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RESEARCH ARTICLE

Adaptations in muscle oxidative capacity, fiber size, and oxygen supply capacity after repeated-sprint training in hypoxia combined with chronic hypoxic exposure

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van der Zwaard S, Brocherie F, Kom BLG, Millet GP, Deldicque L, van der Laarse WJ, Girard O, Jaspers RT. Adaptations in muscle oxidative capacity, fiber size, and oxygen supply capacity after repeated-sprint training in hypoxia combined with chronic hypoxic exposure. *J Appl Physiol* 124: 1403–1412, 2018. First published February 8, 2018; doi:10.1152/japplphysiol.00946.2017.—In this study, we investigate adaptations in muscle oxidative capacity, fiber size and oxygen supply capacity in team-sport athletes after six repeated-sprint sessions in normobaric hypoxia or normoxia combined with 14 days of chronic normobaric hypoxic exposure. Lowland elite field hockey players resided at simulated altitude (≥14 h/day at 2,800–3,000 m) and performed regular training plus six repeated-sprint sessions in normobaric hypoxia (3,000 m; LHTLH; $n = 6$) or normoxia (0 m; LHTL; $n = 6$) or lived at sea level with regular training only (LLTL; $n = 6$). Muscle biopsies were obtained from the m. vastus lateralis before (pre), immediately after (post-1), and 3 wk after the intervention (post-2). Changes over time between groups were compared, including likelihood of the effect size (ES). Succinate dehydrogenase activity in LHTLH largely increased from pre to post-1 (~35%), likely more than LHTL and LLTL (ESs = large-very large), and remained elevated in LHTLH at post-2 (~12%) vs. LHTL (ESs = moderate-large). Fiber cross-sectional area remained fairly similar in LHTLH from pre to post-1 and post-2 but was increased at post-1 and post-2 in LHTL and LLTL (ES = moderate-large). A unique observation was that LHTLH and LHTL, but not LLTL, improved their combination of fiber size and oxidative capacity. Small-to-moderate differences in oxygen supply capacity (i.e., myoglobin and capillarization) were observed between groups. In conclusion, elite team-sport athletes substantially increased their skeletal muscle oxidative capacity, while maintaining fiber size, after only 14 days of chronic hypoxic residence combined with six repeated-sprint training sessions in hypoxia.

NEW & NOTEWORTHY Our novel findings show that elite team-sport athletes were able to substantially increase the skeletal muscle oxidative capacity in type I and II fibers (+37 and +32%, respectively), while maintaining fiber size after only 14 days of chronic hypoxic residence combined with six repeated-sprint sessions in hypoxia. This increase in oxidative capacity was superior to groups performing chronic hypoxic residence with repeated sprints in normoxia and residence at sea level with regular training only.

altitude training; angiogenesis; hypertrophy; oxygen transport; skeletal muscle

INTRODUCTION

In team sports, maximal to near-maximal intensity sprints are repeated throughout the match, and therefore, repeated-sprint ability is a crucial fitness component of athletes engaged in these disciplines (5). To improve repeated-sprint ability, team-sport athletes must concurrently train to enhance peak power production required during maximal efforts as well as oxidative metabolism to speed up recovery between efforts (4, 5, 20). This may be challenging, as prime skeletal muscle fiber determinants for peak power production (i.e., fiber size) and maximal oxygen consumption (i.e., oxidative capacity) are inversely related (48, 52, 53). A decline in oxidative capacity with increasing fiber size may be prevented if oxygen supply to the mitochondria is enhanced, such that the muscle fiber core is prevented from becoming hypoxic at maximal activation (53). Athletes may therefore seek training strategies to simultaneously increase oxidative capacity and fiber size and enhance oxygen supply capacity to improve their sport-specific performance.

A promising training strategy to enhance repeated-sprint ability is the combination of living at altitude and repeated-sprint training in hypoxia (9, 19, 38). Recently, a 2-wk altitude training using a live high-train low regimen with repeated-sprint training in hypoxia (LHTLH) and normoxia (LHTL) has been shown to improve repeated-sprint ability and incremental field performance in elite field hockey players, with superior changes in repeated-sprint ability in LHTLH vs. LHTL (9). Both groups showed analogous improvement in blood oxygen carrying capacity (+3–4% increases in hemoglobin mass; Ref.

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9), illustrating that chronic hypoxic residence increases red blood cell mass, even in well-trained athletes (31). Muscle adaptations are likely key to the superior improvements in the LHTLH vs. LHTL, as suggested by LHTLH's beneficial changes in muscle transcriptional factors (8). Repeated sprints induce local tissue hypoxia providing an additive training stimulus for skeletal muscle adaptations, likely more when sprints are performed in hypoxia (7, 17). However, it remains unknown what functional and structural adaptations occur in skeletal muscle in response to hypoxic residence with repeated-sprint training in hypoxia and normoxia to facilitate the improvements in sport-specific performance in these athletes.

Sensing of cellular oxygen tension is important for skeletal muscle adaptation. In hypoxic conditions, when cellular oxygen tension is low, hypoxia-induced factor 1α (HIF-1α) is stabilized and induces transcription of genes for red blood cell formation, capillary growth, and energy metabolism (16, 47). Training in hypoxia, more than in normoxia, has been shown to increase HIF-1α mRNA levels with low- and high-intensity training (54). However, only high-intensity training in hypoxia leads to transcription of vascular endothelial growth factor (VEGF) and myoglobin (Mb), which enhance capillary growth and oxygen transport within the muscle fibers, respectively (54). High-intensity training both in hypoxia and normoxia may stimulate oxidative enzyme activities and increase mitochondrial density (10, 37, 50, 54), whereas severe chronic hypoxic exposure (>5,000-m altitude) may reduce them (21, 25, 32). With regard to muscle fiber size, increases have been observed with training in normoxia but generally not with endurance training in hypoxia (54). Hypoxic exposure is presumed to reduce the rate of mRNA translation and the synthesis rate of contractile filaments (43, 53); therefore, hypoxic residence may induce muscle fiber atrophy (21, 25, 34, 39) but not in all cases (15, 26, 33), possibly due to total hypoxic dose (14). Currently, the extent to which skeletal muscle fiber size, oxidative capacity, and oxygen supply capacity are upregulated (or not) by LHTLH and LHTL is unknown.

The aim of this study was to assess changes in muscle oxidative capacity, muscle fiber size, and oxygen supply capacity in team-sport athletes in response to hypoxic residence combined with either repeated-sprint training in hypoxia or normoxia compared with living and training at sea level. Considering the previously observed improvements in sports-specific performance in this cohort of team-sport athletes (8, 9), we hypothesized that 14 days of LHTLH and LHTL with six repeated-sprint sessions will increase muscle fiber oxidative capacity, capillary density, and Mb concentration ([Mb]) yet to a larger extent in LHTLH. Given the short duration of the intervention, we did not expect changes in fiber size in all three groups.

METHODS

Subjects. Eighteen lowland elite male field hockey players provided written informed consent before participating in this study. Subjects were recruited from Belgian, Spanish, and Dutch first division clubs and competed at the (inter)national level. They were excluded if acclimatized or exposed to hypoxia >2,000 m for more than 48 h in the 6 mo before participation and if they had any history of altitude-related sickness or health risks that compromised their safety during training and/or hypoxic exposure (i.e., illness, injury, and insufficient fitness level). The study was conducted according to the Declaration of Helsinki (2013) and was approved by the Anti-Doping Laboratory Qatar Institutional Review Board (Agreement No. SCH-ADL-070). Subject characteristics are reported in Table 1.

Experimental design, chronic hypoxic residence, and repeated-sprint training. This double-blind controlled experiment has been described elsewhere (9). In brief, subjects were randomly assigned to one of three groups, two intervention groups enrolled in a simulated altitude training camp in Qatar and a control group, which were matched for fitness level and hockey playing position. Testing was performed and muscle biopsies were obtained before (pre), 2–3 days after (post-1), and 3 wk after the 14-day intervention (post-2). Intervention groups resided in normobaric hypoxia (≥14 h/day at $F_{I_{O_2}}$: ~14.5–14.2% or 2,800–3,000 m of simulated altitude) and performed regular field hockey training at sea level with the addition of six repeated-sprint sessions in normobaric hypoxia ($F_{I_{O_2}}$: ~14.2%, 3,000 m, LHTLH; $n = 6$) or normoxia ($F_{I_{O_2}}$: 20.9%, 0 m, LHTL; $n = 6$). The control group resided at sea level with regular training only (LLTL; $n = 6$). Chronic normobaric hypoxia in the intervention groups ranged between 2,500 m (*day 1*), 2,800 m (*days 2–5*), and 3,000 m (*days 6–14*), with $F_{I_{O_2}}$ values of 15.1, 14.5, and 14.2%, respectively. In addition to usual field hockey practice (described in Ref. 9), subjects performed six repeated-sprint sessions (with ≥36 h in between) either in normobaric hypoxia or normoxia on indoor synthetic grass within a 45-m inflatable hypoxic marquee (Altitude Technology Solutions, Brisbane, QLD, Australia). Repeated-sprint sessions were ~50 min in duration and consisted of a 15-min warm-up, maximal-effort repeated sprints and 10-min recovery phase. Each session included four sets of 5 × 5-s maximal-effort sprints with 25 s of passive rest between sprints and 5 min of standing rest between sets. For LHTLH, ambient air within the inflatable running lane was mixed with nitrogen (from pressurized tanks) to reduce $F_{I_{O_2}}$ to ~14.2% and simulate an altitude of ~3,000 m. Subjects were blinded to simulated altitude, as both LHTLH and LHTL groups were told to be sprinting in hypoxic conditions, whereas they had no specific information about the simulated altitude levels. To reinforce the blinding effect, intervention groups were divided into four training subgroups, allowing assignment of different teammates for each training day, and roommates who lived in the same hypoxic room did not necessarily belong to the same intervention group. Moreover, in this experiment, neither the researchers (with the exception of the main investigator) nor the club's technical and support staffs knew which subjects belonged to which one of the two experimental groups. Questionnaire results after the experiment indicated that the subjects of LHTLH and LHTL groups were successfully blinded to their hypoxic group classification (9). Ferritin concentrations (142.7 ± 65.6 μg/l) and soluble transferrin receptor (240.7 ± 26.0 mg/dl) obtained during the lead-in period at sea level (means ± SD) indicated that none of the subjects was iron deficient at the start of this study.

Skeletal muscle biopsy. Biopsy samples were taken from mid-portion of the m. vastus lateralis using a modified Bergstrom needle technique. After 48 h without any exercise activity, biopsy sites were subcutaneously anesthetized with 1% xylocaine. Muscle tissue sam-

Table 1. *Subject characteristics*

	LHTLH ($n = 6$)	LHTL ($n = 6$)	LLTL ($n = 6$)
Age, yr	26 ± 3	26 ± 5	22 ± 2
Weight, kg	78.9 ± 8.2	72.9 ± 8.4	74.8 ± 10.8
Height, m	1.78 ± 0.08	1.78 ± 0.04	1.77 ± 0.06
$\dot{V}O_{2max}$, ml·kg^{-1}·min^{-1}	53.6 ± 2.8	52.2 ± 1.4	51.3 ± 1.3
Position	3DF/1MF/2FW	1GK/2DF/1MF/2FW	3DF/2MF/1FW

Values are presented as means ± SD. $\dot{V}O_{2max}$, maximal oxygen uptake was estimated from an incremental running test up to exhaustion (Yo-Yo Intermittent Recovery Test Level 2; Ref 9); DF, defender; MF, midfield; FW, forward; GK, goalkeeper; LHTLH, live high-train low and high (with repeated sprints in hypoxia) group; LHTL, live high-train low (with repeated sprints in normoxia) group; LLTL, live low-train low or control group.

ples were then obtained randomly from opposite legs over the three test sessions (i.e., left-right-left or right-left-right). Samples were frozen in liquid nitrogen, placed in a cryostat, and cut in 10-μm-thick sections at −20°C. Sections were collected on polylysine-coated slides and stored at −80°C until further use.

Fiber type and fiber cross-sectional area. Fiber-type distribution was determined from myosin heavy chain (MHC) expression assessed by immunofluorescence analysis (6). Briefly, biopsy sections were blocked with 10% normal goat serum and incubated with monoclonal primary antibodies (i.e., 1 μg/ml BA-D5, 1 μg/ml SC-71, and 5 μg/ml 6H1 for MHC-I, IIA, and IIX, respectively; Developmental Studies Hybridoma Bank, Iowa City, IA) and incubated in the dark with secondary fluorescent antibodies (i.e., 5 μg/ml Alexa Fluor 488 IgG_{2b}, 488 IgG_1, and 647 IgM antibodies for MHC-I, IIA, and IIX, respectively; Developmental Studies Hybridoma Bank). Muscle fiber basal lamina was stained by incubation with a dilute of wheat germ agglutinin (1:50). Images were captured using a CCD camera (PCO; Sensicam, Kelheim, Germany) connected to a fluorescence microscope (Axiovert 200M; Zeiss, Göttingen, Germany) at ×10 objective and with image processing software (Slidebook 5.5; Intelligent Image Innovations, Denver, CO). Fiber-type composition (type I, IIA, and IIX) and fiber cross-sectional area (FCSA) per fiber type were determined from these images using SMASH, a semiautomatic Matlab application (The MathWorks, Natick, MA), for analysis of immunofluorescent biopsy sections (49). Fiber boundaries were manually confirmed and modified if necessary. Since the proportion of IIX fibers was small (3.3 ± 3.9%) and because in trained populations the MHC IIX isoforms are commonly coexpressed with MHC IIA isoforms (1), histochemical properties of IIX fibers were not analyzed separately. Histochemical analyses for succinate dehydrogenase (SDH) activity, FCSA and myoglobin concentration ([Mb]) were performed for type I fibers and type IIA fibers, now referred to as type II fibers. For analysis of fiber type and FCSA, the fiber number per subject per time point was 199 ± 102 for type I fibers and 211 ± 118 for type II fibers (means ± SD).

Mitochondrial oxidative capacity. SDH activity was determined using quantitative histochemistry (45, 51). Briefly, 10-μm biopsy sections were air dried directly after sectioning and incubated in the dark for 20 min in a medium of 0.4 mM tetranitroblue tetrazolium (Sigma, St. Louis, MO), 75 mM sodium succinate, 5 mM sodium azide, and 37.5 mM sodium phosphate buffer, at 37°C and with pH 7.6 (45). Images were captured with ×10 objective using a CCD camera (Sony XC77CE, Towada, Japan) connected to a LG-3 frame grabber (Scion, Frederick, MD) and a DMRB microscope with calibrated gray filters (Leica, Wetzlar, Germany). Spatially averaged SDH activity, including subsarcolemmal mitochondria, was obtained from 660-nm absorbance measurements in randomly selected cells using ImageJ (30, 51). On average, 24 ± 2 type I fibers and 24 ± 3 type II fibers were analyzed per time point for every subject. Note that SDH activity determined using this quantitative histochemistry method is calibrated to maximal oxygen consumption of isolated muscle fibers and cardiomyocytes under hyperoxic conditions (12, 29). This calibration was used to calculate muscle fiber oxidative capacity (fiber $\dot{V}O_{2max}$, in $nmol \cdot mm^{-3} \cdot s^{-1}$) according to Ref. 51 to express our findings relative to the inverse relationship between fiber size and oxidative capacity (53).

Myoglobin concentration. [Mb] was determined by calibrated histochemistry using a vapor-fixation technique (3, 30). Briefly, sections were freeze dried at −80°C to prevent redistribution or dissolution of peroxidase activity by water condensation and warmed to room temperature. Sections were fixed by paraformaldehyde vapor for 1 h at 70–75°C and fixated by 2.5% glutaraldehyde solution in 0.07 M sodium phosphate buffer for 10 min at room temperature, pH 7.4. Biopsy sections were then incubated for 60 min in 59 ml 50 mM Tris-80 mM KCl buffer consisting of 25 mg ortho-tolidine (Sigma T8533), dissolved in 2 ml 95% ethanol at 50°C, and 1.43 ml 70% tertiary-butyl-hydroperoxide (Fluka Chemie 19995, Buchs, Switzerland), pH 8.0. Images were captured (as described in *Mitochondrial oxidative capacity*), and absorbance was measured at 436 nm (30). Peroxidase activity of hemoglobin was excluded from analysis. [Mb] in each fiber type was obtained after calibration of using gelatin sections containing known concentrations of horse Mb. On average, 23 ± 4 type I fibers and 22 ± 3 type II fibers were analyzed per time point for every subject.

Capillaries. Capillaries were stained with Ulex europaeus agglutinin I lectin (UEA-1), adapted from Ref. 46. In short, sections of 10 μm were air dried, fixed in acetone, and blocked with 1% bovine serum album. Biopsy sections were then incubated with biotinylated UEA-1 (20 μg/ml; Vector Laboratorie, Burlingame, CA), incubated with VECTASTAIN Elite ABC Kit PK-6100 (Vector Laboratories), and incubated with Red Peroxidase Substrate SK-4285 (ImmPACT AMEC; Vector Laboratories). Images were collected (Zeiss AxioCam MRc color camera; Zeiss Axioskop microscope and image-capture software Zeiss KS 300 version 3.0; Carl ZeissImaging Solutions), and photomicrographs were taken from multiple areas of the biopsy section to count muscle fibers and capillaries. Capillaries were analyzed in ImageJ to obtain capillaries around each fiber (CAF), capillary-to-fiber ratio (C/F), and capillaries per mm^2 muscle tissue (CD). Longitudinally cut capillaries were counted as one at each muscle fiber junction (2). With regard to the C/F ratio, muscle fiber fragments cut by the right and lower margins and associated capillaries were included in the analysis and those cut by left and upper margins were rejected (55). On average, 266 ± 82 capillaries and 127 ± 35 muscle fibers were analyzed per subject per time point.

Data and statistical analysis. Data are reported as means ± SD. Missing values were due to freeze damage or insufficient muscle tissue for histology, which reduced the sample size of LHTLH ($n = 5$ at pre and post-1) and LLTL ($n = 5$ at pre); sample size of the other time points and LHTL remained unaltered ($n = 6$). One-way ANOVA test or nonparametric Kruskal-Wallis tests were used to detect changes in mean values over time between groups, and least significant difference post hoc tests or Mann-Whitney tests were used to localize the differences. The magnitude-based inferences approach (5) was used to provide a more nuanced quantification of effects sizes (ESs) with respect to the smallest worthwhile effect. ESs were calculated from standardized differences (Cohen's d units) and were considered to be small (0.20–0.60), moderate (0.60–1.20), large (1.20–2.0), very large (2.0–4.0), or extremely large (>4.0) (24). ESs were reported in standardized Cohen's d units ± 90% confidence interval (90% CI). The likelihood that effects are beneficial, trivial, or harmful was quantified using the ESs, confidence interval, and the smallest worthwhile change of 0.20 (i.e., effect size of 0.20 Cohen's d) with the following scale: <0.5%, most unlikely; 0.5–5%, very unlikely; 5–25% unlikely; 25–75%, possibly; 75–95%, likely; 95–99.5%, very likely; and >99.5%, most likely (24). The average percentage of change was also obtained from pooled data of type I and II fibers. The inverse relationship between fiber size and mitochondrial oxidative capacity was obtained from literature (53). Results were considered significant if $P < 0.05$. Tendencies were also reported if $P < 0.10$.

RESULTS

Table 2 presents group comparisons for changes in SDH activity, FCSA, [Mb], and capillarization between pre and post-1 and between pre and post-2. Figure 1 displays muscle fiber histochemistry results. Fiber type distribution remained similar within all groups throughout the study (46 ± 4, 51 ± 3, and 49 ± 2% for percentage type I fibers in LHTLH, LHTL, and LLTL, respectively).

Mitochondrial oxidative capacity and muscle fiber size. Figure 2 shows group changes in SDH activity and FCSA in type I and type II fibers. In LHTLH, the change in SDH activity

Table 2. *Fiber-type-specific changes in SDH activity, FCSA, [Mb], and capillarization over time compared between groups using effect sizes*

	Δ(Post-1 − Pre)			Δ(Post-2 − Pre)		
Effect Sizes	LHTL-LLTL	LHTLH-LLTL	LHTLH-LHTL	LHTL-LLTL	LHTLH-LLTL	LHTLH-LHTL
Magnitude ES	Cohen's d ± 90%CI	Cohen's d ± 90%CI	Cohen's d ± 90%CI	Cohen's d ± 90%CI	Cohen's d ± 90%CI	Cohen's d ± 90%CI
SDH activity I	1.64 ± 1.22	3.72 ± 2.03	1.71 ± 1.68	−1.80 ± 1.25	0.19 ± 1.84	1.63 ± 1.45
SDH activity II	1.02 ± 0.90	2.87 ± 2.45	1.37 ± 2.11	−0.97 ± 0.90	0.02 ± 1.85	1.05 ± 1.49
FCSA I	0.07 ± 1.04	−1.32 ± 0.80	−1.10 ± 0.92	0.15 ± 1.92	−0.96 ± 1.80	−0.88 ± 1.31
FCSA II	−0.01 ± 2.52	−1.51 ± 0.68	−1.46 ± 1.30	0.98 ± 3.34	−1.04 ± 1.28	−1.50 ± 1.31
[Mb] I	−0.13 ± 0.48	−0.19 ± 0.40	−0.08 ± 0.34	−0.75 ± 0.59	−0.24 ± 0.31	0.38 ± 0.46
[Mb] II	−0.10 ± 0.48	−0.61 ± 0.46	−0.58 ± 0.48	−1.18 ± 1.03	−0.84 ± 0.65	0.19 ± 0.98
CD	1.05 ± 1.44	1.53 ± 2.12	0.40 ± 1.69	−0.28 ± 0.86	1.00 ± 1.40	0.98 ± 1.02
CAF	0.99 ± 1.10	−0.11 ± 1.36	−1.01 ± 1.13	1.09 ± 1.10	−0.25 ± 1.28	−1.19 ± 0.91
C/F	1.01 ± 1.69	−0.07 ± 1.03	−0.84 ± 1.34	0.76 ± 1.44	−0.34 ± 0.85	−0.85 ± 1.08

Likelihood ES	+	~	-	+	~	-	+	~	-	+	~	-	+	~	-	+	~	-
SDH activity I	97	2	1	99	0	1	93	3	4	1	1	98	50	15	35	95	3	3
SDH activity II	94	5	2	96	1	3	86	5	9	2	5	93	43	16	41	85	7	8
FCSA I	40	28	32	1	1	98	2	4	95	48	15	37	13	9	78	8	10	82
FCSA II	44	12	44	0	0	100	2	3	94	66	7	26	5	7	88	3	4	93
[Mb] I	12	48	40	5	46	49	8	66	25	1	5	94	1	39	60	76	21	2
[Mb] II	14	50	36	1	6	93	1	7	92	2	4	94	1	4	95	49	27	24
CD	85	8	7	87	5	8	59	16	25	17	27	56	85	8	7	91	6	3
CAF	89	7	4	34	21	45	4	7	89	91	6	3	26	21	53	1	3	96
C/F	81	9	11	32	27	41	9	10	81	76	12	12	14	25	62	5	9	86

Changes over time (between pre and post-1 and between pre and post-2) were compared between groups using effect sizes (ESs). ESs were calculated from standardized differences (Cohen's d) and were considered to be small (0.20–0.60), moderate (0.60–1.20), large (1.20–2.0), very large (2.0–4.0), or extremely large (>4.0) (24). ESs were reported in standardized Cohen's d units ± 90% confidence interval (90%CI). The likelihood that effects are beneficial, trivial, or harmful was quantified using the ESs, confidence interval, and the smallest worthwhile change of 0.20 (i.e., ESs of 0.20 Cohen's d) with the following scale: <0.5%, most unlikely; 0.5–5%, very unlikely; 5–25%, unlikely; 25–75%, possibly; 75–95%, likely; 95–99.5%, very likely; and >99.5%, most likely (24). Pre, post-1, and post-2, before, immediately after, and 3 wk after the intervention; SDH, succinate dehydrogenase; FCSA, fiber cross-sectional area; [Mb], myoglobin concentration; CD, capillary density; CAF, capillary number around the fiber; C/F, capillary-to-fiber ratio; LHTLH, live high-train low and high (with repeated-sprints in hypoxia) group; LHTL, live high-train low (with repeated-sprints in normoxia) group; LLTL, live low-train low or control group.

in type I and II fibers from pre to post-1 (+37 and +32%, respectively) was significantly larger than in LHTL ($P < 0.05$, ES = large for type I fibers and $P = 0.08$, ES = large for type II fibers, respectively) and in LLTL ($P < 0.05$, ESs = very large and $P < 0.05$, ESs = very large, respectively). At post-2, SDH activity in type I fibers remained elevated with respect to pre in LHTLH (+12%) vs. LHTL ($P < 0.05$, ESs = large). Notably, SDH activity at pre was already higher in LHTL compared with that in the other groups ($P \leq 0.06$, ESs = moderate-large, in type I and type II fibers). Compared with LHTLH, with FCSA remaining fairly similar in type I and type II fibers between pre and post-1, FCSA increased in type I and type II fibers from pre to post-1 in LHTL and LLTL ($P < 0.05$, ESs = moderate-large and $P < 0.05$, ESs = large, respectively). Compared with LHTLH, the increase in FCSA of type II fibers from pre to post-2 tended to be larger in LHTL ($P = 0.07$, ESs = large). The fiber-specific analyses of changes in SDH activity and FCSA demonstrated similar patterns for type I and type II fibers. Overall, these results indicate that in LHTLH SDH activity was substantially increased during the intervention, whereas FCSA remained fairly constant.

[Mb] and capillaries. Changes in [Mb] showed only small-to-moderate effects between groups (see Table 2), revealing similar patterns for type I and type II fibers (Fig. 3). In LHTLH, [Mb] in type II fibers decreased from pre to post-1, whereas [Mb] remained constant in LHTL and LLTL ($P = 0.06$, ESs = small and $P < 0.05$, ESs = moderate, respectively). From pre to post-2, [Mb] of type II fibers was similar in LHTLH and decreased in LHTL vs. increased in LLTL ($P = 0.09$, ESs = moderate and $P < 0.05$, ESs = moderate, respectively). LHTL also showed decreases in [Mb] within type I fibers between pre to post-2 in contrast to LLTL ($P < 0.05$, ESs = moderate). In summary, [Mb] in LHTLH decreased from pre and post-1 but returned near baseline levels at post-2. LHTL showed similar [Mb] at pre and post-1, whereas [Mb] decreased from post-1 to post-2.

Similar to [Mb], changes in CD, C/F, and CAF revealed mostly small-to-moderate effects between groups (see Table 2 and Fig. 4). From pre up to post-2, CAF showed likely larger increases in LHTL compared with LHTLH and LLTL ($P < 0.05$, ESs = moderate and $P = 0.05$, ESs = moderate, respectively). However, given the changes in FCSA, CD was increased in LHTLH from pre to post-2 but decreased in LHTL and LLTL ($P < 0.05$, ESs = moderate and $P = 0.20$, ESs = moderate). Note that at pre, CAF was likely lower in LHTL compared with that in LLTL ($P = 0.09$, ESs = moderate). These results suggest that capillaries per fiber increased in LHTL but not in LHTLH and LLTL. However, with the changes in FCSA, LHTLH showed superior improvements in CD compared with LHTL and LLTL.

Combination of oxidative capacity and fiber size. Our findings show that team-sport athletes possessed high combinations of fiber size and oxidative capacity with respect to the reported inverse relationship between fiber size and mitochondrial oxidative capacity from scientific literature (53). A unique observation was that the LHTLH and LHTL, but not the LLTL, substantially improved their combination of fiber size and oxidative capacity after the intervention (Fig. 5).

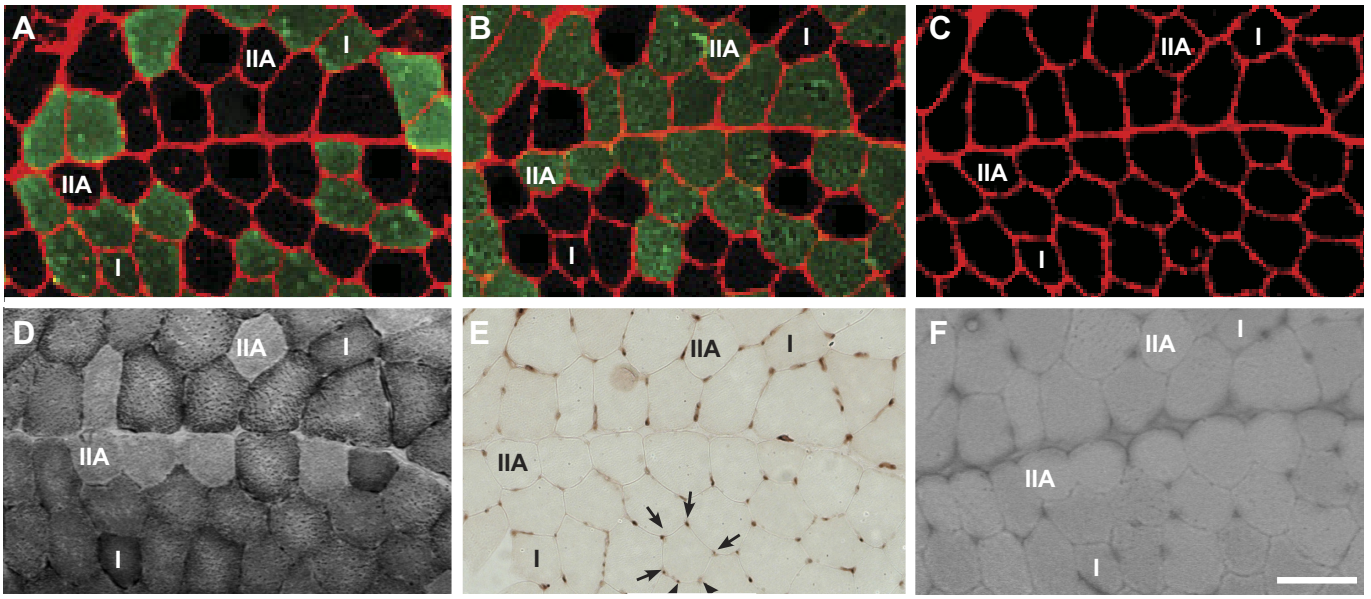

Fig. 1. Immunohistochemical stainings for myosin heavy chain type I, IIA, and IIX expression (*A–C*), enzyme histochemical stainings for succinate dehydrogenase activity (*D*), Ulex europaeus agglutinin I lectin for capillaries (*E*), and myoglobin concentration (*F*) from a representative subject immediately after intervention for type I and II fibers (post-1). Cross sections of 10 μm were obtained from the human m. vastus lateralis. *A–C*: muscle fiber type I, IIA, and IIAX are identified. *D*: type IIX was not present in this subject. *E*: capillaries were indicated by arrows in 1 muscle fiber. *F*: histochemical assays for myoglobin contain black spots that indicate peroxidase activity of hemoglobin, which were excluded from analysis. Scale bar = 100 μm.

DISCUSSION

The present study shows that elite team-sport athletes are able to substantially increase their mitochondrial oxidative capacity (+35%), while maintaining similar fiber size, after living high-training low and high (the so-called LHTLH; i.e., chronic hypoxic residence combined with six repeated-sprint sessions in hypoxia) for 14 days. As expected, the increase in oxidative capacity was larger in LHTLH compared with that in LHTL and LLTL, whereas [Mb], CAF, and C/F showed small-to-moderate effects and no superior changes for LHTLH. In contrast to our hypothesis, muscle fiber size increased in LHTL and LLTL from pre to post-1 and post-2 but remained fairly constant in LHTLH. Given the changes in muscle fiber size, LHTLH showed superior improvements in CD.

Mitochondrial oxidative capacity. SDH activity substantially increased in LHTLH from pre to post-1 and remained elevated at post-2 in LHTLH vs. LHTL. Therefore, LHTLH seems to be a superior strategy to improve skeletal muscle fiber oxidative capacity. These adaptations support the superior improvements in repeated-sprint ability in LHTLH compared with LHTL and LLTL previously observed in this cohort of team-sport athletes (9) and complement their reported molecular adaptations (8). Team-sport athletes have to perform many high-intensity sprints during a match, often with incomplete recoveries. The ability to recover from high-intensity sprints is predominantly limited by the resynthesis rate of phosphocreatine (5, 17). High oxidative capacity strongly relates to fast oxygen consumption recovery kinetics (56) and appears to be essential for fast phosphocreatine resynthesis rates (42). We could therefore speculate that the increased oxidative capacity likely facilitated the improved repeated-sprint ability in LHTLH and, to a lower extent, in LHTL (9).

In our elite team-sport athletes, average SDH activity values at pre were ~20% higher than those reported in highly trained track and road cyclists (51). Nevertheless, the LHTLH and LHTL groups were able to increase their SDH activity during the intervention (pooled improvement of type I and II fibers was ~35 and ~9%, for LHTLH and LHTL respectively). Skeletal muscle adaptations with exercise are thought to be augmented when performed under hypoxia (7, 17). Local tissue hypoxia results in a mismatch between oxygen supply and demand, which triggers skeletal muscle adaptations, primarily via the HIF pathways, to maintain homeostasis of oxygen availability (32). In a companion paper, we reported that HIF-1α mRNA expression levels were increased from pre to post-1 in LHTLH but not in LHTL and LLTL (8). Moreover, the HIF-1α protein is stabilized by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (40), which was also upregulated in LHTLH during the intervention and not in LHTL and LLTL (8). PGC-1α plays an important role in the mitochondrial biogenesis and oxidative phosphorylation (16). Therefore, both PGC-1α and HIF-1α seem critical for skeletal muscle adaptations to increase oxygen consumption (40) and may explain these superior increases in SDH activity in LHTLH vs. LHTL and LLTL.

Chronic hypoxic exposure at high altitude (>5,000 m) may reduce mitochondrial oxidative enzyme activities (21, 25). However, these reductions could be avoided at lower altitude levels (26, 39), such as the altitude in the present study (~3,000 m). Similar to our results in LHTLH and LHTL, adaptations of mitochondrial density and oxidative enzyme activity are augmented with high-intensity bouts of continuous cycling in hypoxia compared with normoxia (37, 50, 54), although not in all cases (13, 35, 44) (for review, see Ref. 32). Part of the difference in SDH activity between LHTLH and LHTL may be due to increases in FCSA in LHTL that could have repressed the increases in SDH activity during the intervention. In recreationally active subjects, sprint-interval training in nor-

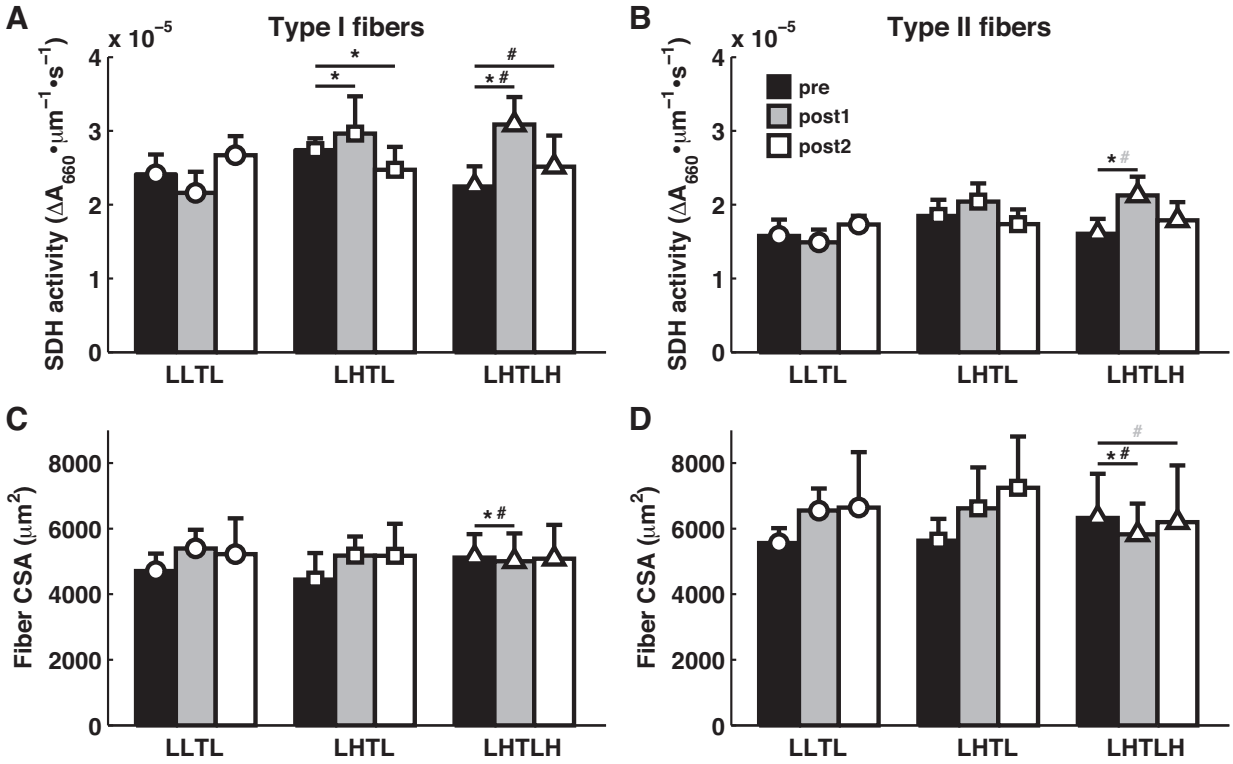

Fig. 2. Succinate dehydrogenase (SDH; *A* and *B*) activity and fiber cross-sectional area (CSA; *C* and *D*) measured before (pre), immediately after (post-1), and 3 wk after the intervention (post-2) for type I (*A* and *C*) and II (*B* and *D*) fibers. Histograms show mean group data at pre, post-1, and post-2 for live high-train low and high (with repeated-sprints in hypoxia) group (LHTLH; △), live high-train low (with repeated-sprints in normoxia) group (LHTL; □), and live low-train low or control group (LLTL; ○). Black symbols show significant effects ($P < 0.05$), and gray symbols indicate a tendency ($P < 0.10$). *Different from LLTL. #Different from LHTL.

moxic conditions has been shown to increase oxidative potential (reflected by an 38% increase in citrate synthase activity) and endurance capacity (10). The novel findings of this study show that elite team-sport athletes are able to reach similar improvements in oxidative capacity of both type I and type II fibers (~37 and ~32%, respectively) after only 14 days of LHTL combined with as few as six repeated-sprint training sessions in hypoxia.

Muscle fiber size. Fiber size remained fairly constant during the intervention in LHTLH (pooled change of type I and II fibers was −5%), whereas FCSA was increased in LHTL (+17%) and LLTL (+16%). Similar to our findings, FCSA of vastus lateralis muscle remained fairly constant (−8%) in recreationally trained subjects after 6 wk of hypoxic endurance training at 3,850 m, whereas FCSA increased (+43%) after normoxic endurance training (54). Although prolonged resi-

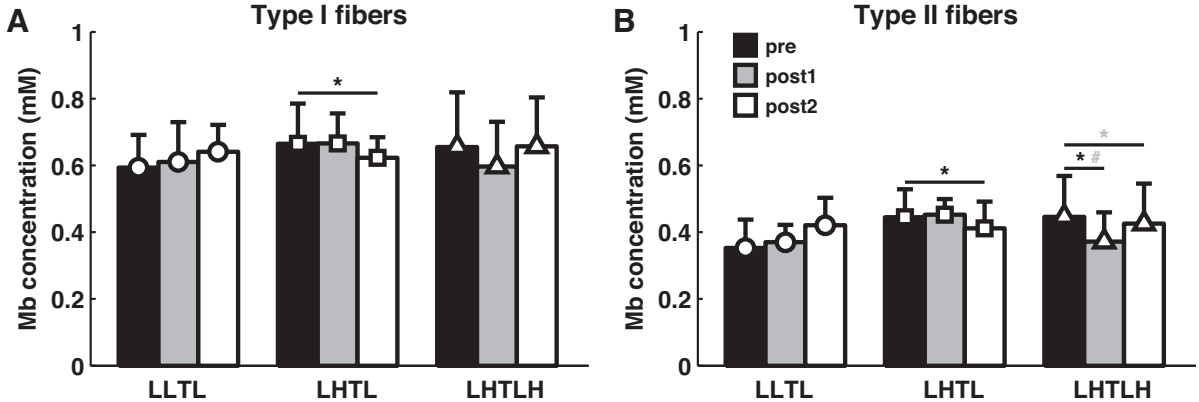

Fig. 3. Myoglobin concentration ([Mb]) measured before (pre), immediately after (post-1), and 3 wk after the intervention (post-2) for type I and type II fibers (*A* and *B*, respectively). Histograms show mean group data at pre, post-1, and post-2 for live high-train low and high (with repeated-sprints in hypoxia) group (LHTLH; △), live high-train low (with repeated-sprints in normoxia) group (LHTL; □), and live low-train low or control group (LLTL; ○). Changes over time (between pre and post-1 and between pre and post-2) were compared between groups using effect sizes. Black symbols show significant effects ($P < 0.05$), and gray symbols indicate a tendency ($P < 0.10$). *Different from LLTL. #Different from LHTL.

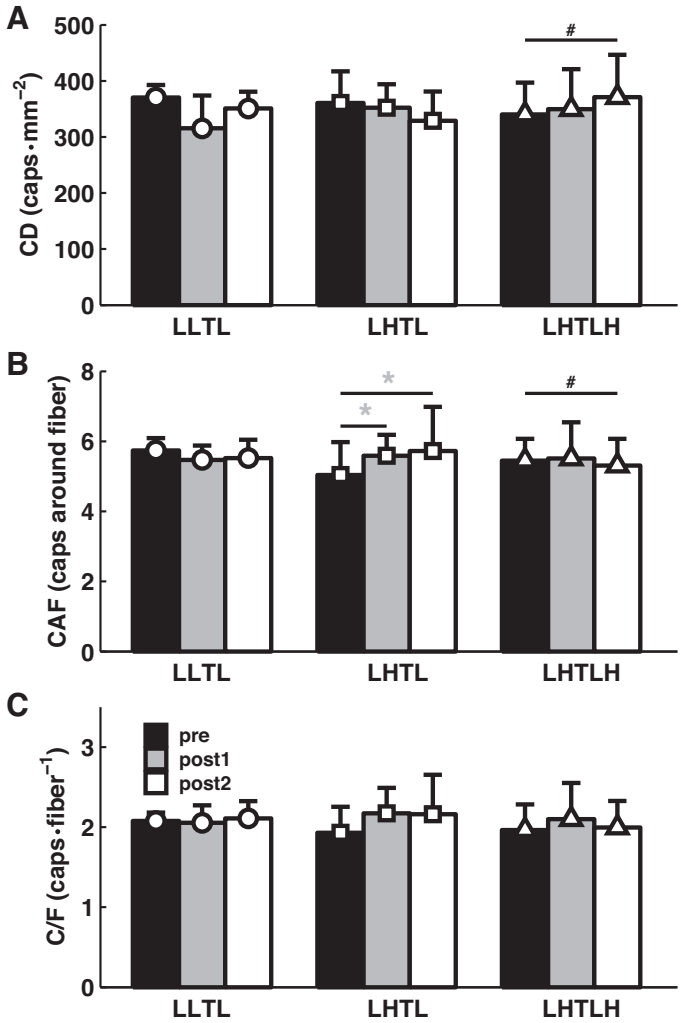

Fig. 4. Capillary density (CD; *A*), capillary number around the fiber (CAF; *B*), capillary-to-fiber ratio (C/F; *C*) measured before (pre), immediately after (post-1), and 3 wk after the intervention (post-2). Histograms show mean group data at pre, post-1 and post-2 for live high-train low and high (with repeated-sprints in hypoxia) group (LHTLH; △), live high-train low (with repeated-sprints in normoxia) group (LHTL; □), and live low-train low or control group (LLTL; ○). Changes over time (between pre and post-1 and between pre and post-2) were compared between groups using effect sizes. Black symbols show significant effects ($P < 0.05$), and gray symbols indicate a tendency ($P < 0.10$). *Different from LLTL. #Different from LHTL.

dence in severe chronic hypoxia (≥40 days and at >5,000 m) without physical activity has been shown to result in ~15–25% reductions in FCSA (21, 25, 34, 39), no changes in FCSA were reported with shorter chronic hypoxic exposure (15–28 days) and/or lower altitude levels (3,200–4,100 m) (15, 26, 33). It has been suggested that "hypoxic dose" (i.e., severity and duration of hypoxic exposure) determines the magnitude of atrophy (14, 18), although muscle atrophy in hypoxia is certainly more complex (43). Hypoxic dose was similar in LHTLH and LHTL (9); however, the combination of hypoxia and high-intensity exercise may have induced (extreme) local tissue hypoxia that affected FCSA in LHTLH vs. LHTL, probably as a consequence of oxidative stress and/or lower cellular energy state (AMP:ATP ratio) (53). Still, both LHTLH and LHTL were able to maintain their fiber size.

The moderate-to-large increases in FCSA in LHTL and LLTL vs. LHTLH after the 14-day intervention period and another 3 wk of supervised training were quite surprising. In general, increases in skeletal muscle FCSA are not observed until at least 6–7 wk of resistance training (36). Although enhancement of protein synthesis starts immediately after exercise in normoxia, expansion of FCSA may generally be delayed due to initial increases in myofibrillar density (36). Alternatively, the increases in FCSA may not reach significance because of measurement error or a lack of statistical power that is commonly observed in studies with muscle biopsies (36). In addition, hypertrophy may relate to edema associated with muscle damage (11) or increased glycogen storage (41). Glycogen content has been shown to increase after only 2 wk of sprint interval training in recreationally trained subjects (10). Therefore, changes in FCSA may be explained by myofibrillar hypertrophy and/or cellular edema, while it should be noted that these changes were not accompanied by functional improvements in jump performance (9).

Oxygen supply capacity. Changes in capillarization and [Mb] during the study showed mostly small-to-moderate effects between groups. In a companion study, in LHTLH the mRNA expression for VEGF and Mb was shown to be increased from pre to post-1, but not in LHTL and LLTL (8). These observations agree with findings showing that mRNA expression for VEGF and Mb is stimulated during high-intensity endurance training in hypoxia rather than in normoxia (54), resulting in increased capillary length density with endurance training in hypoxia (+19%). Moreover, given changes in FCSA, LHTLH revealed superior increases in CD from pre to post-2 (+9%) compared with LHTL and LLTL. Capillary growth was observed from pre to post-2 in LHTL and not in LHTLH, whereas in our companion paper VEGF mRNA levels increased from pre to post-1 in LHTLH and not in LHTL (8). Here, the discrepancy in LHTLH and LHTL may be explained by the present study including only a subpanel ($n = 6$ in each group) of subjects from the companion papers (8, 9). Alternatively, severity of hypoxia during exercise may be important for the lack of capillary growth observed in LHTLH. Recent findings suggest that endurance training at supramaximal intensities abolishes improvements in capillarization and VEGF protein expression, even though VEGF mRNA expression levels still increase (22, 23). Accordingly, the combination of chronic hypoxic exposure with maximal-intensity sprints in hypoxia in LHTLH may have induced severe local tissue hypoxia that could have led to abolished angiogenesis, despite the increased VEGF mRNA expression (8). Nevertheless, LHTLH showed superior improvements in CD, which facilitates oxygen diffusion to the mitochondria.

The results show that [Mb] was reduced in LHTLH from pre to post-1 but returned near baseline levels at post-2. In contrast, LHTL showed constant [Mb] from pre to post-1 but reduced [Mb] at post-2. The reductions in [Mb] in LHTLH and LHTL were unexpected, as changes in ferritin concentration during the intervention did not indicate iron deficiencies among our subjects. We assessed [Mb] by its peroxidase activity, reflecting functional Mb incorporating a heme molecule. Erythropoiesis, which was observed in both LHTLH and LHTL (9), poses high iron demands associated with the synthesis of hemoglobin and may eventually lead to reductions in skeletal muscle iron stores, reducing functional Mb protein expression (32). Mb

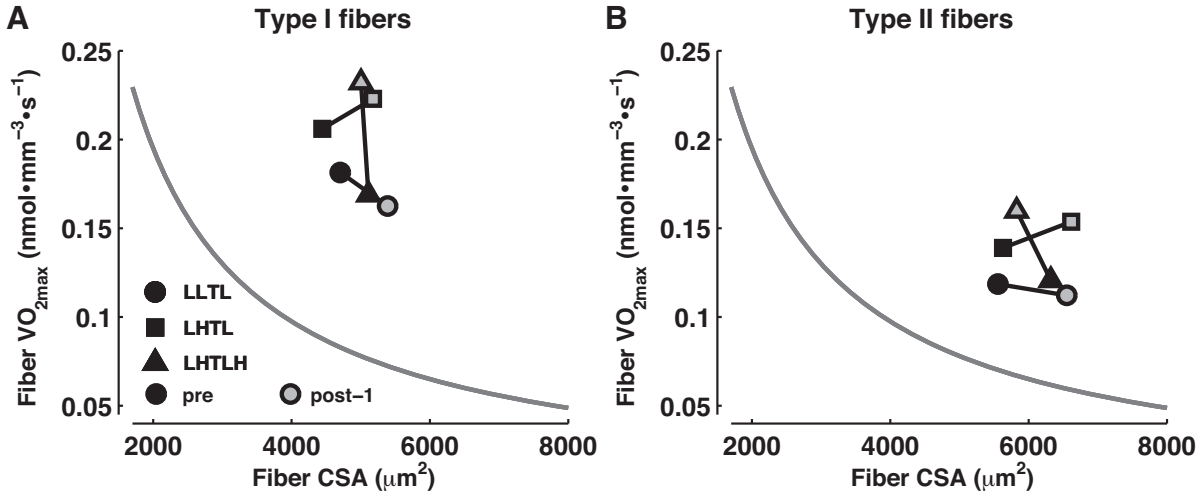

Fig. 5. Fiber size and oxidative capacity measured at before (pre) and immediately after (post-1) were plotted against the inverse relationship between fiber cross-sectional area (FCSA) and oxidative capacity as previously reported in scientific literature. *A*: FCSA and oxidative capacity of type I fibers. *B*: FCSA and oxidative capacity of type II fibers. Group averages were shown for live high-train low and high (with repeated sprints in hypoxia) group (LHTLH; triangles), live high-train low (with repeated sprints in normoxia) group (LHTL; squares), and live low-train low or control group (LLTL; circles) and at pre (black symbols) and post-1 (gray symbols). From literature, the inverse relationship between fiber size and fiber oxidative capacity across species was adapted from Van Wessel et al. (53). Our elite team-sport athletes showed combinations of fiber size and oxidative capacity that were already above this reported inverse relationship at the start of the intervention (pre). Nevertheless, our subjects even further increased their combination of FCSA and oxidative capacity in LHTLH (improved oxidative capacity) and LHTL (improved oxidative capacity and FCSA) but not in LLTL.

protein synthesis is stimulated only when hypoxia is combined with a secondary stimulus, such as exercise (28). Previous observations in LHTLH, combining hypoxia with exercise, reported increased Mb mRNA expression immediately after the intervention, whereas the hypoxic stimulus may be insufficient in LHTL for increasing Mb mRNA expression (8). However, here, LHTLH did not increase Mb protein until 3 wk postintervention. A delayed increase in Mb protein (at post-2) may result from the time needed to translate Mb mRNA into functional protein, as shown in zebrafish that were maintained under chronic hypoxia (27). Note that the magnitudes of increases in Mb protein concentration are not the same as those in functional Mb, as Mb protein may already increase before the incorporation of a heme molecule, necessary for Mb to become functional. Previously, hypoxic training has been shown to induce significant increases in [Mb] (+8%) or nonsignificant increases in [Mb] (+7%) after 4 and 8 wk of high-intensity endurance training, respectively (35, 50). Thus future studies should focus on the regulation of functional [Mb] in dependence of severity of hypoxia and exercise type as well as availability of iron.

Fiber size and mitochondrial oxidative capacity are known to be inversely related (48, 52, 53). While the team-sport athletes already possessed a high product of muscle fiber size and oxidative capacity (0.133 ± 0.023 in $\Delta A_{660} \cdot \mu m \cdot s^{-1}$), comparable to well-trained cyclists (52), a unique observation was that the athletes performing LHTLH and LHTL substantially improved their integrated SDH activity after the intervention (both by ~30%). In these athletes, concurrent improvement of determinants of peak power production and oxidative metabolism likely facilitated the improvement in physical performance (9). We speculate that increases in SDH activity are critical to the improved repeated-sprint performance (9), as SDH activity has shown to be proportional to muscle fiber $\dot{V}O_{2max}$ and whole body $\dot{V}O_{2max}$ (12, 29, 51), facilitating enhanced resynthesis of phosphocreatine and recovery from maximal-intensity sprints. This increase in oxygen demand of the muscle fiber requires concomitant increases in oxygen supply capacity. Although changes in [Mb] and capillarization during the intervention may not have fully accommodated changes in integrated SDH activity, hemoglobin mass was also increased (9) and may have contributed to an improved oxygen supply capacity as well. Future studies are warranted to gain insight in muscle adaptations at mRNA and protein level in response to chronic hypoxic exposure and training in hypoxic conditions. This may also help elucidating the time course and interactions of these adaptations for oxygen supply capacity to the mitochondria and oxygen demands of the muscle fiber.

Limitations

To further elucidate separate effects of chronic hypoxic residence and repeated-sprint training, it would have been interesting to compare the results of LHTLH and LHTL to another group that lived at sea level and performed repeated-sprint training in normoxia in addition to their regular training. However, this was not feasible in the present study, due to the limited population of elite (field hockey) athletes and difficulty to obtain multiple biopsy samples from such elite athletes.

In addition to statistical testing for significance, the magnitude-based inferences approach provides a more nuanced quantification of effects. This approach interprets the findings with respect to the smallest worthwhile effect in this population of elite athletes and quantifies the effects into more finely graded magnitudes of likelihood of benefit, triviality, and harm. Therefore, also nonsignificant effects may be captured that are worthwhile to these elite athletes, which may serve as indicators of training adaptations. Of note is that both statistical approaches revealed similar findings (see Figs. 2–4 and Table 2).

Conclusion

Our findings show that after 14 days of chronic hypoxic residence combined with six repeated-sprint sessions in hypoxia, elite team-sport athletes substantially improved the oxidative capacity in their type I and type II fibers with respect to the start of the intervention. Muscle fiber size remained fairly constant in LHTLH, whereas FCSA increased in LHTL and LLTL. Changes in oxygen supply capacity were less prominent, showing mostly small-to-moderate effects between groups. A unique observation was that LHTLH and LHTL, but not LLTL, improved their combination of fiber size and oxidative capacity. Therefore, LHTLH and LHTL are adequate training strategies to improve the combination of prime skeletal muscle fiber determinants for peak power production and maximal oxygen consumption.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.B., G.P.M., and O.G. conceived and designed research; F.B., G.P.M., L.D., and O.G. performed experiments; S.v.d.Z., B.L.K., W.J.v.d.L., and R.T.J. analyzed data; S.v.d.Z., F.B., B.L.K., G.P.M., L.D., W.J.v.d.L., O.G., and R.T.J. interpreted results of experiments; S.v.d.Z., B.L.K., W.J.v.d.L., and R.T.J. prepared figures; S.v.d.Z., B.L.K., W.J.v.d.L., and R.T.J. drafted manuscript; S.v.d.Z., F.B., B.L.K., G.P.M., L.D., W.J.v.d.L., O.G., and R.T.J. edited and revised manuscript; S.v.d.Z., F.B., B.L.K., G.P.M., L.D., W.J.v.d.L., O.G., and R.T.J. approved final version of manuscript.

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