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High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: implications for exercise performance

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ABSTRACT: The purpose of the present study was to investigate whether exercise training-induced adaptations in human skeletal muscle mitochondrial bioenergetics are magnified under thermal conditions resembling sustained intense contractile activity and whether training-induced changes in mitochondrial oxidative phosphorylation (OXPHOS) efficiency influence exercise efficiency. Twenty healthy men performed 6 wk of high-intensity exercise training [*i.e.*, speed endurance training (SET; $n = 10$)], or maintained their usual lifestyle ($n = 10$). Before and after the intervention, mitochondrial respiratory function was determined *ex vivo* in permeabilized muscle fibers under experimentally-induced normothermia (35°C) and hyperthermia (40°C) mimicking *in vivo* muscle temperature at rest and during intense exercise, respectively. In addition, activity and content of muscle mitochondrial enzymes and proteins were quantified. Exercising muscle efficiency was determined *in vivo* by measurements of leg hemodynamics and blood parameters during one-legged knee-extensor exercise. SET enhanced maximal OXPHOS capacity and OXPHOS efficiency at 40°C, but not at 35°C, and attenuated hyperthermia-induced decline in OXPHOS efficiency. Furthermore, SET increased expression of markers of mitochondrial content and up-regulated content of MFN2, DRP1, and ANT1. Also, SET improved exercise efficiency and capacity. These findings indicate that muscle mitochondrial bioenergetics adapts to high-intensity exercise training in a temperature-dependent manner and that enhancements in mitochondrial OXPHOS efficiency may contribute to improving exercise performance.—Fiorenza, M., Lemminger, A. K., Marker, M., Eibye, K., Iaia, F. M., Bangsbo, J., Hostrup, M. High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: implications for exercise performance. FASEB J. 33, 8976–8989 (2019). www.fasebj.org

KEY WORDS: mitochondrial respiratory function · mitochondrial efficiency · high-intensity interval training (HIIT) · uncoupling proteins · exercise efficiency

Exercise training promotes multiple changes in skeletal muscle cells (fibers), including substantial adaptations at the mitochondrial level that culminate in expanded mitochondrial volume and increased oxidative capacity (1). Although the beneficial effects of exercise training on human skeletal muscle mitochondrial volume are well

ABBREVIATIONS: ANT1, adenine nucleotide translocase 1; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein-3; BSA, bovine serum albumin; CI_P, maximal OXPHOS capacity through complex I; $CI+CII_P$, maximal OXPHOS capacity through complexes I and II combined; CII_P, maximal OXPHOS capacity through complex II; CON, control; CS, citrate synthase; DRP1, dynamin related protein 1; DXA, dual-energy X-ray absorptiometry; EEE, energy expenditure during exercise; *ETS*, electron transport system respiratory capacity; FAO_P, fatty acid oxidative capacity; HAD, 3-hydroxyacyl-CoA dehydrogenase; HR_{max}, maximal heart rate; HSP70, heat shock protein 70; IDH, isocitrate dehydrogenase; iPPO, incremental peak power output; L_N, normal leak respiration; L_{Omy}, oligomycin-induced leak respiration; MFN2, mitofusin 2; OXPHOS, oxidative phosphorylation; P/O ratio, molar amount of ATP produced per mole of atomic oxygen consumed; Pre, before the intervention; Post, after the intervention; RCR, respiratory control ratio; REE, energy expenditure at rest; SET, speed endurance training; SUIT, substrate-uncoupler-inhibitor titration; UCP3, uncoupling protein 3; $\dot{V}_{CO2}$, CO_2 release; $\dot{V}_{O2}$, oxygen consumption; $\dot{V}_{O2max}$, maximal $\dot{V}_{O2}$

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documented (2), less is known about training-induced adaptations in mitochondrial bioenergetics and their implications for exercise performance.

Mitochondria generate ATP *via* oxidation of substrates [oxidative phosphorylation (OXPHOS)]. Thus, mitochondrial respiratory function can be assessed from rate of mitochondrial oxygen consumption. For such a purpose, *in vitro* polarographic measurements of oxygen consumption from isolated mitochondria have been utilized for over 60 years (3). However, recent advances in the field of high-resolution respirometry allow for *in situ* assessments of mitochondrial respiratory function from very small samples of permeabilized muscle fibers, being a more physiologically relevant measure of mitochondrial functional properties within an integrated cellular system (4, 5).

Skeletal muscle mitochondrial OXPHOS capacity has been identified as an important determinant of endurance performance in humans (6). Existing evidence supports the beneficial impact of exercise training on mitochondrial respiratory function (7), with training-induced enhancements in maximal OXPHOS capacity being associated with improvements in exercise performance (8–16). On the other hand, intrinsic mitochondrial maximal OXPHOS capacity (*i.e.*, mitochondrial respiratory capacity normalized to mitochondrial content) has been shown to be either unaltered or depressed in response to exercise training (9–11, 17, 18), suggesting that training-induced enhancements in mitochondrial respiratory function are related to an expansion in mitochondrial volume rather than to improvements in mitochondrial respiration *per se*.

Mitochondrial OXPHOS system relies on the tight coupling between electron transport and ATP synthesis. However, a part of the energy generated by electron transport is uncoupled from ATP synthesis resulting in heat dissipation. Accordingly, mitochondrial OXPHOS coupling efficiency, defined as the molar amount of ATP produced per mole of atomic oxygen consumed (*i.e.*, P/O ratio), or alternatively as the ratio of state 3 to state 4 respiration [*i.e.*, respiratory control ratio (RCR)], is a critical feature of mitochondrial function. In contrast to maximal OXPHOS capacity, OXPHOS efficiency seems less responsive to exercise training, with most (9, 10, 15–17, 19–22) but not all (8) human studies failing to report training-induced enhancements in indices of mitochondrial coupling efficiency.

Notably, OXPHOS efficiency is highly affected by changes in muscle temperature (23). In mitochondria isolated from rat skeletal muscle, both P/O ratio and RCR decrease with increasing temperature (24). In view of these temperature-dependent alterations, a recent study from Zoladz *et al.* (25) demonstrated that endurance training attenuated the decline in OXPHOS efficiency occurring at high assay temperature (42°C) in isolated rat skeletal muscle mitochondria. A shortcoming of studies conducted in humans is that muscle mitochondrial respiratory function is commonly assessed at a standardized temperature of 37°C. However, although human muscle temperature at rest is within a range of 35–36°C, it may rise up to 40°C during intense exercise (26–28). Accordingly, it should be investigated whether changes in mitochondrial respiratory function attained by a period of exercise training are temperature dependent in human skeletal muscle. In addition, in light of the exercise intensity-dependency of the training-induced mitochondrial qualitative changes (8, 10, 12) and in view of the transient heat stress incurred during high-intensity exercise (29), it is conceivable that high-intensity exercise training promotes adaptations attenuating the hyperthermia-induced decline in OXPHOS efficiency.

Lastly, given that the overall efficiency of the exercising muscle is determined by the efficiency of the processes providing energy (*i.e.*, mitochondrial coupling) and converting energy to external work (*i.e.*, contractile coupling) (30, 31), it is relevant to examine whether training-induced enhancements in OXPHOS efficiency translate to a better efficiency of the contracting muscle during sustained exercise.

Thus, in the present study, we investigated the effect of high-intensity exercise training on skeletal muscle mitochondrial respiratory function under experimentally-induced hyperthermia resembling muscle temperature during intense exercise. In addition, to examine whether muscle mitochondrial adaptations attained by high-intensity exercise training are associated with changes in exercise efficiency, we integrated *ex vivo* measurements of mitochondrial respiratory function with *in vivo* assessments of exercising muscle efficiency. Our hypotheses were that high-intensity exercise training would improve muscle mitochondrial respiratory capacity and coupling efficiency in a temperature-specific manner and that these adaptations would be accompanied by improvements in both exercise efficiency and capacity.

MATERIALS AND METHODS

Human subjects and ethics

Twenty-one healthy men were initially included, of whom 20 completed the study. Prior to inclusion, subjects were informed of risks and discomforts associated with the experimental procedures. Each subject gave his oral and written informed consent. Inclusion criteria were male sex, age of 18–40 yr, a maximal oxygen consumption ($\dot{V}_{O2max}$) between 45 and 55 ml/min/kg, and a body mass index of 19–26 kg/m^2. Exclusion criteria were abnormal electrocardiogram, chronic disease, ongoing pharmacologic treatment, and smoking. Included subjects were allocated in either a control (CON; $n = 10$) or training (SET; $n = 10$) group. All subjects were recreationally active, and their characteristics are presented in **Table 1**. The study was approved by the regional research ethics committee of Copenhagen, Denmark, (H-17004045) and adheres to the principles of the Declaration of Helsinki. The study was registered at *https://clinicaltrials.gov/* (NCT03317704).

Study design and preliminary tests

This study was designed as a longitudinal randomized controlled trial (**Fig. 1**). Prior to enrolment in the study, each subject underwent an examination for assessment of eligibility criteria, including an incremental test to exhaustion performed on a mechanically braked cycle-ergometer (LC4; Monark Exercise, Vansbro, Sweden) for determination of $\dot{V}_{O2max}$. The test protocol consisted of a 4-min bout at 100 W followed by an incremental graded test with increments of 25 W/min until volitional exhaustion. Pulmonary gas exchanges

TABLE 1. *Subject characteristics before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period*

Subject characteristic	CON (n = 10)		SET (n = 10)	
	Pre	Post	Pre	Post
Age (yr)	25.7 ± 1.2		23.0 ± 1.1	
Height (m)	1.84 ± 0.02		1.86 ± 0.02	
Weight (kg)	76.0 ± 2.5	75.9 ± 2.6	79.6 ± 3.8	79.5 ± 3.7
Body mass index (kg/m^2)	22.4 ± 0.5	22.4 ± 0.5	23.0 ± 0.8	23.0 ± 0.8
Whole-body muscle mass (kg)	61.5 ± 2.7	61.5 ± 2.7	60.4 ± 2.8	60.8 ± 2.8
Whole-body fat mass (kg)	11.7 ± 1.1	11.5 ± 1.1	16.2 ± 1.7†	15.7 ± 1.5†
Whole-body fat (%)	15.5 ± 1.6	15.3 ± 1.6	20.0 ± 1.5†	19.5 ± 1.5
Experimental thigh muscle mass (kg)§	7.05 ± 0.37	7.03 ± 0.36	6.94 ± 0.40	7.13 ± 0.39*
Experimental thigh fat mass (kg)	1.21 ± 0.12	1.20 ± 0.13	1.61 ± 0.17	1.57 ± 0.17
Experimental thigh fat (%)	14.8 ± 1.6	14.7 ± 1.6	18.6 ± 1.4	17.9 ± 1.4*
REE (kcal/min)	1.50 ± 0.10	1.53 ± 0.06	1.65 ± 0.11	1.80 ± 0.14
$\dot{V}_{O2max}$ (ml/min)§	4053 ± 262	3932 ± 206	3976 ± 192	4377 ± 184*
$\dot{V}_{O2max}$ (ml/min/kg)§	52.8 ± 2.0	51.5 ± 1.5	50.2 ± 1.4	55.3 ± 1.4*

Values are means ± SEM. *Significantly different from Pre ($P < 0.05$). †Significantly different from CON ($P < 0.05$). §Significant group-time interaction ($P < 0.05$).

were measured breath-by-breath using an online gas analysis system (Oxycon Pro; Vyaire Medical, Mettawa, IL, USA). $\dot{V}_{O2max}$ was determined as the highest value achieved during a 30-s period. Criteria used for achievement of $\dot{V}_{O2max}$ were a plateau in pulmonary oxygen consumption ($\dot{V}_{O2}$) despite an increase in workload and a respiratory exchange ratio above 1.10. Then, subjects returned to the laboratory on a different day to perform an incremental graded test with one leg in a one-legged knee-extensor ergometer for determination of the time to exhaustion and incremental peak power output (iPPO). The test started at 12 W and progressed with increments of 6 W/min. Subjects were instructed to keep a constant cadence of 60 rpm, and the test was terminated when cadence dropped below 55 rpm.

Experimental setup

Two experimental days, separated by 2–4 d, were conducted before (Pre) and after (Post) a 6-wk intervention period, with the experimental days at Post being carried out within 5 d after the last training session in SET (Fig. 1). Subjects refrained from caffeine, alcohol, and exercise for 24 h prior to the experimental days. In addition, subjects were asked to record their food intake for the 48 h before experimental day 1 so that they could replicate the same food intake during the 48 h preceding experimental day 2 as well as during the 48 h preceding experimental day 1 and 2 at Post.

Experimental day 1

Subjects reported to the laboratory in the morning after an overnight fast. After 10 min of supine rest, subjects were scanned with whole-body dual-energy X-ray absorptiometry (DXA). After the scan, subjects were provided with a standardized breakfast consisting of 18.7 g of carbohydrates, 6.8 g of fats, 17.0 g of proteins, and water *ad libitum*. After 15 min of rest in the supine position, a 3-mm incision was made over the lateral portion of the experimental thigh under local anesthesia (2 ml lidocaine without epinephrine, 20 mg/ml Xylocain; AstraZeneca Pharmaceuticals, Wilmington, DE, USA), and a biopsy was obtained from the vastus lateralis muscle by using a percutaneous Bergstrom needle with suction. At this point, subjects included in SET went

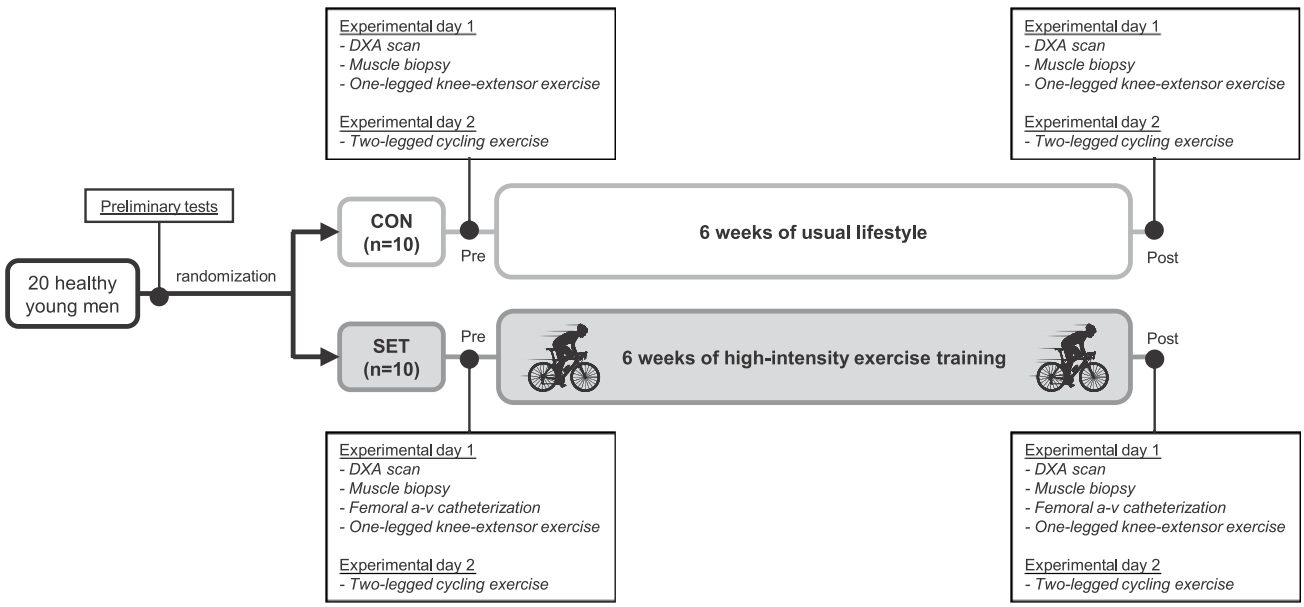

Figure 1. Schematic presentation of the study design.

through an experimental procedure aimed at determining exercising muscle efficiency.

After local anesthesia (2 ml lidocaine without epinephrine, 20 mg/ml Xylocain), Arrow catheters (20 gauge; Teleflex, Wayne, PA, USA) were placed in the femoral artery and vein of the experimental leg. The catheters were placed 2–3 cm below the inguinal ligament and advanced 10 cm in the proximal direction. The correct placement of the catheters was verified by ultrasound Doppler (Vivid E9; GE Healthcare, Waukesha, WI, USA). After ~60 min of rest, subjects were seated in a semirecumbent position with a hip-angle fixed at ~110° and completed a one-legged knee-extensor exercise test. The test protocol included three submaximal exercise bouts performed at low- (5 min at 30% iPPO), moderate- (5 min at 50% iPPO), and high-intensity (4 min at 80% iPPO). Upon completion of the high-intensity bout, an incremental test was carried out with increments of 6 W/min. Subjects were instructed to keep a constant cadence of 60 rpm throughout the test, and the incremental test terminated when subjects were no longer able to maintain a cadence above 55 rpm. Blood was drawn from the femoral artery and vein during the last minute of each submaximal exercise bout. In order to account for the transit time of blood from the artery through the muscle capillary bed and to the collection point at the vein (32), the arterial samples were taken ~5 s before the venous samples. In addition, femoral artery blood flow was measured at rest and during the last minute of each submaximal exercise bout.

Subjects included in CON completed the one-legged knee-extensor exercise test as described for SET but without assessments of exercising muscle efficiency.

Experimental day 2

Subjects reported to the laboratory on a second occasion. Upon arrival in the morning, after an overnight fast, subjects were provided with the same standardized breakfast consumed on experimental day 1. Thirty minutes after the ingestion of the meal, subjects rested in the supine position for 10 min to measure pulmonary gas exchanges at rest. After that, subjects performed a two-legged cycling exercise test on a mechanically braked cycle-ergometer (LC4; Monark Exercise). The test protocol included 12 min of warm-up followed by an incremental graded test with increments of 20 W/min until volitional exhaustion. During the incremental test, pulmonary gas exchanges were measured breath-by-breath using an online gas analysis system (Oxycon Pro; Vyaire Medical). $\dot{V}_{O2max}$ was determined as described for the preliminary visit. In SET, time to exhaustion and iPPO were also recorded. Heart rate was monitored throughout the test, and maximal heart rate (HR_{max}) was established as the highest value achieved during the test.

Exercise training intervention

Subjects included in CON were instructed to maintain their usual lifestyle and not to change their physical activity level.

Subjects included in SET performed 6 wk of cycling-based supramaximal-intensity interval training [*i.e.*, speed endurance training (SET)] consisting of repeated 20-s bouts of all-out sprinting interspersed with 2 min of recovery. Subjects were instructed to pedal as fast as possible during the 20-s sprints and to cycle at low intensity (50–100 W) during the recovery periods. The number of sprints performed throughout a training session was progressively increased from wk 1 to 5, and it was reduced during wk 6 to reduce accumulated fatigue induced by the intense training period while maximizing physiologic and performance adaptations (**Fig. 2**). SET was chosen in view of evidence indicating its effectiveness to increase mitochondrial respiratory capacity, up-regulate mitochondrial proteins, and enhance performance (7, 33, 34). All training sessions were preceded by a 7-min warm-up at a relative intensity corresponding

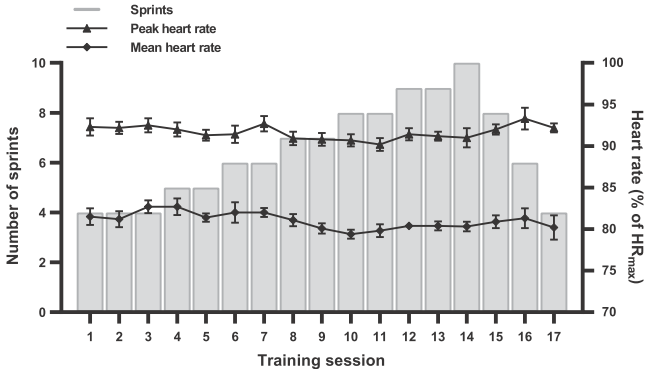

Figure 2. Number of sprints and heart rate during the high-intensity exercise training period. Heart rate values are means ± SEM.

to ~70% HR_{max}. During the first and the last training session, the power outputs during each sprint were recorded as previously described (35). All training sessions were supervised, and subjects wore heart rate monitors (Team2 Wearlink+; Polar, Bethpage, NY, USA) throughout the whole training session. Overall, subjects completed 17 training sessions, with training compliance being 100% aside from one subject who missed one training session. On average, the mean heart rate during a training session (*i.e.*, exercise and recovery intervals included) was 81 ± 1% of HR_{max} and peaked at 91 ± 1% HR_{max} (Fig. 2).

Measurements and data analysis

Body composition

Whole-body muscle mass, fat mass, and fat percentage were measured by DXA (Lunar iDXA; GE Healthcare). The scanner was calibrated prior to measurements in accordance with the manufacturer's guidelines. Before each scan, subjects rested for 10 min in the supine position to accommodate fluid distribution, thereby minimizing estimation error of muscle mass (36, 37). In addition, the composition of the experimental thigh, defined as the area from ischial tuberosity to the patellar groove, was determined using the DXA software (Encore Forma, v.15; GE Healthcare). All scans and analyses were conducted by the same operator.

Muscle biopsies

Sampled biopsies were divided in two portions. The first portion was immediately placed in ice-cold biopsy preservation solution and prepared for assessment of mitochondrial respiratory function, as previously described (5). The second biopsy portion was snap-frozen in liquid N_2 and stored at −80°C ready to be freeze-dried. Muscle specimens were weighed before and after freeze-drying to determine the water content. After freeze-drying the samples, connective tissue, visible fat, and blood were carefully dissected away. Then, the dissected muscle samples were stored at −80°C until analysis for enzymatic activity and protein content. Both enzymatic activity and protein content were determined in duplicates (*i.e.*, two different samples were obtained from the same muscle specimen after dissection and the mean value of the two samples was used as result). One subject from the CON group did not undertake the muscle biopsy sampling at Post because of discomfort associated with the procedure.

Mitochondrial respiratory function

Mitochondrial respiratory function was measured in permeabilized muscle fibers by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). A

substrate-uncoupler-inhibitor titration (SUIT) protocol was applied to measure specific features of mitochondrial respiration. Respirometric analyses were carried out in duplicate at a chamber temperature of either 35 or 40°C (*i.e.*, four respiration chambers were used, two chambers at 35°C and two chambers at 40°C). Instrumental and chemical oxygen background fluxes were calibrated as a function of oxygen concentration and subtracted from the total volume-specific oxygen flux (Datlab v.6.1 software; Oroboros Instruments). Oxygen levels were maintained between 200 and 500 μM to prevent potential oxygen diffusion limitation.

Mitochondrial respiratory capacity The SUIT protocol was specific for the analysis of multiple mitochondrial respiratory states induced *via* separate titrations, as previously described (38). The SUIT protocol started with addition of malate (2 mM) and octanoyl carnitine (0.2 mM) to induce normal leak respiration (L_N) in absence of adenylates. Then, ADP (5 mM) without the addition of Mg^{2+} was titrated to determine fatty acid oxidative capacity (FAO_P). Pyruvate (5 mM) and glutamate (10 mM) were added for measurement of maximal OXPHOS capacity through complex I (CI_P). Succinate (10 mM) was then added to induce maximal OXPHOS capacity through complex I and II combined ($CI+CII_P$), corresponding to maximal state 3 respiration. Addition of cytochrome C (Cyt C; 10 μM) allowed for assessment of mitochondrial outer membrane intactness and was used as an internal control for compromised integrity of the mitochondrial preparation. If mitochondrial respiratory capacity increased more than 10% after addition of Cyt C, the measurements were not used in the analyses. Oligomycin (1 μM) was then added to inhibit ATP synthase and to measure oligomycin-induced leak respiration (L_{Omy}), which is the leak state corresponding to $CI+CII_P$ and is comparable to state 4 respiration (39). After that, a series of stepwise carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) titrations (1.5–3.0 μM) were carried out to reach the electron transport system respiratory capacity (*ETS*) state. Rotenone (0.5 μM) was then added to inhibit complex I so that maximal OXPHOS capacity through complex II (CII_P) could be measured. Finally, antimycin A (2.5 μM) was added to terminate respiration and allow for the determination and correction of residual oxygen consumption, indicative of nonmitochondrial oxygen consumption in the chamber. One experiment from the CON group was not used in the analyses because of high Cyt C response.

Mitochondrial OXPHOS efficiency The RCR, as determined by the ratio of state 3 (*i.e.*, $CI+CII_P$) to state 4 (*i.e.*, L_{Omy}) respiration, was used as an index of OXPHOS coupling efficiency. For statistical and conceptual reasons, the RCR was replaced by $1\text{-}1/RCR = 1\text{-}L_{Omy}/CI+CII_P$ (40).

Enzymatic activity

Maximal enzyme activity of citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HAD) were quantified in muscle homogenates using fluorometric method (Fluoroscan Ascent; Thermo Fisher Scientific, Waltham, MA, USA) at 25°C, as previously described (41). Enzymatic activity was normalized to grams of total protein.

Protein content in homogenate lysate

Protein content in muscle homogenate lysates was determined by SDS-PAGE and Western blot analyses. In short, freeze-dried muscle samples (~2 mg dry weight) were homogenized for 1 min at 29 Hz (Qiagen Tissuelyser II; Retsch, Haan, Germany) in a fresh batch of ice-cold buffer [10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM sodium orthovanadate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 3 mM benzamidine]. Afterwards, samples were rotated end over end for 1 h at 4°C, followed by centrifugation at 17,500 *g* for 20 min at 4°C. The supernatant (lysate) was collected, and total protein concentration in each sample was determined by a bovine serum albumin (BSA) standard kit (Thermo Fisher Scientific) assayed in triplicate. Then, each lysate sample was mixed with 6× Laemmli buffer (7 ml 0.5 M Tris-base, 3 ml glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue) and double-distilled H_2O to reach equal protein concentration. Equal amounts of protein (range: 6–12 μg) were loaded in each well of either 4–15 or 16.5% precast gels (Bio-Rad, Hercules, CA, USA). Samples from each subject were loaded on the same gel, with the Pre samples being placed adjacent to the Post samples. The same pool of a mixed human muscle standard lysate was loaded in three different wells per gel, and the mean intensity of these samples was used for normalization to allow gel-to-gel comparison, as previously described (42). Proteins were separated according to their molecular weight by SDS-PAGE gel electrophoresis and semidry transferred to a PVDF membrane (MilliporeSigma, Burlington, MA, USA). The membranes were blocked in either 2–5% skim milk or 3% BSA in a mixture of tris-buffered saline and Tween 20 (TBST) before being incubated overnight at 4°C in primary antibody diluted in either 2–5% skim milk or 3% BSA (see Supplemental Table S1). After washing in TBST, membranes were incubated with a secondary antibody for ~1 h at room temperature. The secondary horseradish peroxidase-conjugated antibodies used were diluted 1:5000 in 2–5% skim milk or 3% BSA depending on the primary antibody (P-0447, P-0448, and P-0449; Agilent Technologies, Santa Clara, CA, USA). The membrane staining was visualized by incubation with a chemiluminescent horseradish peroxidase substrate (MilliporeSigma) before image digitalization on a Chemi Doc MP (Bio-Rad). Western blot band intensity was determined by densitometry quantification (total band intensity adjusted for background intensity) using Image Lab v.4.0 (Bio-Rad).

Blood variables, leg $\dot{V}_{O2}$ and leg $\dot{V}_{CO2}$

Blood sample analyses Femoral arterial and venous blood samples were drawn in heparinized tubes for immediate analyses of partial pressure of O_2 (P_{O2}) and CO_2 (P_{CO2}), O_2 saturation, hemoglobin concentration, bicarbonate ion concentration, and pH using an ABL800 Flex (Radiometer, Copenhagen, Denmark).

Femoral artery blood flow Femoral artery blood flow was measured with ultrasound Doppler (Vivid E9; GE Healthcare) equipped with a linear probe operating at an imaging frequency of 8.0 MHz and Doppler frequency of 3.1 MHz as previously described (43).

Calculations Leg O_2 consumption ($\dot{V}_{O2}$) and CO_2 release ($\dot{V}_{CO2}$) were calculated as the femoral artery blood flow (F) times the arteriovenous difference in content of the given gas in accordance to Fick's principle, as follows:

$$\dot{V}_{O_2} = F \times (Ca_{O_2} - Cv_{O_2})$$

$$\dot{V}_{CO_2} = F \times (Ca_{CO_2} - Cv_{CO_2})$$

Content of O_2 and CO_2 in arterial blood (Ca_{O2} and Ca_{CO2}, respectively) and venous blood (Cv_{O2} and Cv_{CO2}, respectively) were computed using the equations from Siggaard-Andersen *et al.* (44).

Exercise efficiency

Gross and net efficiency during one-legged knee-extensor exercise were determined using work performed, energy expenditure during exercise (EEE) and energy expenditure at rest (REE) expressed in kcal/min, as follows:

$$\text{Gross efficiency } (\%) = [\text{Work}/\ \text{EEE}] \times 100$$

$$\text{Net efficiency } (\%) = [\text{Work}/\ (\text{EEE} - \text{REE})] \times 100$$

REE was calculated from the resting values of leg $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ using the table of nonprotein respiratory quotient proposed by Peronnet and Massicotte (45). To account for the glycogen oxidation occurring during exercise, EEE was calculated from the leg $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ measured during the final minute of low-intensity exercise (LI_{ex}), moderate-intensity exercise (MI_{ex}), and high-intensity exercise (HI_{ex}) using the equations proposed by Jeukendrup and Wallis (46), as follows:

$$EEE_{LI_{ex}} = 0.575 \times \dot{V}_{CO_2} - 4.435 \times \dot{V}_{O_2}$$

$$EEE_{MI_{ex},\ HI_{ex}} = 0.550 \times \dot{V}_{CO_2} - 4.471 \times \dot{V}_{O_2}$$

Statistics

Between-group differences in subject characteristics at baseline and after the intervention period were determined using a linear mixed model with group (CON, SET) and time (Pre, Post) as fixed factors and subjects as random factor. To estimate between-group differences in the training-induced changes, as well as within-time (Pre *vs.* Post) and between-group (CON *vs.* SET) differences, a linear mixed model was used with group-time interaction as a fixed factor, subjects as random factor, and with baseline $\dot{V}_{O2max}$ and baseline value of the outcome variable included as covariates. For mitochondrial respiratory function measurements, a linear mixed model was used with group-time-temperature interaction as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O2max}$ and baseline value of the outcome variable included as covariates. To estimate differences in the hyperthermia-induced changes in mitochondrial OXPHOS capacity and efficiency, a linear mixed model was used with group-time-temperature interaction as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O2max}$ and 35°C value of the outcome variable included as covariates. For exercise efficiency measurements, a linear mixed model was used with time as a fixed factor, subjects as random factor, and baseline $\dot{V}_{O2max}$ and baseline value of the outcome variable included as covariates. Model checking was based on Shapiro Wilk's test and quantile-quantile plots. In case of heteroscedasticity (*i.e.*, unequal variance), log-transformation was applied prior to analysis. Model-based Student's *t* tests were used in pairwise comparisons to identify between- and within-group differences. The level of significance for all analyses was defined as $P < 0.05$. Statistical analyses were carried out with R v.3.4.1 (*https://www.r-project.org/*) and the extension packages *lme4* and *multcomp*. Absolute values are presented as means ± SEM. Fold-changes are presented as means ± 95% confidence intervals.

RESULTS

Anthropometrics and cardiorespiratory fitness

Age, weight, height, body mass index, and $\dot{V}_{O2max}$ were not different between the SET and the CON group at baseline (Pre) (Table 1). Muscle mass and fat percentage of the experimental thigh increased by 0.2 ± 0.0 kg ($P = 0.001$) and decreased by 0.8 ± 0.3% points ($P = 0.015$), respectively, with training in SET, whereas no changes were observed in CON (Table 1). $\dot{V}_{O2max}$ (in ml/min/kg body mass) increased by 10 ± 1% with training in SET ($P < 0.001$) and remained unchanged in CON, with the change being greater in SET than in CON ($P < 0.001$) (Table 1).

Mitochondrial respiratory capacity

Mitochondrial respiration values are reported in **Table 2**, whereas training- and hyperthermia-induced changes in mitochondrial respiratory function are presented in **Fig. 3**.

In SET, L_N decreased with training when analyzed at 40°C ($P < 0.001$) but not at 35°C, with the change at 40°C being greater than that observed at 35°C (temperature-time interaction; $P < 0.001$) as well as than that observed in CON (group-time interaction; $P < 0.001$) (Fig. 3*B*). In addition, temperature-dependent training-induced change in L_N was different between SET and CON (group-time-temperature interaction; $P < 0.001$). In SET, $CI+CII_P$ increased with training at 40°C only ($P < 0.001$), with the change being greater than that observed in CON (group-time interaction; $P = 0.004$) and with the temperature-dependent training-induced change being different between SET and CON (group-time-temperature interaction; $P = 0.005$). In SET, L_{Omy} increased with training at both 35°C ($P = 0.002$) and 40°C ($P = 0.008$).

Mitochondrial respiratory capacity did not change in CON at either 35°C or 40°C during the 6 wk.

Mitochondrial OXPHOS efficiency

OXPHOS efficiency (*i.e.*, $1\text{-}L_{Omy}/CI+CII_P$) increased with training in SET when analyzed at 40°C, but not at 35°C, and was unchanged in CON, with the training-induced change in OXPHOS efficiency at 40°C being greater than that observed at 35°C (temperature-time interaction; $P = 0.001$) as well as than that observed in CON (group-time interaction; $P < 0.001$) (Fig. 3*C*). In addition, temperature-dependent training-induced change in OXPHOS efficiency was greater in SET than CON (group-time-temperature interaction; $P = 0.039$) (Fig. 3*C*).

OXPHOS efficiency was lower at 40°C than at 35°C in SET and CON at both Pre and Post ($P < 0.01$) (Fig. 3*D*). In SET, hyperthermia-induced decrease in OXPHOS efficiency was smaller at Post than Pre (temperature-time interaction; $P = 0.038$), and temperature-dependent training-induced change was greater in SET than CON (group-time-temperature interaction; $P = 0.001$) (Fig. 3*D*).

Enzymes and proteins modulating mitochondrial function

Maximal activity of CS and HAD increased with training in SET ($P < 0.001$, $P = 0.017$) and did not change in CON, and the changes in SET were larger than in CON ($P = 0.001$, $P = 0.003$) (**Table 3**).

Protein content of subunits from the electron transport chain complexes I, II, III, and IV increased with training in SET ($P < 0.001$, $P = 0.003$, $P = 0.001$, $P = 0.007$) and were unchanged in CON, with changes in subunits from complexes I, II, and III being greater in SET than in CON ($P < 0.001$, $P = 0.005$, $P = 0.030$) (**Fig. 4**). Isocitrate dehydrogenase (IDH) did not change with the intervention in either SET or CON. Mitofusin 2 (MFN2) and dynamin related protein 1 (DRP1) increased with training in SET ($P = 0.001$, $P = 0.004$), but not in CON, with the changes

TABLE 2. *Mitochondrial respiration values before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period*

Mitochondrial respiration value		Normothermia (35°C)		Hyperthermia (40°C)	
		CON (n = 8)	SET (n = 10)	CON (n = 8)	SET (n = 10)
J_{O2} (pmol O_2/s/mg)					
L_N	Pre	6.7 ± 0.7	4.4 ± 0.5†	9.0 ± 1.1	9.2 ± 0.7$^{\#}$
	Post	7.0 ± 0.5	4.9 ± 0.5	10.9 ± 1.0$^{\#}$	5.6 ± 0.9*,†
FAO_P	Pre	31.8 ± 3.3	31.3 ± 4.1	35.4 ± 3.1$^{\#}$	30.6 ± 3.9
	Post	34.0 ± 3.4	31.4 ± 2.6	39.3 ± 3.6$^{\#}$	28.5 ± 2.8$^{\dagger,\#}$
CI_P	Pre	64.8 ± 6.3	47.5 ± 4.6†	61.7 ± 5.4	41.7 ± 4.4$^{\dagger,\#}$
	Post	65.3 ± 5.9	52.4 ± 4.3	61.1 ± 4.9	45.8 ± 3.6$^{\#}$
CI+CII_P	Pre	97.7 ± 8.7	79.3 ± 7.0	98.4 ± 7.9	70.0 ± 7.5$^{\dagger,\#}$
	Post	98.4 ± 8.1	88.0 ± 6.8	97.3 ± 7.5	87.0 ± 5.4*
L_{Omy}	Pre	17.4 ± 1.6	15.7 ± 1.0	23.0 ± 1.9$^{\#}$	22.9 ± 1.0$^{\#}$
	Post	18.5 ± 2.0	19.0 ± 1.4*	24.5 ± 1.5$^{\#}$	27.2 ± 1.9$^{*,\#}$
ETS	Pre	63.6 ± 5.5	71.1 ± 7.5	50.4 ± 3.4$^{\#}$	56.1 ± 4.5$^{\#}$
	Post	68.7 ± 8.1	76.7 ± 5.4	52.0 ± 6.4$^{\#}$	59.3 ± 4.7$^{\#}$
CII_P	Pre	38.1 ± 3.9	48.1 ± 3.9†	32.8 ± 2.9	39.7 ± 2.7$^{\#}$
	Post	40.4 ± 4.1	50.8 ± 3.5†	32.1 ± 3.6$^{\#}$	41.8 ± 3.3$^{\dagger,\#}$
OXPHOS efficiency					
1-L_{Omy}/CI+CII_P	Pre	0.82 ± 0.01	0.80 ± 0.01	0.76 ± 0.02$^{\#}$	0.64 ± 0.03$^{\dagger,\#}$
	Post	0.81 ± 0.01	0.78 ± 0.01	0.74 ± 0.02$^{\#}$	0.68 ± 0.02 $^{*,\dagger,\#}$

Values are means ± SEM. *Significantly different from Pre ($P < 0.05$). †Significantly different from CON ($P < 0.05$). $^{\#}$Significantly different from 35°C ($P < 0.05$).

being greater in SET than in CON ($P = 0.041$, $P = 0.017$). Bcl-2/adenovirus E1B 19 kDa-interacting protein-3 (BNIP3), uncoupling protein 3 (UCP3), and heat shock protein 70 (HSP70) were unaffected by the intervention in either SET or CON, whereas adenine nucleotide translocase 1 (ANT1) was up-regulated in SET only ($P = 0.029$).

Exercise efficiency

Femoral artery blood flow during one-legged knee-extensor exercise at low-, moderate-, and high-intensity was lower after than before the training intervention in SET (all $P < 0.05$) (**Table 4**). Leg $\dot{V}_{O2}$ (in ml/min) and leg $\dot{V}_{O2}$ relative to thigh muscle mass (in ml/min/kg muscle) during one-legged knee-extensor exercise at low-, moderate-, and high-intensity decreased with training in SET (all $P < 0.001$). Gross efficiency and net efficiency during one-legged knee-extensor exercise at low-, moderate-, and high-intensity improved with training in SET (gross efficiency, $P = 0.001$, $P < 0.001$, $P < 0.001$; net efficiency, $P = 0.003$, $P = 0.001$, $P < 0.001$).

Exercise performance

Time to exhaustion and iPPO achieved during incremental one-legged knee-extensor exercise increased with training in SET (all $P < 0.001$) and were unchanged in CON, with the change being greater in SET than CON (all $P < 0.001$) (**Table 5**). In SET, the training intervention increased time to exhaustion and iPPO achieved during incremental two-legged cycling exercise from 526 ± 26 to 612 ± 18 s ($P < 0.001$) and from 355 ± 14 to 384 ± 12 W ($P < 0.001$), respectively.

In SET, the peak and the mean power output achieved during the first *vs.* the last training session increased from 1025 ± 55 to 1094 ± 56 W ($P = 0.021$) and from 796 ± 44 to 827 ± 39 W ($P = 0.030$), respectively. Likewise, the mechanical work performed during the first *vs.* the last training session increased from 48.6 ± 3.4 to 53.3 ± 3.1 kJ ($P < 0.001$).

DISCUSSION

Herein we demonstrate that 6 wk of high-intensity exercise training promotes an increase in mitochondrial maximal OXPHOS capacity and OXPHOS efficiency under experimentally-induced hyperthermia (40°C) but not in normothermia (35°C). Furthermore, the high-intensity exercise training attenuated hyperthermia-induced decline in OXPHOS efficiency. The training period increased expression of muscle markers of mitochondrial content and up-regulated abundance of MFN2, DRP1, and ANT1. Hyperthermia-specific training-induced enhancements in mitochondrial maximal OXPHOS capacity and OXPHOS efficiency were accompanied by improvements in gross and net efficiency during low-, moderate- and high-intensity one-legged knee-extensor exercise, as well as by improvements in performance during both incremental one-legged knee-extensor and two-legged cycling exercise.

Temperature-specific training-induced adaptations in muscle mitochondrial respiratory function

To the best of our knowledge, the present study is the first to show training-induced enhancements in OXPHOS efficiency in permeabilized fibers from human skeletal muscle, with prior evidence failing to detect substantial changes in indices of mitochondrial coupling efficiency following a period of exercise training (9, 10, 15, 17, 19, 20). However, in view of the observed discrepancy between normothermia- and hyperthermia-related training

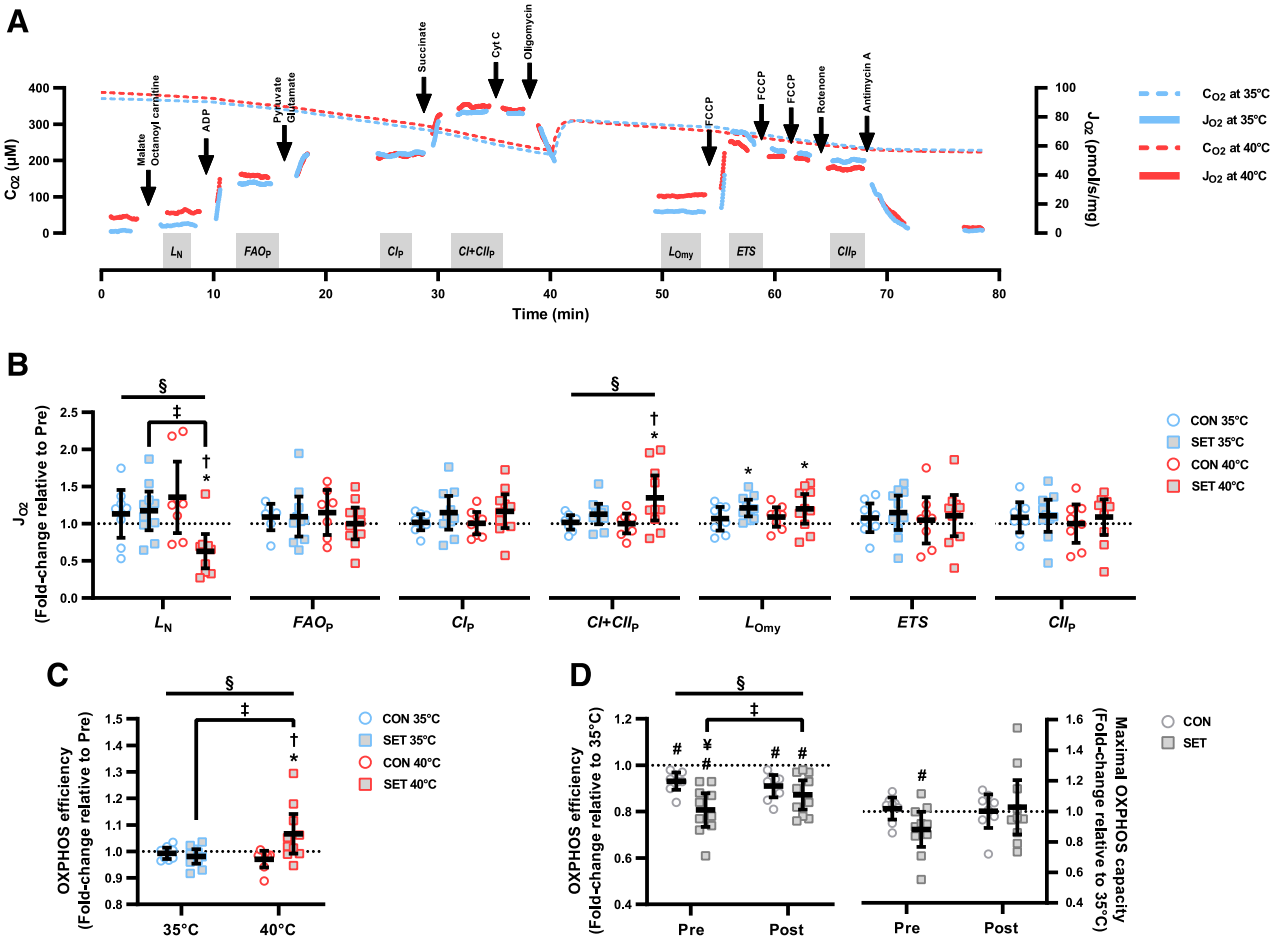

Figure 3. Mitochondrial respiratory function. *A*) Representative track of oxygen concentration (C_{O2}) and mass-specific mitochondrial respiration [O_2 flux (J_{O2})] measured in permeabilized fibers from human vastus lateralis at 35 and 40°C. *B*) Training-induced changes in mass-specific mitochondrial respiratory capacity measured at 35 and 40°C in the control (CON) and the high-intensity exercise training (SET) group. *C*) Training-induced changes in OXPHOS efficiency (1-L_{Omy}/*CI+CII*$_P$) measured at 35 and 40°C in the control (CON) and the high-intensity exercise training (SET) group. *D*) Hyperthermia-induced changes in OXPHOS efficiency (1-L_{Omy}/*CI+CII*$_P$) and mass-specific maximal OXPHOS capacity (*CI+CII*$_P$) before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period. Data presented as individual values with means ± 95% confidence intervals; n = 8 (CON) and n = 10 (SET). *$P < 0.05$, significant difference between Pre and Post. †$P < 0.05$, significant difference between CON and SET in the training-induced change (group-time interaction). ‡$P < 0.05$, significant difference between 35 and 40°C in the training-induced change (temperature-time interaction). §$P < 0.05$, significant difference between CON and SET in the temperature-dependent training-induced change (group-time-temperature interaction). #$P < 0.05$, significant difference between 35 and 40°C. ¥$P < 0.05$, significant difference between CON and SET in the hyperthermia-induced change (group-temperature interaction).

adaptations, comparisons with other studies using different assay temperatures should be made carefully.

The training-induced increase in maximal OXPHOS capacity observed at 40°C is in line with the increased *CI+CII*$_P$ (measured at 37°C) reported following a period of either high-intensity interval training (9, 11, 15, 18, 20), resistance training (19), or a combination of different training modalities (14, 20, 47). At the same time, the lack of training-induced changes in maximal OXPHOS capacity observed at 35°C is consistent with the absence of alterations in *CI+CII*$_P$ (measured at 37°C) documented following either moderate-intensity continuous (10, 12, 13, 17, 47), high-intensity interval (9, 10, 12, 48), or resistance training (14, 20, 47). Of note, the few studies utilizing

TABLE 3. *Maximal activity of mitochondrial enzymes before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period*

	CON (n = 9)		SET (n = 10)	
Mitochondrial enzyme	Pre	Post	Pre	Post
CS (μmol/min/g protein)§	77.8 ± 6.1	74.1 ± 6.5	76.2 ± 6.1	90.1 ± 4.7*,†
HAD (μmol/min/g protein)§	27.3 ± 2.1	24.5 ± 1.9	27.5 ± 2.0	31.0 ± 1.6*,†

Values are means ± SEM. *Significantly different from Pre ($P < 0.05$). †Significantly different from CON ($P < 0.05$). §Significant group-time interaction ($P < 0.05$).

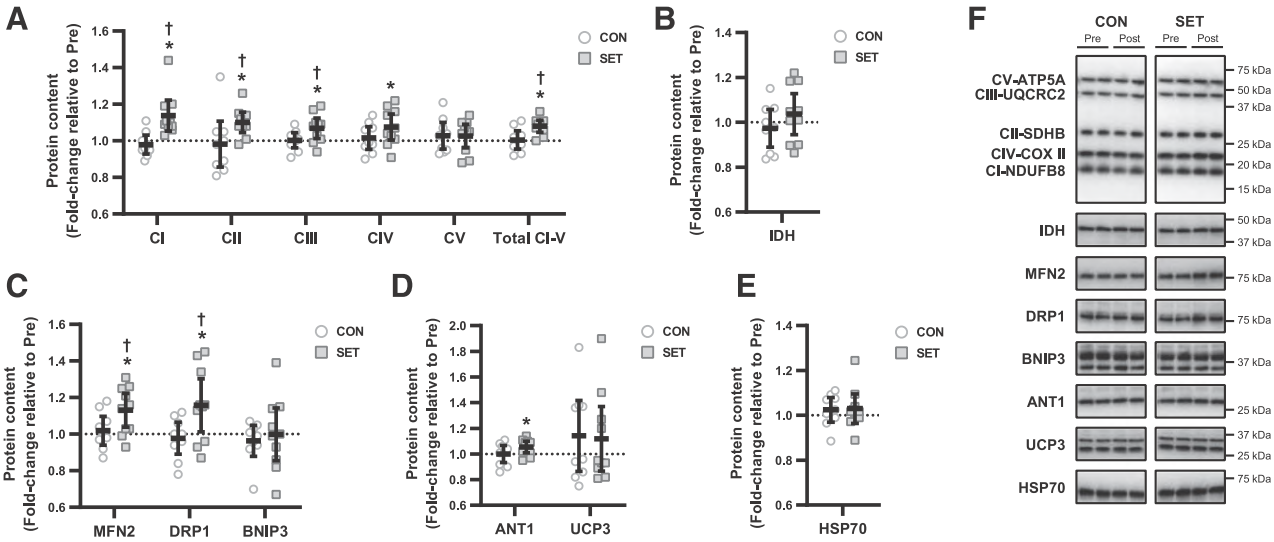

Figure 4. Proteins modulating mitochondrial function. *A–E*) Training-induced changes in protein content of subunits of the electron transport chain complexes I–V (CI, CII, CIII, CIV, CV) (*A*), IDH (*B*), markers of mitochondrial turnover (*C*), uncoupling proteins (*D*), and HSP70 (*E*). *F*) Representative western blots. Data presented as individual values with means ± 95% confidence intervals; $n = 8$ (CON) and $n = 10$ (SET). $^{*}P < 0.05$, significant difference between Pre and Post. $^{\dagger}P < 0.05$, significant difference between CON and SET in training-induced change (group-time interaction).

a training regimen that included brief supramaximal-intensity efforts, as in the current study, reported either an increase in *CI+CII*$_P$ in permeabilized fibers (10) or a decrease in *CI+CII*$_P$ in isolated mitochondria (49), suggesting that between-study comparisons should account for the methodological approach employed for measuring mitochondrial OXPHOS capacity.

Overall, the enhancements in OXPHOS efficiency and maximal OXPHOS capacity were only apparent at 40°C, thus implying thermal specificity of the training-induced adaptations in mitochondrial respiratory function.

Besides training-induced alterations in OXPHOS capacity and efficiency, our results show that L_N decreased with training under hyperthermia only, whereas L_{Omy} increased with training irrespective of assay temperature. Because L_N represents mitochondrial oxygen consumption in absence of adenylates, thereby resembling mitochondrial respiration rate in a resting state, it can be argued that the training intervention promoted an adaptive response lowering energy dissipation when resting muscle is exposed to heat stress. Conversely, given that L_{Omy} represents leak respiration associated with maximal OXPHOS capacity, it appears that the training intervention was ineffective in reducing energy dispersion of the contracting muscle at either normothermic or hyperthermic conditions. However, it should be noted that, under hyperthermia, the training-induced increase in L_{Omy} was paralleled by an increase in maximal OXPHOS capacity (*CI+CII*$_P$), resulting in higher OXPHOS efficiency. In spite of training-induced enhancements in *CI+CII*$_P$, the high-intensity exercise training did not affect maximal OXPHOS capacity through either complex I (*CI*$_P$) or complex II (*CII*$_P$) as well as maximal fat oxidation capacity (*FAO*$_P$) and electron transport system capacity (*ETS*), a finding partly consistent with studies reporting a mismatch between changes in these respiratory states and increments in *CI+CII*$_P$ (10, 11, 15, 18). From a methodological point of view, it is worth noting that *ETS* was lower than *CI+CII*$_P$ (see Table 2), an observation that may be related to the inhibitory effect of oligomycin on maximal uncoupled respiration (50, 51).

A new aspect of the present study was to investigate the effects of experimentally-induced hyperthermia *per se* on mitochondrial respiratory function in permeabilized fibers from human skeletal muscle. The observation that maximal OXPHOS capacity (*CI+CII*$_P$) did not increase with increasing assay temperature (see Fig. 3*D*) contrasts with the hyperthermia-induced increments in state 3 respiration documented in rodents (24, 25, 52). This dissimilarity, besides possibly depending on interspecies differences, may be attributed to the different methodological approaches employed to measure mitochondrial respiration (*i.e.*, high-resolution respirometry in permeabilized fibers *vs.* Clark-type electrodes in isolated mitochondria). It is worth mentioning that although maximal OXPHOS capacity decreased with hyperthermia in SET at baseline, this was not the case for CON. Such a finding, considering that SET exhibited a trend for a lower maximal OXPHOS capacity than CON in normothermia at baseline (see Table 2), suggests that skeletal muscle mitochondria characterized by lower maximal OXPHOS capacity are more prone to be affected by heat stress. This assumption is further supported by the observation that hyperthermia-induced impairments in maximal OXPHOS capacity disappeared concomitantly with an increase in maximal OXPHOS capacity following the intervention in SET.

The observed hyperthermia-induced decrease in OXPHOS efficiency is in line with the decline in P/O ratio and RCR occurring at high temperature in isolated rat skeletal muscle mitochondria (24, 25, 52). Overall, the present temperature-dependent alterations in mitochondrial respiratory function are in accordance with studies in isolated mitochondria from rat cardiac and hepatic tissue reporting no hyperthermia-induced increments in maximal OXPHOS capacity in the heart (53)

TABLE 4. *Leg hemodynamics, $\dot{V}_{O2}$, and efficiency during one-legged knee-extensor exercise before (Pre) and after (Post) the high-intensity exercise training (SET) period*

	SET (n = 10)	
Measurement	Pre	Post
Low-intensity exercise (30% iPPO)		
Workload (W)	22 ± 1	
Femoral artery blood flow (L/min)	3.04 ± 0.21	2.65 ± 0.12*
a-v O_2 difference (ml/L)	151 ± 6	147 ± 3
Leg $\dot{V}_{O2}$ (ml/min)	456 ± 30	391 ± 23*
Leg $\dot{V}_{O2}$ (ml/min/kg muscle)	65.6 ± 1.9	55.0 ± 1.5*
Leg RQ	0.71 ± 0.06	0.72 ± 0.06
Gross efficiency (%)	17.8 ± 1.3	20.5 ± 1.1*
Net efficiency (%)	19.4 ± 1.4	22.5 ± 1.3*
Moderate-intensity exercise (50% iPPO)		
Workload (W)	37 ± 2	
Femoral artery blood flow (L/min)	3.80 ± 0.21	3.59 ± 0.19*
a-v O_2 difference (ml/L)	167 ± 6	158 ± 4*
Leg $\dot{V}_{O2}$ (ml/min)	635 ± 44	570 ± 37*
Leg $\dot{V}_{O2}$ (ml/min/kg muscle)	91.7 ± 4.6	79.8 ± 2.8*
Leg RQ	0.82 ± 0.03	0.83 ± 0.05
Gross efficiency (%)	21.1 ± 0.9	23.5 ± 0.8*
Net efficiency (%)	22.4 ± 1.0	25.1 ± 1.0*
High-intensity exercise (80% iPPO)		
Workload (W)	59 ± 3	
Femoral artery blood flow (L/min)	4.56 ± 0.23	4.08 ± 0.21*
a-v O_2 difference (ml/L)	178 ± 6	174 ± 4
Leg $\dot{V}_{O2}$ (ml/min)	808 ± 45	714 ± 48*
Leg $\dot{V}_{O2}$ (ml/min/kg muscle)	115.1 ± 5.6	100.5 ± 5.3*
Leg RQ	0.97 ± 0.03	0.93 ± 0.04
Gross efficiency (%)	27.8 ± 0.8	30.3 ± 0.9*
Net efficiency (%)	29.2 ± 0.8	31.9 ± 1.0*

Values are means ± SEM. a-v O_2 difference, arteriovenous difference in oxygen content; RQ, respiratory quotient. *Significantly different from Pre ($P < 0.05$).

and hyperthermia-induced decrements in mitochondrial coupling in both the heart and the liver (53, 54).

Notably, we observed that the high-intensity exercise training attenuated the hyperthermia-induced decline in OXPHOS efficiency; an adaptation in accordance with the dampened hyperthermia-induced impairments in OXPHOS efficiency documented following a period of endurance exercise training in rats (25).

Taken together, the present outcomes not only support the effectiveness of high-intensity exercise training in promoting mitochondrial qualitative changes but also highlight that these adaptations may only occur or be detected within a thermal environment resembling that associated with sustained intense muscle contraction. Thus, our results suggest that assay temperatures similar to those achieved by the exercising muscle should be utilized during *ex vivo* assessments of training-induced changes in mitochondrial respiratory function. From a physiologic standpoint, our findings possibly indicate a mitochondrial hormetic response to heat stress, whose magnitude is amplified under experimentally-induced hyperthermia.

High-intensity exercise training up-regulates muscle proteins modulating mitochondrial function

The high-intensity exercise training period increased protein content of subunits from complexes I, II, III, and IV of the electron transport chain as well as CS activity, indicating that increments in mitochondrial content occurred in concert with the observed modifications in mitochondrial respiratory function. Such a finding is

TABLE 5. *One-legged knee-extensor exercise performance before (Pre) and after (Post) the control (CON) and the high-intensity exercise training (SET) period*

	CON (n = 10)		SET (n = 10)	
Measure of exercise performance	Pre	Post	Pre	Post
Time to exhaustion (s)[§]	315 ± 15	306 ± 14	315 ± 34	451 ± 37*,†
iPPO (W/kg muscle)[§]	10.1 ± 0.9	10.0 ± 0.8	11.4 ± 0.9	14.6 ± 1.1*,†

Values are means ± SEM. *Significantly different from Pre ($P < 0.05$). †Significantly different from CON ($P < 0.05$). §Significant group-time interaction ($P < 0.05$).

supported by reports showing concomitant increments in mitochondrial content and mitochondrial respiratory capacity (9, 11, 12, 17, 18) and points against the previously proposed dissociation between mitochondrial content and respiratory function (10, 55). On the other hand, we observed no training-induced changes in content of IDH, an observation in contrast to other research showing an increase in IDH following a period of intense interval training (34).

Aside from adaptations in mitochondrial content, we observed that expression of MFN2 and DRP1 increased with the training intervention, possibly indicating an enhanced capacity for mitochondrial dynamic remodeling. These adaptations are in line with prior data indicating upregulated MFN2 and DRP1 mRNA content in response to a single bout of high-intensity exercise in human skeletal muscle (56). In addition, although the observed training-induced increase in MFN2 is in agreement with other studies (10, 12, 17), this is not the case for the training-induced increase in DRP1 (10), suggesting that the training intervention adopted in the current study enhanced the capacity of the mitochondria to undergo both fusion and fission events. On the other hand, we observed no apparent changes in BNIP3 with the training intervention, which may indicate that the capacity for mitophagy regulation was unaltered by the training undertaken. Other studies have observed increased BNIP3 levels following 8 wk of exercise training in young healthy men (57), with the different response being possibly attributed to the shorter duration and the lower volume of the training intervention utilized in the present study.

Given that temperature-dependent impairments in mitochondrial coupling efficiency have been proposed to depend on hyperthermia-induced proton leak (52), we explored whether protein levels of ANT1 and UCP3, which are involved in mitochondrial proton leak (58), were altered by the training intervention. We observed that the high-intensity exercise training intervention increased content of ANT1, which is consistent with that reported in response to 6 wk of endurance training (59). The training-induced concomitant increase in ANT1 and maximal OXPHOS capacity coincides with observations in other species showing a connection between rise in ANT1 and increase in OXPHOS capacity (60). Indeed, upregulation of ANT1 abundance may serve as a protective mechanism to prevent excess reactive oxygen species (ROS) production *via* a mild uncoupling lowering the protonmotive force (58). Moreover, it is relevant to point out that, besides being involved in proton leak, ANT1 affects mitochondrial ADP sensitivity by driving ADP import across the inner mitochondrial membrane (61). Thus, it may be that, in light of evidence indicating temperature-dependent changes in mitochondrial ADP sensitivity (62), the observed hyperthermia-specific training-induced enhancement in OXPHOS efficiency occurred *via* ANT1-mediated alterations in ADP sensitivity.

The observation that UCP3 abundance was unaltered by the training intervention suggests that training-induced improvements in mitochondrial coupling efficiency were likely not related to a reduced UCP3–mediated proton leak. In accordance with the present study, unaltered UCP3 content has been reported following a period of either endurance (59) or high-intensity intermittent exercise training (63). However, it cannot be excluded that the training intervention lowered UCP3 activity rather than abundance, as previously observed in rats (25). Thus, future studies should investigate the importance of both content and activity of ANT1 and UCP3 for training-induced adaptations in mitochondrial respiratory function.

To further explore the potential mechanisms underlying the temperature-specificity of the observed training-induced mitochondrial adaptations, we quantified changes in HSP70, a molecular chaperone and cytoprotective protein mediating enhancement of mitochondrial respiratory capacity in diseased animal models (64, 65). Interestingly, protein levels of HSP70 were not altered by exercise training in the present study, suggesting a minor role for HSP70 in modulating changes in mitochondrial bioenergetics in healthy skeletal muscle.

High-intensity exercise training enhances exercise efficiency and performance

The present study shows that enhancements in mitochondrial OXPHOS efficiency elicited by high-intensity exercise training are accompanied by improvements in exercise efficiency and that this occurs independently of reductions in abundance of uncoupling proteins. This is in contrast to studies indicating that improvements in mitochondrial coupling and exercise efficiency are associated with reductions in protein content of ANT1 (66) and that higher exercise efficiency positively correlates with lower mRNA and protein content of UCP3 (67–71).

Although a lack of association between mitochondrial coupling efficiency and cycling efficiency has been reported in a cross-sectional study comparing untrained and trained individuals (67), the concomitant enhancements in mitochondrial coupling and exercise efficiency observed in the present study suggest that training-induced changes in mitochondrial respiratory function may have contributed to augmenting exercise efficiency. Given that increments in pulmonary $\dot{V}_{O2}$ closely reflect increments in the exercising muscle $\dot{V}_{O2}$ (72), training-induced changes in exercise efficiency are commonly inferred from measurements of pulmonary gas exchange during cycle ergometry (73). However, in the present study, we measured leg $\dot{V}_{O2}$ during one-legged knee-extensor exercise, a methodological approach that allows for accurate assessments of efficiency of the contracting skeletal muscle by excluding possible increments in O_2 cost associated with processes occurring outside the exercising muscle (*e.g.*, respiratory and cardiac muscle work).

Consistent with the well-established importance of exercise efficiency for endurance performance (74) and given the purported association between fatigue and muscle inefficiency during high-intensity exercise (75), it is conceivable that the observed improvements in exercise efficiency along with the ~5% increase in $\dot{V}_{O2max}$ partly explain the greater peak power and time to exhaustion achieved during the incremental exercise. Taken together, the mitochondrial qualitative changes elicited by the high-

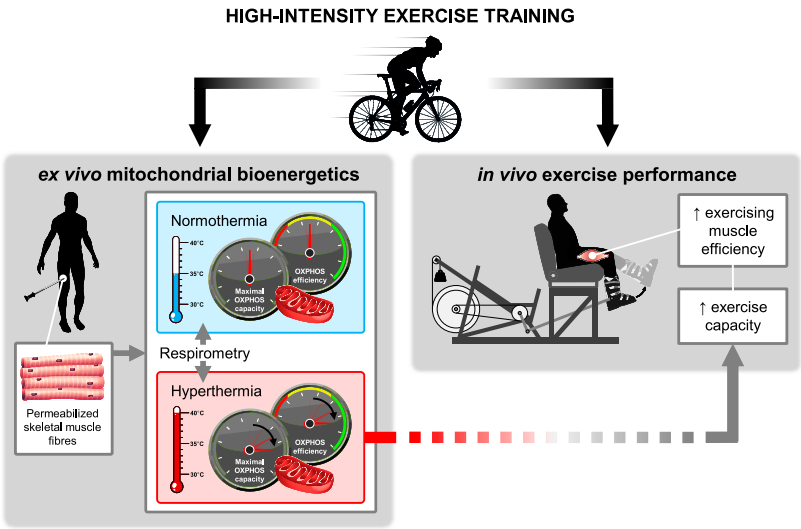

Figure 5. Proposed association between high-intensity exercise training-induced adaptations in muscle mitochondrial bioenergetics and exercise performance. The effects of high-intensity exercise training on *ex vivo* mitochondrial respiratory function are magnified under experimentally-induced hyperthermia mimicking *in vivo* muscle temperature during intense exercise. The hyperthermia-specific training-induced changes in mitochondrial OXPHOS efficiency are accompanied by enhancements in overall efficiency of the exercising muscle, which possibly contribute to improving the capacity for sustained exercise.

intensity exercise training period may have played a prominent role in enhancing exercise performance.

Summary and perspectives

The present study demonstrates that high-intensity exercise training promotes beneficial adaptations in human muscle mitochondrial OXPHOS capacity and efficiency, but only when *ex vivo* mitochondrial respiratory function is assessed under experimentally-induced hyperthermia mimicking *in vivo* muscle temperature during intense exercise. These findings not only indicate temperature-dependency of training-induced mitochondrial qualitative changes but also provide novel methodological insights into assessments of mitochondrial respiratory function in human permeabilized muscle fibers. Furthermore, the exercise training period attenuated the hyperthermia-induced decline in mitochondrial coupling efficiency, suggesting that high-intensity exercise training improves the bioenergetic efficiency of skeletal muscle during sustained intense contractile activity. These mitochondrial qualitative changes were associated with quantitative changes in enzymes and proteins important for mitochondrial function, including regulators of either mitochondrial dynamics or mitochondrial uncoupling. Importantly, we report the novel observation that the mitochondrial qualitative and quantitative changes elicited by the high-intensity exercise training were accompanied by enhancements in both exercise efficiency and capacity, thus highlighting the physiologic relevance of the observed mitochondrial adaptations (**Fig. 5**).

Given that the P/O ratio can only be directly measured in isolated mitochondria, and in view of the intended integrative approach of the present study, mitochondrial OXPHOS efficiency was assessed in a preserved myocellular system (*i.e.*, permeabilized fiber bundles) *via* a methodological approach indirectly determining P/O ratio by assuming absolute ATP production from a known amount of ADP. In view of this limitation, alternative methods to quantify P/O ratio in permeabilized muscle fibers have been recently proposed (76); however, further methodological development is necessary to account for other factors possibly affecting P/O ratio, including ATP-ADP recycling, proton leak and slip, cation cycling, and ROS production.

In addition, it should be noted that muscle temperature represents only one of the physiologic factors to be considered when modeling *in vivo* conditions during *ex vivo* respiratory assessments (77). Thus, future studies should evaluate the impact of further parameters (*i.e.*, O_2 availability and metabolite and ion concentrations) in mimicking *in vivo* skeletal muscle conditions. Lastly, simultaneous assessments of changes in mitochondrial dynamics, ultrastructure (*i.e.*, cristae density), and supercomplex formation are warranted to unravel the specific mechanisms whereby exercise training enhances the efficiency of mitochondrial energy transduction. FJ

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AUTHOR CONTRIBUTIONS

M. Fiorenza, A. K. Lemminger, J. Bangsbo, and M. Hostrup designed the study; M. Fiorenza, A. K. Lemminger, and K. Eibye conducted the experiments; M. Marker conducted the training and collected data; M. Fiorenza and A. K. Lemminger analyzed data; M. Fiorenza, A. K. Lemminger, J. Bangsbo, and M. Hostrup drafted the manuscript; all authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript; and all authors agree to be accountable for all aspects of the manuscript in ensuring that questions related to the

accuracy or integrity of any part of the manuscript are appropriately investigated and resolved.

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