

## Reduced efficiency, but increased fat oxidation, in mitochondria from human skeletal muscle after 24-h ultraendurance exercise

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**Fernström M, Bakkman L, Tonkonogi M, Shabalina IG, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B, Sahlin K.** Reduced efficiency, but increased fat oxidation, in mitochondria from human skeletal muscle after 24-h ultraendurance exercise. *J Appl Physiol* 102: 1844–1849, 2007. First published January 18, 2007; doi:10.1152/jappphysiol.01173.2006.—The hypothesis that ultraendurance exercise influences muscle mitochondrial function has been investigated. Athletes in ultraendurance performance performed running, kayaking, and cycling at 60% of their peak O<sub>2</sub> consumption for 24 h. Muscle biopsies were taken preexercise (Pre-Ex), postexercise (Post-Ex), and after 28 h of recovery (Rec). Respiration was analyzed in isolated mitochondria during state 3 (coupled to ATP synthesis) and state 4 (noncoupled respiration), with fatty acids alone [palmitoyl carnitine (PC)] or together with pyruvate (Pyr). Electron transport chain activity was measured with NADH in permeabilized mitochondria. State 3 respiration with PC increased Post-Ex by 39 and 41% ( $P < 0.05$ ) when related to mitochondrial protein and to electron transport chain activity, respectively. State 3 respiration with Pyr was not changed ( $P > 0.05$ ). State 4 respiration with PC increased Post-Ex but was lower than Pre-Ex at Rec ( $P < 0.05$  vs. Pre-Ex). Mitochondrial efficiency [amount of added ADP divided by oxygen consumed during state 3 (P/O ratio)] decreased Post-Ex by 9 and 6% ( $P < 0.05$ ) with PC and PC + Pyr, respectively. P/O ratio remained reduced at Rec. Muscle uncoupling protein 3, measured with Western blotting, was not changed Post-Ex but tended to decrease at Rec ( $P = 0.07$  vs. Pre-Ex). In conclusion, extreme endurance exercise decreases mitochondrial efficiency. This will increase oxygen demand and may partly explain the observed elevation in whole body oxygen consumption during standardized exercise (+13%). The increased mitochondrial capacity for PC oxidation indicates plasticity in substrate oxidation at the mitochondrial level, which may be of advantage during prolonged exercise.

P/O ratio; uncoupling protein 3; fatty acid oxidation

DURING THE LAST FEW YEARS, the importance of mitochondrial function for health and performance has been highlighted (17). It is well known that oxidation of fatty acids (FA) is augmented and lactate formation is reduced during exercise after endurance training. This is explained by an increased mitochondrial density in skeletal muscle and a concomitant increased activity of oxidative enzymes (15). Whole body lipid oxidation increases progressively during prolonged exercise, even when work rate is maintained constant. The increased lipid oxidation is in part related to increased plasma levels of FA and thus

increased availability of FA. The control of lipid oxidation during exercise is likely exerted at several steps (31), but it is not completely understood. Recent studies have shown that the capacity of mitochondria to oxidize FA in vitro was correlated to whole body FA oxidation during low-intensity exercise (16, 28). This demonstrates that not only mitochondrial quantity but also mitochondrial quality influences whole body FA oxidation and suggests that intrinsic differences in mitochondrial quality between subjects can influence fuel utilization in vivo. The performance during prolonged exercise is dependent on the ability to use FA as a fuel, and an increased capacity of mitochondria to oxidize FA would, therefore, be advantageous. We hypothesized that ultraendurance exercise can upregulate the mitochondrial capacity to oxidize FA.

Strenuous exercise is associated with increased oxidative stress (18), which results in increased lipid peroxidation (8, 23) and tissue damage (8, 23). Both in vitro and in vivo studies suggest that mitochondrial function can be affected by oxidative stress, but there is considerable controversy as to whether exercise can impair mitochondrial function (for references, see Ref. 18). A phenomenon that has attained considerable interest is the slow increase in oxygen consumption ( $\dot{V}O_2$ ) (oxygen drift), which occurs during submaximal exercise at a constant work rate. Several hypotheses have been proposed to explain this finding, including decreased efficiency of oxidative phosphorylation, i.e., mitochondrial efficiency (38, 39). Previous studies in rats demonstrate that mitochondrial efficiency was unchanged after exhaustive running for 2 h (32). However, the effect of prolonged exercise on mitochondrial efficiency has not been studied previously in humans.

Specific proteins present in the mitochondrial membrane [e.g., uncoupling protein 3 (UCP3)] may facilitate back-leakage of protons and thus decrease the efficiency of oxidative phosphorylation. Expression of UCP3 is increased during conditions of high plasma levels of FA, such as during starvation or after high-fat diet (13), and decreased by endurance training (12, 14). The adaptation is rapid, and an increase in UCP3 protein has been observed in human muscle already 36 h after pharmacological interference with FA metabolism (29). UCP3 mRNA have been reported to be increased after exercise (25), but protein expression of UCP3 has not been shown to be changed by acute exercise (12, 14). Long-term strenuous exercise is associated with prolonged exposure to increased levels

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of FA, which makes this an interesting model for studies of UCP3 protein expression and mitochondrial uncoupling.

In the present study, we have investigated ultraendurance athletes who performed exercise for 24 h at ~60% of their individual peak  $\dot{V}O_2$  ( $\dot{V}O_{2\text{ peak}}$ ). The purpose of the study was to investigate functional aspects of mitochondria isolated from muscle biopsies taken Pre-Ex and Post-Ex. We have especially investigated the hypothesis that, after ultraendurance exercise, 1) mitochondrial efficiency (measured in vitro) is reduced; 2) UCP3 protein expression and mitochondrial noncoupled respiration (state 4) is increased; and 3) the capacity of mitochondria to oxidize fat is increased.

## MATERIALS AND METHODS

All subjects were fully informed about the procedure and of possible risks and discomfort involved in the experiment; they were also informed about their right to terminate the experiment at any time point. The Ethics Committee of the Karolinska Institutet, Stockholm, Sweden, approved the design of the study.

**Subjects.** All participants of the study were men and belong to the elite Swedish ultraendurance performance athletes. Details of subject characteristics are shown in Table 1. The subjects have previously belonged to the Swedish elite in various sports and have been training for 3–9 yr within extreme endurance exercise. All subjects belong to the Swedish top 10 in extreme endurance exercise, and eight of the subjects have recent merits in world championship within the top 10.

**Pretests.** The athletes performed pretests on a kayak ergometer (Dansprint aps, Hovide, Denmark), cycle ergometer (Monark ergo-med 893E, Monark Exercise, Varberg, Sweden), and treadmill (Rodby Electronics, Vansbro, Sweden). After a brief warm-up, the work rate was raised every minute above the estimated work rate to reach  $\dot{V}O_{2\text{ peak}}$ .  $\dot{V}O_2$  was measured with an online system (AMIS 2001, Inovision A/S Odense, Denmark). All subjects also performed lactate threshold tests (4 mmol/l) during kayaking, cycling, and running, to make sure that they worked below their individual threshold during the main study.

**Test protocol for the main study.** The athletes arrived at the test laboratory in the morning after 1 night of fasting (with access to water) and preceding 3 days of standardized food intake, 4,250 kcal/day [52% carbohydrates (CHO), 31% fat, and 18% protein]. Body composition was measured using the air displacement methodology (Dempster, 1995, no. 29) on BodPod S/T (Life Measuring USA). A polyethylene catheter was inserted into a forearm vein before exercise, and blood samples were taken in heparinized tubes. Subjects exercised for 24 h and performed 12 blocks of exercise (4× running, 4× kayaking, and 4× cycling) at a work rate corresponding to ~60% of their individual  $\dot{V}O_{2\text{ peak}}$ . Each block consisted of 110 min of exercise followed by 10 min of rest. The last exercise bout was cycling. The subjects were allowed to eat standardized food during the

10-min breaks (59% CHO, 29% fat, and 12% protein), aiming to give each person 50% of the estimated energy expenditure. They were allowed to drink water freely during the experiment. Heart rate was recorded using a heart rate monitor (Polar S610i, Polar Electro Oy, Kempele, Finland).  $\dot{V}O_2$  and respiratory exchange ratio (RER) were during the main test measured on standardized load with the Douglas bag technique. The volume of the expired air was measured with a Tissot Spirometer (WE Collins, Braintree, MA), and fractions of oxygen and carbon dioxide were determined using a Beckman S-3A and LB2 gas analyzer (Beckman Instruments, Fullerton, CA). Subjects performed a standardized exercise test before (Pre-Ex, 10-min duration), immediately after (Post-Ex, 20-min duration), and 28 h postexercise (Rec, 10-min duration). Subjects cycled at the same absolute work rate (40–50% of  $\dot{V}O_{2\text{ peak}}$ ), and  $\dot{V}O_2$  and  $CO_2$  production were measured during steady-state conditions. Blood samples were taken at the end of the standardized exercise tests and during the last 15 min of the 24-h endurance exercise period.

**Muscle biopsies and isolation of mitochondria.** A muscle biopsy was taken 5–12 days before the main experiment (Pre-Ex), <30 min after terminating the last block (i.e., cycling exercise) of ultraendurance exercise (Post-Ex), and 28 h after termination of exercise (Rec). During a period of 24 h before the pretest and 28 h before the postexercise biopsy, athletes had standardized food containing 58% CHO, 25% fat, and 17% protein. None of the subjects had any food 3 h before the biopsy. All muscle biopsy samples were taken from the vastus lateralis muscle. After local anesthesia (1–2 ml Carbocain; 20 mg/ml, Astra), an incision was made through the skin and fascia, and the biopsy was taken using a Weil Blackesly conchotome (Wisex, Mölndal, Sweden). The muscle biopsy was divided into portions. One portion (average 46 mg) was used for assay of proteins [UCP3, adenine nucleotide translocator, and myosin heavy chain (MHC)] and was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Another portion (average 110 mg) was used to isolate mitochondria as previously described (34). Muscle specimens were disintegrated with scissors and treated with 0.4 mg/ml protease (Sigma P-4789), followed by homogenization and differential centrifugation. The final mitochondria pellet was resuspended in a buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris-base, 0.1 mM EDTA, and 0.2% bovine serum albumin, pH 7.4) and kept on ice until analysis of respiratory activity.

**Mitochondrial respiration.**  $\dot{V}O_2$  was measured using a Clark-type electrode (Hansatech DW1; Hansatech, King's Lynn, Norfolk, UK) at  $25^\circ\text{C}$ . Respiration was analyzed in an oxygraph medium (225 mM mannitol, 75 mM sucrose, 10 mM Tris-base, 10 mM  $K_2HPO_4$ , 0.1 mM EDTA, 80  $\mu\text{M}$   $MgCl_2$ , and 0.2% bovine serum albumin, pH 7.1). Mitochondrial suspension (~60  $\mu\text{g}$  protein) was added to the reaction medium, and coupled respiration (state 3) was initiated by the addition of ADP (final concentration 0.3 mM). State 3 respiration was measured twice, first with 10  $\mu\text{M}$  palmitoyl carnitine (PC) and 2 mM malate, and next after further addition of 5  $\mu\text{M}$  PC and 4 mM pyruvate (Pyr). The rationale for using PC instead of palmitate is to bypass the influence of PC transferase-1 (CPT-1) on respiration rate and to enable measurements of P/O ratio (amount of added ADP divided by oxygen consumed during state 3). The respiratory control index was calculated as the ratio between state 3 and state 4 respirations. The efficiency of oxidative phosphorylation (P/O ratio) was calculated as the amount of added ADP divided by the oxygen consumed during state 3 (6). Respiration data have been corrected for electrode drift. After measurements of respiration, the oxygraph solution with mitochondria was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analyzed for maximal electron transport chain (ETC) activity. ETC activity was measured (2–3 days after the main experiment) after permeabilizing mitochondria with 10  $\mu\text{g}/\text{ml}$  alamethicin (Sigma A-3665), which was added to the oxygraph solution, together with NADH (450  $\mu\text{mol}/\text{l}$ ) and cytochrome *c* (2  $\mu\text{mol}/\text{l}$ ) (19). Respiration rate, measured at  $25^\circ\text{C}$ , increased rapidly (within 1 min) to a stable value. Maximal ETC activity was three to four times higher than state 3 respiration with Pyr, which is consistent with that reported in

Table 1. Subject characteristics

Age, yr	27 (24–32)
Body weight, kg	80.5 (73.4–85.4)
Height, cm	182 (175–186)
BMI, $\text{kg}/\text{m}^2$	24.2 (22.3–28.8)
Body fat, %weight	16.9 (10.8–26.1)
$\dot{V}O_{2\text{ peak}}$ , $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	61.9 (52.7–69.8)
Type I fibers, MHC I%	56.3 (48.0–74.0)

Values are means and ranges (in parentheses) from 9 male subjects. Myosin heavy chain (MHC) I% is the average value from three biopsies [preexercise (Pre-Ex), postexercise (Post-Ex), and 28 h Post-Ex]. All subjects belong to the Swedish top 10 in extreme endurance exercise, and 8 of the subjects have recent merits in world championship within the top 10. BMI, body mass index;  $\dot{V}O_{2\text{ peak}}$ , peak  $O_2$  consumption.

freeze-permeabilized mitochondria from human muscle (27). However, freeze permeabilization appears to be a less reliable technique, since the number of freeze-thaw cycles required to obtain peak values of respiration varies between different samples (27).

**Protein expression, glucose, and FA.** Portions of freeze-dried muscle were cleaned from blood, fat, and connective tissue and homogenized in cold lyses buffer with protease inhibitors. Protein concentration was determined in muscle homogenate and in the mitochondrial suspension using Pierce Protein Assay Kit (kit no. 23223, Pierce, Rockford, IL). All respiratory parameters were expressed per mitochondrial protein. Values have not been corrected for the protein content of the oxygraph solution (0.2% BSA), which corresponds to 1.4–1.6% of the measured protein. Plasma glucose was measured by the Synchron LX system that determines glucose concentration by an oxygen rate method with Beckman oxygen electrode. Plasma FA content was measured with Wako NEFA C test kit (no. 994-75409 D).

**Fiber-type distribution.** MHC composition was analyzed as previously described (7) and modified for humans (1). Briefly, muscle homogenate (80  $\mu$ l) was mixed with 200  $\mu$ l of sample buffer (10% glycerol, 5%  $\beta$ -mercaptoethanol, 2.3% SDS, 62.5 mM Tris-base, and 0.2% bromophenol blue at pH 6.8). The homogenate was denatured by boiling at 100°C for 3 min and loaded on a SDS-PAGE gel [8% polyacrylamide (100:1 acrylamide-bisacrylamide), 30% glycerol, 67.5 mM Tris-base, 0.4% SDS, and 0.1 M glycine]. Gels were run at 80 V for at least 42 h at 4°C, and MHC bands were made visible by Coomassie staining (Sigma B-0149). The gels were scanned on Gel Doc 2000 (Bio-Rad). MHC II and MHC I bands were quantified using Quantity One 1-D Analysing software (Bio-Rad). MHC I is expressed in percentage of total MHC.

**Measurements of UCP3.** Muscle homogenate was solubilized in Laemmli sample buffer with  $\beta$ -mercaptoethanol and denatured by boiling. Protein (100  $\mu$ g) was added per lane on 12% polyacrylamide gels and separated by SDS-PAGE for 60 min at 135 V. The separated polypeptides were transferred to a polyvinylidene difluoride membrane at 10 V for 60 min and blocked in Tris-buffered saline (20 mmol/l Tris, 140 mmol/l NaCl, pH 7.6) with 5% nonfat milk. Membranes were incubated overnight with polyclonal antibody against UCP3 (Chemicon AB 3046), diluted 1:1,000, washed, and incubated with secondary antibody goat anti-rabbit (IgG-horseradish peroxidase, no. sc-2030, Santa Cruz). The membrane was again washed and incubated with chemiluminescence detection reagent enhanced chemiluminescence (no. RPN 2106, Amersham). Finally, an X-ray film was exposed to the membrane for 10 min. The optical density of the bands was quantified by using Quantity One 1-D analyzing software (Bio-Rad). The intensity of bands will vary between different gels, due to methodological factors. Samples from the same subject (Pre-Ex, Post-Ex, and Rec) were analyzed on the same gel. To enable comparison between different gels (i.e., between subjects), the density of the bands was related to a standard muscle sample, loaded on each gel. Samples were analyzed in duplicate.

**Statistics.** All data are presented as means  $\pm$  SE. Data were tested for normal distribution before parametric statistics were performed. Differences between time points were tested with one-way repeated-measures ANOVA. If a difference was detected, the location of significance was determined with Fisher least significant difference post hoc test. Correlation between two variables was tested with correlation analysis. Statistic significance was set to  $P < 0.05$ .

## RESULTS

Plasma FA increased fourfold at the end of endurance exercise ( $P < 0.05$ ) and returned to the Pre-Ex value 28 h Post-Ex, whereas plasma glucose was unchanged (Table 2).  $\dot{V}O_2$ , measured during cycling at the same absolute work rate for each subject (40–50% of  $\dot{V}O_{2\text{peak}}$ ), was 13% higher at the end of endurance exercise and remained 7% higher 28 h

Table 2. Effect of ultraendurance exercise on metabolic parameters during a standard exercise test

	Pre-Ex	Post-Ex	28 h Post-Ex
FA, mmol/l	0.30 $\pm$ 0.04	1.24 $\pm$ 0.12*	0.24 $\pm$ 0.03†
Glucose, mmol/l	4.6 $\pm$ 0.1	4.4 $\pm$ 0.4	4.7 $\pm$ 0.1
RER	0.89 $\pm$ 0.02	0.84 $\pm$ 0.01*	0.89 $\pm$ 0.01†
$\dot{V}O_2$ , l/min	2.26 $\pm$ 0.06	2.55 $\pm$ 0.07*	2.41 $\pm$ 0.07*†

Values are means  $\pm$  SE from 9 subjects. The parameters were measured during cycle exercise at the same absolute work rate (125–175 W), corresponding to 40–50% of  $\dot{V}O_{2\text{peak}}$ . All measurements were made during steady-state conditions with exercise duration of 10–20 min (for details see MATERIALS AND METHODS). Fatty acid (FA) and glucose were measured in plasma. RER, respiratory exchange ratio;  $\dot{V}O_2$ , O<sub>2</sub> consumption. \* $P < 0.05$  vs. Pre-Ex. † $P < 0.05$  vs. Post-Ex.

Post-Ex (both  $P < 0.05$  vs. Pre-Ex; Table 2). RER decreased during endurance exercise ( $P < 0.05$  vs. Pre-Ex) but was reversed to the initial value 28 h Post-Ex (not significant vs. Pre-Ex).

Mitochondrial ADP stimulated respiration (state 3) and non-coupled respiration (state 4) were first measured with PC alone and then together with Pyr. The rationale for using PC instead of palmitate is to bypass the influence of CPT-1 on respiration rate and to enable measurements of P/O ratio. State 3 respiration with PC increased by 39% Post-Ex ( $P < 0.05$ ) but was reversed to the Pre-Ex value at Rec (Fig. 1). State 3 respiration with PC + Pyr was not significantly changed after exercise. State 4 respiration was higher Post-Ex with PC ( $P < 0.05$  vs. Pre-Ex; Fig. 1) but decreased below the Pre-Ex value at Rec with both PC and PC + Pyr ( $P < 0.05$ ). The relative mitochondrial FA oxidation [PC/(PC + Pyr)] was 0.68  $\pm$  0.05 Pre-Ex and varied greatly between subjects (range 0.46–0.92). The relative mitochondrial FA oxidation increased markedly Post-Ex to 0.87  $\pm$  0.04 ( $P < 0.05$ ) but was not different from the initial level at Rec (0.74  $\pm$  0.06; nonsignificant vs. Pre-Ex) (Table 3). Relative FA oxidation was not significantly correlated to fiber-type composition measured as MHC I ( $r = 0.20$  Pre-Ex;  $r = 0.20$  Post-Ex;  $r = 0.34$  Rec). ETC activity was not significantly changed by ultraendurance exercise (Table 3). State 3 respiration (with PC) related to ETC activity increased Post-Ex ( $P < 0.05$  vs. Pre-Ex) and remained elevated at Rec. Mitochondrial efficiency (P/O ratio) decreased Post-Ex when measured both with PC (–9%,  $P < 0.05$ ) and with PC + Pyr (–6%,  $P < 0.05$ ) and remained reduced at Rec (Fig. 2).

Protein expression of UCP3 (arbitrary units) related to the Pre-Ex value (=100%) was not different Post-Ex (97  $\pm$  12%) but tended to be lower at Rec (81  $\pm$  13%) ( $P = 0.07$  vs. Pre-Ex) (Fig. 3). No correlation was observed between UCP3 and MHC I ( $r = 0.18$ ), or between UCP3 and relative FA oxidation ( $r = 0.04$  Pre-Ex,  $r = 0.53$  Post-Ex, and  $r = 0.09$  Rec), or between UCP3 and state 4 respiration ( $r = -0.24$  with PC,  $r = -0.04$  with PC + Pyr).

## DISCUSSION

The most important novel findings of the present study were that 1) mitochondrial efficiency decreased after ultraendurance exercise and remained reduced after 28 h recovery; 2) mitochondrial FA oxidation (state 3) and relative FA oxidation [PC/(PC + Pyr)] increased Post-Ex; and 3) noncoupled respiration rate (state 4) was reduced after 28 h of recovery.



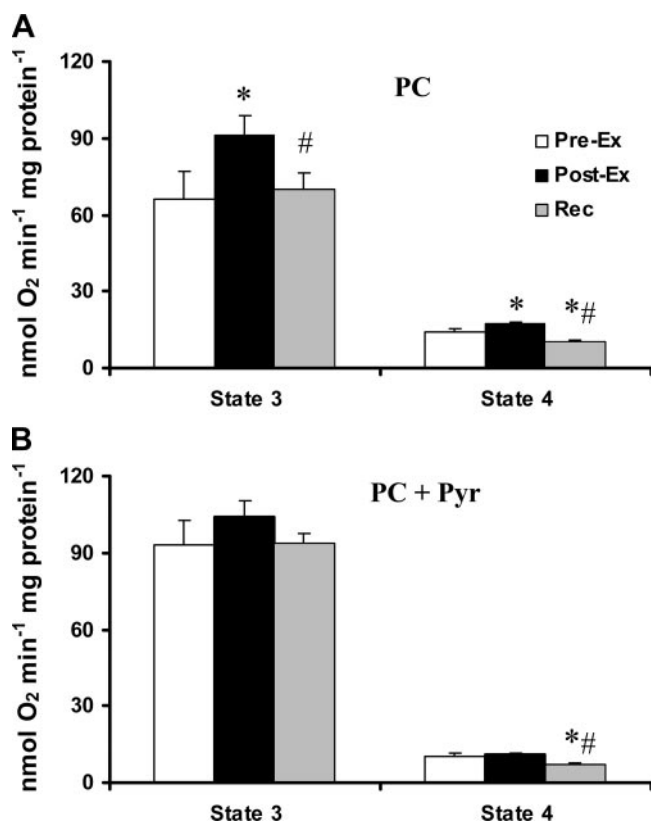


Fig. 1. Effect of ultraendurance exercise on mitochondrial respiration. State 3 corresponds to maximal ADP-stimulated respiration rate and state 4 to the respiration rate in the absence of ADP. Respiration was measured with palmitoyl carnitine (PC) + malate and after further addition of PC and pyruvate (Pyr). Pre-Ex, before exercise; Post-Ex, after exercise; Rec, 28 h after exercise. Values are means ± SE from 9 subjects. \**P* < 0.05 vs. Pre-Ex. #*P* < 0.05 vs. Post-Ex.

*Reduced mitochondrial efficiency.* A major finding of the present study was that mitochondrial efficiency (P/O ratio measured in vitro) was reduced after ultraendurance exercise. Previous studies have shown that mitochondrial P/O ratio is a conservative parameter, which remains stable after high-intensity exhaustive exercise (26, 36), intermittent static contractions to fatigue (28), and after endurance training (35). To our

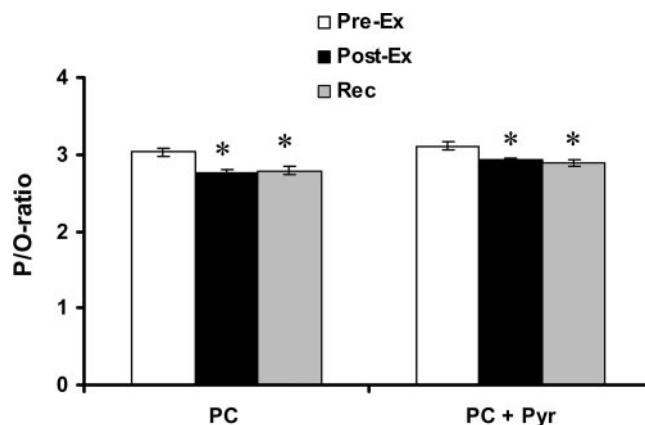


Fig. 2. Effect of ultraendurance exercise on mitochondrial efficiency (P/O ratio). P/O ratio was calculated as the ratio between added ADP and consumed oxygen during respiration with PC + malate and with PC and Pyr. Values are means ± SE from 9 subjects (*n* = 8 at Rec). \**P* < 0.05 vs. Pre-Ex.

knowledge, there is no previous study in humans where values of P/O ratio have been reported after prolonged exercise. Exercise is known to be associated with increased generation of reactive oxygen species (18), which, if maintained for a prolonged period of time, may exhaust the antioxidative defense and lead to oxidative damage of cellular components. Previous studies have shown that mitochondria are more vulnerable to oxidative stress after training in both rats (20) and humans (35). Although the present subjects were well trained, belonging to the Swedish national team in this sport, they may, therefore, still be susceptible to oxidative stress at the mitochondrial level. Additionally, plasma FA were increased four-fold at the end of exercise (Table 2) and maintained high during a large part of the 24-h exercise period (data not shown). It is well known that FA may uncouple oxidative phosphorylation in vitro, and we have previously shown that

Table 3. Effects of ultraendurance exercise on mitochondrial respiratory parameters

Respiratory Parameter	Substrate	Pre-Ex	Post-Ex	Rec
Maximal ETC activity, nmol O <sub>2</sub> ·min <sup>-1</sup> ·mg protein <sup>-1</sup>	NADH	358±30	357±24	312±18
State 3/ETC, %	PC	18.1±2.1	25.6±1.6*	22.9±2.5*
	PC + Pyr	26.2±1.9	29.4±1.0	30.6±1.9
State 4/ETC, %	PC	4.0±0.5	4.8±0.2	3.4±0.3†
	PC + Pyr	2.9±0.2	3.2±0.2	2.3±0.3†
Relative FA oxidation, state 3 PC/[state 3 (PC + Pyr)]		0.68±0.05	0.87±0.04*	0.74±0.06

Values are means ± SE from 9 subjects. Maximal electron transport chain (ETC) activity was measured in permeabilized mitochondria. State 3 is maximal ADP stimulated respiration rate with palmitoyl carnitine alone (PC) or together with pyruvate (PC + Pyr). State 4 is respiration rate when all added ADP is phosphorylated to ATP. \**P* < 0.05 vs. Pre-Ex. †*P* < 0.05 vs. Post-Ex.

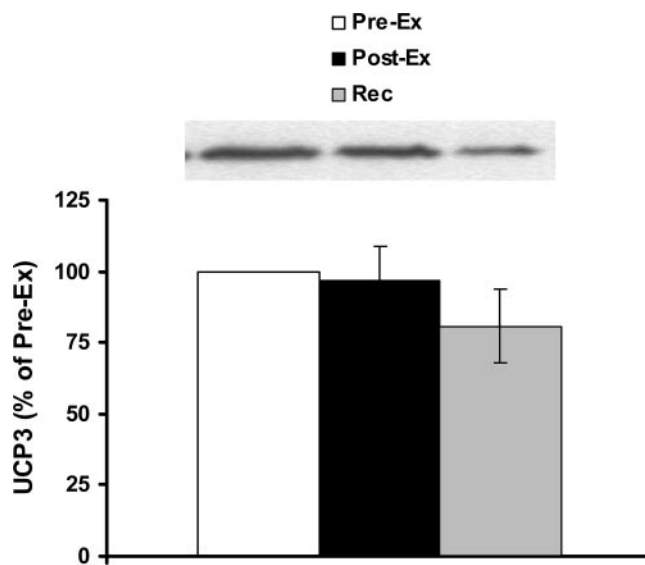


Fig. 3. Effect of ultraendurance exercise on uncoupling protein 3 (UCP3) protein expression. UCP3 protein expression is expressed in arbitrary units and referred to the Pre-Ex value (=100%). Values are means ± SE from 9 subjects. *P* = 0.07 vs. Pre-Ex. Inset above bars shows original traces of Western blot from one subject.

FA-induced increase in state 4 respiration of isolated mitochondria is augmented after training (33). The combination of prolonged exposure to oxidative stress and elevated FA may deteriorate mitochondrial protein or lipid components and might be the cause of reduced P/O ratio.

Decreased P/O ratio could result from increased back leakage of protons, which is reflected by state 4 respiration. However, the extent of proton leakage is influenced by the electrochemical proton gradient, which will be lower during state 3 (during which P/O ratio is measured) than during state 4. Furthermore, P/O ratio is, in addition to proton leakage, influenced by the degree of electron slippage in ETC (i.e., decreased proton efflux per flux of electrons) and the efficiency of ATP synthase complex (proton flux through ATP synthase per ATP formed). The link between state 4 respiration and P/O ratio is, therefore, indirect and not compulsory. The results from this study show that P/O ratio was reduced 28 h Post-Ex, despite a reduction in state 4 respiration. The exercise-induced decrease in P/O ratio can thus not be explained by an increased proton conductance of the mitochondrial membrane as reflected by state 4 respiration.

*Increased capacity of mitochondrial FA oxidation.* Another intriguing finding was that mitochondrial capacity of FA oxidation was increased in muscle samples taken Post-Ex when related to mitochondrial protein (+39%), ETC activity (+41%), and PC + Pyr respiration (+28%). The upregulation of FA oxidation in isolated mitochondria may be caused by an increased transport of FA into mitochondria or by increased metabolism within mitochondria. The present results demonstrate that maximal ETC activity was unchanged, which suggests that complexes I, III, and IV were not affected by exercise. The activity of complex II and  $\beta$ -oxidation was not investigated, and they remain potential sites of control. CPT-1 is involved in the inward transport of long-chain FA and is considered to be a key control site of mitochondrial FA oxidation. However, when PC is used as a mitochondrial substrate, CPT-1 is bypassed, and the site of control is located downstream. FAT/CD36 is a well-characterized protein located in intracellular pools, which can be recruited to the plasma membrane in response to muscle contraction (4) or insulin (21). Recently, FAT/CD36 was found in human skeletal muscle mitochondria (3). FAT/CD36 may work in conjunction with CPT-1 to facilitate uptake and oxidation of long-chain FA in human skeletal muscle during endurance exercise. Indeed, it has been shown that 120-min cycling profoundly increases FAT/CD36 in the mitochondrial membrane and that the increase correlates to mitochondrial oxidation of palmitate and whole body lipid oxidation (16). FAT/CD36 was not measured in this study, but an increased translocation to mitochondria is a plausible explanation for the increased mitochondrial relative FA oxidation after ultraendurance exercise. However, the presence of FAT/CD36 in mitochondria is debated (37), and further studies are required to confirm the presence of FAT/CD36 in mitochondria.

*Role of UCP3.* The capacity of UCP3 to uncouple respiration from ATP synthesis and thereby influence energy expenditure is debated (2, 9, 24). The lower state 4 respiration 28 h Post-Ex is consistent with the tendency toward reduced UCP3 protein expression ( $P = 0.07$  vs. Pre-Ex) but, as discussed above, not compatible with the reduced P/O ratio. There is evidence that UCP3 is activated by superoxide and FA (5, 11).

It can, therefore, not be excluded that the degree of UCP3-related uncoupling in vivo is different than that expected from changes in UCP3 protein expression and state 4 respiration measured in isolated mitochondria. An alternative suggested role for UCP3 is to facilitate FA oxidation (22). Relative FA oxidation showed a large variability between subjects, but it was not correlated to UCP3 protein expression in this study. Furthermore, relative FA oxidation increased Post-Ex, but UCP3 protein was not changed. These findings do not support a direct role of UCP3 in FA oxidation.

*Physiological perspectives.* Assuming that the ATP demand remains constant, one would expect that the observed 6–9% decrease in P/O ratio after ultraendurance exercise should be expressed as an increased oxygen demand during exercise. Indeed, whole body  $\dot{V}O_2$  measured during steady-state cycling at a standardized work rate was 13% higher Post-Ex and 7% higher 28 h Post-Ex, respectively. The duration of the standard exercise test was longer when the test was performed Post-Ex than Pre-Ex and 28 h Post-Ex (20 vs. 10 min), but this is unlikely to affect the results due to the low work rate (40–50% of  $\dot{V}O_{2\text{ peak}}$ ) and prevailing steady-state conditions. The lower RER at the end of ultraendurance exercise corresponds to an increase in FA oxidation from 37 to 53%. The oxygen demand in vivo is ~10% higher with FA than with CHO, and the increased FA oxidation would thus, in this case, correspond to an additional increase in Post-Ex oxygen cost by ~2%.

There is evidence that mitochondrial ability to oxidize FA influences whole body FA oxidation during low-moderate-intensity exercise. First, the rate of mitochondrial palmitate oxidation during state 4 correlated with whole body fat oxidation during exercise at 60%  $\dot{V}O_{2\text{ peak}}$  (16). Second, we have recently observed a correlation between relative FA oxidation during state 3 in isolated mitochondria and whole body relative FA oxidation during low-intensity exercise (28). The increase in relative rate of FA oxidation, observed in isolated mitochondria after 24-h endurance exercise, may, therefore, be expressed in vivo and, together with increased supply of FA (plasma FA increased fourfold), contribute to a shift in fuel utilization. An increased fat oxidation during exercise is of physiological advantage, since this would spare the limited stores of CHO.

*Summary.* This is the first study of mitochondrial function in response to ultraendurance exercise. Mitochondrial efficiency during state 3 was reduced after ultraendurance exercise and does confirm our hypothesis. State 4 respiration was lower 28 h Post-Ex, and a trend toward reduced UCP3 protein expression was observed ( $P = 0.07$ ). The increased oxygen cost during exercise, which was observed, may, in part, be explained by reduced mitochondrial efficiency, but it cannot be explained by an increased expression of UCP3 protein. Mitochondrial capacity for FA oxidation was markedly increased Post-Ex and may be of physiological advantage by influencing whole body fuel utilization.

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