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

Maria Fernström,1,2 Linda Bakkman,1,2 Michail Tonkonogi,2,3 Irina G. Shabalina,2 Zinaida Rozhdestvenskaya,2 C. Mikael Mattsson,1,2 Jonas K. Enqvist,1,2 Björn Ekblom,1,2 and Kent Sahlin1,2,4

1Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm; 2Åstrands Laboratory, The Swedish School of Sport and Health Sciences (GIH), Stockholm; and 3University of Dalarna, Falun, Sweden; and 4Institute of Sport Sciences and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark

Submitted 18 October 2006; accepted in final form 8 January 2007

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/japplphysiol.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 O2 consumption for 24 h. Muscle biopsies were taken preexercise (Pre-Ex), postexercise (Post-Ex), and after 28 h of recovery (Rec). Respirations 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). Mitochrondial 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 (VO2) (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
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 VO2 (VO2peak). 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 timepoint. 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 ergomedic 893E, Monark Exercise, Varberg, Sweden), and treadmill (Rodby Electronics, Vansbro, Sweden). After a brief warm-up, the exercise was raised every minute above the estimated work rate to reach VO2peak. VO2 was measured with an online system (AMIS 2001, Inovision A/S Odense, Denmark). 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 the test and 10 min of rest. The last exercise bout was performed 10 min 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 Carabocain; 20 mg/ml, Astra), an incision was made through the skin and fascia, and the biopsy was taken using a Weil Blackleys conchothome (Wisex, Mölndal, Sweden). The muscle biopsy was divided into portions. One portion (average 46 mg) was used for assay of proteins [UCP3, adenosine nucleotide translocator, and myosin heavy chain (MHC)] and was frozen in liquid nitrogen and stored at ~80°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 mitochondrial suspension 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.** VO2 was measured using a Clark-type electrode (Hansatech DW1; Hansatech, King’s Lynn, Norfolk, UK) at 25°C. Respiration was analyzed in an oxygraph medium (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 activity was measured (2–3 days after the main experiment) after permeabilization with mitochondria was frozen in liquid nitrogen and stored at ~80°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 μg/ml alamethicin (Sigma A-3665), which was added to the oxygraph solution, together with NADH (450 μmol/l) and cytochrome c (2 μmol/l) (19). Respiratory rate, measured at 25°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**

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>27 (24–32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>80.5 (73.4–85.4)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>182 (175–186)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.2 (22.3–28.8)</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>16.9 (10.8–26.1)</td>
</tr>
<tr>
<td>VO2peak, ml kg⁻¹min⁻¹</td>
<td>61.9 (52.7–69.8)</td>
</tr>
<tr>
<td>Type I fibers, MHC %</td>
<td>56.3 (48.0–74.0)</td>
</tr>
</tbody>
</table>

Values are means and ranges (in parentheses) from 9 male subjects. Myosin heavy chain (MHC) 1% 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; VO2peak, peak VO2 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. 087780).

Mitochondrial FA oxidation (state 3) and relative FA oxidation (state 4) were measured using PC instead of palmitate 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 initial value 28 h Post-Ex (not significant vs. Pre-Ex).

Mitochondrial efficiency (P/O ratio) decreased Post-Ex when respiratory exchange ratio; V˙O2, O2 consumption. *P < 0.05 vs. Pre-Ex. †P < 0.05 vs. Post-Ex.

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

<table>
<thead>
<tr>
<th></th>
<th>Pre-Ex</th>
<th>Post-Ex</th>
<th>28 h Post-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, mmol/l</td>
<td>0.3±0.04</td>
<td>1.24±0.12*</td>
<td>0.24±0.03†</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.6±0.1</td>
<td>4.4±0.4</td>
<td>4.7±0.1</td>
</tr>
<tr>
<td>RER</td>
<td>0.89±0.02</td>
<td>0.84±0.01*</td>
<td>0.89±0.01†</td>
</tr>
<tr>
<td>V˙O2, l/min</td>
<td>2.26±0.06</td>
<td>2.55±0.07*</td>
<td>2.41±0.07†</td>
</tr>
</tbody>
</table>

Values are means ± 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 V˙O2peak). 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; V˙O2, O2 consumption. *P < 0.05 vs. Pre-Ex. †P < 0.05 vs. Post-Ex.

### 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). V˙O2, measured during cycling at the same absolute work rate for each subject (40–50% of V˙O2peak), was 13% higher at the end of endurance exercise and remained 7% higher 28 h 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).

### 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.
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 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

<table>
<thead>
<tr>
<th>Respiratory Parameter</th>
<th>Substrate</th>
<th>Pre-Ex</th>
<th>Post-Ex</th>
<th>Rec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal ETC activity,</td>
<td>NADH</td>
<td>358±30</td>
<td>357±24</td>
<td>312±18</td>
</tr>
<tr>
<td>mmol O₂·min⁻¹·mg</td>
<td>PC</td>
<td>18.1±2.1</td>
<td>25.6±1.6*</td>
<td>22.9±2.5*</td>
</tr>
<tr>
<td>protein⁻¹</td>
<td>PC + Pyr</td>
<td>26.2±1.9</td>
<td>29.4±1.0</td>
<td>30.6±1.9</td>
</tr>
<tr>
<td>State 3/ETC, %</td>
<td>PC</td>
<td>4.0±0.5</td>
<td>4.8±0.2</td>
<td>3.4±0.3†</td>
</tr>
<tr>
<td></td>
<td>PC + Pyr</td>
<td>2.9±0.2</td>
<td>3.2±0.2</td>
<td>2.3±0.3†</td>
</tr>
<tr>
<td>State 4/ETC, %</td>
<td>PC</td>
<td>0.68±0.05</td>
<td>0.87±0.04*</td>
<td>0.74±0.06</td>
</tr>
<tr>
<td>Relative FA oxidation,</td>
<td>(PC + Pyr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>state 3 PC/[state 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(PC + Pyr)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
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 β-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 roll 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 VO₂ 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 VO₂ 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-intensity exercise. First, the rate of mitochondrial palmitate oxidation during state 4 correlated with whole body fat oxidation during exercise at 60% VO₂ 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.

**REFERENCES**

REduced mitochondrial efficiency after ultraendurance exercise


