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Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes

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Jacobs RA, Lundby C. Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. *J Appl Physiol* 114: 344–350, 2013. First published December 6, 2012; doi:10.1152/japplphysiol.01081.2012.—Changes in skeletal muscle respiratory capacity parallel that of aerobic fitness. It is unknown whether mitochondrial content, alone, can fully account for these differences in skeletal muscle respiratory capacity. The aim of the present study was to examine quantitative and qualitative mitochondrial characteristics across four different groups ($n = 6$ each), separated by cardiorespiratory fitness. High-resolution respirometry was performed on muscle samples to compare respiratory capacity and efficiency in active, well-trained, highly trained, and elite individuals. Maximal exercise capacity (ml $O_2 \cdot min^{-1} \cdot kg^{-1}$) differed across all groups, with mean ± SD values of 51 ± 4, 64 ± 5, 71 ± 2, and 77 ± 3, respectively. Mitochondrial content assessed by citrate synthase activity was higher in elite trained compared with active and well-trained (29 ± 7 vs. 16 ± 4 and 19 ± 4 $nmol \cdot min^{-1} \cdot mg\ wet\ wt^{-1}$, respectively). When normalizing respiration to mitochondrial content, the respiratory capacities during maximal fatty acid oxidation ($P = 0.003$), maximal state 3 respiration ($P = 0.021$), and total electron transport system capacity ($P = 0.008$) improved with respect to maximal exercise capacity. The coupling efficiency of β-oxidation, however, expressed no difference across groups. These data demonstrate the quantitative and qualitative differences that exist in skeletal muscle mitochondrial respiratory capacity and efficiency across individuals that differ in aerobic capacity. Mitochondrial-specific respiration capacities during β-oxidation, maximal oxidative phosphorylation, and electron transport system capacity all correspondingly improve with aerobic capacity, independent of mitochondrial content in human skeletal muscle.

exercise; skeletal muscle; fat oxidation

MEASURES OF WHOLE BODY CARDIORESPIRATORY fitness ostensibly represent the state of health in humans (1, 12, 24–26, 31, 34). Although maximal aerobic capacity is primarily limited by the oxygen transport system (3, 44), i.e., maximal cardiac output and oxygen-carrying capacity of the blood, mitochondrial content (7, 18) and skeletal muscle respiratory capacity (9) both also share a strong positive correlation with maximal aerobic capacity in humans. Morphometric examination and analysis of biochemical expression show that mitochondrial density and content increase in response to training (15, 16, 18) and differ between untrained and trained individuals (20, 28, 33, 46). Skeletal muscle oxidative capacity also increases with training (36) and varies across groups differing in activity level (28, 33, 46). There is debate whether the increase in skeletal muscle respiration capacity that parallels aerobic fitness can be explained by quantitative differences in mitochondrial content alone (41, 45), or whether qualitative adaptations, such as functional modifications in respiratory control and capacity, also improve along with whole body aerobic capacity (13).

We previously determined that mitochondrial respiratory capacity, particularly oxidative phosphorylation capacity (P) and electron transport system capacity (ETS), are strongly predictive of overall exercise capacity in highly trained athletes (22). Over many other physiological variables, including total hemoglobin mass and cerebral oxygenation, P, exclusively, was identified as the strongest determinant of endurance performance (22). Mitochondrial content does not always differ between individuals who display significantly different exercise capacities (38, 40), and yet skeletal muscle P is the strongest predictor of endurance performance (22). Moreover, prolonged exposure to hypoxia can diminish respiratory capacity and enhance mitochondrial coupling efficiency, independent from any change in mitochondrial content (23). Together, these data suggest that qualitative differences in mitochondria may exist independent from mitochondrial content.

Previous reported differences in skeletal muscle mitochondria respiratory capacity across groups that differed in level of cardiorespiratory fitness did not control for the significant variation in mitochondrial content between the groups (33, 46). No study has compared functional differences in mitochondria between normal, healthy individuals vs. elite athletes. The aim of this study is to analyze mitochondrial differences, both quantitative and qualitative, across four different groups of healthy and physically active subjects who differ in aerobic capacity. As P is the strongest determining factor in endurance performance (22) among a more homogenous group of athletes with negligible differences in mitochondrial content (38), we hypothesize that differences in mitochondria from AT to ET individuals will possess distinct qualitative differences.

METHODS

Ethical Approval

All experimental protocols involving human subjects were approved by the Eidgenössische Technische Hochschule Zürich for Kanton Zurich (2010–066/0 and EK 2011-N-51) and Kanton Vaud (215/10), Switzerland, in accordance with the declaration of Helsinki. Before the start of the experiments, informed oral and written consents were obtained from all participants.

Subjects and Experimental Design

Subject characteristics are shown in Table 1. Twenty-four young and physically active subjects (23 men and 1 woman) voluntarily participated in this study. No subjects were taking any prescription medications nor had any known family history of type 2 diabetes, severe obesity, or cardiovascular diseases. Previous studies

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Table 1. *Subject characteristics*

Group	Age, yr	Height, cm	Weight, kg	Absolute $\dot{V}O_{2max}$, l/min	Absolute W_{max}, W	Relative W_{max}, W/kg	Years of Exercise	Time Training/Year, h	Time Training/Week, h
AT	26 ± 4	181 ± 5	77 ± 5	3.96 ± 0.39	326 ± 27	4.2 ± 0.4	8.8 ± 5.6	190 ± 88[b,c,d]	3.7 ± 1.7[b,c,d]
WT	32 ± 8	180 ± 7	73 ± 10	4.68 ± 0.60	378 ± 59	5.2 ± 0.5[a]	13.9 ± 5.3	493 ± 141[a,d]	9.5 ± 2.7[a,d,g]
HT	28 ± 2	175 ± 7	68 ± 10	4.82 ± 0.67[e]	389 ± 45	5.8 ± 0.3[a,f]	9 ± 4.8	636 ± 43[a,d]	12.3 ± 0.8[a,d,f]
ET	28 ± 6	179 ± 8	68 ± 6	5.24 ± 0.52[a]	417 ± 41[a]	6.2 ± 0.3[a,b]	16 ± 6.2	847 ± 126[a,b,c]	16.4 ± 2.6[a,b,c]

Physiological characteristics for all subjects ($n = 24$) are means ± SD. AT, active subjects ($n = 6$); WT, well-trained subjects ($n = 6$); HT, highly trained subjects ($n = 6$); ET, elite athletes ($n = 6$); $\dot{V}O_{2max}$, maximal oxygen consumption; W_{max}, maximal power output. Difference ($P < 0.05$) from [a]AT, [b]WT, [c]HT, and [d]ET. Tendency of difference ($0.05 < P < 0.1$) from [e]AT, [f]WT, [g]HT, and [h]ET.

show no main effect of sex on skeletal muscle oxidative capacity and, consequently, grouped all data according to training status (28, 33, 46), as we have done in the present study. We divided the 24 subjects into four different tiers, dependent on their aerobic capacity: active (AT), well-trained (WT), highly trained (HT), and elite (ET). The AT group performed physical activity or sport (climbing, mountain biking, tennis, karate, road cycling, hockey, skating, running, swimming, or tour skiing) sporadically for an average of 1.58 days/wk, with no regular routine; the WT group participated in some variation of a regular exercise or sport program (climbing, running, Thai boxing, or road cycling) for an average of 2.46 days out of the week; and HT and ET athletes engaged in endurance exercise (triathlon training or road/mountain/cyclo-cross cycling and road or mountain cycling for HT and ET, respectively) on average for 2.49 and 2.81 days/wk, respectively. All individuals were separated into their respective groups based on aerobic capacities, which were obtained via tests of maximal oxygen consumption ($\dot{V}O_{2\,max}$).

Exercise Tests

Exercise tests to obtain values of $\dot{V}O_{2\,max}$ were completed on an electronically braked cycle ergometer (Monark, Varberg, Sweden). The exercise protocol started with a 5-min collection of resting oxygen consumption ($\dot{V}O_2$), followed by two consecutive absolute submaximal workloads, 100 W and 150 W, which were maintained for 5 min each. Thereafter, the workload increased 25 W/min until voluntary exhaustion. During the last minutes of the test, subjects were vigorously encouraged to perform to complete exhaustion, and the achievement of $\dot{V}O_{2\,max}$ was established by standard criteria in all tests (2). Subjects wore a face mask covering their mouth and nose for breath collection (Hans Rudolph, Kansas City, MO), and O_2 and CO_2 concentration in the expired gas was continuously measured and monitored as breath-by-breath values (Quark, Cosmed, Rome, Italy and Innocor, Innovision, Odense, Denmark). The gas analyzers and flowmeters of each applied spirometer were calibrated before each test.

Skeletal Muscle Sampling

Skeletal muscle biopsies were obtained from the *m. vastus lateralis* under local anesthesia (1% lidocaine) of the skin and superficial muscle fascia, using the Bergström technique (6), with a needle modified for suction at rest in the morning, and in a fasted state at least 24 h after the last bout of exercise. The biopsy was immediately dissected free of fat and connective tissue and divided into sections for measurements of mitochondrial respiration.

Skeletal Muscle Preparation

The skeletal muscle samples were sectioned into parts to measure mitochondrial respiration. Each part was immediately placed in ice-cold biopsy preservation solution (BIOPS) containing 2.77 mM CaK_2EGTA buffer, 7.23 mM K_2EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM 2-(*N*-morpholino)ethanesulfonic acid hydrate, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2{\cdot}6H_2O$, 5.77 mM ATP, and 15 mM phosphocreatine (pH 7.1). Muscle samples were then gently dissected with the tip of two 18-gauge needles, achieving a high degree of fiber separation verified microscopically. Chemical permeabilization followed via incubation in 2 ml of BIOPS with saponin (50 μg/ml) for 30 min in 4°C (27). Lastly, samples were washed with a mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H_2O$, 60 mM potassium-lactobionate, 20 mM taurine, 10 mM KH_2P0_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l bovine serum albumin (pH 7.1) for 10 min in 4°C.

Mitochondrial Respiration Measurements

Muscle bundles were blotted dry and measured for wet weight in a balance-controlled scale (XS205 DualRange Analytical Balance, Mettler-Toledo), maintaining constant relative humidity and providing hydration consistency as well as stability of weight measurements. Respiration measurements were performed in mitochondrial respiration medium 06 (MiR05 + catalase 280 IU/ml). Measurements of $\dot{V}O_2$ were performed at 37°C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria) with all additions of each substrate, uncoupler, and inhibitor titration (SUIT) protocol added in series. Standardized instrumental were performed to correct for back-diffusion of oxygen into the chamber from the various components, leak from the exterior, $\dot{V}O_2$ by the chemical medium, and sensor $\dot{V}O_2$. Oxygen flux was resolved by software allowing nonlinear changes in the negative time derivative of the oxygen concentration signal (Oxygraph 2k, Oroboros, Innsbruck, Austria). All experiments were carried out in a hyperoxygenated environment to prevent any potential oxygen diffusion limitation.

Respiratory Titration Protocol

The SUIT protocol applied in the study has been previously described (22). The protocol was specific to the examination of individual aspects of respiratory control through a sequence of coupling and substrate states induced via separate titrations. All respirometric analyses were made in duplicates, and all titrations were added in series as presented. The concentrations of substrates, uncouplers, and inhibitors used were based on prior experiments conducted for optimization of the titration protocols. Leak respiration in absence of adenylates (L_N) was induced with the addition of malate (2 mM) and octanoyl carnitine (0.2 mM). The L_N state represents the resting $\dot{V}O_2$ of an unaltered and intact electron transport system free of adenylates. Maximal electron flow through electron transferring-flavoprotein (ETF) and fatty acid oxidative capacity (P_{ETF}) were both determined following the addition of ADP (5 mM). In the P_{ETF} state, the ETF linked transfer of electrons requires the metabolism of acetyl-CoA, hence the addition of malate, to facilitate convergent electron flow into the Q-junction from both complex I (C1) and ETF, allowing β-oxidation to proceed. The contribution of electron flow through C1 is far below capacity, and so here the rate-limiting metabolic branch is electron transport through ETF, such that malate + octanoyl carnitine + ADP-stimulated respiration is representative of, rather than specific to, electron capacity through ETF (11, 13, 35, 36, 39). State 3 respiratory capacity specific to C1, NADH dehydrogenase (P_{C1}), was induced following the additions of pyruvate (5 mM) and

glutamate (10 mM). P was then induced with the addition of succinate (10 mM). P demonstrates a naturally intact electron transport system's capacity to catalyze a sequential set of redox reactions that are partially coupled to the production of ATP via ATP synthase. P maintains an electrochemical gradient across the inner mitochondrial membrane dictated by the degree of coupling to the phosphorylation system (13, 35). As an internal control for compromised integrity of the mitochondrial preparation, the mitochondrial outer membrane was assessed with the addition of cytochrome *c* (10 μM). There was no indication of mitochondrial damage indicated by the average change in respiration of 1.1% across all subjects ($P = 0.361$) following addition of cytochrome *c*. Phosphorylative restraint of electron transport was assessed by uncoupling ATP synthase (complex V) from the electron transport system with the titration of the proton ionophore, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (0.5 μM per addition up to optimum concentrations ranging from 1.5 to 3 μM), reaching ETS. Finally, rotenone (0.5 μM) and antimycin A (2.5 μM) were added, in sequence, to terminate respiration by inhibiting C1 and complex III (cytochrome bc_1 complex), respectively. With C1 inhibited, electron flow specific to complex II (C2), succinate dehydrogenase, and state 3 respiration through C2 (P_{C2}) can be measured. Inhibition of respiration with antimycin A then allows for the determination and correction of residual $\dot{V}O_2$, indicative of nonmitochondrial $\dot{V}O_2$ in the chamber.

Citrate Synthase Activities

Citrate synthase (CS) activities were assayed in homogenates of the skeletal muscle samples used in respiration measurements. The contents of the Oxygraph-2k chambers (2 ml each) were removed after each respiration experiment and washed once with 2 ml of MiR05. One percent Triton X-100 and 2 μl of a protease inhibitor cocktail (Sigma Aldrich cat. no. 539134) were added to the combined solutions (content and wash) and then homogenized for 30 s with a T10 basic ULTRA-TURRAX homogenizer near maximum speed. The homogenate was then centrifuged for 15 min at 4°C, and the supernatant was removed, frozen in liquid nitrogen, and stored at −80°C. As has been previously described (42), CS activity was measured fluorometrically at 412 nm and 25°C (Citrate Synthase Assay Kit, Sigma-Aldrich), according to the manufacturer.

Data Analysis

Significance was set at $P = 0.05$, but P values of < 0.10 are also noted. Data are presented as means ± SD. Mitochondria-specific respiration was calculated by dividing mass-specific respiration (pmol $O_2 \cdot min^{-1} \cdot mg$ wet wt^{-1}) by the corresponding CS activity (nmol $\cdot min^{-1} \cdot mg$ wt^{-1}). Comparisons of age, weight, height, absolute and relative $\dot{V}O_{2\,max}$, absolute and relative maximal power (W_{max}), years of exercise, respiratory capacities, CS, and indexes of mitochondrial coupling control were compared using a one-way ANOVA (SPSS Statistics 17.0, SPSS, Chicago, IL). Significant main effects or interactions were further analyzed by Tukey's post hoc test. The time of training per year and per week did not, however, display a Gaussian distribution, and, therefore, a Kruskal-Wallis ANOVA and Mann-Whitney *U*-tests were used to reveal differences between groups. An analysis of covariance was also run on mass-specific respirometric values with CS activities included as the covariant being controlled for. Multiple linear regression analysis with backward elimination was used to identify the strongest association between measurements of mass-specific and mitochondrial-specific respiration to cardiorespiratory fitness.

RESULTS

$\dot{V}O_{2\,max}$

All groups differed in $\dot{V}O_{2\,max}$ (Fig. 1). Group $\dot{V}O_{2\,max}$ values presented as means ± SD, maximum and minimum, respectively, in ml $\cdot min^{-1} \cdot kg^{-1}$, were as follows: AT (51.2 ± 4.1, 46.9 and 55.5); WT (63.9 ± 4.7, 58.9 and 68.8); HT (71.3 ± 1.7, 69.5 and 73.1); and ET (77.3 ± 3.0, 74.1 and 80.4). The AT group was lower ($P < 0.001$) than all other groups. The WT group was higher than AT ($P < 0.001$), but lower than HT ($P = 0.009$) and ET ($P < 0.001$). The HT group was higher than both AT ($P < 0.001$) and WT ($P = 0.009$) groups, but lower than the ET group ($P = 0.04$).

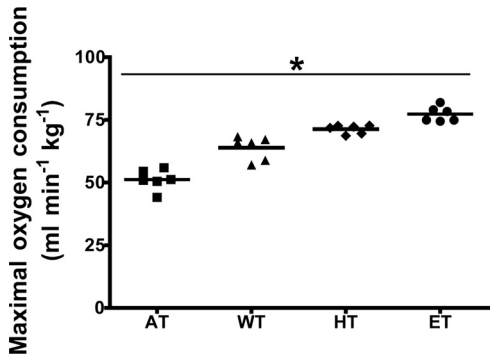

Fig. 1. Aerobic capacities across groups. Individual values of maximal oxygen consumption ($\dot{V}O_{2\,max}$) across groups are shown. AT, active; WT, well trained; HT, highly trained; ET, elite. *Significance of $P < 0.05$.

Subject Characteristics

All subject characteristics are presented in Table 1. There were no differences in age, height, weight, or years of exercise across groups. The absolute W_{max} only differed between the ET and AT groups ($P = 0.01$); however, the relative W_{max} for the AT group was lower than WT, HT, and ET ($P = 0.004$, $P < 0.001$, and $P < 0.001$, respectively). The ET also had a greater relative W_{max} than the WT group ($P = 0.002$), and the HT group showed a tendency for a higher relative W_{max} than the WT group ($P = 0.071$). The time-spent training per week and year demonstrated a progressive increase across groups, as the AT group exhibited the least time-spent participating in some form of physical activity, and the ET group had the most time-spent.

CS Activity

CS values (nmol $\cdot min^{-1} \cdot mg$ wet wt^{-1}) across groups (mean ± SD) were as follows: AT (16.2 ± 4.9), WT (18.6 ± 4.0), HT (25.1 ± 7.1), and ET (28.7 ± 7.0). The only differences detected in CS activity between groups were observed in ET compared with AT ($P = 0.008$) and WT groups ($P = 0.035$). There was a tendency for difference between AT and HT ($P = 0.072$). CS activity is an empirically established and reliable biomarker of mitochondrial content (30). CS activities are presented in Fig. 2.

Respiratory Capacity

Mass-specific respiration. There were no differences in mass-specific respiration (Fig. 3*A*) between AT and WT, WT and HT, or between HT and ET groups at any respiratory states, although P_{ETF} had a tendency to increase from AT to WT ($P = 0.056$). The AT group had lower fat respiration, P_{ETF} ($P = 0.012$ and $P < 0.001$), P ($P = 0.015$ and $P < 0.001$), ETS ($P = 0.003$ and $P < 0.001$), and submaximal P_{C2} ($P = 0.005$ and $P = 0.002$) than the HT and ET groups, respectively. The AT group also expressed lower L_N ($P = 0.019$) and submaxi-

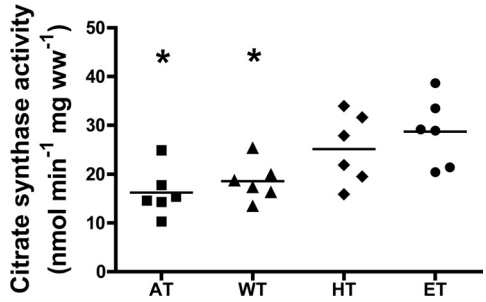

Fig. 2. Citrate synthase (CS) activities. Individual values of CS activities are presented across group as a biomarker of mitochondrial content. *Significance from ET of $P < 0.05$.

mal P_{C1} ($P = 0.033$) vs. the ET group. The WT group expressed lower respiration compared with the ET group during P ($P = 0.009$) and ETS ($P = 0.001$) and had a tendency to have lower P_{ETF} ($P = 0.052$).

Mass-specific respiration when controlling for mitochondrial content. Significant differences in mass-specific respiration were altered when controlling for CS activity as a covariate (data not shown). The lower respiratory capacities of the AT group vs. the HT group were only apparent during ETS and P_{C2} ($P = 0.049$), but showed a tendency for lower P_{ETF}, P, and P_{C2} ($P = 0.069$, 0.079, and 0.052, respectively). The AT group expressed lower respiration compared with the ET group at P_{ETF} ($P = 0.004$), P ($P = 0.005$), and ETS ($P = 0.003$) and had a tendency for lower P_{C2} ($P = 0.067$). The WT group expressed lower ETS than the ET group ($P = 0.033$) and also had a tendency for lower P ($P = 0.067$). There were no differences across groups during L_N or P_{CI}.

Mitochondria-specific respiration. Mass-specific respiration does not take into account differences in mitochondrial content between samples. Accordingly, respirometric analyses were adjusted for CS activity, a biomarker shown to express strong concordance with mitochondrial content and total cristae area, as measured by transmission electron microscopy as well as myocellular respiratory capacity (30). Figure 3*B* illustrates mitochondrial-specific respiration across all groups. There were no differences in mitochondrial-specific respiration between AT, WT, and HT groups across all respiratory states, although there was a tendency for lower respiration in the AT vs. the HT during P_{ETF} ($P = 0.056$). When normalizing respiration to mitochondrial content, P was greater in ET vs. only AT ($P = 0.031$), while P_{ETF} ($P = 0.003$ and 0.048) and ETS ($P = 0.015$ and 0.029) were greater in ET compared with both AT and WT, respectively. There was a tendency for P to be lower in the WT vs. ET group ($P = 0.066$). There were no differences across all groups during L_N, P_{C1}, or P_{C2}.

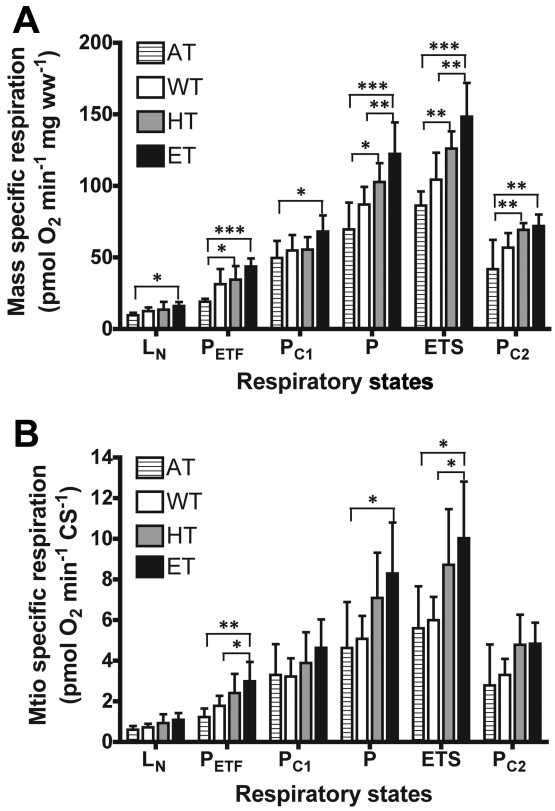

Fig. 3. Mitochondrial respiration control across groups. *A*: mass-specific respiratory capacity. *B*: mitochondria-specific respiratory capacity across groups. L_N, leak respiration in absence of adenylates; P_{ETF}, maximal electron flow through electron-transferring flavoprotein (ETF) and fatty acid oxidative capacity; P_{C1}, submaximal state 3 respiratory capacity specific to complex I; P, maximal state 3 respiration and oxidative phosphorylation capacity; ETS, electron transport system capacity; P_{C2}, submaximal state 3 respiratory capacity specific to complex II. Values are means ± SD. Significant difference of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Mitochondrial Coupling Efficiency and Respiratory Control

We calculated the leak control ratio (LCR) as indication of electron coupling efficiency during β-oxidation (LCR_{ETF}), as has been fully explained previously (23). The LCR_{ETF} did not express any difference across groups (Fig. 4*A*). The phosphorylation system control ratio (PSCR), ratio of P to ETS expressing the degree of maximal P constraint by the phosphorylation

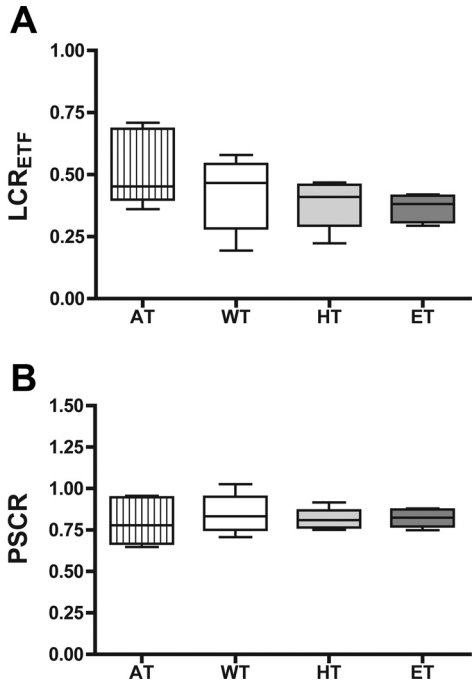

Fig. 4. Mitochondrial coupling efficiency and respiratory control. *A*: leak control ratio (LCR), electron coupling control during β-oxidation (LCR_{ETF}) across groups. *B*: phosphorylation system control ratio (PSCR) across groups.

system or ATP synthase (13), was also determined. While skeletal muscle PSCR differs significantly between mice and humans (21), it did not differ between groups that varied in aerobic capacity (Fig. 4*B*).

Correlation Between Maximal Exercise Respiratory Capacities

All mitochondrial parameters examined in this study expressed a significant correlation to $\dot{V}O_{2\,max}$, except for the PSCR ($R = 0.077$, $P = 0.361$). The second weakest correlation (behind L_N respiration) was between values of mitochondrial content and exercise capacity ($R = 0.635$, $P < 0.001$). The LCR_{ETF} was the only value showing a significant negative correlation ($R = -0.462$, $P = 0.012$). Finally, multiple linear regression analysis using backward elimination for mass-specific (Fig. 5*A*) and mitochondria-specific respiration (Fig. 5*B*), respectively, calculated ETS as the best predictive mitochondrial parameter of $\dot{V}O_{2\,max}$.

DISCUSSION

In this study we divided one large group of healthy subjects ($n = 24$) into four different groups ($n = 6$), dependent on their aerobic capacity and studied mitochondrial function via respirometric analysis. This study presents several novel findings. First, qualitative, independent from quantitative, differences in mitochondrial characteristics are apparent across groups of healthy humans in accordance with $\dot{V}O_{2\,max}$. Fat respiration (P_{ETF}), P, and electron transport across the entire respiratory system (ETS) all improve, in combination with aerobic capacity. Second, efficiency of coupling control during β-oxidation and phophorylative restraint of ATP synthase on electron transport do not appear to differ across individuals with markedly disparate measures of $\dot{V}O_{2\,max}$. Finally, ETS correlates best, among all other respiratory states, to cardiorespiratory fitness in humans.

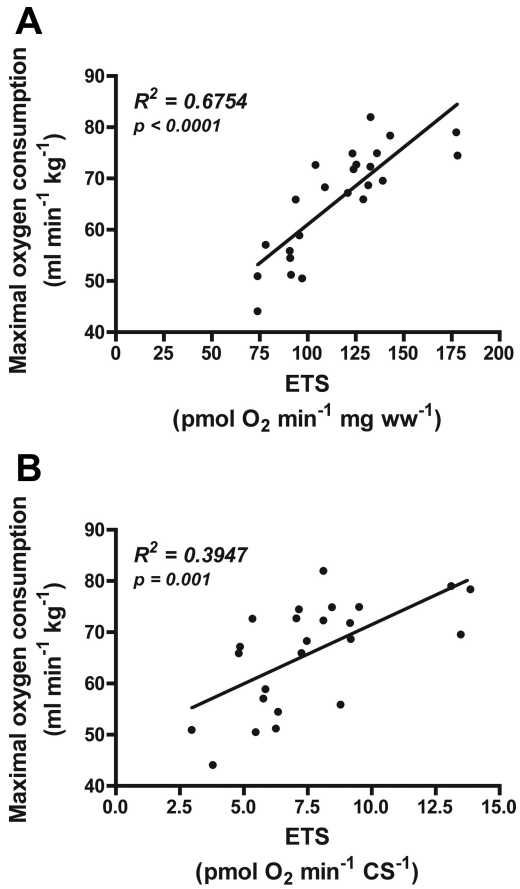

Fig. 5. Correlation of ETS capacity and $\dot{V}O_{2\,max}$ across groups. *A*: mass-specific respiration. *B*: mitochondrial-specific respiration.

There is a strong correlation between mitochondrial content and maximal aerobic capacity in both humans and animals (17, 18, 20, 32). The subjects included in this study displayed this correlation between CS and $\dot{V}O_{2\,max}$ ($P < 0.001$), although differences in mitochondrial content were not apparent between all groups (Fig. 2). It has been suggested that oxidative capacity of skeletal muscle is dependent purely on the quantity of mitochondria in the muscle (19, 41). Here we present strong evidence to suggest that there are qualitative improvements in mitochondria that correspond with whole body aerobic capacity, independent of mitochondrial content. The seemingly inconsistent results between those presented here and past studies (41) may be due to the fact that the former study used substrates for respiration specific to either mitochondrial C1 or C2 individually, but never collectively (41). Our data support these previous findings, as we also failed to identify any respiratory differences between any groups at P_{C1} or P_{C2} respiratory states when normalizing respiration to mitochondrial content (Fig. 3*B*). We stimulated P with saturating concentrations of ADP and substrate supply for both C1 and C2. This convergent electron input of both complexes provides higher respiratory values compared with the isolated respiration of either C1 (pyruvate/glutamate + malate or glutamate + malate) or C2 (succinate + rotenone) (13, 37). Accordingly, P presents with more physiological relevance to the study of mitochondrial function, as substrate provision for both complexes is necessary to confirm a complete and intact electron transport system and measure maximal respiratory capacity in skeletal muscle (8).

Although the capacity for fat respiration differed between AT and ET groups and also showed a tendency for improvement in HT over AT ($P = 0.056$), mitochondrial coupling efficiency during fatty acid oxidation was the same across all groups (Figs. 3*B* and 4*A*). Fat oxidation during exercise has been reported as greater in trained vs. untrained individuals at the same relative workload (43). Moreover, a greater percentage of energy expenditure during exercise comes from fat oxidation in trained vs. untrained subjects at the same absolute workload (43). There is also a greater intramuscular triglyceride content in trained vs. untrained humans (20), which also increases and becomes localized next to the mitochondria in response to exercise training (18) and is increased in active vs. nonactive individuals (14). One training study has reported improvements in mass-specific mitochondrial respiratory capacity, along with improvements in mitochondrial fat metabolism when sedentary individuals trained for 10 wk, while mitochondrial content, as assessed by mitochondrial DNA, did not increase (36). Unfortunately, mitochondrial DNA does not strongly correlate with measures of mitochondrial content and total cristae area, as assessed by transmission electron microscopy or myocellular respiratory capacity (30), and thus does not adequately serve as biomarker of mitochondrial content. Therefore, satisfactory mitochondrial-specific analysis of P_{ETF}

could not be analyzed (36). We extend on previous findings and show that the capacity for fat oxidation improves with cardiorespiratory fitness; however, efficiency appears unaltered.

Another qualitative difference in mitochondria observed across groups differing in aerobic fitness was ETS (Fig. 3*B*). Oxidative capacity of skeletal muscle is known to improve with overall fitness (28), although specific modifications in mitochondrial function in relation to fitness are much less understood. Differences in mass-specific respiration capacity in young and older subjects matched for aerobic capacity are negligible until respiration is normalized to mitochondrial content, where a reduction in respiratory capacity becomes apparent (29). Endurance-trained individuals present with a greater substrate flux through the tricarboxylic acid cycle over sedentary individuals, independent from any difference in ATP synthesis (5). The greater flux through the tricarboxylic acid cycle without an increase in ATP led authors to speculate that resting mitochondrial respiration in endurance-trained individuals is uncoupled from ATP synthesis to a greater degree than their sedentary counterparts (5). Here we show that ET athletes have a greater capacity for electron transport across the entire respiratory chain vs. AT and WT subjects, even though they present with similar phosphorylative restraint of electron transport (Fig. 5*B*). In the ETS state, the inner mitochondrial membrane potential is completely collapsed with an open transmembrane proton circuit. The uninhibited flow of electrons through the respiratory system can, therefore, indirectly serve as an indication of maximal attainable mitochondrial membrane potential. A larger capacity for electron transport with similar phosphorylative restraint allows for a superior uncoupling capacity, which appears to correspond with aerobic capacity. Across all states of respiration measured, ETS fit best with cardiorespiratory fitness (Fig. 5*A*). This correlation remained even when controlling for differences in mitochondrial content across all groups (Fig. 5*B*), further demonstrating the qualitative differences in mitochondrial function that are associated with whole body aerobic fitness in humans.

This study specifically highlights significant qualitative changes in mitochondria by means of respiratory capacity. Less is known in regards to how mitochondrial efficiency fluctuates with aerobic capacity. While an improvement in the coupling efficiency of electron transport during β-oxidation was not apparent across groups, the coupling control across the entire respiratory chain could not be determined with this SUIT protocol. Previous studies utilizing mitochondrial isolation techniques report no change in coupling control (4), while others report an improvement in mitochondrial efficiency between trained vs. sedentary individuals (10, 46). The malleability of mitochondria in response to training merits further attention.

One possible limitation in the present study is the supraphysiological concentrations of oxygen that are required during respirometric analysis to overcome diffusive limitations of the samples (35), and the potential alterations in mitochondrial function that may accompany this hyperoxygenated environment. This, however, is not believed to have influenced our results or conclusions, as all samples were treated similarly and exposed to the same oxygen concentrations.

In summary, both mitochondrial quantity and quality improve with aerobic capacity. Qualitative differences in mitochondria across levels of cardiorespiratory fitness were not fully understood and questioned to even exist. As such, we divided 24 healthy and aerobically fit individuals into four groups that differed in whole body aerobic exercise capacity. Across these healthy individuals, both quantitative and qualitative mitochondrial variations were evident. Those with elite aerobic capacities displayed superior respiratory capacity over those who were active or well-trained. Specifically, these qualitative differences observed across groups were specific to greater capacities for fat oxidation, oxidative phosphorylation, and electron transport across the entire respiratory system. These results make it apparent that mitochondrial modifications with improving cardiorespiratory fitness comprise more than just an increase in mitochondrial content. It is important and necessary to differentiate mitochondrial content from function in future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.A.J. and C.L. conception and design of research; R.A.J. performed experiments; R.A.J. analyzed data; R.A.J. and C.L. interpreted results of experiments; R.A.J. prepared figures; R.A.J. drafted manuscript; R.A.J. and C.L. edited and revised manuscript; R.A.J. and C.L. approved final version of manuscript.

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