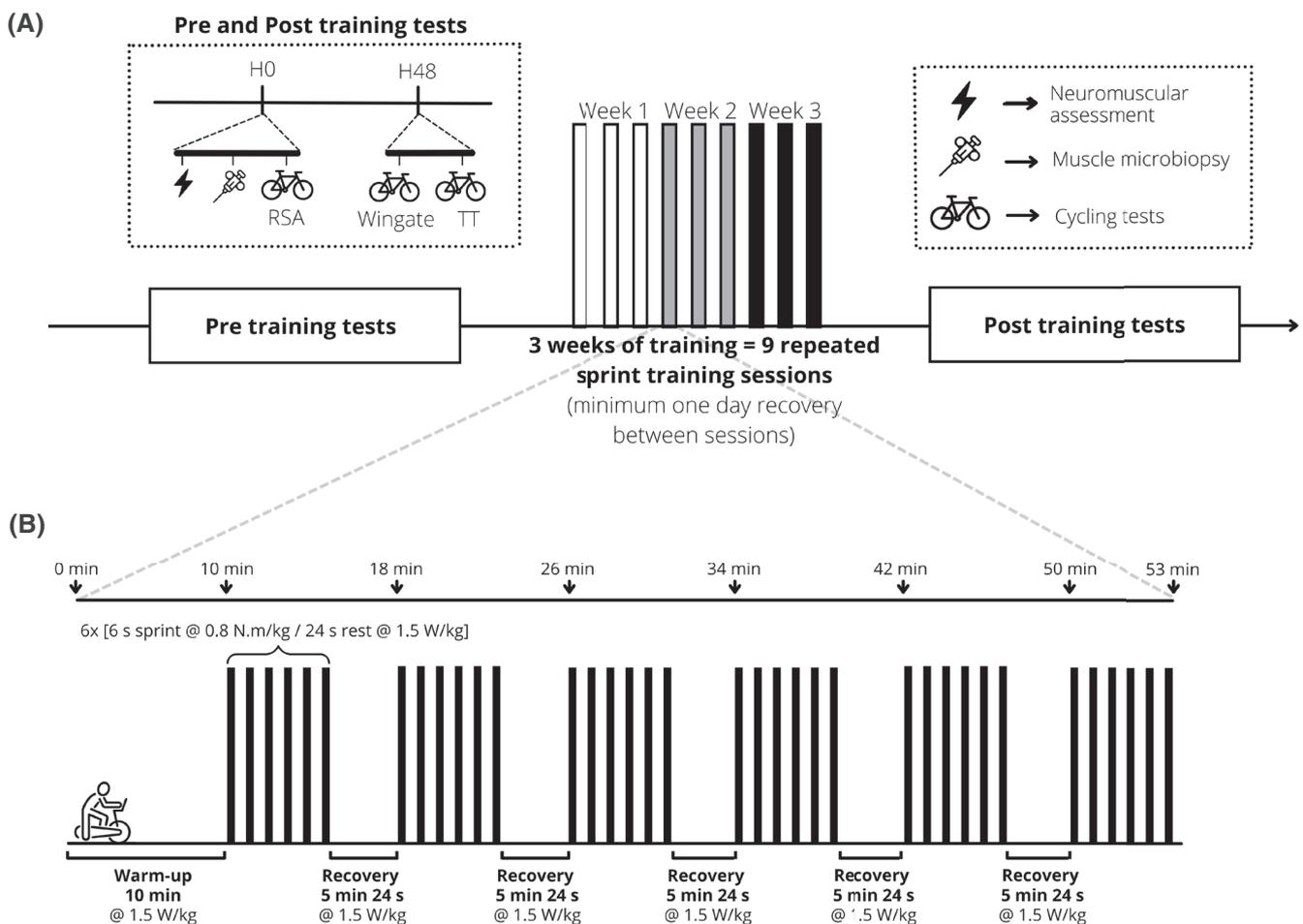


FIGURE 1 Graphical representation of the experimental procedures. (A) general overview of the protocol. (B) representation of a repeated sprint training session.

angle of 90° and the trunk-thigh at 100° (180° = full extension). The dynamometer axis was aligned with the knee extension axis, and the lever arm was fixed to the shank with a strap. The upper body was strapped to the chair with a belt across the abdomen and two cross-shoulder harnesses to limit compensatory movements. Participants were asked to maintain their arms crossed on their chest during the entire assessment. Measurements were made on the same leg (right) throughout the study using the same chair position settings.

The EMG activity of VL was recorded using pairs of circular silver chloride (Ag/AgCl) electrodes (recording diameter: 10 mm, inter-electrode distance: 20 mm, Kendall Meditrace 100, Tyco, Cork, Ireland) positioned on the muscle belly according to the SENIAM recommendations.¹⁸ The reference electrode was placed over the patella. Low resistance between electrodes was obtained by shaving and cleaning the skin with alcohol. EMG signals were amplified (gain = 1000), filtered through a 10–500 Hz band pass filter, recorded at a sampling rate of 2 kHz, and stored for off-line analysis with the same hardware and software used for the force signal.

2.2.3 | Neuromuscular assessments

A standardized warm-up entailing several submaximal contractions (from 20% to 80% of the maximal estimated strength) was followed by a test battery consisting of a 5-s maximal voluntary isometric contraction (MVC) of the knee extensors with one superimposed doublet at 100 Hz (paired stimuli) (to quantify MVC force, MVC EMG root mean square, and voluntary activation level (VAL) according to the twitch interpolation technique), followed by one doublet at 100 Hz, one doublet at 10 Hz [to quantify 100 Hz doublet peak force and 10/100 Hz ratio], and a single stimulus [to quantify the muscle compound action potential (M-wave)] delivered at every 2 s after the MVC. Paired and single stimuli of 1 ms were delivered at 400 V through a circular cathode (diameter: 2 cm, same model as electrodes used for EMG recordings) positioned at the femoral triangle level over the femoral nerve with a transcutaneous constant current electrical stimulator (model DS7AH, Digitimer, Welwyn Garden City, UK). The anode (10 × 5 cm, Compex Sa, Ecublens, Switzerland) was placed in the gluteal fold opposite to the cathode. For MVC, participants were asked to raise the force progressively (1–2 s) until a plateau was reached and then maintain this force for 3–4 s. The superimposed paired stimuli were delivered manually during the plateau phase. The investigator provided strong verbal encouragement during the MVC. A supramaximal current intensity (120% of the optimal intensity) was used during the entire experiment to maintain

an optimal stimulation of the femoral nerve.¹⁹ The optimal stimulation intensity was assessed incrementally at the beginning of each test session and was reached when a higher intensity of stimulation did not exert a further increase in peak twitch torque and M-wave amplitude.

2.2.4 | Repeated sprint ability test (RSA)

Participants warmed-up for 1 min at a constant resistance of 1.5 W.kg⁻¹ followed by two 6 s sprints 1 min apart. The RSA test consisted of all-out sprints (6 s effort—24 s active recovery with 20 W resistance between sprints) on a cycle ergometer (Lode Excalibur Sport Ergometer, Lode B.V., Netherlands) at a fixed torque factor of 0.8 N.m.kg⁻¹. Participants repeated the sprints until exhaustion or were stopped after the twentieth repetition. The power developed by the participants was continuously recorded during the RSA test. Lactatemia was measured from a capillary fingertip sample (Lactate Pro, Arkray, Japan) immediately after the test.

2.2.5 | Wingate test and 10 km time trial

First, participants cycled during 1 min with no resistance at 80 rpm. Immediately thereafter, participants performed the Wingate test, consisting in one 30 s all-out sprint at a fixed torque factor of 0.8 N.m.kg⁻¹. Lactatemia was measured immediately at the end of the test. After a 15-min recovery period off, participants warmed-up freely for 2 min and then performed the 10 km time-trial test. Participants cycled as fast as possible for a virtual distance of 10 km. Participants were informed of 25%, 50%, 75%, and 90% of test completion. Each participant was strongly verbally encouraged during all tests.

2.2.6 | Training protocol

The training protocol was completed in a normobaric hypoxic room using O₂ extraction to simulate altitude (ATS Altitude Training, Sydney, Australia). The hypoxic device simulated an altitude of 3800 m above sea level, with a F_IO₂ of ~0.136 for the RSH group, while the RSN group trained at the laboratory terrestrial altitude of 372 m, with a F_IO₂ of ~0.209. The training protocol was single blinded as participants had no information about the condition of training.

After a standardized 10 min warm-up at 1.5 W.kg⁻¹, the participants performed six sets of six sprint repetitions (6 s effort—24 s active recovery) with the same resistance as for the RSA test. A 5-min easy spinning recovery at 1.5 W.kg⁻¹ separated each set. Participants were told to cycle as

fast as possible while remaining seated during each sprint and were strongly encouraged by the experimenters. To standardize the beginning of each sprint, the pre-sprint cycling cadence was imposed at ~80 RPM.

2.3 | Data analysis

2.3.1 | Analysis of power output

The mean power was calculated for the RSA test, the Wingate test, and the 10 km time trial. For the RSA test, the power of each sprint was averaged over the entire test. In addition, the sprint decrement (Sdec) score was also calculated during the RSA test as the ability to resist to fatigue using the following formula²⁰:

$$\text{Sdec (\%)} = \left[1 - \frac{S_1 + S_2 + S_3 + \dots + S_{20}}{S_{\text{best}} \times 20} \right] \times 100,$$

where S_1 = sprint 1 mean power, etc., and S_{best} = best sprint mean power (usually the first repetition).

2.3.2 | Analysis of EMG and force signals

The MVC force was considered as the highest level of force attained during contraction. The twitch interpolation technique was used to evaluate the VAL, which was estimated using the following formula²¹:

$$\text{VAL} = [100 - D \times (\text{MVC}_{\text{@stim}} \div \text{MVC}) \div \text{PD} \times 100],$$

where D = difference between the force just before ($\text{MVC}_{\text{@stim}}$) and during the superimposed doublet and PD = potentiated doublet force at rest.

The peak force evoked by the 100 Hz doublet was quantified to assess contractile properties.²²

The M-wave first phase amplitude in response to the single stimulation was measured as an index of sarcolemmal excitability.²³

2.3.3 | Proteomics

Proteomic analysis was performed as previously described,²⁴ with slight modifications.

Due to limited sample processing capacity, six participants per group were included for proteomics analysis. Tissue samples (~10 mg) were homogenized and then allowed to dry. The pellet was resuspended, heated, and used for all subsequent steps. Protein concentrations were determined by the tryptophane fluorescence method

(Wisniewski et al., 2015).²⁵ Samples were digested following a modified version of the modified version of the in-StageTip method (named miST method).²⁶ Completeness of digestion was assessed qualitatively by SDS-PAGE migration.

For peptide fractionation, aliquots of 5 μg of all samples were mixed to create a pool, which was manually separated into seven fractions by off-line basic reversed-phase (bRP). The fractions collected were purified, and dried bRP fractions were redissolved and injected for LC-MS/MS analysis. Data-dependent (DDA) and data-independent acquisition (DIA) measurements were carried out using identical LC gradients.

For creation of spectral libraries, DDA was carried out on the seven bRP fractions using a standard Trapped Ion Mobility Spectrometry Parallel Accumulation-Serial Fragmentation (TIMS PASEF) method.²⁷ Up to 10 precursors were targeted per TIMS scan cycle. The diaPASEF method used the same instrument parameters as the DDA methods and was as reported previously.²⁸

A library was constructed from the DDA data for the fractions by searching the reference human proteome (RefProt, <http://www.UNIPROT.org>) database of September 3, 2020 (75 777 sequences). Two subset-specific libraries were first created for the RSN and RSH sample groups. These were subsequently combined to yield a global library. The two sample subsets were all compared to the unified global library to increase consistency of protein identification.

Protein quantitation by DIA was done using the library described above. Both MS1 and MS2 data were used for quantitation.²⁹ Quantities for protein groups were obtained by summing all assigned peptide intensities after filtering. Global normalization of runs/samples was done based on the median of peptides. Total normalized intensities for protein groups were exported to .txt files for further analysis.

Intensity values were \log_2 -transformed. After removal of contaminants and assignment to groups, Student's t -tests were carried out among all conditions, with Benjamini-Hochberg correction for multiple testing (q -value threshold $>.05$). The difference of means obtained from the test was used for 1D enrichment analysis on associated Gene Ontology Biological Processes (GoBP) / Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation as described elsewhere.³⁰ The enrichment analysis was also FDR-filtered (Benjamini-Hochberg, q -val $<.02$). Using annotations from the HUGO Gene Nomenclature Committee, we searched for proteins involved in each mitochondrial complex and created a heatmap (Figure 3H) representing the change of protein expression after RSN and RSH training. We also used STRING protein-protein interaction network (<http://www.string-db.org>) to represent the protein clusters in Figures 5F and 6G,H.

Raw data deposition

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD040779.

2.3.4 | Western blot analysis

Muscle samples were suspended in a lysis buffer (100 μ L/5 mg of tissue) containing the following: 20 mM Tris/HCl (pH 6.8), 2 mM EDTA (pH 8), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM beta-glycerophosphate, 1 mM KH_2PO_4 , 1 mM PMSF, 1 mM NaVO_3 , 50 mM NaF, and a protease inhibitor mixture (Roche, Complete Mini, Basel, Switzerland). The preparation was homogenized with a potter, incubated for 1 h at 4°C on wheel, and sonicated. The nuclei and debris were removed by centrifugation at 9300 \times g at 4°C for 10 min and protein quantification performed in duplicate using the BCA Kit (Thermo Fisher Scientific, Ecublens, Switzerland). 2 \times Laemmli sample buffer containing SDS and 2-mercapto-ethanol (Bio-Rad, Hercules, CA, USA) was added to 10 or 20 μ g of protein. For OXPHOS, peroxisome proliferator-activated receptor coactivator-1 α (PGC1 α), mitochondrial import receptor subunit TOM20 homolog, and vascular endothelial growth factor A (VEGFa) analysis, samples were not heated while for other proteins an incubation of 3 min at 95°C was performed. Then, samples were electrophoresed 40 min on 4%–15% SDS-precast gradient gels (Bio-Rad, Hercules, CA, USA) and wet transferred for 1 h onto pre-activated PVDF membranes. Total protein staining was detected on membranes with Red Ponceau staining, and bands were quantified using the Image Studio software v.5.2.5 (LI-COR, Lincoln, NE, USA). Red Ponceau was washed out using PBS, and membranes were incubated for 1 h at room temperature with PBS-LI-COR blocking buffer (LI-COR, Lincoln, NE, USA). Then, membranes were incubated overnight at 4°C with primary antibody prepared in blocking buffer (see Table S1 for details). Membranes were washed in PBS-Tween-20 and incubated for 1 h at room temperature with secondary antibody IRDye 680-conjugated donkey anti-mouse and IRDye 800-conjugated donkey anti-rabbit IgG (LI-COR, Lincoln, NE, USA, 1:10000 and 1:5000, respectively) or IRDye 680-conjugated goat anti-mouse and IRDye 800-conjugated goat anti-rabbit IgG (LI-COR, Lincoln, NE, USA, 1:100000 and 1:10000, respectively). Immunoreactive bands were visualized using infrared fluorescence (IR-Odyssey scanner, LI-COR, Lincoln, NE, USA), and band densities were quantified using Image Studio v 5.2.5 (LI-COR, Lincoln, NE, USA). Protein intensity signal was normalized to that of total protein staining, and protein quantifications were expressed as a percentage of the pre-RSN values.

2.4 | Statistical analysis

Normality of the data was checked using the Shapiro–Wilk test. To compare the training effect (pre vs. post) in the two different conditions (RSN vs. RSH), a linear mixed model with time and training condition as fixed effect and participant as random effect was used. When the interaction was significant, multiple comparisons were conducted to test differences among pairs of means using the Sidak post hoc test. The variation in the n values for blood lactate concentration data were due to omission of measurement during data collection. For the neuromuscular assessment-related variables, the missing values were due to noise-contaminated signal or technical issues with electrical stimulations. Finally, for western blot analysis, muscle sample volume was limited for some participants. Data were analyzed using *Jamovi software* (version 2.3, Sydney, Australia). The significance level was set at $p \leq .05$. Data are presented as means with individual values \pm standard deviation. Data from western blot analysis are presented as relative to pre-RSN.

3 | RESULTS

3.1 | RSN and RSH training similarly improve human exercise performance

Compliance with training was very good (no sessions were missed). At the end of the last testing session, participants were asked to tell under which condition they thought they had trained. ~70% of the participants did not guess correctly under which conditions they had been training suggesting adequate blinding. Exercise performance was improved in a similar manner for both RSN and RSH groups (Figure 2). The mean power was significantly increased for the RSA test (RSN: +7.2% \pm 7.7% vs. RSH: +7.9% \pm 6.6%, $p < .001$, Figure 2A). To quantify fatigue resistance to sprint repetitions, which is an important parameter for performance, Sdec was calculated during the RSA test. Sdec was reduced in both groups (RSN: -40.1% \pm 25.9% vs. RSH: -12.5% \pm 64.1%, $p = .004$, Figure 2B) without significant changes in the best sprint mean power (Figure 2C). Blood lactate concentration showed no significant changes after training (Figure 2D). The time to completion of the 10-km time trial was significantly reduced from 1083 \pm 244 s to 965 \pm 181 s after RSN and from 1057 \pm 254 s to 934 \pm 191 s after RSH (RSN: -10.2% \pm 6.5% vs. RSH: -10.7% \pm 6.1%, $p < .0001$). The mean power significantly increased during both the 10-km time trial (RSN: +11.9% \pm 8.3% vs. RSH: +12.6% \pm 7.4%, $p < .0001$, Figure 2E) and the Wingate test (RSN: +1.3% \pm 3.6% vs. RSH: +4.4% \pm 5.0%,

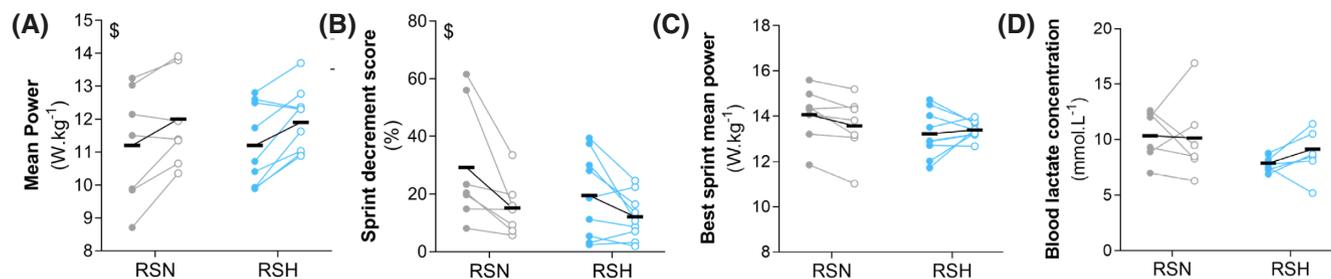
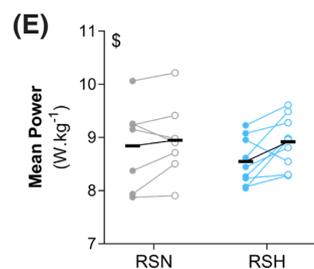
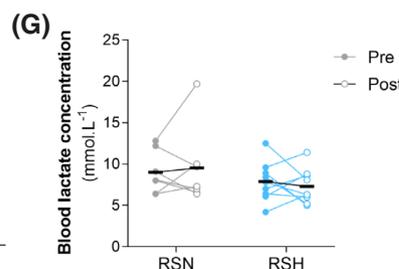
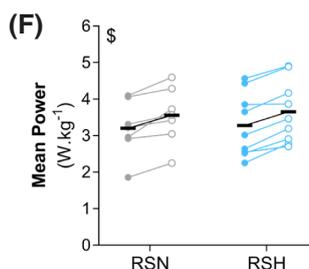
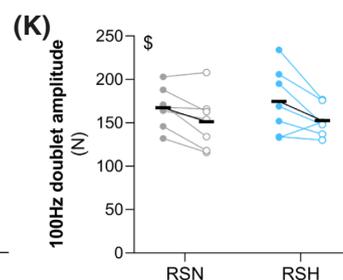
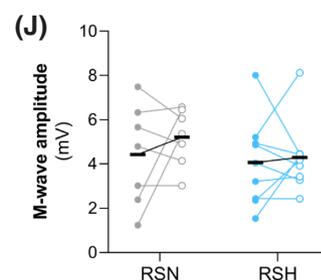
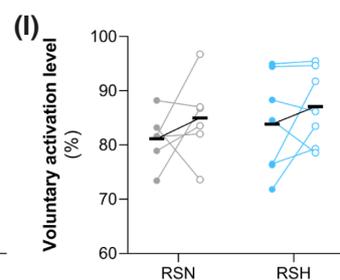
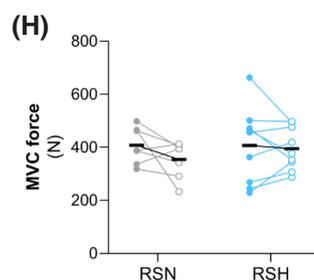
RSA test**10 km time trial test****Wingate test****Neuromuscular testing**

FIGURE 2 RSN and RSH training similarly improved human performance to various cycling tests. (A–C) Measurements of the mean power (A), sprint decrement score (B), best sprint mean power (C), and blood lactate concentration (D) in response to the repeated sprint ability (RSA) test; $n = 7$ and 9 participants (A–C) and $n = 6$ and 8 participants (D) for repeated sprint training in normoxia (RSN) and repeated sprint training in hypoxia (RSH), respectively. (E–G) Measurements of the mean power developed during the 10 km time-trial test (E) and measurements of the mean power (F) and the blood lactate concentration (G) in response to the Wingate test; $n = 7$ and 9 participants for RSN and RSH, respectively. (H) Assessment of knee extensor maximal voluntary contraction (MVC) force; $n = 7$ and 9 participants for RSN and RSH, respectively. (I) Voluntary activation level; $n = 6$ and 7 participants for RSN and RSH, respectively. (J) Amplitude of M-wave; $n = 7$ and 9 participants for RSN and RSH, respectively. (K) Electrically evoked force at 100 Hz double stimulation; $n = 7$ participants per group. Dots linked by lines represent individual participants and black thick lines represent the mean before (pre) and after (post) training. $^{\$}p \leq .05$ main effect of time.

$p = .025$, Figure 2F). Furthermore, blood lactate concentration was not significantly affected immediately after the Wingate test (Figure 2G).

Knee extensor neuromuscular assessments were performed to quantify the potential neural and contractile adaptations in response to training. While RSN and RSH training did not affect MVC force, VAL, nor M-wave amplitude, both groups showed a similar slight reduction in PD by $\sim 11\%$ (Figure 2H–K).

Together, these results show similar performance improvements following RSN and RSH training.

3.2 | RSN and RSH training reduce proteins involved in mitochondrial oxidative phosphorylation

Investigation of muscle metabolic adaptations triggered by RSN and RSH training was done by first using an unbiased proteomic analysis. The results indicated significant decreases in proteins related to the OXPHOS pathway. The biological processes “oxidative phosphorylation,” “cellular respiration,” and “generation of precursor metabolites and energy” were attenuated in response to both RSN and

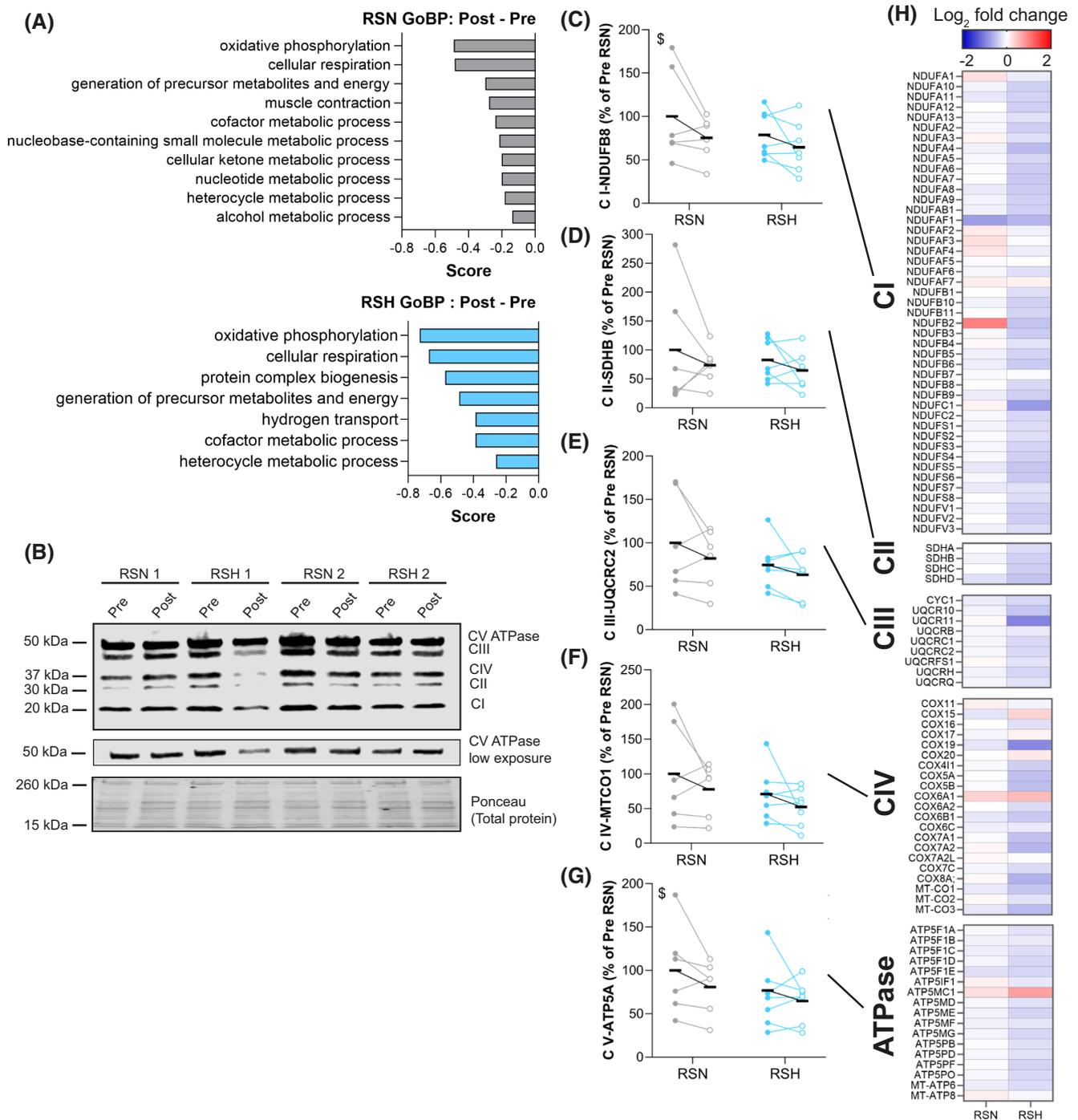


FIGURE 3 Reduced oxidative phosphorylation proteins in response to RSN and RSH training. (A) Protein groups related to Gene Ontology Biological Processes (GoBP) that are significantly decreased after three weeks of repeated sprint training in normoxia (RSN) and repeated sprint training in hypoxia (RSH). The median values of the difference of post training–pre training were calculated, and a score affected to the amplitude of the difference, $n = 6$ per group. Benjamini–Hochberg corrected t test. (B) Representative immunoblots of mitochondrial OXPHOS proteins. (C–G) Quantifications of the proteins in (B) related to total protein; $n = 6$ and 7 participants for RSN and RSH, respectively. Dots linked by lines represent individual participants and black thick lines represent the mean before (pre) and after (post) training. (H) Heat map of changes in protein expression of mitochondrial complexes from pre to post RSN and RSH training. $^{\$}p \leq .05$ main effect of time.

RSN training, with a stronger score for RSH (Figure 3A). Subsequently, western blot showed significant decreases in mitochondrial complex I and ATP synthase proteins

(Figure 3C–G). Detailed proteomic analysis showed a decrease in several OXPHOS proteins beyond those validated by western blot, especially after RSH training (Figure 3H).

As mitochondrial oxidative capacity can also be modulated by mitochondrial biogenesis, of which one of the main regulators is PGC-1 α ,³¹ its protein expression was quantified. Using an antibody recognizing all its isoforms,³² our results showed a significant increase in PGC-1 α protein levels in both training conditions (Figure 4B). While the classical mitochondrial outer membrane protein TOM20 was not altered (Figure S1B) the levels of multiple proteins involved in mitochondrial translation were increased in our proteomics dataset (Figure 4C), suggesting that both RSN and RSH triggered mitochondrial biogenesis processes in skeletal muscle.

PGC-1 α expression can be modulated by different factors among which Ca²⁺ through the activation of calcineurin and calmodulin^{33,34} (Figure 4F). Calcineurin protein levels were significantly increased (Figure 4E), with no modifications of the sarcoplasmic reticulum Ca²⁺ chelator calsequestrin (Figure S2B). No significant increase in the calcium/calmodulin-dependent protein kinase II (CamKII) phosphorylation levels was observed in response to either RSN or RSH training (Figure S2C).

We then explored potential PGC-1 α -dependent adaptations (Figure 4D). During hypoxia, PGC-1 α expression increases and the hypoxia-inducible factor-1 α (HIF-1 α) protein is stabilized. Both PGC-1 α and HIF-1 α can trigger the expression of VEGFa^{35,36}, a key regulator of angiogenesis.³⁷ On the other hand, exercise alone can directly induce angiogenesis through PGC-1 α regulation.^{35,38} HIF-1 α and VEGFa protein levels were significantly increased after both training modalities (Figure 4G,H), suggesting a contribution from HIF-1 α to the VEGFa increase.

Together, our results show that RSN and RSH training induced a reduction in proteins involved in OXPHOS, while triggering the machinery of mitochondrial biogenesis and angiogenesis.

3.3 | RSN and RSH training improve glycolytic phenotype with enhanced adaptations after RSH training

During glycolysis, the end-product pyruvate undergoes diverse outcomes based on various factors such as oxygen availability. The results presented above suggest that skeletal muscle exposed to either RSN or RSH training undergoes hypoxic stress with potentially more pyruvate being converted into lactate through mass effect. The muscle monocarboxylate transporter MCT1, predominantly expressed in oxidative muscle fibers and which favors lactate uptake for use as an energy substrate,³⁹ was not modified (Figure 5A,B). Protein levels of MCT4, expressed in more glycolytic muscle fibers where it favors extrusion of

lactate, were however significantly increased in response to both RSN and RSH training modalities (Figure 5A,C). Investigation of key glycolytic enzymes showed a significant increase in the protein levels of hexokinase II, a limiting enzyme of glycolysis (Figure 5D) while lactate dehydrogenase A (LDHa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels were not significantly modified (Figure S3B,C). These results suggest an increase in glycolytic flux in RSN and RSH groups. To explore whether glucose uptake was also altered, we measured the protein levels of the main glucose transporter in response to exercise, glucose transporter 4 (GLUT4),⁴⁰ which were significantly increased only in RSH (Figure 5E), suggesting better glycolytic adaptations in response to RSH. Our proteomics data further supported these results as multiple proteins from the KEGG pathway “Glycolysis/Gluconeogenesis” (significantly enriched only after RSH) were increased (Figure 5F).

These results indicate an improvement of muscle glycolytic phenotype in response to both RSN and RSH training, with possibly superior adaptations after RSH training.

3.4 | RSH training activates the S100A13 pathway to potentiate specific muscle adaptations

We then aimed at investigating what may support the potential superior muscle glycolytic adaptations in RSH group. The top proteins that increased in response to RSH training from our proteomics data belong to the S100A family (S100A13, S100A16, S100A10, S100A1, S100A6, Figure 6A). S100 proteins have recently been shown to promote glycolytic phenotype of cancer cells.⁴¹ The S100 protein family, comprising 25 known members, are Ca²⁺-sensitive proteins mediating a wide range of physiological functions such as proliferation, differentiation, apoptosis, Ca²⁺ homeostasis, energy metabolism, inflammation, and migration/invasion.⁴² When released in the extracellular space, S100A proteins bind to different receptors such as the receptor for advanced-glycation endproducts (RAGE), which is known to activate the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) pathway⁴³ (Figure 6B), a potent pathway in skeletal muscle response to exercise.⁴⁴

We validated by western blot a significant increase in S100A13 protein levels in response to RSH training but not RSN (Figure 6C,D). The levels of Akt phosphorylation tended to increase in RSH (+36%) and not RSN (time x group interaction, $p = .01$, Figure 6E,F). PI3K/Akt axis mediates, among other processes, protein synthesis through the expression of translation initiation and elongation factors.⁴⁵ Our proteomics data showed a significant increase in

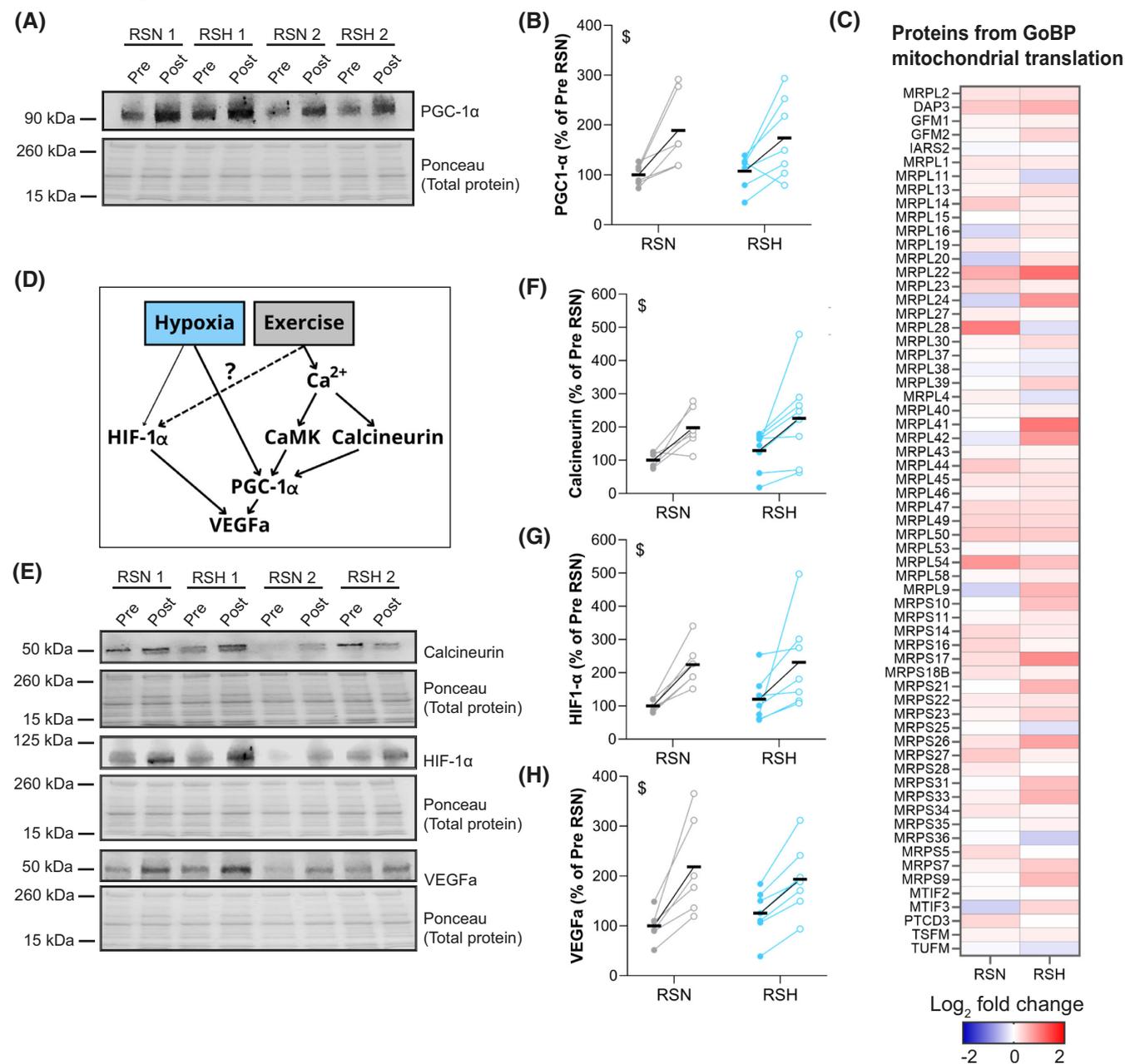


FIGURE 4 RSN and RSH training concurrently increased PGC-1 α protein levels, as well as its upstream regulators and its downstream targets. (A) Representative immunoblots of peroxisome proliferator-associated receptor coactivator-1 α (PGC-1 α) protein. (B) Quantifications of PGC-1 α ; $n = 6$ and 7 participants for repeated sprint training in normoxia (RSN) and repeated sprint training in hypoxia (RSH), respectively. (C) Proteins related to the Gene Ontology Biological Process (GoBP) mitochondrial translation changing in response RSN and RSH training. (D) Graphical representation of PGC-1 α regulators and effectors adapted from Ref. [40]. (E) Representative immunoblots of calcineurin, hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor A (VEGFa) proteins. (F–H) Quantification of proteins in (E); $n = 6$ and 8 participants for RSN and RSH, respectively (calcineurin) and 6 and 7 participants for RSN and RSH, respectively (HIF-1 α and VEGFa). Dots linked by lines represent individual participants, and black thick lines represent the mean before (pre) and after (post) training. $^{\$}p \leq .05$ main effect of time.

proteins involved in translation initiation and elongation in response to RSH but not RSN training (Figure 6G,H), confirming a potential activation of the Akt signaling pathway and its downstream target of protein synthesis in RSH.

Our results suggest that RSH training potentially activates the S100A/Akt axis in muscle fibers, which may trigger further adaptations such as protein synthesis as compared to RSN training.

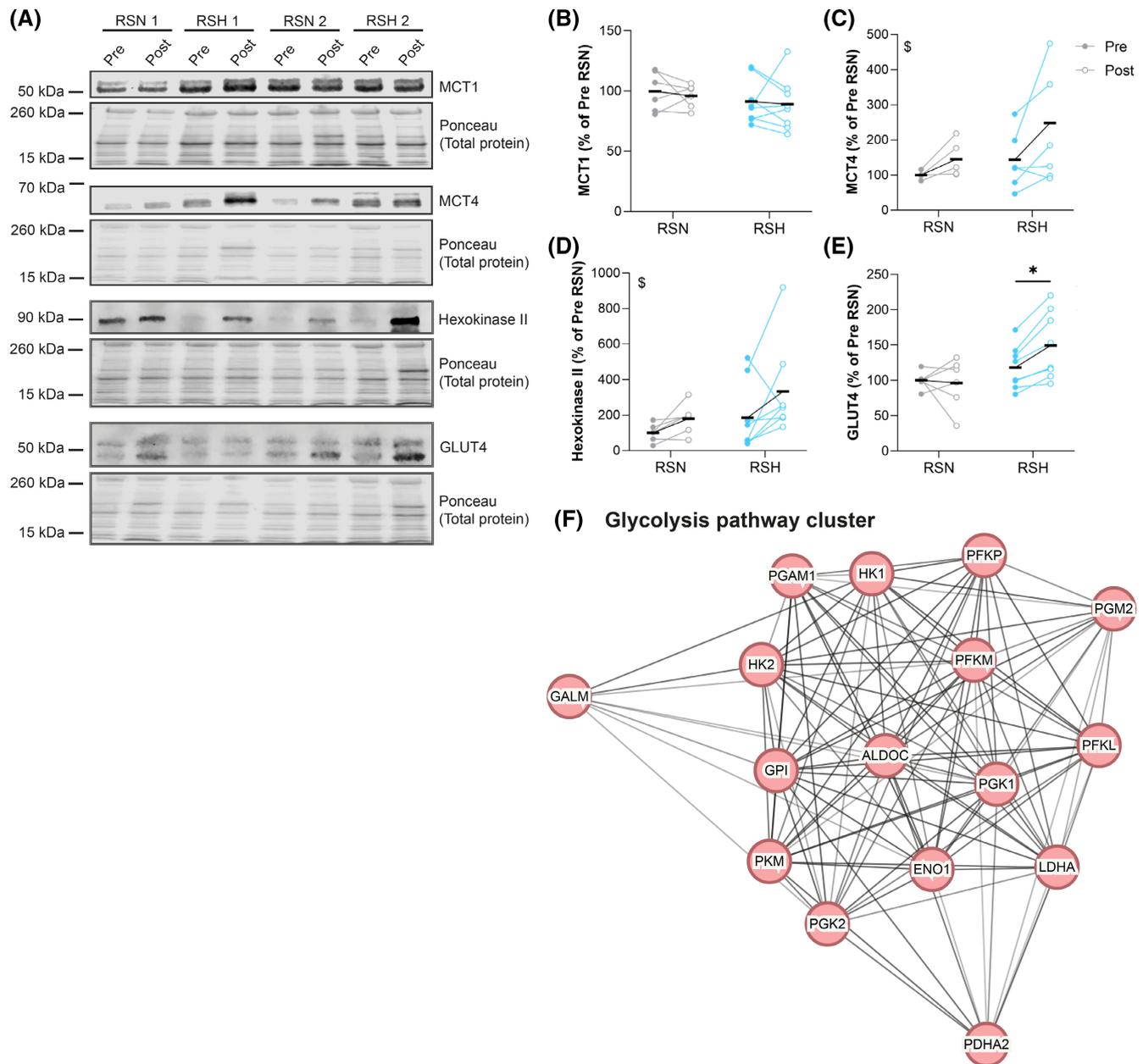


FIGURE 5 RSN and RSH training improve muscle glycolytic phenotype. (A) Representative immunoblots of monocarboxylate transporters 1 (MCT1) and 4 (MCT4), Hexokinase II, and glucose transporter 4 (GLUT4) protein levels. (B–E) Quantification of proteins in (A); 5 and 6 participants for repeated sprint training in normoxia (RSN) and repeated sprint training in hypoxia (RSH), respectively (MCT4) and 6 and 8 participants for RSN and RSH, respectively (MCT1, Hexokinase II, and GLUT4). Dots linked by lines represent individual participants, and black thick lines represent the mean before (pre) and after (post) training. (F) STRING protein–protein interaction network of proteins derived from the KEGG “Glycolysis/Gluconeogenesis” pathway enriched in our proteomics data in response to RSH training. § $p \leq .05$ main effect of time, * $p \leq .05$.

4 | DISCUSSION

Previous studies reported greater performance improvement after RSH as compared to RSN training, but its extent varied between studies, as different tests ranging from aerobic to RSA were used to determine effectiveness. In the present study, three weeks of RSN and RSH training similarly improved RSA, Wingate and time-trial performance.

The previously reported heterogeneity between studies regarding performance is likely due to differences in training modalities, in particular the exercise:recovery ratio, which affects the oxidative-glycolytic balance during sprint repetitions.⁴⁶

It is not clear if RSH differently alters muscle glycolytic or oxidative adaptations as compared to RSN.^{14,15} We here report reduced levels of proteins involved in OXPHOS after

FIGURE 6 RSH training increases the expression of S100A family proteins and translation factors. (A) Volcano plot showing the overview of muscle proteins identified in the proteomic analysis in response to repeated sprint training in hypoxia (RSH). Each point represents an individual protein, and the red dashed line represents the cutoff from where proteins indicated in blue show significant modifications. (B) Graphical representation of the PI3K/Akt pathway activation through binding of RAGE receptor by S100A proteins family. (C) Representative immunoblots of S100A13. (D) Quantifications of S100A13. (E) Representative immunoblots of phospho Akt Ser473 (P Akt Ser⁴⁷³) protein levels and Akt proteins. (F) Quantifications of P Akt Ser⁴⁷³ related to total Akt; $n = 6$ and 8 participants for RSN and RSH, respectively. Dots linked by lines represent individual participants, and black thick lines represent the mean before (pre) and after (post) training. (G, H) STRING protein–protein interaction networks of proteins involved in translation initiation (G) and cytoplasmic ribosome subunits (H) that were significantly increased in the gene ontology enrichment analysis from proteomics in response to RSH training. * $p \leq .05$.

both training modalities. As the levels of TOM20 were not altered, the OXPHOS protein downregulation seems not to result from a decrease in mitochondrial content but rather suggests a specific downregulation of the oxidative pathway, possibly due to reduced OXPHOS flux under hypoxic conditions.⁴⁷ Previous studies reported either no change or an increase in electron transport system subunits following, respectively, HIIT (4 min cycling/2 min recovery)⁴⁸ or sprint interval training (30s all-out sprints).⁴⁹ Again, this variability may be attributed to some methodological differences, particularly the training modalities employed and the timing of muscle biopsies.

The decrease in OXPHOS proteins was concomitant to increased levels of PGC-1 α protein and of protein groups involved in mitochondrial protein translation, suggesting an induction of mitochondrial biogenesis. This process would eventually ensure a metabolic reversion toward OXPHOS once O₂ supply is restored after hypoxia exposure, as previously reported.⁵⁰

We observed increased levels of glycolysis proteins in both RSN and RSH. Under hypoxic conditions, cells reduce their reliance upon O₂-dependent mitochondrial OXPHOS to preferentially use the O₂-independent glycolytic pathway to maintain sufficient ATP production for energy demand. At least two mechanisms lead to the switch from oxidative to glycolytic phenotype under hypoxia: (1) The decreased ATP production by mitochondria in an O₂ limiting environment releases the allosteric inhibition of ATP on glycolysis enzymes to maintain glycolytic flux⁵¹; (2) the stabilization of HIF-1 α protein under hypoxic conditions induces a transcriptional regulation of genes involved in glycolysis and glucose transport,⁵² lowering the conversion of pyruvate into acetyl Co-A by increasing PDK1 to reduce OXPHOS flux.⁶ Little is known about the effect of high-intensity exercise training on HIF-1 α protein levels in human muscle in normoxia. Whether RSN also leads to HIF-1 α protein stabilization and accumulation (as observed in RSH) was not clarified in the literature, even though it has been recently reported that muscle deoxygenation and local hypoxia occur during repeated sprint exercise.⁵³ We found increased HIF-1 α protein also in response to RSN, suggesting that repeated sprint training

can activate the hypoxia pathway even when performed in normoxia. This finding corroborates the increases in glycolytic enzymes and lactate transport proteins observed in response to both RSN and RSH.

RSN and RSH showed increases in VEGFa protein levels to potentially trigger angiogenesis, a result which is supported by the induction of HIF-1 α , as VEGFa is a well-established downstream target of HIF-1 α .³⁷

RSH showed superior muscle glycolytic adaptations as compared to RSN, possibly through the activation of the S100 proteins pathway. S100A10 protein was recently found to favor tumor growth in gastric cancer through the acceleration of glycolysis via the Akt signaling pathway.⁴¹ We observed a significant increase in the levels of S100A13 protein, Akt phosphorylation and its downstream targets involved in protein translation, associated with an increase in the levels of GLUT4 and other proteins involved in glycolysis. Together, this suggests the involvement of the S100A13/Akt pathway in the increase in muscle mass and glycolytic fate in response to RSH. Exercise and hypoxia are known to trigger angiogenesis,^{38,54,55} which is also potentially induced in our study as shown by VEGFa data; thus, an involvement of the S100A proteins in this process is not excluded in response to RSH.

This study highlights a potential role of the S100 proteins pathway in the muscle adaptive response to repeated sprint in hypoxia that was not reported before, opening new research avenues in the field.

While the present study provides valuable insights into the mechanisms of muscle adaptations to RSH, caution should be taken when interpreting the results due to several limitations. The significant improvement in 10-km performance observed in our study after three weeks of training should result from physiological changes. However, we cannot exclude that a learning effect on pacing ability partly contributed to this improvement. Working with human muscle biopsies introduces inherent constraints that impact the robustness of our conclusions. Notably, parameters such as glucose uptake, lactate production, or muscle O₂ consumption were not measured in the muscle samples, limiting our ability to comprehensively assess metabolic responses. Furthermore, we acknowledge a

certain level of heterogeneity in some outcome measures and are mindful of the relatively small sample size, with $n = 6$ or 7 for several outcomes, and of the relatively short duration of the training intervention. We cannot exclude that specific individual responses may influence some results. Due to the absence of cross-sectional analyses of the muscle samples, we can conclude neither on the fiber-type representation within our samples, nor on the fiber-type specific adaptations in response to training.

Future investigations could benefit from analyzing more extensive participant cohorts, employing direct measurements of key metabolic parameters, and exploring additional analyses, such as single-fiber molecular assessments, to gain a more comprehensive and robust insight into the precise mechanisms underlying muscle adaptations to RSH.

5 | CONCLUSION

This study shows quite similar performance improvements in response to RSN and RSH training. Both RSN and RSH elicited a metabolic shift toward glycolytic muscle phenotype, with more pronounced adaptations observed in response to RSH. This was evidenced by a reduction in the levels of proteins associated with the OXPHOS pathway and an increase in the levels of proteins related to the glycolytic pathway, stronger in RSH. In addition, RSH training upregulated multiple proteins involved in protein synthesis. These adaptations likely involve the expression and activation of the S100A proteins/Akt axis.

AUTHOR CONTRIBUTIONS

Conceptualization: Sarah J. Willis, Grégoire P. Millet, Nicolas Place, Nadège Zanou, and Bengt Kayser. Methodology: Sarah J. Willis, Grégoire P. Millet, Louis Laramée, Nadège Zanou, Nicolas Place, and Bengt Kayser. Investigation: Sarah J. Willis, Louis Laramée, Nadège Zanou, Sonia Conde Alonso, and Bengt Kayser. Formal analysis: Clément Lanfranchi, Louis Laramée, and Nadège Zanou. Writing: Clément Lanfranchi, Nadège Zanou, and Louis Laramée wrote the manuscript, all authors contributed to review and editing. Project supervision: Nadège Zanou, Grégoire P. Millet, Nicolas Place, and Bengt Kayser.

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DISCLOSURES

None declared.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD040779. All other data can be provided upon request.

ETHICS APPROVAL

The study protocol was approved by the Ethical Commission for Human Research (CER-VD, protocol 2018-02298).

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SUPPORTING INFORMATION

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