A short period of high-intensity interval training improves skeletal muscle mitochondrial function and pulmonary oxygen uptake kinetics

Peter M. Christensen¹², Robert A. Jacobs³⁴, Thomas Bonne¹, Daniela Flück⁵, Jens Bangsbo¹, Carsten Lundby⁵

¹ Department of Nutrition, Exercise and Sports, Section of Integrated Physiology, University of Copenhagen, Denmark

² Team Danmark (Danish elite sport organization), Copenhagen, Denmark

³ Health and Physical Education, School of Teaching and Learning, Western Carolina University, Cullowhee, NC, USA,

⁴ Department of Physical Therapy, Western Carolina University, Cullowhee, NC, USA,

⁵ Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

Corresponding author:

Carsten Lundby

University of Zürich, Inst. für Physiologie, 23 H 06

Winterthurerstrasse 190, 8057 Zürich, Switzerland

Email: carsten.lundby@access.uzh.ch

Running head: VO₂ kinetics and measures of mitochondrial function

Keywords: High intensity training, oxygen uptake, enzyme activity, OXPHOS, cycling economy
Author contributions:

PMC: Design of study, data collection, data analysis, interpretation of data, manuscript preparation
RAJ: Design of study, data collection, data analysis, interpretation of data, manuscript preparation
TB: Data collection, manuscript preparation
DF: Data collection, manuscript preparation
JB: Data analysis, manuscript preparation, interpretation of data, manuscript preparation
CL: Design of study, data collection, data analysis, interpretation of data, manuscript preparation
Abstract
The aim of the present study was to examine whether improvements in pulmonary VO₂ kinetics following a short period of high-intensity training (HIT) would be associated with improved skeletal muscle mitochondrial function. Ten untrained male volunteers (age: 26 ± 2; mean ± SD) performed six HIT sessions (8-12 x 60 s at incremental test peak power; 271 ± 52 W) over a 2-week period. Before and after the HIT-period, VO₂ kinetics was modelled during moderate intensity cycling (110 ± 19 W). Mitochondrial function was assessed with high-resolution respirometry (HRR) and maximal activities of oxidative enzymes citrate synthase (CS) and cytochrome c oxidase (COX) were accordingly determined. In response to HIT, VO₂ kinetics became faster (τ: 20.4 ± 4.4 vs. 28.9 ± 6.1 s; P<0.01) and fatty acid oxidation (ETFₚ) and leak respiration (Lₙ) both became elevated (P<0.05). Activity of CS and COX did not increase in response to training. Both before and after the HIT-period fast VO₂ kinetics (low τ values) was associated with large values for ETFₚ, electron transport system capacity (ETS) and electron flow specific to complex II (CIIₚ) (P<0.05). Collectively these findings support that selected measures of mitochondrial function obtained with HRR are important for fast VO₂ kinetics and better markers than maximal oxidative enzyme activity in describing the speed of the VO₂ response during moderate intensity exercise.

News and noteworthy
The mechanism(s) facilitating pulmonary VO₂ kinetics following a training intervention remains incompletely understood. Here we provide evidence that exercise training induced improvement in skeletal muscle mitochondrial function coincide with concurrent improvements in pulmonary VO₂ kinetics.
Introduction

Pulmonary oxygen uptake (\(\dot{V}O_2\)) increases at the onset of constant load moderate intensity exercise and reaches a steady state within a few minutes (31, 56). Following the initial cardio-dynamic phase (the delay time for de-oxygenated blood from the exercising muscles to reach the pulmonary circulation), the increase in pulmonary \(\dot{V}O_2\) follows a mono-exponential pattern during moderate intensity exercise providing a close reflection of the \(\dot{V}O_2\) response in the exercising limbs (33). Early training studies demonstrated that exercise training facilitates a more rapid adjustment of the primary \(\dot{V}O_2\) response in previously untrained humans, i.e. faster \(\dot{V}O_2\) kinetics (19, 20). Two main factors, have been proposed to determine the speed of the \(\dot{V}O_2\) kinetics: 1) activation of the skeletal muscle respiration (often referred to as metabolic inertia) due to an increase in muscle ADP and inorganic phosphate; and 2) delivery of oxygen to the skeletal muscle mitochondria, as discussed extensively in numerous reviews (21, 28, 48) and computer simulation modelling (30).

The oxidative enzyme citrate synthase (CS) activity is a marker of mitochondrial density (35), and exercise training for 10 days has been shown to increase CS activity (53) which since then has been reproduced several times with various types of training (55). Thus, untrained individuals typically experience an increase in maximal mitochondrial oxidative enzyme activity concomitant with faster \(\dot{V}O_2\) kinetics following a period of training lasting one to five months (2, 32, 58, 59). In line herewith, cessation of exercise training in soccer players results in reduced maximal mitochondrial enzyme activity (e.g. CS) together with slower \(\dot{V}O_2\) kinetics (9). Collectively these findings suggest that maximal oxidative enzyme activity may serve as an appropriate biomarker(s) reflecting the oxidative capacity of skeletal muscles, which appears to influence the degree of metabolic inertia and hence \(\dot{V}O_2\) kinetics. However, untrained individuals have been reported to achieve faster \(\dot{V}O_2\) kinetics after only two (38) and four training sessions (42), which usually does not facilitate changes in muscle oxidative enzyme activity (18, 42). Thus, the faster \(\dot{V}O_2\) response may not be caused solely by a higher muscle oxidative capacity. Nevertheless, a 2-week period encompassing six sessions of repeated 30-s (7) and 60-s (36) sprint intervals has been reported to increase CS activity in untrained humans. \(\dot{V}O_2\) kinetics was not determined in these studies, but \(\dot{V}O_2\) kinetics was faster after a similar training intervention due to a faster extraction of oxygen as determined by near-infrared spectroscopy (NIRS), suggesting that reduced metabolic inertia was implicated in the faster \(\dot{V}O_2\) response (1). Since muscle biopsies were not obtained in the latter study, a possible link between muscular oxidative adaptations and faster \(\dot{V}O_2\) kinetics following a brief and intense training period with a low volume could not be determined.

During the maximal phase of incremental exercise, CS activity has been found to be ~10 fold higher than the estimated flux through the Krebs cycle leading the authors to suggest that this enzyme likely is a poor predictor of Krebs cycle capacity (4). Therefore, total mitochondrial content or maximal activities of...
oxidative enzymes may lack the sensitivity necessary to serve as precise markers of alterations in metabolic inertia, and hence, changes in VO$_2$ kinetics following training interventions. At least if the enzyme activity is abundant in relation to system capacity. In contrast, high-resolution respirometry (HRR) allows for the integrative study of respiratory capacities in intact mitochondria from permeabilized muscle fibres subjected to a specific titration protocol of various substrates in combination with high abundance of oxygen in the respiratory chamber (41). Thus, HRR can differentiate between simple changes in enzymatic expression vs. an alteration in the efficiency as well as functional capacity of mitochondria. In support, skeletal muscle oxidative capacity (OXPHOS capacity) and electron system capacity (ETS), obtained via HRR, correlates with time-trial performance in trained cyclists whereas CS activity does not (23). Other studies performed on trained cyclists also indicate that CS activity is not a key determinant of performance (10, 54). Hence, HRR may reflect endurance capacity of the muscle better than any single measure or collection of isolated enzyme activities, yet it is unclear if this is also the case when studying pulmonary VO$_2$ kinetics which provides a close reflection of muscle VO$_2$ (33).

Accordingly, the aims of the present study were to (I) examine whether improvements in mitochondrial function, assessed by HRR in addition to maximal oxidative enzyme activities would be found together with faster pulmonary VO$_2$ kinetics following a short period of high-intensity training in previously untrained individuals, and (II) evaluate if fast VO$_2$ kinetics is associated with measures of mitochondrial function. We hypothesized that training would lead to an increased skeletal muscle respiratory capacity (OXPHOS and ETS) allowing for a faster VO$_2$ response when substrate for mitochondrial respiration (ADP, inorganic phosphate and oxygen) is present. Moreover, that oxidative enzyme activity would increase, and that measures of mitochondrial function obtained via HRR would reveal a greater association with VO$_2$ kinetics than the maximal activity of oxidative enzymes.

Methods

Subjects

Ten untrained male volunteers (age: 26 ± 2 years, body mass: 77 ± 9 kg; mean ± SD) participated in the present study. This group of subjects was a subset of a larger group (n = 17) that participated in a study that investigated the mechanisms explaining the rapid improvements in exercise performance in untrained subjects following high intensity training (26). The study procedures were approved by the Ethical committee for the ETH Zürich (EK 2011-N-24), in accordance with the declaration of Helsinki, and all subjects gave their oral and written informed consent to participate in the study after receiving oral and written information describing the study procedures.
Study overview

Subjects performed testing for VO₂ kinetics and had a muscle biopsy taken before and after a ~2-week period with high-intensity training (HIT). A stationary cycle ergometer (Monark 839E, Varberg, Sweden) was used for testing of VO₂ kinetics. The ergometer was set in power mode (meaning that power was fixed irrespective of cadence). Change in load on the flywheel was controlled automatically by an external computer connected to the cycle ergometer which registered cadence every second. The HIT sessions was also performed on a cycle ergometer with a fixed power mode (Cycle Ops 420 Pro Indoor, Saris Cycling Group, WI, USA).

Training intervention

During the 2-week training period subjects performed a total of six training sessions separated by 1-2 days of recovery as described previously (26). Training sessions encompassed repeated 60-s intervals at an fixed intensity corresponding to peak power during incremental testing before HIT (271 ± 52 W; protocol consisting of three steps at 50, 100 and 150 W for 5 min each followed by 30 W increments every 90 s. Subjects were given verbal support during the test and the test terminated when subjects stopped or when cadence dropped below 60 rpm. In the training sessions, each interval was separated by 75 s of recovery at 30 W. Eight intervals were performed in the first two training sessions, ten intervals in the third and fourth session, and twelve intervals in the last two sessions.

VO₂ kinetics

Pulmonary VO₂ was assessed breath by breath prior to and again following HIT training using the Innocor system (Innovision, Odense, Denmark) which has a reported average “within day” variation during moderate intensity cycling at 130 W (~2000 ml min⁻¹) of 20 ml min⁻¹ (95% confidence interval: -20 – 60 ml min⁻¹) and “day to day” variation of 10 ml min⁻¹ (-50 – 60 ml min⁻¹) (14). Before each test, the equipment was calibrated using a 3-L syringe for ventilation and O₂ and CO₂ concentration by using a calibration gas mixture. While wearing a nose-clip, subjects breathed through a mouthpiece during the exercise tests. Subjects performed three constant load intervals at moderate intensity (110 ± 19 W; 40% incremental test peak power) lasting 6 min and separated by 20 min of recovery. All intervals were preceded by 2-min baseline cycling at 20 W. Subjects were free to choose the cadence they cycled during their first interval (64 ± 7 rpm), which was then maintained (± ~5 rpm) throughout the remaining intervals. For the determination of VO₂ kinetics, errant breaths, defined as any value lying more than 4 standard deviations away from the local mean, (e.g. caused by swallowing and coughing), were removed. Subsequently the VO₂ responses for the three transitions were linearly interpolated to give 1-s values, and then averaged. The initial
cardiodynamic component was ignored by eliminating the first 20 s of data after the onset of exercise. The data were fitted using a mono exponential model:

\[ \dot{V}O_2(t) = \dot{V}O_2 \text{ baseline} + A (1 - e^{-\frac{(t-t_d)}{\tau}}) \]

with \( \dot{V}O_2(t) \) being oxygen uptake to a given time (s). \( \dot{V}O_2 \) baseline was calculated as average from 30 to 90 s of the 120 s baseline cycling at 20 W. A, \( T_d \), and \( \tau \) are the amplitude, time delay, and time constant, respectively, for the primary response. Three intervals were chosen as this number of repetitions is known to reduce the 95% confidence interval surrounding the calculation of \( \tau \) relative to using only a single transition (3, 34).

Muscle sampling

Muscle biopsies were obtained under standardized conditions from m. vastus lateralis at baseline and again after the 2-week regimen of HIT. Samples were collected under local anesthesia (1% lidocaine) of the skin and superficial muscle fascia, using the Bergström technique with a needle modified for suction. The biopsy was immediately dissected free of fat and connective tissue and divided into sections for high-resolution respirometry and protein quantification with the latter sections being frozen immediately in liquid nitrogen and stored at -80° C until analysis. All biopsies were obtained 48 h following the last bout of exercise.

Respirometric Analysis

All procedures regarding skeletal muscle preparation, and HRR have been previously described in detail (26). Briefly, fat and connective tissue was first removed from the biopsies. Then the muscle tissue was added to the respiration chamber using a mitochondrial respiration medium 06 (MiR06; MiR05 + catalase 280 IU ml\(^{-1}\) at 37°C (Oroboros, Innsbruck, Austria), with standardized instrumental and chemical calibrations performed prior to all measurements. Respirometric analyses were done in duplicate and all titrations were added in series as presented (Fig. 1). To avoid a diffusional limitation, the oxygen concentration in the chamber was kept between 250 – 420 nmol ml\(^{-1}\). 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 oxygen consumption of an unaltered and intact electron transport system free of adenylates. Maximal electron flow through electron transferring- flavoprotein (ETF) representing fatty acid oxidative capacity (ETF\(_P\)) was determined following the addition of ADP (5 mM). In the ETF\(_P\) state, the ETF-linked transfer of electrons requires the metabolism of acetyl-CoA, hence, the addition of malate, in order to facilitate convergent electron flow into the Q junction from both complex I (CI) and ETF, allowing beta-oxidation to proceed. The contribution of electron flow through CI 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 (24). Submaximal state 3 respiratory capacity (P) specific to complex I (CIₚ) was induced following the additions of pyruvate (5 mM) and glutamate (10 mM). Maximal state 3 respiration, oxidative phosphorylation capacity, was then induced with the addition of succinate (10 mM). This maximal state 3 respiratory state represents the cellular respiration that is resultant to saturating concentrations of ADP and substrate supply for both complex 1 and complex 2 (CI+CIIP; OXPHOS capacity). Convergent electron input to complex 1 and 2 provides higher respiratory values compared to the isolated respiration of either complex 1 (pyruvate/glutamate + malate or glutamate + malate) or complex 2 (succinate + rotenone) (12, 45). Consequently, CI+CIIP presents more physiological relevance to the study of mitochondrial function (6) and is necessary to establish confirmation of a complete and intact electron transport system. CI+CIIP 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. Compared to a correspondent leak state with an equivalent substrate supply, CI+CIIP maintains a lower electrochemical gradient across the inner mitochondrial membrane. That gradient is dictated by the degree of coupling to the phosphorylation system (12, 41). The mitochondrial outer membrane was assessed with the addition of cytochrome c (10 uM). There was no evidence of any compromised mitochondrial membrane integrity across samples measured at baseline with the titration of exogenous cytochrome c (87.4 ± 18.7 to 87.2 ± 18.7 pmol O₂ min⁻¹ mg ww⁻¹, P = 0.56) or following 2 wk of HIT (100.4 ± 23.6 to 100.82 ± 23.8 pmol O₂ min⁻¹ mg ww⁻¹, P = 0.19). Oligomycin was added inhibiting ATP synthase to achieve oligomycin-induced leak respiration (LOMY). The LOMY state is the corresponding leak state to CI+CIIP and is comparable to classic state 4 respiration (8). In LOMY, the chemiosmotic gradient is at maximum, resultant to the combination of maximal substrate supply and inhibition of ATP synthase. Oxygen flux is at a minimum and is representative of proton leak, slip, cation cycling, and overall dyscoupling (6, 12, 45). Phosphorylative restraint of electron transport was assessed by uncoupling ATP synthase (complex 5) from the electron transport system with the titration of the proton ionophore, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP; steps of 0.5 uM) reaching electron transport system (ETS) capacity. The inner mitochondrial membrane potential is completely collapsed, with an open transmembrane proton circuit, in the ETS respiratory state. The uninhibited flow of electrons through the respiratory system can, therefore, indirectly serve as an indication of maximal mitochondrial membrane potential. Rotenone (0.5 uM) and antimycin A (2.5 uM) were added, in sequence, to terminate respiration by inhibiting complex 1 and complex 3 (cytochrome bc₁ complex), respectively. With complex 1 inhibited, electron flow specific to complex 2 (CIIP) can be measured. Prior uncoupling with FCCP has no effect on oxygen flux during CIIP (24), as individual electron input to succinate dehydrogenase does not saturate the Q-cycle. Finally, ascorbate (2 mM) and TMPD (0.5 mM) were simultaneously titrated into the chambers to assess cytochrome c oxidase (COX), complex IV, activity.
CS activity was assayed in muscle lysates using a commercially available CS assay kit (CS0720, Sigma-Aldrich) according to the manufacturer guidelines with minor adjustments. All activities were normalized to mg protein.

Statistics

Changes in VO2 kinetics, respiratory states, and aerobic enzymes following the training period were examined with a Wilcoxon signed rank test given our small sample size as a conservative approach to lower the chance of type I error. Pearson's correlation coefficient (r) was calculated between variables before and after HIT and also between changes in the speed of the VO2 response and changes in HRR variables and enzyme activity. To evaluate if a significant association was present between variables a one tailed test was used throughout the study with the a priori hypothesis that fast VO2 kinetics would be associated with high values for the various variables describing mitochondrial function.

Results

Pulmonary VO2 kinetics

The primary VO2 response was faster after than before HIT (τ: 20.4 ± 4.4 vs. 28.9 ± 6.1 s; P=0.004) whereas end-exercise VO2 (4-6 min: 2261 ± 311 vs. 2304 ± 304 ml min^-1; P=0.16) was not altered (Fig. 2 & 3 and table 1).

High resolution respirometry of skeletal muscle tissue

Higher values relative to before HIT was observed for LN (10 ± 3 vs. 8 ± 1 pmol O2 min^-1 mg ww^-1; P=0.049) (Fig. 4A) and ETFp (26 ± 6 vs. 21 ± 4 pmol O2 min^-1 mg ww^-1; P=0.02) (Fig. 4B). No significant increase in response to HIT was observed for CIp (68 ± 14 vs. 60 ± 13 pmol O2 min^-1 mg ww^-1; P=0.13) (Fig. 4C), CI+CIIP (100 ± 24 vs. 87 ± 19 pmol O2 min^-1 mg ww^-1; P=0.13) (Fig. 4D), LOMY (38 ± 8 vs. 34 ± 6 pmol O2 min^-1 mg ww^-1; P=0.38) (Fig. 4E), ETS (118 ± 30 vs. 99 ± 14 pmol O2 min^-1 mg ww^-1; P=0.06) (Fig. 4F) and CIIP (74 ± 17 vs. 66 ± 11 pmol O2 min^-1 mg ww^-1; P=0.19) (Fig. 4G).

Skeletal muscle oxidative enzyme activity

COX (196 ± 51 vs. 160 ± 35 pmol O2 min^-1 mg ww^-1; P=0.10) (Fig. 4H) and CS (120 ± 35 vs. 128 ± 19 µmol min^-1 mg protein^-1; P=0.65; n=9) (Fig. 4I) was not increased with HIT.

Correlations

Both before and after HIT, fast VO2 kinetics (low values of τ) was associated with large values for ETFp (P<0.05) (Fig. 4B), ETS (P<0.05) (Fig. 4F), and CIIP (P<0.05) (Fig. 4G). Changes following HIT in τ was
associated with changes in CII\(_P\) (P<0.05) (Fig. 5C), but not ETF\(_P\) (Fig. 5A) and ETS (Fig. 5B). In addition, V\(\dot{O}\)\(_2\) kinetics was associated with large values for L\(_N\) before HIT (P<0.01) (Fig. 4A) and large values for LOMY (P<0.05) (Fig. 4E) and CS (P<0.05) (Fig. 2I) after HIT. COX (Fig. 4H) was a poor predictor of fast V\(\dot{O}\)\(_2\) kinetics and no significant associations were present either with Cl\(_P\) (Fig. 4C) or Cl+CIIP (Fig. 4D). The functional gain was only associated with LOMY pre HIT (P<0.05).

**Discussion**

The primary and novel finding in the present study was that dynamic measures of mitochondrial function in the form of high electron transport system capacity (ETS), fatty acid oxidation (ETF\(_P\)) and respiratory capacity specific to complex 2 (CII\(_P\)) was associated with the speed of the primary V\(\dot{O}\)\(_2\) response during moderate intensity cycling, both before and after a 2-week high intensity training intervention in previously untrained subjects. Moreover, ETF\(_P\) increased by training and ETS strongly tended to increase together with the attainment of faster V\(\dot{O}\)\(_2\) kinetics.

The faster V\(\dot{O}\)\(_2\) kinetics during moderate exercise was observed after just six high-intensity training sessions with a total training volume of ~60 min for the entire training period. This is in line with previous studies in which a speeding of the V\(\dot{O}\)\(_2\) response has been observed as little as two to six sessions with either high-intensity low-volume training (1, 38) or low-intensity high-volume training (38, 42). In the present study the faster V\(\dot{O}\)\(_2\) kinetics was attained together with improved oxidative muscle capacity in the form of increased leak respiration in the absence of adenylates (L\(_N\)) (Fig. 4A) and fatty acid oxidative capacity (ETF\(_P\)) (Fig. 4B). In turn the majority of the remaining variables assessed with HRR tended to increase (P>0.05, <0.15), demonstrating that the intense albeit brief training period provided a potent stimulus for the variables measured with HRR. Moreover the observation of associations both before and after HIT between fast V\(\dot{O}\)\(_2\) kinetics and selected HRR measures (Fig. 4B, F, G) do suggest that these associations were not incidental, also in light of the faster V\(\dot{O}\)\(_2\) response attained after HIT together with increases in ETF\(_P\) (Fig. 4B) and the strong tendency for an increase in ETS (Fig. 4F). In future studies it will be of general interest to see if similar findings are observed, and also with the use of higher exercise intensities as well as different subject groups (elite athletes, patient groups). In addition an even larger sample size to increase the statistical power is also considered of importance. But based on the present findings, HRR appear to be a promising tool in elucidating the various components encompassing metabolic inertia and their relation to the speed of primary V\(\dot{O}\)\(_2\) response. Collectively, the present findings suggest that a reduced metabolic inertia was implicated in the faster V\(\dot{O}\)\(_2\) kinetics following the training period. Nonetheless, it cannot be ruled out that the faster V\(\dot{O}\)\(_2\) response following training was at least partly due to higher bulk blood flow (32, 52) and/or improved local distribution of blood flow to the contracting fibres (38) during the initial phase of exercise, or though limited evidence supports that increases in oxygen delivery speeds V\(\dot{O}\)\(_2\) kinetics at moderate intensity (22, 37, 40,
Nevertheless, other factors than HRR derived measures could be important for fast VO₂ kinetics at moderate intensity which is supported by the observation that single HRR measures in the present study “only” explained ~30-50% of the variation in the speed of the VO₂ kinetics. A large proportion of slow twitch fibres could be considered of importance for fast VO₂ kinetics since this fibre type appear to contain more aerobic enzymes than fast twitch fibres (12). This is supported by findings from a cross sectional study in which a large proportion of slow twitch fibres was associated with fast VO₂ kinetics during heavy but not moderate intensity exercise (43). In line herewith, it is worth emphasizing that it likely is the quality (training) of the mitochondria rather than fibre type per se which is of physiologic functional importance, since highly-trained subjects have been found to have similar aerobic enzyme activity in pools of slow- and fast twitch muscles (27). Irrespective of “mitochondrial training status”, creatine phosphate content in the various fibre types may also influence VO₂ kinetics. Accordingly, slow twitch fibres have less creatine phosphate than fast twitch fibres (17) and recently it has been observed that an increase in the creatine phosphate content through dietary interventions leads to slower VO₂ kinetics during intense exercise (11). Moreover, middle distance runners (800-1500-m) had slower VO₂ kinetics during moderate intensity running than long distance runners (5000-10000-m) (29) which may relate in part to an expected higher FT composition in the former group (46) and hence a higher creatine phosphate pool. Change in muscle fibre composition was not assessed in the present study so the potential influence from this cannot be evaluated. Uncoupling protein 3 (UCP3) may influence metabolism during rest and exercise (50). Therefore, faster VO₂ kinetics in theory could arise from higher UCP3 levels (higher VO₂ for the same work load in both the transient phase and during steady state), but that seems unlikely since UCP3 has been found to be lowered in the endurance trained relative to the untrained state (49).

An increase in LN in response to training (Fig. 4A) suggests loss of coupling efficiency across the electron transport system. Change in mitochondrial efficiency in response to exercise training is far from understood. LN positively correlates with training status ranging from normal individuals to elite athletes when measured near sea level (25). Both findings suggest that improvements in respiratory capacity with training may come at the expense of some loss of mitochondrial efficiency in environments near sea-level. However, the functional significance of a lowering of mitochondrial efficiency (increase in LN) is unclear in light of the unchanged steady state VO₂ after the HIT-period and the finding that highly trained cyclist do not appear to have a lower energy requirement during exercise than untrained subjects (39). Electron coupling efficiency was observed to improve while respiratory capacities decreased in individuals staying at high altitude for 4 weeks (24). Throughout that study subjects maintained their level of fitness. Collectively these findings suggest that the regulation of mitochondrial efficiency may be dependent on the environmental oxygen pressure. To evaluate whether oxygen pressure during training or recovery impacts on changes in VO₂ kinetics through distinct response on LN it will be of interest to conduct a training study on a group of untrained subjects - having a large improvement potential - exposed to either normoxia and hypoxia.
Whether the observed increased fatty acid oxidative capacity after HIT (Fig. 4B) was of importance for facilitating fast VO2 kinetics is not clear, owing to the fact that studies typically evaluated fatty acid turnover either from the bloodstream or muscular stores later in exercise (>5 min) (13, 47) which is a time when the rapid increase in VO2 (0-1 min) has surpassed. Breakdown of intramuscular triacylglycerol during moderate intensity exercise was not apparent after 90 min in one study (47) whereas it tended to be lowered (with ~20%) after 5 min in another study also reporting that uptake of free-fatty acids from the blood amounted to 11% of the energy turnover during 60 min of exercise (13). Thus, the relative contribution to the aerobic energy turnover in the first minute of exercise from fatty acid metabolism is probably low. However, a diet high on fat has recently been reported to slow VO2 kinetics (44), hereby showing that fat as a substrate likely impacts on the speed of the VO2 kinetics. Therefore it seems plausible that a high mitochondrial capacity for oxidizing fat may play a role for the speed of the VO2 response. Moreover, it may be that intramuscular triacylglycerol is a substrate that actually contributes more than previously expected to the increase in VO2 during a rest to moderate exercise transition, which could be caused by the close vicinity to the muscle mitochondria.

ETS represents maximal uncoupled respiration and hence the maximal velocity that electrons can travel from complex I through to IV in mitochondria, independent of efficiency, and this likely reflects maximal oxidative capacity of the muscle (24). In turn it also appears to be a valid indicator of how rapid oxidative metabolism can be accelerated based on the present findings (Fig. 4F). During exercise, respiration is considered to be coupled to ADP phosphorylation, thus it needs to be considered whether oxidative capacity (CI+CIIP, maximal state 3 respiration) is more relevant to an in vivo situation. Nevertheless, ETS and not CI+CIIP was linked with fast VO2 kinetics but is worth noting that ETS and CI+CIIP were associated in the present study (r2=0.58 and 0.90 pre and post HIT, respectively). The correlation of fast VO2 kinetics with the ETS respiratory state, opposed to CI+CIIP, may reiterate implications of our data suggested by the correlation of LN. Adaptation to exercise training, especially that observed following several sessions of high-intensity low-volume exercise near sea-level, may come at the expense of mitochondrial efficiency and thus, fast VO2 kinetics and ETS correlate better than measures of CI+CIIP. These interpretations require further research.

Maximal activities of COX and CS were not augmented by training, although a significant increase in COX was identified in the larger sample size that the subjects in the present study was a part of (26). In training studies of similar duration, COX activity has been observed to increase with training (7, 36). CS was not elevated by training which has been observed by some (42) but not all training studies of similar short duration (7, 36, 53). In the present study COX, an established biomarker of mitochondrial volume density (35), was a poor predictor of fast VO2 kinetics both before and after the intervention suggesting that this enzyme per se is not a good marker of the metabolic inertia in untrained subjects. CS activity after the
training period was associated with fast \( \dot{V}O_2 \) kinetics but since this was not the case before the intervention this may reflect that the association post HIT was incidental or measuring error in CS activity pre HIT. During aerobic metabolism, CS is a catalyst early in the Krebs cycle whereas COX is a catalyst later in the oxidative phosphorylation. The lack of association between COX and the speed of the \( \dot{V}O_2 \) response do suggest that this step late in oxidative phosphorylation is not limiting but as mentioned previously caution is warranted due to the moderate sample size (n=10), and thus it will be of interest to see if a similar finding is present in future studies with a larger sample size. Studies performed on trained cyclists have reported that the maximal enzyme activity is an insensitive measure of endurance performance (10, 23, 54). Since HRR derived measures but not enzyme activity improved together with faster \( \dot{V}O_2 \) kinetics it could be argued that HRR provide a better reflection of changes occurring in vivo than the isolated study of maximal activities of oxidative enzymes.

Pulmonary \( \dot{V}O_2 \) during steady state was not reduced with training (Fig. 2 & 3B), but eight of the ten subjects had a lower \( \dot{V}O_2 \) relative to before HIT (range 13-155 ml min\(^{-1}\)). A cross-sectional study has found that the energy requirement for constant load exercise is not different between world-class cyclists and untrained individuals (39), although a lowering of steady state \( \dot{V}O_2 \) has been reported in some training studies (38, 58). The absolute \( \dot{V}O_2 \) during steady state (~2300 ml min\(^{-1}\) for 110 W) was high in the present study compared with other studies with similar work rates (1, 14, 44). This likely relates to the \( \dot{V}O_2 \) equipment measuring to high values since no subjects experienced any slow component (delta \( \dot{V}O_2 \) from 4-6 min) being 21 ± 49 pre HIT (range -53 to 107 ml min\(^{-1}\)) and -20 ± 42 post HIT (range: -98 to 51 ml min\(^{-1}\)) (Table 1). In turn, since the \( \dot{V}O_2 \) baseline and amplitude are used to calculate tau (time to attain 63% of the amplitude), any small measuring noise in absolute values is not expected to affect the calculation of tau and hence the associations with HRR variables in the present study.

During HRR the respiratory chambers are hyper-oxygenated and do not represent a limitation for respiration of the muscle fibres (15). Likewise, oxygen delivery appears to more than adequate in the exercising human muscle during moderate intensity exercise (5, 16, 40), thus oxygen delivery to the contracting fibres may therefore be comparable in HRR and moderate cycling as in the present study. Still, if training improved either bulk oxygen delivery or distribution - as suggested in other studies (32, 28, 52) - the abundance of oxygen during HRR is not truly reflective of in vivo conditions, at least in the untrained state. Thus, we can not exclude that an improvement in local oxygenation can have impacted on in vivo conditions for respiration (e.g. ADP levels and reactive oxygen species) in the mitochondria during cycle exercise post HIT, whereas conditions in vitro during HRR has been similar both pre and post HIT. Nevertheless, the HRR data do show that a number of the respiratory steps are improved with training (Fig. 4A, B) suggesting that the mitochondria are capable of a higher respiration rate and this is also mirrored by the faster \( \dot{V}O_2 \) kinetics post HIT.
In a training study, several factors are changed. Therefore the precise contribution from a single variable (eg. in HRR) to the measured change in the in vivo VO₂ response is not easy to quantify. As such, the present study do not provide the possibility to manipulate with variables as in computer modelling studies of respiration (30). Nevertheless, the findings do point towards which links in the respiratory chain assessed with HRR appear to impact the most on the speed of the VO₂ response measured in vivo. Respiration during HRR is expressed in relation to the amount of muscle. It is considered unlikely that all fibres in the quadriceps muscle are active during moderate exercise as used in the present study. In turn, the intense nature of the HIT intervention likely resulted in mitochondrial adaptations in muscle fibres in high order motor units (51), but even with this limitation HRR appear to reflect the functional capacity of the muscle in terms of obtaining a rapid increase in VO₂ during exercise. Yet, it could be that associations between HRR variables and VO₂ kinetics would be stronger after a moderate intensity training intervention since the confounding influence from adaptations in high order motor units would be minimized.

In summary, two weeks of high intensity training in untrained subjects resulted in faster VO₂ kinetics during moderate intensity cycling together with improved fatty acid oxidation and leak respiration in the absence of adenylates assessed with high-resolution respirometry, whereas maximal activity of CS and COX-4 and energy turnover during steady state conditions was unchanged. In turn, electron transport system capacity, fatty acid oxidation and electron transport specific to complex 2 appeared to be good predictors of fast VO₂ kinetics both before and after the training intervention.


Figure & Table legends

Figure 1:
Changes in oxygen concentration (left; high starting values) and oxygen flux (right; low starting values) during high-resolution respirometry used for evaluation of mitochondrial function in a representative subject. See methods section for details.

Figure 2:
TOP: Pulmonary oxygen uptake (\(\dot{V}O_2\)) shown in 5-s intervals for graphical clarity with exponential fits of the response during 6-min of cycling at moderate intensity (110 ± 19 W) before (open circles; A) and after (filled circles; B) a short period of high-intensity training in ten untrained subjects. BOTTOM: The modelled \(\dot{V}O_2\) response shown before (dashed line) and after training (full line) in absolute values (C) and expressed relative to end exercise \(\dot{V}O_2\) (4-6 min of exercise; D).

Figure 3:
Mean and individual values for the primary pulmonary oxygen uptake (\(\dot{V}O_2\)) response (\(\tau\); being the time to attain 63% of the amplitude) (A) and steady state energy expenditure from 4-6 min of exercise (B) during 6-min of cycling at moderate intensity (110 ± 19 W) in ten untrained subjects before (pre; open bars) and after (post; filled bars) a short period of high-intensity training.

**Value post significantly different from pre (P<0.01).

Figure 4:
Associations between the speed of the primary pulmonary oxygen uptake response (\(\tau\); being the time to attain 63% of the amplitude) cycling at moderate intensity (110 ± 19 W) and variables describing mitochondrial function in ten untrained subjects before (pre; open circles and bars) and after (post; filled circles and bars) a short period of high-intensity training.

\(L_N\): leak respiration without adenylates (A), \(ETF_p\): capacity for fatty acid oxidation (B), \(CI_p\): respiration capacity specific to complex I in the mitochondria (C), \(CI + CIi\): maximal state 3 respiration OXPHOS capacity (D), \(L_{OMY}\): oligomycin-induced leak respiration (E), \(ETS\): electron transport system capacity (F), \(CII_p\): electron flow specific to complex 2 in the mitochondria (G), \(COX\): cytochrome c oxidase activity in complex IV (H), \(CS\): citrate synthase activity (I, n=9).

*Value post significantly different from pre (P<0.05).
**Figure 5:**

Associations between changes in the speed of the primary pulmonary oxygen uptake response ($\tau$; being the time to attain 63% of the amplitude) cycling at moderate intensity ($110 \pm 19$ W) and changes in ETF$_p$ ($A$; capacity for fatty acid oxidation, ETS (B; electron transport system capacity) and CII$_p$ (C; electron flow specific to complex II in the mitochondria) in ten untrained subjects after a short period of high-intensity training.

**Table 1:**

Changes in pulmonary oxygen uptake ($VO_2$) kinetics during cycling exercise for 6 min at moderate intensity ($110 \pm 19$ W) in ten untrained subjects before (pre) and after (post) a short period of high-intensity training.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (ml min$^{-1}$)</td>
<td>909 ± 121</td>
<td>923 ± 100</td>
</tr>
<tr>
<td>$Td$ (s)</td>
<td>11.4 ± 4.0</td>
<td>17.5 ± 3.3**</td>
</tr>
<tr>
<td>$\tau$ (s)</td>
<td>28.9 ± 6.1</td>
<td>20.4 ± 4.4**</td>
</tr>
<tr>
<td>$A$ (ml min$^{-1}$)</td>
<td>1393 ± 256</td>
<td>1337 ± 286</td>
</tr>
<tr>
<td>End exercise $VO_2$ 4-6 min (ml min$^{-1}$)</td>
<td>2304 ± 304</td>
<td>2261 ± 311</td>
</tr>
<tr>
<td>Delta $VO_2$ 4-6 min (ml min$^{-1}$)</td>
<td>21 ± 49</td>
<td>-20 ± 42</td>
</tr>
</tbody>
</table>

$Td$: Time delay, $\tau$: the time to attain 63% of the amplitude of the response, $A$: amplitude

** Value post significantly different from pre ($P<0.01$).