# Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle

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**Mitochondrial proteins such as uncoupling protein 3 (UCP3) and adenine nucleotide translocase (ANT) may mediate back-leakage of protons and serve as uncouplers of oxidative phosphorylation. We hypothesized that UCP3 and ANT increase after prolonged exercise and/or endurance training, resulting in increased uncoupled respiration (UCR). Subjects were investigated with muscle biopsies before and after acute exercise (75 min of cycling at 70% of $\dot{V}_{O_2peak}$) or 6 weeks endurance training. Mitochondria were isolated and respiration measured in the absence (UCR or state 4) and presence of ADP (coupled respiration or state 3). Protein expression of UCP3 and ANT was measured with Western blotting. After endurance training $\dot{V}_{O_2peak}$, citrate synthase activity (CS), state 3 respiration and ANT increased by 24, 47, 40 and 95%, respectively (all $P < 0.05$), whereas UCP3 remained unchanged. When expressed per unit of CS (a marker of mitochondrial volume) UCP3 and UCR decreased by 54% and 18% ($P < 0.05$). CS increased by 43% after acute exercise and remained elevated after 3 h of recovery ($P < 0.05$), whereas the other muscle parameters remained unchanged. An intriguing finding was that acute exercise reversibly enhanced the capacity of mitochondria to accumulate $Ca^{2+}$ ($P < 0.05$) before opening of permeability transition pores. In conclusion, UCP3 protein and UCR decrease after endurance training when related to mitochondrial volume. These changes may prevent excessive basal thermogenesis. Acute exercise enhances mitochondrial resistance to $Ca^{2+}$ overload but does not influence UCR or protein expression of UCP3 and ANT. The increased $Ca^{2+}$ resistance may prevent mitochondrial degradation and the mechanism needs to be further explored.**

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The coupling between oxygen utilization and ATP formation is central in muscle energetics. However, the coupling is not perfect and part of the oxygen consumption is due to mitochondrial uncoupling, i.e. uncoupled respiration (UCR). UCR is related to an energy-demanding proton cycle by which the released energy is dissipated as heat instead of being trapped in the form of ATP. The contribution of UCR to metabolic rate is considered to be significant and 20% of the basal oxygen consumption in rats has been attributed to UCR (Rolfe & Brand, 1996).

The mechanism behind UCR and its physiological role is not fully understood but is currently an intensive research field. One hypothesis is that specific mitochondrial proteins mediate back-leakage of protons over the inner mitochondrial membrane and therefore have the capacity to uncouple respiration from ATP synthesis (Hagen & Vidal-Puig, 2002). The presence of such proteins is well documented in brown adipose tissue where uncoupling protein 1 (UCP1) mediates non-shivering thermogenesis (Klaus *et al.* 1991). Homologous proteins have been identified in other tissues and the major forms found in skeletal muscle have been named uncoupling protein 2 (UCP2) and 3 (UCP3) (Gong *et al.* 1997; Giacobino, 2001). The functional role of UCP2 and UCP3 (Garvey, 2003) is under debate.

Previous studies in humans have shown that UCP3 mRNA expression is increased after acute exercise (Pilegaard *et al.* 2000) but unchanged after endurance training (Tonkonogi *et al.* 2000*a*). Cross-sectional studies have shown reduced expression of UCP3 mRNA (Boss *et al.* 2000) and UCP3 protein (Schrauwen *et al.* 1999;

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Russell *et al.* 2003) in skeletal muscle from trained subjects. In contrast, recent data from Holloszy's group show that during short-term training in rats UCP3 protein increases in parallel with other mitochondrial proteins and thus in accordance with mitochondrial biogenesis (Jones *et al.* 2003). It was considered unlikely that the relative composition of mitochondrial proteins could change considerable (Jones *et al.* 2003). A longitudinal training study in human subjects would solve the controversy but remains to be done. Furthermore, the effect of acute exercise on UCP3 protein expression has not been studied in humans.

Proton leakage through the phospholipid bilayer accounts for only a small proportion of total UCR (Brookes *et al.* 1997) and is more likely to be mediated by mitochondrial proteins such as UCP3, adenine nucleotide translocase (ANT) and ATP synthase. ANT mediates ADP influx into mitochondrial matrix and efflux of ATP and is considered to contribute to uncoupled respiration (Tikhonova *et al.* 1994; Skarka *et al.* 2003). The effect of acute exercise and endurance training on ANT protein expression in humans is not known.

Leakage of protons may also occur by intermittent opening of mitochondrial permeability transition (MPT) pores. MPT pores are large mitochondrial membrane channels formed as a complex between mitochondrial proteins (porin, ANT and cyclophilin-D). Oxidative stress, elevation of plasma $Ca^{2+}$ and low ATP favour the opening of MPT pores (Bowser *et al.* 2002), which leads to release of mitochondrial substances from the matrix, decreased membrane potential and increased UCR. The effect of acute exercise on MPT pore opening in human skeletal muscle mitochondria has previously not been studied.

During prolonged exercise above the lactate threshold, oxygen uptake ($\dot{V}_{O_2}$) increases slowly despite maintained power output. This phenomenon has been named the slow component of $\dot{V}_{O_2}$ kinetics or the $\dot{V}_{O_2}$ drift. $\dot{V}_{O_2}$ drift may in part be explained by increased body temperature, elevated levels of adrenaline (epinephrine), increased recruitment of fast-twitch fibres and an increased reliance on fat oxidation. However, the reasons for oxygen drift remain elusive (Zoladz & Korzeniewski, 2001). After strenuous exercise, oxygen consumption remains elevated for several hours and is referred to as the slow component of excess post-exercise oxygen consumption (EPOC) (Gaesser & Brooks, 1984). The mechanism for the slow component of EPOC is not fully understood. We hypothesize that increased UCR can contribute to EPOC and oxygen drift.

The purpose of this study was (i) to investigate the effects of acute exercise and training on the protein expression of UCP3 and ANT, (ii) to investigate the effect of acute exercise and training on UCR in isolated mitochondria and relate these findings to $\dot{V}_{O_2}$ drift, EPOC and putative mediators of proton leakage (i.e. UCP3 and ANT), and (iii) to investigate the effect of exercise on the vulnerability of mitochondria to $Ca^{2+}$ overload.

## Methods

### Subjects

**Acute exercise.** Nine healthy subjects (5 male and 4 female) performed one single bout of cycle exercise. The age, height, weight and $\dot{V}_{O_2peak}$ of the subjects in this group were (mean ± s.e.m.) 25 ± 1 years, 176 ± 3 cm, 72 ± 3.9 kg and 50 ± 2.1 ml $min^{-1}$ $kg^{-1}$.

**Endurance training.** Eight healthy subjects (4 male and 4 female) participated in the endurance-training program. The age, height, weight and $\dot{V}_{O_2peak}$ of the subjects were 26 ± 2 years, 173 ± 3 cm, 70 ± 4.7 kg and 38 ± 2.5 ml $min^{-1}$ $kg^{-1}$. Results on oxidative capacities have been reported previously (Tonkonogi *et al.* 2000*a*).

The subjects were fully informed of the possible risks and discomforts involved in the experiment before giving their written voluntary consent. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The experimental design of the study was approved by the Ethics Committee of the Karolinska Institutet, Stockholm, Sweden.

### Determination of $\dot{V}_{O_2peak}$

$\dot{V}_{O_2peak}$ test was conducted on an ergometer cycle (Monark 829e, Varberg, Sweden). An incremental submaximal exercise test was performed to estimate $\dot{V}_{O_2peak}$. After a brief period of rest, the workrate was raised rapidly above that estimated to elicit $\dot{V}_{O_2peak}$ and thereafter raised in small steps until exhaustion. Expired air was analysed for $O_2$ and $CO_2$ online using the AMIS cardiopulmonary function test system (Innovision A/S DK-5260 Odense S, Denmark).

### Experimental protocol

**Acute exercise.** At least 2 days after the $\dot{V}_{O_2peak}$ test the subjects reported to the laboratory to perform the experiment. They were instructed to abstain from heavy physical exercise and from alcohol on the day before the experiment. They were also instructed to avoid breakfast on the day for the experiment and were not allowed to eat until the experiment was completed as it is known that UCP3 is influenced by food intake (Schrauwen *et al.*

2002). Cardiorespiratory parameters were measured pre- and 3 h post-exercise (after the subjects had rested in the supine position for 30 min). The expired air was collected using the Douglas bags technique and analysed for $O_2$ and $CO_2$ using a Beckman S-3 A and LB-2 analyser (Beckman Instruments, Fullerton, CA, USA). Subjects cycled on a Monark ergometer cycle for 75 min at an intensity of approximately 70% of their $\dot{V}_{O_2peak}$. Expired air was collected online every 15 min and analysed for $O_2$, $CO_2$ and RER (respiratory exchange ratio) on the AMIS cardiopulmonary function test system. Heart rate was registered continuously with a Polar sport tester (Polar Electro, Kemple, Finland). Muscle biopsies were taken from m. vastus lateralis pre-exercise, immediately post-exercise (within 5 min) and 3 h post-exercise. The biopsies taken pre-exercise and 3 h post-exercise were taken after measurements of cardiorespiratory parameters. The first two biopsies were taken from the same leg. Blood samples were taken from an antecubital vein prior to the muscle biopsies (pre- and 3 h post-exercise) and during the last minutes of exercise.

**Endurance training.** Eight untrained subjects completed a six-week training program consisting of four sessions per week. Prior to the first training session measurements of $\dot{V}_{O_2peak}$ were conducted. Each training session was performed on a Monark cycle ergometer and lasted one hour. The subjects cycled at 70% of $\dot{V}_{O_2peak}$ for the first 30 min and performed interval training for the remaining 30 min. After 2 and 4 weeks, the workrate was increased by 5% to account for the estimated increase in $\dot{V}_{O_2peak}$. Two to three days following the last training session, the test of $\dot{V}_{O_2peak}$ was repeated. Muscle biopsies were taken from m. vastus lateralis pre- and post-training (2 days after the $\dot{V}_{O_2peak}$ test).

## Muscle biopsies and isolation of mitochondria

Muscle biopsies were taken from the lateral aspect of the quadriceps femoris muscle, at a depth of 2–3 cm. After local anaesthesia (1–2 ml Carbocain; 20 mg $ml^{-1}$, AstraZeneca), an incision was made through the skin and fascia and the biopsy was taken using a Bergström needle with suction. The biopsy was divided into portions. One portion was frozen in liquid nitrogen and stored at −80°C until determination of CS and protein expression of UCP3 and ANT. Another portion was used for preparation of isolated mitochondria according to the method of Tonkonogi & Sahlin (1997). Briefly, a muscle specimen was minced with scissors and muscle mitochondria were isolated by protease treatment (Nagarse, 0.4 mg $ml^{-1}$, Sigma P4789), followed by homogenization and differential centrifugation. The final mitochondria pellet was resuspended in a medium consisting of (mM) 225 mannitol, 75 sucrose, and 10 Tris, 0.1 EDTA (pH 7.40), and kept on ice until analysis of respiratory activity. An aliquot of the suspension (10 $\mu$l) was taken for measurements of mitochondrial citrate synthase activity (CS) as previously described (Tonkonogi *et al.* 1997).

## Analytical methods

**Measurements of mitochondrial respiratory activity.** Oxygen consumption was measured using a Clark-type electrode (Hansatech DW1; Hansatech, King's Lynn, Norfolk, UK), at 25°C. Respiration was analysed in medium containing (mM): mannitol 225, sucrose 75, Tris 10, KCL 10, $K_2HPO_4$ 10, EDTA 0.1, pyruvate 5, malate 2 (pH 7.35). The mitochondrial suspension was added to the reaction medium and coupled respiration (state 3) was initiated by the addition of 200 $\mu$M ADP. The respiratory rate returned to that prior to the addition of ADP when all ADP was phosphorylated to ATP and corresponds to UCR or state 4.

The effect of free fatty acids on UCR was determined by addition of oleate in increasing concentrations to the mitochondrial suspension in the respiration medium as above but including oligomycin (0.5 mg $ml^{-1}$), fatty acid-free BSA (0.2%), 0.05 mM hypoxanthine and 0.003 U $ml^{-1}$ xanthine oxidase. Oligomycin was added to block proton leakage through ATP synthase. Hypoxanthine and xanthine oxidase were added to generate superoxide, since this has been reported to activate proton leakage through UCP3. Oleate was added to final concentrations of 40, 80 and 100 $\mu$M corresponding to calculated free concentrations of 0.02, 0.24 and 8.36 $\mu$M (Richieri *et al.* 1993).

**Muscle citrate synthase activity.** CS was measured by spectrophotometry in isolated mitochondria and in freeze-dried muscle dissected free from non-muscle constituents using a technique previously described (Tonkonogi *et al.* 1997).

**UCP3.** Portions of freeze-dried muscle were homogenized in ice-cold lysis buffer and the protein concentration was determined (BCA protein assay 23223 Pierc Cat. 1610737, Bio Rad Laboratories, Hercules, CA, USA). Homogenates were solubilized in Laemmli sample buffer and denaturated by boiling. A constant amount of protein was added per lane (75 $\mu$g) on 12% polyacrylamide

gels and separated by SDS-PAGE for 60 min at 135 V. The separated polypeptides were transferred to a PVDF membrane at 10 V for 60 min, and blocked in tris-buffered saline (TBS) with 5% non-fat milk. Membranes were incubated overnight with polyclonal antibody against UCP3 (Chemicon AB3046), diluted 1 : 1000, washed and incubated with secondary antibody goat antirabbit (IgG-HRP, NO.sc-2030 Santa Cruz). The membrane was again washed and incubated with the chemiluminescence detection reagent ECL, No RPN 2106 (Amersham). Finally, an X-ray film was exposed to the membrane for 50 min. The optical density of the bands was quantified by using Molecular Analyst 1.5 (Bio-Rad). The analytical procedure for analysis of UCP3 has previously been described in detail (Tonkonogi *et al.* 2003).

**ANT.** Homogenization, measurement of protein concentration and denaturation was performed as for UCP3. A constant amount of protein was added per lane (75 $\mu$g) on 12% polyacrylamide gels and separated by SDS-PAGE for 60 min at 135 V. Polypeptides were transferred to a PVDF membrane at 100 V for 120 min, and blocked in TBS with 5% non-fat milk. Membranes were incubated 2 h with polyclonal antibody against ANT (ANT-1, Q-18, No,sc-9300, Santa Cruz) diluted 1 : 200, washed and incubated with secondary antibody bovine antigoat (IgG-HRP, No. sc-2350 Santa Cruz). After washing the membrane was incubated with chemiluminescence detection reagent ECL (No RPN 2106, Amersham), and exposed to a film for 50 min. The optical density of the bands was quantified by using Molecular Analyst 1.5 (Bio-Rad). The Western blot procedure for ANT has previously been described in detail (Tonkonogi *et al.* 2003).

**Mitochondrial permeability transition pores.** Mitochondrial swelling was monitored continuously as the change in absorbance at 540 nm. An aliquot of mitochondrial suspension equivalent to $5 \times 10^{-2}$ U CS corresponding to 19.1 $\mu$g protein (3.15–7.47 $\mu$l mitochondrial suspension) was diluted to a final volume of 7.5 $\mu$l and resuspended in 100 $\mu$l of buffer (pH 7.4) containing (mM) 150 KCl, 5 Tris, 5 $K_2HPO_4$, 5 malate, 5 pyruvate. Equivalent pulses of $CaCl_2$ (2 $\mu$mol of $Ca^{2+}$ (U CS)$^{-1}$ = 10 nmol) were added to the mitochondria every 3 min until a rapid decrease in absorbance occurred. This condition indicates progressive swelling of mitochondria due to opening of MPT pores. The time point for massive MPT pore opening was defined as the point when the rate of decrease in absorbance exceeded 0.0175 absorbance units per minute. Results from preliminary experiments on human muscle mitochondria using $Ca^{2+}$-sensitive electrodes showed that 3 min was sufficient for complete uptake of $Ca^{2+}$ from the medium during initial calcium pulses.

**NEFA and blood glucose.** Glucose concentration was measured in whole blood with Accu-Chek (Roche Diagnostics AB). Blood for analysis of nonesterified fatty acids (NEFA) was centrifuged and the supernatant was immediately frozen. NEFA was measured in plasma using the Wako NEFA *C*-test kit. Nr: 994–75409 D (Waco Chemicals Inc, Richmond, USA).

## Data analysis

Data are presented as means ± S.E.M. Statistical significance of the difference between means was tested with either Student's paired *t* test or repeated measures analysis of variance (ANOVA). ANOVA was followed by a *post hoc* test with Greenhouse-Geisser and Huynh-Feldt adjustments. Significance of differences was set as $P < 0.05$.

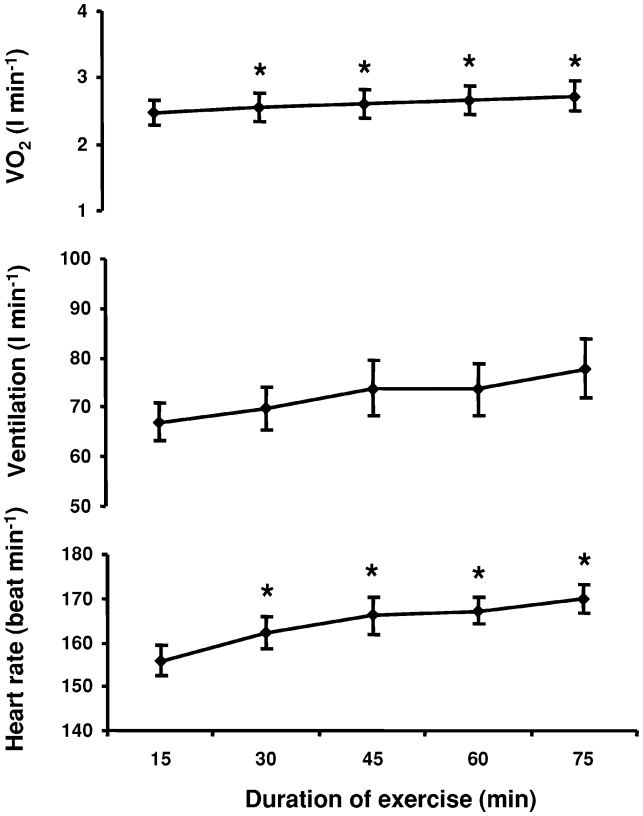


**Figure 1. Cardiorespiratory parameters during exercise**
Values are mean ± S.E.M. from 9 subjects. Subjects exercised for 75 min at 70% of $\dot{V}_{O_2peak}$. *Significant difference from value at 15 min.

**Table 1. Effect of acute exercise on parameters of skeletal muscle mitochondria and substrates in blood**

| | Pre-exercise | Post-exercise | 3 h post-exercise |
|---|---|---|---|
| Muscle CS (mmol $min^{-1}$ (kg wet wt)$^{-1}$) | 19.0 ± 1.3 | 27.3 ± 2.2* | 27.2 ± 3.4* |
| UCP3 (AU (mg protein)$^{-1}$) | 117 ± 29 | 109 ± 41 | 135 ± 46 |
| ANT (AU (mg protein)$^{-1}$) | 152 ± 25 | 169 ± 24 | 177 ± 22 |
| State 3 (nmol $O_2$ $min^{-1}$ (mg protein)$^{-1}$) | 187 ± 16 | 201 ± 22 | 205 ± 20 |
| State 4 (nmol $O_2$ $min^{-1}$ (mg protein)$^{-1}$) | 21 ± 3 | 24 ± 4 | 25 ± 3 |
| NEFA (mM) | 0.22 ± 0.03 | 0.86 ± 0.11* | 1.05 ± 0.1* |
| Glucose (mM) | 4.5 ± 0.2 | 3.8 ± 0.3 | 4.1 ± 0.3 |

Values are mean ± S.E.M. from 9 subjects. *Significant difference from value at rest ($P < 0.05$). UCP3 and ANT are expressed in arbitrary units (AU) per mg of muscle protein. State 3 (coupled respiration) and state 4 (UCR) correspond to mitochondrial respiration in the presence and absence of ADP and are expressed per mg mitochondrial protein. NEFA = nonesterified fatty acids in plasma.

## Results

### Acute exercise

The subjects cycled for 75 min at a constant workrate corresponding to about 70% of their individual $\dot{V}_{O_2peak}$. Despite the constant power output there was a slow increase (Fig. 1) in $\dot{V}_{O_2}$ (from 2.47 ± 0.20 l $min^{-1}$ at 15 min to 2.72 ± 0.23 l $min^{-1}$ at 75 min, $P < 0.05$) and heart rate (156 ± 3 *versus* 170 ± 3 beats $min^{-1}$, $P < 0.05$). $\dot{V}_{O_2}$ drift between 15 and 75 min averaged 10% (range 3–16%) of $\dot{V}_{O_2}$ at 15 min. $\dot{V}_{O_2}$ drift was neither correlated to the increase in heart rate nor to the increase in ventilation. The respiratory exchange ratio decreased from 0.95 ± 0.01 after 15 min exercise to 0.92 ± 0.01 after 75 min of exercise ($P < 0.05$), and corresponds to an increased relative fat oxidation from 17% (15 min) to 27% (75 min). The observed shift in substrate oxidation would increase $\dot{V}_{O_2}$ by 1% (assuming 10% higher P/O ratio for fat oxidation). $\dot{V}_{O_2}$, measured while the subjects rested in the supine position was 17% higher 3 h post-exercise than pre-exercise (0.28 ± 0.02 *versus* 0.24 ± 0.01 l $min^{-1}$, $P < 0.05$) and corresponds to the slow phase of EPOC.

Muscle CS activity increased after acute exercise by 43% and remained elevated in the recovery state (Table 1), whereas protein expression of putative mediators of proton leak (UCP3 and ANT) was unchanged. Due to the increase in muscle CS activity, which is unlikely to be related to an increase in mitochondrial density (cf. Discussion), mitochondrial CS could not be used as a reference base for mitochondrial respiration. Coupled respiration (state 3) and UCR (state 4) were unchanged when expressed per milligram of mitochondrial protein.

As already known and also shown in Fig. 2, mitochondrial proton leak and UCR increase after exposure to NEFA. Plasma NEFA increased after exercise (286% of the value at rest) and was further increased after 3 h of recovery (369% of the value at rest) (Table 1). However, the individual increase in NEFA was unrelated to either the subject's oxygen drift during exercise ($r = 0.075$) or the elevated $\dot{V}_{O_2}$ 3 h post-exercise ($r = 0.15$). Furthermore, measurements in isolated mitochondria demonstrated that the sensitivity of mitochondrial respiration to oleate was similar pre- and post-exercise (Fig. 2).

Opening of large permeability transition pores may be another mechanism of mitochondrial uncoupling. Swelling and rupture of mitochondria was observed spectrophotometrically after successive additions of $Ca^{2+}$. Interestingly mitochondria isolated from muscle samples taken immediately post-exercise could buffer more $Ca^{2+}$ before swelling and rupture than samples taken pre-exercise ($P < 0.05$). The change was reversed 3 h post-exercise (Fig. 3).

### Endurance training

As previously reported (Tonkonogi *et al.* 2000*b*), endurance training for 6 weeks increased oxidative power at the whole body level (24% increase in $\dot{V}_{O_2peak}$, $P < 0.05$) and at the muscle level as demonstrated by increases in CS (47% increase in CS, $P < 0.05$) and ADP-stimulated mitochondrial respiration (40% increase in state 3 respiration, $P < 0.05$). When mitochondrial respiration was expressed in relation to a marker of mitochondrial volume (CS), state 3 respiration remained unchanged, whereas state 4 respiration (UCR) decreased by 18% ($P < 0.05$, Table 2).

Protein expression of ANT was increased to a similar extent to CS by training (Table 2, Fig. 4). In contrast, protein expression of UCP3 was not significantly changed by training (Table 2), and decreased by 53% ($P < 0.05$) when related to CS (Fig. 4).

## Discussion

One of the major findings in this study was that protein expression of UCP3 does not increase in parallel with

other mitochondrial proteins (CS and ANT) during training-induced mitochondrial biogenesis. Both UCP3 and UCR decreased after endurance training when related to CS, a marker of mitochondrial volume. The reduced UCR reflects a reduced proton leak, possibly due to the lower level of UCP3. The decrease in UCP3 after training is consistent with previous findings of lower UCP3 protein in trained human subjects (Schrauwen *et al.* 1999) but different from that observed in rats, where UCP3 protein increased in parallel with other proteins during short-term training (Jones *et al.* 2003). UCP3 protein is known to be higher in fast-twitch fibres than in slow-twitch fibres (Hesselink *et al.* 2001) and endurance-trained subjects tend to have a larger proportion of slow-twitch fibres. Differences in fibre type composition may therefore contribute to the lower levels of UCP3 protein observed in trained subjects (Schrauwen *et al.* 1999). Although fibre type composition was not measured in the present

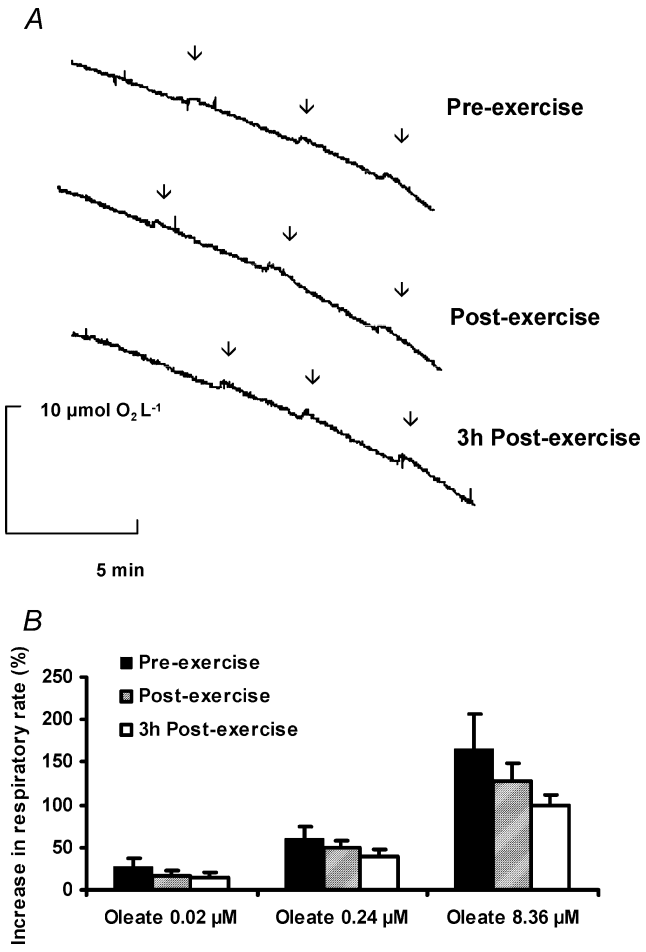


**Figure 2. Effect of prolonged exercise on uncoupled respiration induced by fatty acids**

*A*, original oxygraph recordings from a representative subject showing oxygen tension *versus* time. The arrows denote additions of free fatty acids (oleate) at concentrations of 0.02, 0.24 and 8.4 $\mu$M. Mitochondria were isolated from human skeletal muscle samples taken pre-exercise, post-exercise and 3 h post-exercise. Respiration was measured in the presence of pyruvate, malate, oligomycin (specific inhibitor of ATP-synthase), hypoxanthine and xanthine oxidase. *B*, the graph shows the relative increase in mitochondrial respiration after addition of oleate in increasing concentrations. Values are means ± S.E.M., $n = 9$. The effect of oleate was statistically significant but the difference between muscle samples was not statistically significant.

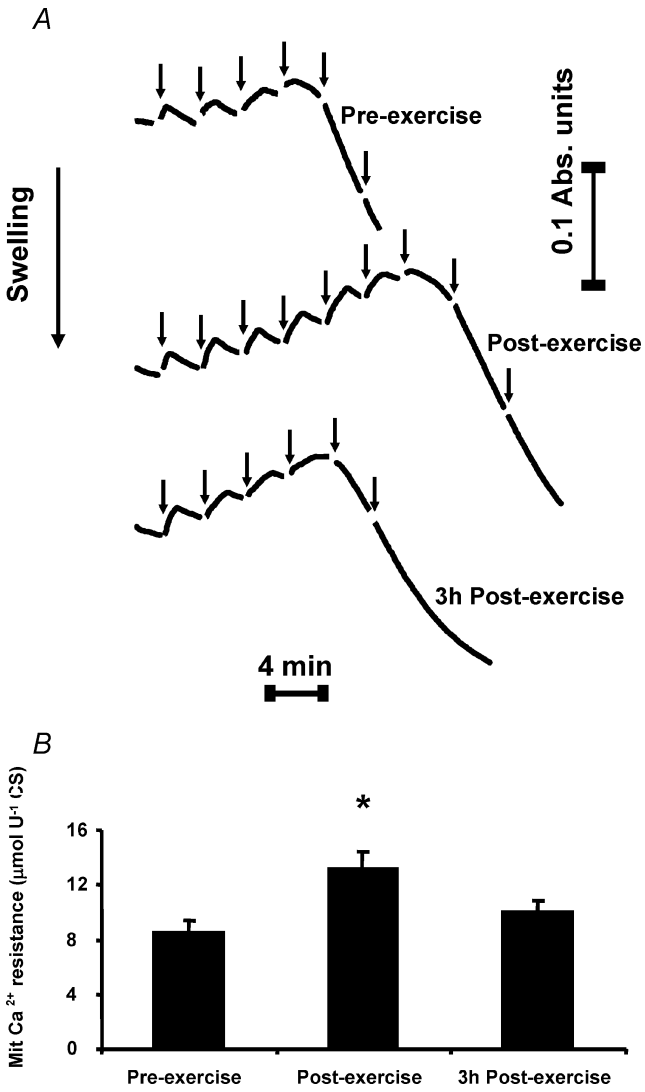


**Figure 3. Effect of acute exercise on mitochondrial resistance to $Ca^{2+}$-induced MPT (mitochondrial permeability transition) pore opening**

Pore opening was induced by subsequent additions of $Ca^{2+}$ pulses to isolated muscle mitochondria. *A*, original recordings of absorbance measured at 540 nm. Arrows denote additions of $Ca^{2+}$ (2 $\mu$mol (U CS)$^{-1}$). *B*, mitochondrial $Ca^{2+}$ resistance denotes the accumulated amount of $Ca^{2+}$ required to initiate rapid progressive swelling of mitochondria indicating opening of MPT pores. Subjects cycled at 70% of $\dot{V}_{O_2peak}$ for 75 min. Values are mean ± S.E.M., $n = 7$, $^*P < 0.05$.

study, it is unlikely that 6 weeks of training would alter fibre type composition (Thayer *et al.* 2000) and explain the reduced UCP3 expression observed in the present longitudinal study. The reasons for the divergent effects on UCP3 expression in this study and that of Jones *et al.* (2003) is unclear but may relate to the short training period in the latter study (10 days *versus* 6 weeks in present study), differences in type of muscles and fibre types involved in the exercise, and/or differences between species.

The physiological role of UCP3 is under debate and suggested roles include mitochondrial uncoupling, reduced formation of reactive oxygen species (ROS) (Vidal-Puig *et al.* 2000), transport of ionized fatty acids out of the mitochondrial matrix (Schrauwen *et al.* 2001) and control of oxidative phosphorylation (Toukouogi *et al.* 2000*a*). These suggested functional roles are independent but could all be correct. Evidence for a role of UCP3 in uncoupling comes from studies on UCP3 knock-out mice, which have lower state 4 respiration (Harper & Himms-Hagen, 2001). It has also been reported that obese diet-resistant women have decreased mitochondrial proton leak and reduced expression of UCP3 in skeletal muscle (Harper *et al.* 2002). Evidence against UCP3 being an uncoupler includes the finding that fasting increases mRNA for UCP3 in skeletal muscle, despite a reduced metabolic rate (Millet *et al.* 1997). Furthermore, elevation of UCP3 by a high-fat diet did not change the efficiency of mitochondrial energy conversion (measured as the rate of PCr resynthesis after exercise) and it was concluded that the primary role of UCP3 was not uncoupling (Hesselink *et al.* 2003). However, actively phosphorylating mitochondria have a lower membrane potential than mitochondria in state 4 and it seems likely that this would reduce or abolish proton leak. Therefore a maintained level of mitochondrial efficiency during exercise does not exclude the possibility that UCP3 functions as a mild uncoupler during basal conditions.

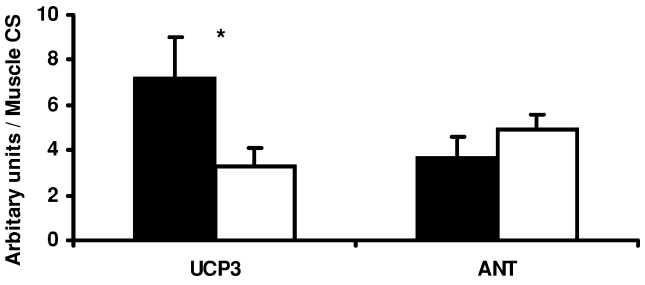


**Figure 4. Effect of training on ANT and UCP3 protein in skeletal muscle**

Protein content pre- (filled bars) and post-training (open bars) expressed as arbitrary units per mg of muscle protein and related to muscle CS. Values are means ± S.E.M. from 8 subjects. * $P < 0.05$ *versus* pretraining.

Mitochondrial density was increased by about 40% after 6 weeks of training and maintenance of the intrinsic proton leakage per mitochondrion would correspond to a large increase in basal metabolic rate. Providing that UCP3 has a role in uncoupling, the observed reduction in UCP3 in response to training could be an important compensatory mechanism to avoid excessive heat production and energy expenditure during basal conditions. Given the role of UCP3 in reducing mitochondrial ROS formation one could argue that training should increase vulnerability to oxidative damage. However, the system is complex and compensatory mechanisms are likely to be involved.

Acute exercise had no effect on UCP3 and ANT protein expression or on UCR, measured in isolated mitochondria. The hypothesis that increased UCR could explain oxygen drift or the slow component of EPOC cannot therefore be supported. However, metabolic conditions and the control of UCR are different *in vivo* than during the experimental conditions used *in vitro*. Previous studies on isolated mitochondria have shown that state 4 respiration increases (Brooks *et al.* 1971) and that the efficiency in energy transfer decreases (Brooks *et al.* 1971; Willis & Jackman, 1994) during hyperthermia. Furthermore, fatty acids are known to increase UCR and both fatty acids and superoxide are potent stimulators of proton leakage through UCP3 (Echtay *et al.* 2002). Plasma levels of NEFA increased 3–4 times during exercise and continued to increase during the post-exercise period. Furthermore, since mitochondrial formation of superoxide is elevated during exercise it is possible that mitochondrial uncoupling will be augmented.

An interesting finding in this study was that the resistance of isolated mitochondria to $Ca^{2+}$ overload was increased in samples taken immediately post-exercise. The results are surprising since mitochondrial $Ca^{2+}$ content is known to increase after prolonged exercise (Madsen *et al.* 1996). The present data do not provide any clue as to the mechanism behind the protective effect of exercise on MPT. Opening of MPT is associated with dysfunction and eventually degradation of mitochondria. Mitochondrial degradation can in its turn lead to destruction of the nucleus in the vicinity and thereby to muscle hypotrophy (Primeau *et al.* 2002). The increased $Ca^{2+}$ tolerance of mitochondria post-exercise could serve as a protective mechanism, resulting in a decrease in the rate of mitochondrial degradation and instead favouring an increased rate of mitochondrial biogenesis. Ischaemic injury is known to be associated with opening of MPT (Xu *et al.* 2001). The association between ischaemic

**Table 2. Effect of 6 weeks of endurance training on muscle mitochondrial parameters**

| | Pre-training | Post-training | Significance |
|---|---|---|---|
| Muscle CS (mmol $min^{-1}$ (kg wet wt)$^{-1}$) | 21.1 ± 1.0 | 31.0 ± 1.5 | $P < 0.05$ |
| UCP3 (AU (mg protein)$^{-1}$) | 142 ± 28 | 98 ± 18 | n.s. |
| ANT (AU (mg protein)$^{-1}$) | 76 ± 20 | 149 ± 19 | $P < 0.05$. |
| State 3 (nmol $O_2$ $min^{-1}$ (unit of CS)$^{-1}$) | 65.7 ± 3.3 | 62.5 ± 2.4 | n.s. |
| State 4 (nmol $O_2$ $min^{-1}$ (unit of CS)$^{-1}$) | 7.7 ± 0.6 | 6.3 ± 0.3 | $P < 0.05$ |

Values are mean ± S.E.M. from 8 subjects. UCP3 and ANT are expressed in arbitrary units (AU) per mg of muscle protein. State 3 (coupled respiration) and state 4 (UCR) represent mitochondrial respiration in the presence and absence of ADP and are expressed per CS activity in the mitochondrial suspension.

preconditioning and mitochondrial resistance to $Ca^{2+}$ overload should be further investigated.

Acute exercise resulted in a large increase in muscle CS (+43%) and is consistent with that observed in previous studies (Tonkonogi *et al.* 1997; Leek *et al.* 2001). However, the high CS maintained after 3 h of recovery is a new intriguing finding which opens up new perspectives in training and athletic performance. *De novo* enzyme synthesis seems unlikely due to the short time period and an increased specific activity of CS appears to be more plausible. The mechanism for this is however, unknown (see Tonkonogi *et al.* 1997 for a full discussion).

In summary, the present study demonstrated that the density and oxidative power of mitochondria increases after endurance training but that UCP3 protein and UCR are reduced in relation to mitochondrial volume. The reduction in UCR may relate to the reduction in UCP3 and serve as prevention against excessive basal thermogenesis due to increased mitochondrial density. An intriguing finding was the increase in mitochondrial $Ca^{2+}$ tolerance immediately post-exercise, which may serve as a protective mechanism against exercise-induced mitochondrial degradation. The mechanism behind this needs to be further explored.

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