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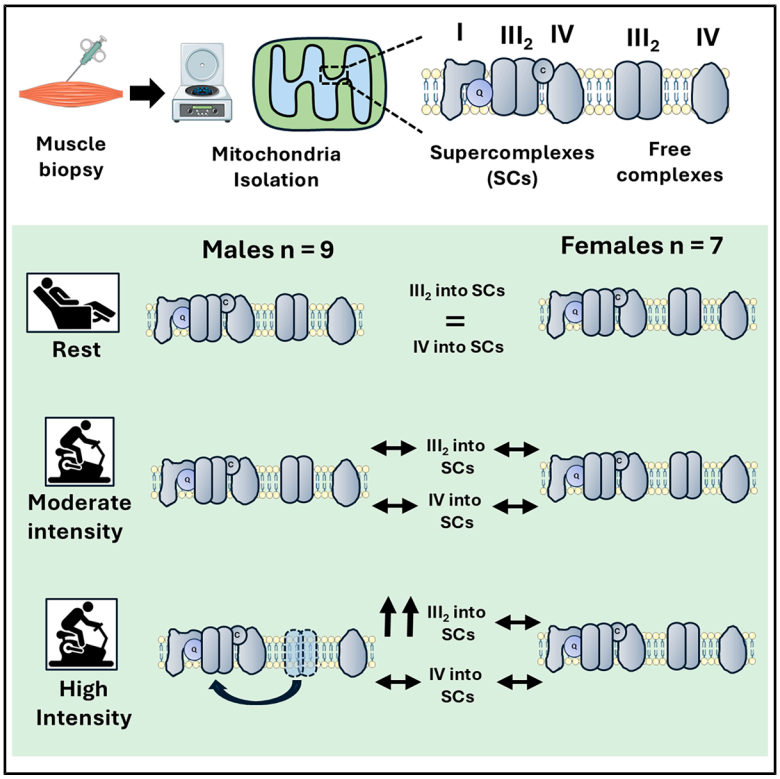
Exercise induces sex-specific assembly of mitochondrial supercomplexes

Graphical abstract



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In brief

Huertas et al. demonstrate that exercise dynamically modulates the assembly of mitochondrial respiratory complexes into supercomplexes in human skeletal muscle, with this remodeling being sex dependent. This highlights the importance of accounting for biological sex when studying mitochondrial adaptations to exercise.

Highlights

- Females maintain a stable ETC supercomplex profile in response to exercise
- Males increase the abundance of supercomplexes after exercise
- SC abundance inversely correlates with lactate during exercise in males

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Exercise induces sex-specific assembly of mitochondrial supercomplexes

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SUMMARY

The mitochondrial respiratory complexes of the electron transport chain (ETC) form supramolecular structures known as supercomplexes (SCs) whose functions remain partially understood. An increase in carbohydrate oxidation, such as that induced by high-intensity contractions within skeletal muscle (SKM), has been proposed to promote the assembly of high molecular weight SCs (HMWSCs). Here, healthy, active young subjects (7 females and 9 males) performed a moderate- followed by a high-intensity exercise bout. We found that males increased the assembly of complex III (CIII) into SCs, particularly HMWSCs, in an intensity-dependent manner within SKM. Females showed a stable content of both HMWSCs and I+III_2 SCs during exercise. In contrast, the assembly of CIV into SCs was not promoted by exercise in either sex. These findings indicate that the ETC complex organization can be modulated by exercise, and the mitochondrial supercomplex assembly in human SKM appears to be regulated in a sex-specific manner.

INTRODUCTION

Mammalian mitochondria play a central role in maintaining cellular homeostasis by producing adenosine triphosphate through oxidative phosphorylation, as well as by sensing and responding to intracellular signals.[1,2] A long-standing scientific debate concerns the supramolecular organization of the mitochondrial electron transport chain (ETC). Respiratory complexes can exist either as individual entities or assembled into higher-order structures known as supercomplexes (SCs).[3] The physiological relevance of SCs remains under discussion. While some studies suggest that SCs primarily serve a structural role,[4–6] accumulating evidence supports the view that SCs allow for a dynamic reconfiguration of the ETC to meet changing cellular energy demands.[7–10]

The organization of the ETC is influenced by the predominant metabolic substrate.[8] When electrons are mainly derived from pyruvate oxidation (i.e., high NADH flux), the assembly of the N-respirasome (I+III_2+IV) facilitates efficient electron transfer from complex I (CI) to complex III (CIII). In contrast, during fatty acid oxidation—characterized by a high $FADH_2$/NADH ratio—CI is downregulated or degraded, allowing electrons to enter the ETC downstream of CI and directly reach CIII.[8] In this scenario, it has been proposed that SC: III_2+IV assembly in yeast enhances cellular fitness by reducing the diffusion distance for electrons between complexes[11] and later demonstrated that the formation of III_2+IV SC facilitates electron transfer by two-dimensional (2D) diffusion of cytochrome *c*.[12] Notably, some studies support the idea that SC formation optimizes the efficiency of the respiratory chain and correlate with better resistance to fatigue and fasting.[13–15]

However, to date, no study has examined the *in vivo* assembly of ETC complexes into SCs within human skeletal muscle (SKM) during acute exercise, as most evidence comes from training protocols lasting several weeks: endurance training in older adults increased SC assembly,[16] whereas high-intensity interval training in young individuals did not alter SC organization independently of mitochondrial content.[17] In contrast, sprint-interval training promoted assembly of the III_2+IV SCs after 8 weeks.[18] These data suggest that SC remodeling depends on the nature of the exercise stimulus.

SKM is a metabolically flexible tissue that predominantly relies on fatty acids (~90%) during low-intensity contractions and

shifts to carbohydrates (~75%) during high-intensity activity.[19] This substrate switching correlates with the assembly of SCs in response to high-intensity exercise, as previously observed *in vitro* sprint interval training models using C2C12 myotubes.[20] In addition, after a period of exercise training, a relationship between mitochondrial enlargement and the degree of SC assembly has been reported.[21] We have previously shown that high-intensity exercise induces a transient elongation of the SKM mitochondrial network in male subjects.[22] In that study, we also observed an increase in OPA1 protein content during exercise.[22] While we did not quantify different isoforms, it is known that OPA1 promotes cristae remodeling, thereby enabling SC formation.[23] Here, we tested the hypothesis that acute exercise increases the assembly of SCs in human SKM.

Female SKM displays greater mitochondrial efficiency and tighter respiratory control than males,[24,25] together with lower mitochondrial reactive oxygen species (ROS) production.[26] Consistently, females rely more on oxidative metabolism, whereas males exhibit greater carbohydrate utilization at rest and during exercise.[27–29] Accordingly, we included recreationally active females (n = 7) and males (n = 9) to assess potential biological sex differences following dynamic exercise. Our findings show that females did not exhibit changes in ETC configuration during exercise, whereas males demonstrated increased assembly of CIII into high molecular weight SCs (HMWSCs) in response to high-intensity exercise (HIGH). These results indicate a sex-specific regulation of SC assembly during exercise and highlight the importance of considering biological sex when analyzing SKM metabolism.

RESULTS AND DISCUSSION

Comparable lactate responses to HIGH exercise occur despite sex differences in time to exhaustion

During the first laboratory visit, participants performed a graded cycling test to exhaustion to determine lactate threshold, incremental peak power output (W_{peak}), and peak oxygen consumption ($\dot{V}O_2$ peak) (Table 1).

On the second visit, subjects completed two exercise bouts: 30 min of cycling at a moderate intensity corresponding to lactate threshold intensity (MOD), followed by a time-to-exhaustion test (TTE) at a high intensity corresponding to 120% of W_{peak} (HIGH) (Figure 1A). MOD exercise elicited a steady-state blood lactate concentration ($[La^-]$) in both sexes (~5 mM) and stable blood glucose levels (Figures 1B and 1C). In response to HIGH exercise, blood lactate reached 13.7 mM in males and 12.4 mM in females, with no significant sex difference (Figure 1D). Blood glucose levels remained stable in both sexes following HIGH (Figure 1E). Notably, TTE performance was significantly longer in males than in females ($p < 0.001$) (Figure 1F).

Exercise induces a sex-specific assembly of muscle respiratory SC

In addition, muscle biopsies were obtained at rest (PRE), after MOD, and immediately following HIGH exercise (Figure 1A) to perform blue native gel electrophoresis (BNGE). Notably, we were unable to detect any band from one female subject at PRE and MOD (Figure S1). We excluded this subject from the analysis, resulting in a final sample of n = 9 males and n = 7 females (from 8 females initially recruited). As previously described for human SKM,[16] we were able to detect both the dimeric form of complex III (i.e., III_2) and different bands corresponding to SCs (Figure S1). As for complex IV, we detected it as a monomer, a dimer, and bands corresponding to SCs. In addition, we quantified the SCs associated with complex I in 9 subjects (n = 3 females). In this case, we were unable to detect two bands from one female subject (PRE and MOD), one band (PRE) from one male subject, and one band (MOD) from another male subject (Figure S1, red boxes), resulting in a final sample of 7 subjects for this complex. In line with previous data, we were unable to detect free complex I as it is almost fully found as SCs.[16]

Table 1. Participants' characteristics

	Females (n = 7)	Males (n = 9)
Age (years)	23 ± 4	22 ± 2
Height (cm)	169 ± 9	180 ± 7
Weight (kg)	61.6 ± 8.7	76.4 ± 7.3
$\dot{V}O_2$ peak (mL/min/kg)	45.1 ± 6.2	49.4 ± 5.7
Wpeak (W)	197 ± 33	256 ± 19
MOD (W)	128 ± 37	176 ± 16
HIGH (W)	238 ± 40	308 ± 23
MOD work (kJ)	230 ± 67	317 ± 27
HIGH work (kJ)	29.4 ± 7.2	67.6 ± 17.3

W, watts. Mechanical work (kJ) was calculated as power (W) × time (s)/1,000.

We then quantified the incorporation of complex III_2 and CIV into distinct SC assemblies, specifically: (1) the I + III_2 SC, (2) high molecular weight SCs (HMWSCs), and (3) the III_2+IV SCs. The sum of all bands containing SCs is referred to as total SC content (tSCs) (Figures 2A–2H; Figure S1). All values are expressed relative to the distribution of complex III_2 and the monomeric form of complex IV within SCs, as well as the fold change of this distribution relative to PRE. Regarding complex I, due to its nearly complete incorporation into SCs, its value is expressed as the fold change of all bands relative to PRE.

First, we compared the levels of complex III_2 and CIV assembled into SCs between sexes at rest (PRE) and found a similar abundance of SCs in both groups (Figure S2). We then explored the effect of acute exercise on SC abundance within SKM. Regarding complex III_2, we found a significant time × sex interaction at HIGH for both tSCs and HMWSCs (Figures 2B and 2C). After Tukey adjustment, males at HIGH showed increased tSCs compared with both PRE and MOD levels. Similarly, males displayed a higher content of HMWSCs at HIGH compared with PRE. Notably, males exhibited greater increases in both tSCs and HMWSCs than females at HIGH. No significant interaction was observed for the supercomplex I+III_2.

As for complex IV, the abundance of CIV-containing SCs followed a similar sex-related pattern, although this did not reach statistical significance (Figures 2E–2H). Lastly, we analyzed the amount of SCs containing CI and found no time effect; since only two females were included, sex was not modeled in this analysis (Figure S3). Notably, no changes were detected in the

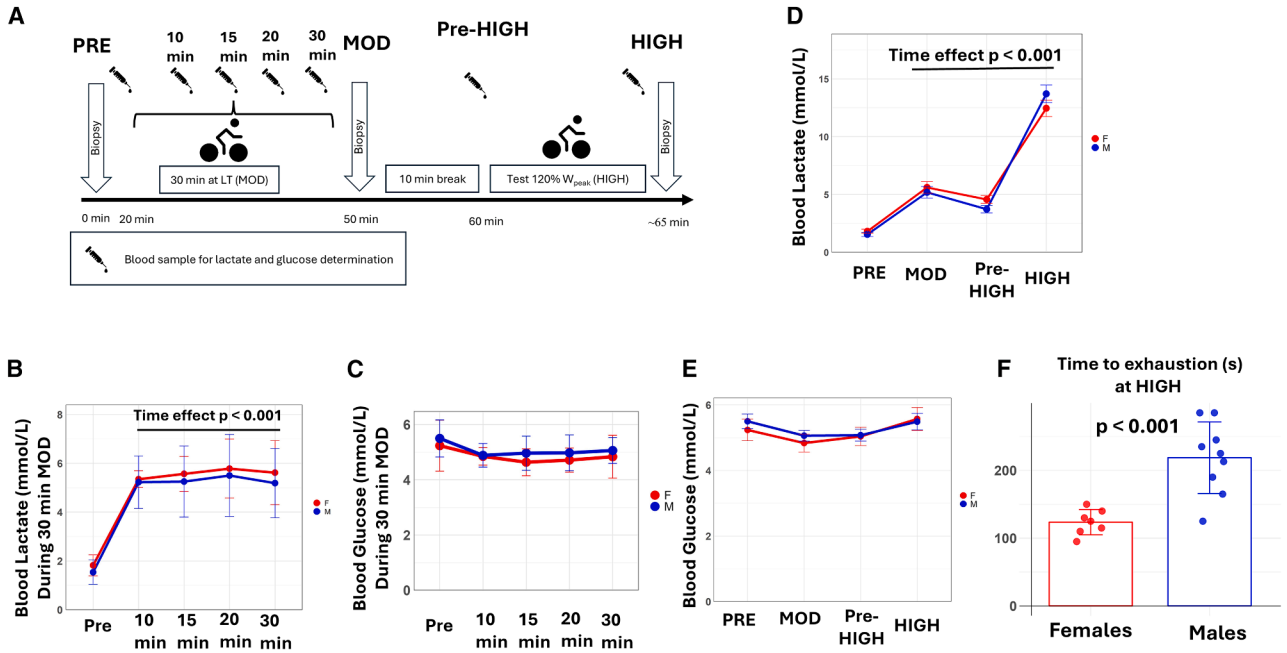

Figure 1. Comparable metabolic responses to exercise despite sex differences in time to exhaustion

Graph representation of the study design (A). Lactate (B) and glucose (C) blood levels during the 30 min at moderate intensity (MOD) exercise. Lactate (D) and glucose (E) blood levels at PRE, after MOD, at pre-HIGH, and after HIGH exercise. Time to exhaustion at high intensity exercise (F).

Data in (B)–(E) were analyzed using linear-mixed effects. Data in (F) were analyzed using an independent *t* test. M, males ($n = 9$) and F, females ($n = 7$). Error bars indicate standard deviations.

abundance of either the dimeric form of complex III or the monomeric form of complex IV. However, we observed a significant interaction when analyzing all bands containing complex III at HIGH, indicating a higher total complex III content in males (Figure S4). This pattern suggests that *de novo* complex III may be assembled into SCs following exercise in males. Such a mechanism may align with the rapid increase in SKM mitochondrial protein synthesis observed after exercise in an intensity-dependent manner.[30,31] Altogether, our results reveal a clear sex-based divergence in mitochondrial SC dynamics during exercise, with males exhibiting intensity-dependent assembly of complex III into SCs—particularly HMWSCs—and females showing no significant modulation.

SC assembly inversely correlates with blood lactate following HIGH exercise in males

We acknowledge that the experiments performed in the present study do not allow us to provide a mechanistic overview of the signals inducing SC assembly in males during exercise. However, several hypotheses could account for our observations.

Among the proposed physiological functions of SCs, one of the most consistently observed is the reduction of ROS production by the ETC.[32,33] In support of this, 6 weeks of endurance training in rats has been shown to increase SC content in SKM, likely reducing markers of oxidative damage.[34] However, this adaptation may not directly apply to acutely exercising muscle. In fact, during high-intensity exercise, mitochondrial ROS production in SKM can decrease to levels even lower than those at rest.[35] Therefore, it is unlikely that the SC assembly observed in males during HIGH represents a protective response against excessive ROS production. An alternative explanation could be that SC formation is linked to the longer time spent at high intensity by males. Nevertheless, no significant correlations were found between TTE and the abundance of complex III in tSCs, HMWSCs, or $I+III_2$ (Figure 3). When all the subjects were pooled together, a positive association was found between TTE and the abundance of complex IV in SCs (Figure 3; Figure S5). These findings suggest that other exercise-induced signals, independent of ROS suppression or time under load, are likely responsible for triggering the SC assembly in males during intense exercise.

Following HIGH, a negative correlation between tSCs and HMWSCs and blood [La^-] was observed in males but not in females (Figure 3; Figure S5). Importantly, circulating lactate concentrations were comparable between sexes. Despite this similarity, the inverse association between the SC assembly and lactate was evident only within males, suggesting that mitochondrial responses to intense exercise may differ between sexes under comparable metabolic conditions. Bootstrapping (5,000 resamples) supported most of the significant associations, with 95% confidence intervals reported in Table S2. One correlation (tSCs/IV vs. lactate in men) showed a confidence interval crossing zero and should therefore be interpreted with caution. Several mechanisms could potentially explain the negative relationship observed between the SC assembly and lactate levels in males.

First, according to the lactate shuttle theory, glycolytic fibers generate lactate, which is either oxidized locally by oxidative

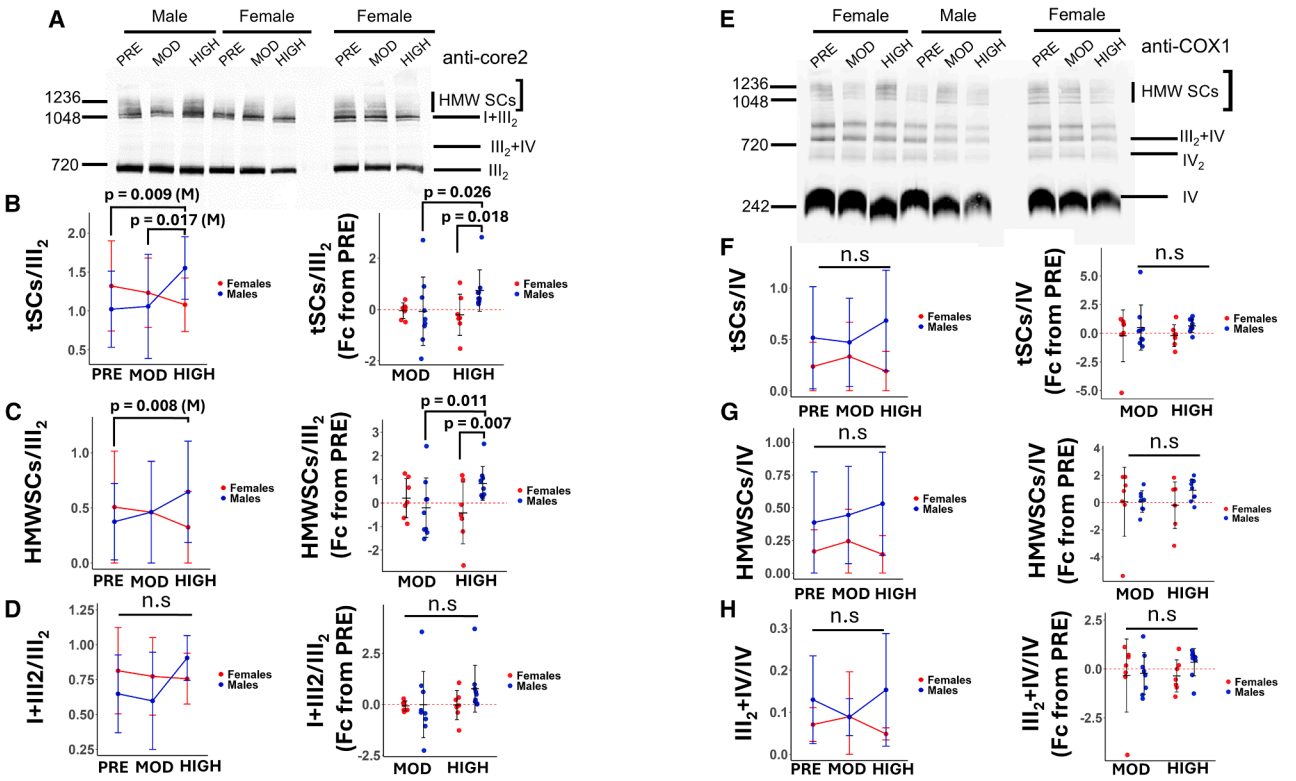

Figure 2. Sexual dimorphism in mitochondrial electron transport chain dynamics during exercise (n = 9 males and n = 7 females)

(A–D) Immunodetection of Core2 (complex III) after blue native (BN)-PAGE electrophoresis of digitonin-solubilized mitochondria from SKM at the indicated time points.

(E–H) Immunodetection of COX1 (complex IV) after BN-PAGE electrophoresis of digitonin-solubilized mitochondria from SKM at the indicated time points.

The left side of (B)–(D) and (F)–(H) shows the densitometric quantification of the proportion of each complex assembled into supercomplexes (SCs), while the right side shows the fold change (log_2-transformed) relative to PRE. tSCs, total SCs and HMWSCs, high molecular weight supercomplexes. Data were analyzed using linear mixed-effects models and adjusted by Tukey's test. Blue lines and points correspond to males, whereas red lines and points correspond to females. Data in (B) and (C) showed significant interaction at HIGH. p values correspond to Tukey-adjusted comparisons. M refers to the male group. All error bars represent standard deviations.

fibers or exported into the circulation.[36] Thus, SC assembly may facilitate mitochondrial lactate oxidation. This could explain the inverse relationship observed between the SC assembly and circulating lactate. Supporting this idea, males subjected to acute high-intensity exercise have been reported to upregulate monocarboxylate transporter 1 (MCT1) expression in SKM.[37] Although subcellular localization was not assessed, this increase may enhance lactate influx into the cell and, potentially, into mitochondria. In contrast, female SKM shows a reduction in MCT1 expression in response to a comparable exercise stimulus.[38] These observations are consistent with the possibility that lactate handling may differ between sexes under high-intensity exercise.

Second, it should be noted that our data support a model in which strenuous exercise in male SKM increases the abundance of HMWSCs containing complex III. An increase in SCs containing CIII may enhance mitochondrial NADH oxidation,[39] which could indirectly influence lactate production by altering cytosolic redox balance. Nonetheless, further mechanistic studies are required to elucidate the signals driving SC assembly and their metabolic implications during intense exercise.

In summary, this study reveals a sexual dimorphism in mitochondrial SC assembly within human SKM in response to exercise. While females maintain a stable ETC configuration during both moderate- and high-intensity exercise, males exhibit a distinct reorganization of the ETC after high-intensity exercise, characterized by increased assembly of complex III into HMWSCs. Notably, our findings point to a potential interplay between lactate metabolism and the regulation of SC assembly during exercise, particularly in males.

Limitations of the study

This study has several limitations that should be acknowledged. First, although SC assembly was observed following HIGH exercise in males, the MOD and HIGH interventions were performed sequentially rather than randomized. Therefore, we cannot exclude the possibility that the response detected after HIGH reflects cumulative or additive metabolic stress from both exercise bouts rather than an isolated effect of intensity per se. Second, due to the substantial tissue requirements of the BNGE procedure, we were unable to measure intracellular lactate concentrations. Therefore, we cannot rule out the possibility that

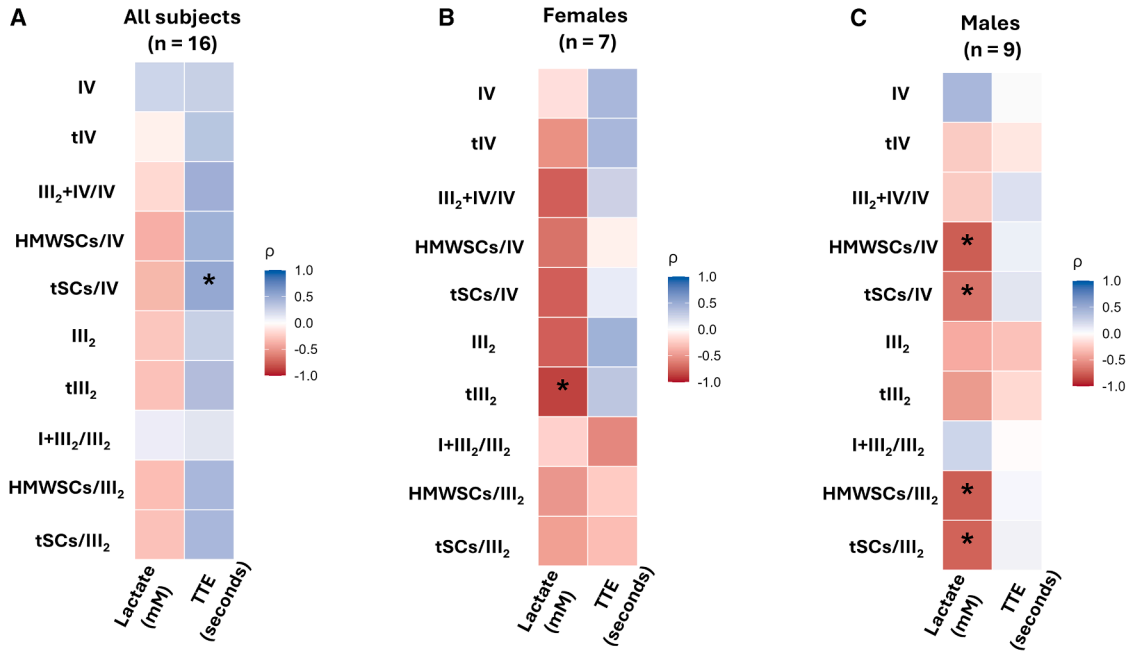

Figure 3. Relationship between blood lactate, TTE at HIGH, and the relative distribution of complexes III2 and IV into SCs

Heatmaps show Spearman's rank correlations (ρ) between blood [La^-] or TTE and the relative distribution of complex III_2 and CIV into SCs, their total content ($tIII_2$ and tIV), and their free forms.

(A) All subjects (n = 16), (B) females (n = 7), and (C) males (n = 9). * indicates significant correlation ($p < 0.05$).

See also Figure S5.

intracellular lactate dynamics differ from those observed in blood. Third, sex-related differences in SKM fiber-type composition (i.e., oxidative vs. fementative profiles) may partially account for the divergent SC assembly responses observed.[29] Additionally, it is plausible that equalizing the time spent at HIGH intensity between sexes might lead to a more comparable SC assembly response in females. Notably, while all male participants exhibited an increase in tSCs containing CII following HIGH, 5 out of 7 females showed a decrease. This divergent response suggests that additional sex-specific factors may contribute to the regulation of SC assembly during high-intensity exercise. Finally, it should be acknowledged that our correlation data are based on a small sample size, making this observation necessary to be explored mechanistically.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Rafael A. Casuso (rcasuso@uloyola.es).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- All data generated or analyzed during this study are included in this published article and its supplemental information files.
- All data reported in this paper will be shared by the lead contact upon request.
- The R scripts used for statistical analyses are available from the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.R.H., S.C., and R.A.C.; formal analysis, S.C. and R.A.C.; funding acquisition, J.R.H., N.B.N., S.C., and R.A.C.; investigation, S.C., J.A.-V., A.B.A., J.B., L.N., N.B.N., and A.R.-C.; methodology, J.R.H., S.C., R.A.C., J.A.-V., A.B.A., J.B., L.N., N.B.N., and A.R.-C.; supervision,: J.R.H., N.B.N., S.C., and R.A.C.; writing – original draft, S.C. and R.A.C.; and writing – review and editing, S.C., A.B.A., J.A.-V., R.A.C., J.A.E., and J.R.H.

DECLARATION OF INTERESTS

S.C. is a member of the advisory board of *Cell Reports*

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-UQCRC2 (CORE2)	ProteinTech	14742-1-AP; RRID: AB_2241442
Rabbit anti-UQCRC1	Sigma	HPA003525; RRID: AB_1080484
Mouse anti-COXI	Invitrogen	459600; RRID:AB_10374492
Rabbit anti-NDUFA5	Merck	SAB1406167 (discontinued)
Goat anti-Rabbit IRDye 680	Invitrogen	A21076; RRID: AB_2535736
Goat anti-Mouse IRDye 800	Invitrogen	SA5-10176; RRID: AB_2556756
Biological samples		
Human skeletal muscle biopsies (vastus lateralis)	This paper	N/A
Human blood samples	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Digitonin	Sigma-Aldrich	D5628-1G
Brilliant Blue G	Serva Scientist. LabClinics	35050.03
Bis-Tris	Sigma-Aldrich	B9754
6-Aminocaproic acid	Sigma-Aldrich	A2504
Software and algorithms		
R software	R Foundation	https://www.r-project.org
lme4 package	Bates et al.[40]	https://CRAN.R-project.org/package=lme4
emmeans package	Lenth et al.[41]	https://CRAN.R-project.org/package=emmeans
LI-COR Odyssey CLx (for imaging)	LI-COR	https://www.licor.com
Other		
Oxycon Pro metabolic system	Viasys Healthcare	N/A
Odyssey CLx Imaging System	LI-COR	N/A
Monark ergometer	Monark Exercise	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All participants received oral and written information about the potential risks and discomforts associated with participation before providing written consent. Eligibility criteria were: 18–30 years, no chronic diseases, non-smoking, and recreationally physically active, with a Body Mass Index <28 kg/m^2. The ethics committees of the University of Granada (23/CEIH72015) and the Regional Ethics Committee of Copenhagen (H-17012051) approved the study, which was conducted in accordance with the Declaration of Helsinki. Seventeen participants (males, $n = 9$; females, $n = 8$) completed the full protocol. However, PRE and MOD (see below) mitochondrial SC data could not be obtained from one female participant, this participant was excluded from the dataset. Consequently, all analyses were performed in a final sample of 9 males and 7 females. The physical characteristics of the analyzed cohort are described in Table 1.

METHOD DETAILS

Experimental design

The participants visited the laboratory twice with each visit separated by at least five days. During the first visit, the subjects performed a graded cycling test to exhaustion to determine lactate threshold (LT), incremental peak power output (W_{peak}), and peak oxygen consumption ($\dot{V}O_2$peak). Each participant completed their respective two trials at a similar time of day (±2 h) to minimize the potential influence of diurnal variations on exercise performance. During the second visit, participants reported to the laboratory in a rested state. Following 30 min of rest, a blood sample was collected to determine resting blood lactate levels and muscle biopsy from the m. vastus lateralis was obtained (PRE). Then, participants performed 30 min of cycling at the intensity corresponding to LT

(MOD; females: 127 ± 35 W; males: 176 ± 16 W), after which a second muscle biopsy was obtained. Ten minutes after the 30 min of MOD cycling, a TTE test was performed at an intensity of 120% of W_{peak} (females: 195 ± 32 W; males: 257 ± 19 W). Immediately after exhaustion, a third muscle biopsy was obtained (HIGH).

Testing procedures and equipment

All cycling tests were performed on an electronically braked ergometer (Monark Exercise, Vansbro, Sweden) with the same specific geometric setup for each test. During the graded exercise test, breath-by-breath pulmonary gas exchange was measured using an online gas analysis system (Oxycon Pro; Viasys Healthcare), with calibration performed before each test. The graded cycling test started at 100 W for males and 75 W for females, and participants had to maintain a continuous cadence of ∼65–85 rpm. The workload was increased by 25 W every three minutes until voluntary fatigue or the inability to maintain >65 rpm despite strong verbal encouragement. $\dot{V}O_2$peak was determined as the highest averaged $\dot{V}O_2$ over a 30 s period and confirmed by either a plateau in $\dot{V}O_2$, a $[La^-]$ >8 mmol/L, a respiratory exchange ratio >1.15, or a combination of these. W_{peak} was computed as 25 W×(t/180) + W_{compl} (t is the time at the last workload before exhaustion [s]; W_{compl} is the last workload completed [W]). $[La^-]$ was determined by collecting capillary blood samples from a heated fingertip (Clinitubes, Radiometer, Denmark) at rest and during the last 30 s during each three-minute step, or immediately after exhaustion. Analysis was performed on a blood-gas analyser (ABL800 Flex, Radiometer, Denmark). The workload at LT was subsequently determined using a breakpoint method based on a deviation from linearity in the $[La^-]$-workload relationship occurring at 4 mmol/L and confirmed with the ventilatory response, characterized by a non-linear increase in ventilation (VE) with a simultaneous rise in VE/$\dot{V}O_2$ and VE/$\dot{V}CO_2$.[42] The exact power output for LT was calculated using linear interpolation between the nearest $[La^-]$ values above and below the 4.0 mmol/L threshold. Participants were asked to prepare similarly for both test days including refraining from strenuous physical activity 24h prior and avoiding caffeine, nicotine, and alcohol 12h prior to each visit. Additionally, participants were instructed to consume the same diet 12 h prior to each test day, to come to the laboratory after consuming a light self-selected breakfast and to refrain from further food intake for at least 1 h prior to testing.

30 min submaximal and time-to-exhaustion test

During the 30 min of cycling at LT (MOD), $[La^-]$ and glucose concentrations were determined prior to exercise and after 10-, 15-, 20-, and 30-min. Glucose and $[La^-]$ were also determined immediately before and after the TTE test. Exhaustion was determined voluntary fatigue or the inability to maintain >65 rpm despite strong verbal encouragement as described for the $\dot{V}O_2$peak test.

Muscle samples

Muscle biopsies (∼80 mg of muscle tissue) were collected under local anesthesia (1–2 mL of 2% lidocaine without epinephrine, 20 mg/mL Xylocain; AstraZeneca, UK) through an incision made in the skin and fascia of the thigh (m. vastus lateralis). Biopsies were collected using a Bergström needle with suction directed proximally, distally, and laterally. This ensured that each sample was obtained from a unique region of the vastus lateralis, avoiding potential confounding effects of inflammation from previous biopsies. The biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Mitochondrial supercomplex assessment by blue native-page (BN-PAGE) electrophoresis

Blue Native Page (BN-PAGE) electrophoresis was performed on crude mitochondrial fractions from muscle tissue as previously outlined.[43] The mitochondrial pellets were resuspended in an appropriate volume of medium C (1 mM aminocaproic acid, 50 mM Bis-Tris-HCl, pH 7.0) to achieve a protein concentration of 10 mg/mL, and membrane proteins were solubilized using digitonin (4 g/g) and incubated on ice for 10 min. After 30 min of centrifugation at 13,000 g at 4°C, the supernatants were collected, and 3 μL of 5% Brilliant Blue G dye prepared in 1 mM aminocaproic acid was added. Mitochondrial proteins (100 μg) were loaded onto a 3–13% gradient native gel for electrophoresis. Following electrophoresis, the samples were electroblotted onto polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-FL, 0.45 μm; Merck Millipore, IPFL00010) for 1 h at 100 V in transfer buffer (48 mM tris, 39 mM glycine, and 20% ethanol). and probed with a specific primary antibody (1:1000 for 2 h at room temperature). UQCRC2 (CORE2) subunit ProteinTech14742-1-AP, UQCRC1 subunit Sigma HPA003525, COXI subunit Invitrogen MTCO1 459600, NDUFA5, Merk SAB1406167 (discontinued). Secondary antibodies used were: α-Rabbit680 (Invitrogen A21076) and α-Mouse800 (Invitrogen SA5-10176) at dilution of 1:5000, for 1 h at room temperature. The Protein bands were visualized using the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

All analyses and graphs were performed using R (version 4.4.2). To account for within-subject variability in repeated measures, mixed-effects linear regression models were applied using the *lmer* function from the *lme4* package.[40,44] Estimated marginal means were calculated and adjusted using the *emmeans* package[41] and Tukey-adjusted pairwise comparisons were conducted within each level of the interacting factors (i.e., time within sex and sex within time). When comparing two groups, independent *t*-tests were used. Statistical significance was set at $p < 0.05$ for all comparisons, and post hoc Tukey adjustments were applied for multiple testing.

Supercomplexes (SCs) were analyzed both as the relative distribution of each complex assembled into SCs and as the fold change (FC) relative to PRE. In addition, the total content of each complex (sum of all bands) and the free forms of complex III_2 and IV were quantified. Complex I was found fully assembled into SCs, with no detectable free form; therefore, relative distribution was not calculated, and the FC of total SCs containing complex I was analyzed relative to PRE. All fold-change values were log_2-transformed before analysis. Correlation analyses were performed using Spearman's rank correlation coefficients (ρ). To assess the robustness of the correlation estimates, bootstrapping (5,000 resamples) was conducted and 95% confidence intervals were derived.