Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: an uncoupling-protein-mediated effect?

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Uncoupled respiration (UCR) is an essential property of muscle mitochondria and has several functions in the cell. We hypothesized that endurance training may alter the magnitude and properties of UCR in human muscle. Isolated mitochondria from muscle biopsies taken before and after 6 weeks of endurance exercise training ($n = 8$) were analysed for UCR. To investigate the role of uncoupling protein 2 (UCP2) and UCP3 in UCR, the sensitivity of UCR to UCP-regulating ligands (non-esterified fatty acids and purine nucleotides) and UCP2 and UCP3 mRNA expression in muscle were examined. Oleate increased the mitochondrial oxygen consumption rate, an effect that was not attenuated by GDP and/or cyclosporin A. The effect of oleate was significantly greater after compared with before training. Training had no effect on UCP2 or UCP3 mRNA levels, but after training the relative increase in respiration rate induced by oleate was positively correlated with the UCP2 mRNA level. In conclusion, we show that the sensitivity of UCR to non-esterified fatty acids is up-regulated by endurance training. This suggests that endurance training causes intrinsic changes in mitochondrial function, which may enhance the potential for regulation of aerobic energy production, prevent excess free radical generation and contribute to a higher basal metabolic rate.

Key words: exercise, human skeletal muscle, oxygen consumption, proton leak, uncoupling.

INTRODUCTION

Skeletal muscle is the single most important contributor to body total metabolic rate in both humans and rodents [1,2]. A substantial part of the resting respiration rate in rat skeletal muscle is due to the leakage of protons across the mitochondrial inner membrane [3,4]. Thus the proton-leak-dependent (uncoupled) oxygen consumption (uncoupled respiration; UCR) in skeletal muscle appears to be a significant determinant of whole-body metabolic rate. In addition to its role as a determining factor of metabolic rate, proton leak through the mitochondrial inner membrane appears to have several important functions in the cell. The cycle of proton pumping and proton leak provides a mechanism for increasing the sensitivity and rate of response of aerobic metabolism to changes in energy demand [1,3]. Furthermore, it has been suggested that mild uncoupling caused by proton leak prevents the one-electron reduction of O_2 by the mitochondrial electron transport chain and the generation of reactive oxygen species (ROS) [5]. Although proton leak is considered to be an important factor in many metabolic processes, the molecular mechanisms involved in the leakage of protons across the mitochondrial membrane in skeletal muscle are still poorly understood.

An increasing amount of evidence suggests that two recently characterized mitochondrial proteins, uncoupling protein 2 (UCP2) and UCP3, may be involved in the proton leak observed in skeletal muscle [6–14]. UCP2 and UCP3 show high amino acid similarity (59 and 57% respectively [6,15]) with UCP1, a well characterized mitochondrial carrier expressed exclusively in brown adipose tissue. UCP1 plays an important role in the regulation of energy expenditure and heat production in rodents by creating a pathway that allows dissipation of the proton electrochemical gradient across the mitochondrial inner membrane without phosphorylation. UCP2 and UCP3 are abundantly expressed in skeletal muscle and have been correlated with energy expenditure in humans [10].

The activity of UCP1 is tightly controlled by two types of ligands: non-esterified ('free') fatty acids (NEFA) and purine di- and tri-nucleotides, such that UCP1 facilitates the transport of a NEFA anion across the mitochondrial inner membrane. NEFA are then protonated in the cytosol and diffuse back to the matrix, thus delivering protons [16]. An alternative model is that UCP1 acts directly as a proton carrier, using fatty acids as cofactors [17]. Binding of purine nucleotide to the putative nucleotide-binding region of UCP1 inhibits the proton transport activity [18]. Using expression systems, both UCP2 and UCP3 have been shown to behave as uncoupling proteins analogous to UCP1 [6–9,12,14], and NEFA are obligatory for proton flux through human UCP2 and UCP3 in a reconstituted system [9]. Studies on human UCP2 and UCP3 reconstituted in proteoliposomes suggest that nucleotide binding inhibits UCP2 and UCP3 proton conductance [9,19,20]. However, the affinity of reconstituted UCP2- and UCP3-mediated proton transport for purine nucleotides appears to be significantly lower than that of UCP1 [9]. The influence of NEFA and purine nucleotides on UCR in human skeletal muscle has not been investigated.

Several studies have suggested that exercise is a major regulator of UCP2 and UCP3 mRNA expression in the skeletal muscle of

Abbreviations used: UCR, uncoupled respiration; UCP, uncoupling protein; NEFA, non-esterified fatty acids; ROS, reactive oxygen species; $V\!O_2$, pulmonary oxygen uptake; $V\!O_2$peak, peak pulmonary oxygen uptake; CS, citrate synthase; RCR, respiratory control ratio.

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rodents and humans [10,21–24], although both the direction and magnitude of the exercise-induced changes in mRNA have differed between studies. The interpretation of altered UCP2 and UCP3 mRNA levels in skeletal muscle requires functional measurements on skeletal muscle mitochondria. The effects of short-term endurance training on UCR and on UCP2 and UCP3 mRNA expression in human skeletal muscle have not been studied previously. We hypothesized that an endurance training programme would change UCP2 and UCP3 mRNA levels in human skeletal muscle, and that this could result in changes in the magnitude and properties of UCR. To test this hypothesis, the effects of 6 weeks of endurance exercise training on proton-leak-dependent mitochondrial oxygen consumption and its sensitivity to UCP-regulating ligands (NEFA and purine nucleotides) were investigated in isolated skeletal muscle mitochondria from eight healthy humans. Moreover, expression of UCP2 and UCP3 mRNAs in skeletal muscle was measured before and after the endurance training period.

EXPERIMENTAL

Subjects

Four female and four male healthy volunteers participated in the study. The subjects' mean (range) age, height and weight were, respectively, 26 (20–37) years, 70.4 (56.5–98) kg and 1.76 (1.65–1.87) m. The subjects had not done any regular physical training in the 6 months preceding the study, although they were recreationally active. The subjects' peak pulmonary oxygen uptake ($V\mathrm{O}_2$peak) was 2.7 (2.0–3.6) $1 \cdot \mathrm{min}^{-1}$, corresponding to 38.5 (27.8–46.9) $\mathrm{ml} \cdot \mathrm{min}^{-1} \cdot (\mathrm{kg\ body\ mass})^{-1}$. The subjects were informed concerning the procedure and risks involved in the experiment before giving their written 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 Institute, Stockholm, Sweden.

Performance tests

Discontinuous, incremental ergometer tests for estimation of $V\mathrm{O}_2$peak and the lactate threshold were conducted before and 2 days after the 6-week training period. For the female subjects the time of the tests was matched to their menstrual cycle. The subjects cycled (Monark 829 E cycle ergometer; Monark, Varberg, Sweden) at three or more submaximal work rates (4 min each) and at a supramaximal work rate until exhaustion. Expired gases were collected in Douglas bags and analysed for oxygen and carbon dioxide concentrations using Beckman S-3A and LB-2 analysers respectively (Beckman Instruments, Fullerton, CA, U.S.A.). Heart rate during the tests was recorded continuously (Polar Sport Tester 3000; Polar Electro, Kempele, Finland). $V\mathrm{O}_2$peak was defined as the highest $V\mathrm{O}_2$ recorded during the test. Blood was sampled from a finger capillary at the end of each work period and analysed for lactate using a YSI 2300 STAT lactate analyser (YSI, Yellow Springs, OH, U.S.A.). The lactate threshold was defined as the interpolated $V\mathrm{O}_2$ corresponding to a blood lactate concentration of 4 $\mathrm{mmol} \cdot \mathrm{l}^{-1}$, and was expressed as a percentage of $V\mathrm{O}_2$peak.

Endurance training

Subjects attended four training sessions per week over a period of 6 weeks. Training sessions consisted of 30 min of cycle exercise (80 rev./min) at a constant workload estimated to correspond to 70 % of $V\mathrm{O}_2$peak, followed by five 2-min bouts of exercise at 100 % of $V\mathrm{O}_2$peak, interspersed with 4-min periods of exercise at 50 % of $V\mathrm{O}_2$peak. Each training session was preceded by a standardized warm-up, which consisted of 5 min of cycling at 50 W. The workloads were increased every second week, assuming an increase in $V\mathrm{O}_2$peak of 2.5 % per week. Each subject's heart rate was monitored continuously during each training session.

Muscle biopsy sampling

Muscle biopsies were taken from the lateral aspect of the quadriceps femoris muscle 48 h after the performance tests. After local skin anaesthesia [1–2 ml of 20 $\mathrm{mg} \cdot \mathrm{ml}^{-1}$ Carbocain (mepivacaine); Astra, Södertälje, Sweden], incisions were made (one on each leg) through the subcutaneous tissue and fascia at a point approx. one-third of the distance from the upper margin of the patella to the anterior superior iliac spine. Biopsies were taken at a depth of 2–3 cm using a Bergströms needle with suction. Biopsies from the two legs were combined. Each muscle sample was divided into two portions. One portion (60–70 mg) was quenched in liquid nitrogen and stored at −70 °C until determination of muscle enzyme activities and UCP2 and UCP3 mRNA levels. A second portion (72–202 mg) was used for the preparation of isolated mitochondria, as described previously [25]. Briefly, muscle specimens were chopped finely with scissors and mitochondria were isolated by proteinase treatment (Nagarse EC 3.4.21.62), followed by homogenization and subsequent differential centrifugation. The final mitochondrial pellet was resuspended (0.4 $\mu\mathrm{l} \cdot \mathrm{mg}^{-1}$ initial muscle) in a medium consisting of (in $\mathrm{mmol} \cdot \mathrm{l}^{-1}$): 225 mannitol, 75 sucrose, 10 Tris and 0.1 EDTA, pH 7.40. The mitochondrial suspension was kept on ice until determination of respiratory activity. An aliquot of the suspension (10 μl) was taken for measurements of citrate synthase (CS) activity, as described previously [25]. CS was used as a mitochondrial marker, and respiration rates of isolated mitochondria were expressed per unit of CS activity.

Measurement of mitochondrial respiratory activity

The rates of respiration of isolated mitochondria were measured with a Clark-type electrode (Hansatech DW 1; Hansatech, King's Lynn, Norfolk, U.K.) in a water-jacketted glass chamber of 0.3-ml capacity. A temperature of 25 °C was maintained in the chamber. The measurements were carried out in a reaction medium containing (in $\mathrm{mmol} \cdot \mathrm{l}^{-1}$): 225 mannitol, 75 sucrose, 10 Tris, 10 KCl, 10 $\mathrm{K_2HPO_4}$, 0.1 EDTA, 5 pyruvate and 2 malate, pH 7.40. The solubility of oxygen in the medium was considered to be equal to 237.5 $\mu\mathrm{mol} \cdot \mathrm{l}^{-1}$.

Respiration was initiated by the addition of mitochondrial suspension to the reaction medium, and a conventional respiratory experiment with transitions from State 4 to State 3 to State 4 was performed. State 3 was initiated by the addition of ADP (final concentration 270 μM). The respiratory control ratio (RCR) was calculated as the ratio of the respiratory rate in State 3 to the rate of oxygen uptake after exhaustion of ADP.

The effects of NEFA and purine nucleotides on leak-dependent mitochondrial oxygen consumption were determined in the above described reaction medium in the presence of 50 μM atractyloside, 1.5 $\mu\mathrm{g} \cdot \mathrm{ml}^{-1}$ oligomycin and 0.2 % fatty-acid-free BSA. Mitochondria respiring in State 4 were subjected to incremental additions of oleate (final concentrations 40, 80 and 100 μM, corresponding to calculated free concentrations of 21, 239 and 8365 nM respectively [26]), followed by two subsequent additions of GDP (final concentrations 1 and 2 mM) and, finally, an addition of cyclosporin A (10 μM final concentration) to the reaction mixture. The concentrations of atractyloside and oligomycin required to completely inhibit ADP/ATP translocase and

ATP synthase were determined in a separate experiment by dose–response analysis performed with mitochondria isolated from rat ($n = 6$) and human ($n = 2$) skeletal muscle.

Extraction of RNA and quantification of UCP2 and UCP3 mRNAs

Muscle specimens (40–45 mg) were removed from liquid nitrogen and homogenized, using a Polytron mixer, in 1 ml of guanidinium thiocyanate/phenol solution (Sigma Tri-reagent; Sigma, St. Louis, MO, U.S.A.), and total RNA was extracted according to the manufacturer's instructions. The integrity of the extracted RNA was verified by gel electrophoresis [27]. Total cDNA was synthesized using random primers. UCP2 and UCP3 mRNAs were quantified by reverse transcription followed by competitive PCR, using a synthetic multispecific standard with target sequences for UCP2, UCP3 and β-microglobin, as described in detail previously [28]. The UCP3 primer pair recognized sequences shared by the long ($UCP3_l$) and short ($UCP3_s$) forms of UCP3 transcripts [29].

Data analysis

All values reported are means $\pm$ S.E.M. Differences between means were tested for statistical significance by using Student's paired *t*-test or by ANOVA with a repeated-measure design, which was followed by Student–Newman–Keul's *post hoc* tests. Bivariate correlation coefficients were computed on the data, and Fisher's *r* to *z* test was used to determine their statistical significance. Significance was accepted at the 5% level.

RESULTS

The 6 weeks of endurance training increased the subjects' $V\dot{O}_2$peak by 24% ($P < 0.05$). The lactate threshold, expressed as a percentage of $V\dot{O}_2$peak, was 63.1 ± 2.1% before the training period, and increased to 75.5 ± 2.9% ($P < 0.05$) after the endurance training programme. These changes, reflecting up-regulation of whole-body and muscular oxidative function, were accompanied by marked increases in the muscle activities of CS and β-hydroxyacyl-CoA dehydrogenase [by 47% ($P < 0.05$) and 19% ($P < 0.05$) respectively], indicating that muscle mitochondrial capacity was enhanced by training. The training programme did not affect the muscle activity of phosphofructokinase, a marker of glycolytic activitiy. Results for muscle enzyme activities and muscle aerobic power will be published in detail elsewhere [30].

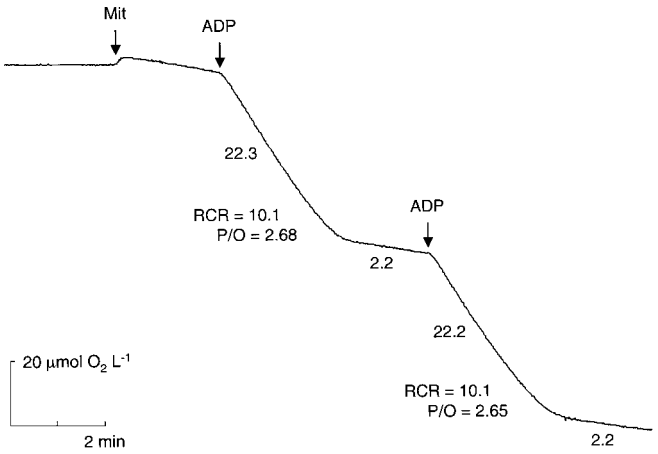

Figure 1 Representative oxygraphic trace of mitochondrial respiration in isolated human skeletal muscle mitochondria

The initial oxygen concentration was 237.5 μM O_2. Pyruvate (5 mM) + malate (2 mM) were used as respiratory substrates. The indicated additions are: Mit, 7.5 μl of mitochondrial suspension; ADP, 270 μM ADP. The numbers indicate rates of oxygen consumption, as μmol of $O_2 \cdot min^{-1} \cdot l^{-1}$. The RCR was calculated as the ratio of the respiratory rate in State 3 to the rate of oxygen uptake after exhaustion of ADP.

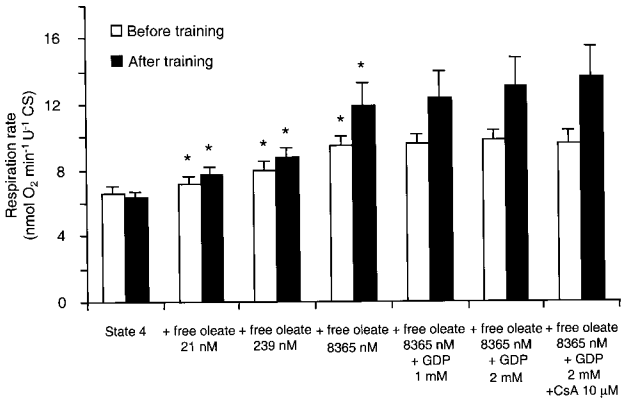

Figure 2 Proton-leak-dependent oxygen consumption rates in isolated human skeletal muscle mitochondria

Oxygen consumption rates of mitochondria isolated from human muscle samples obtained before and after a 6-week endurance training period were measured the presence of 50 μM atractyloside, 1.5 $\mu g \cdot ml^{-1}$ oligomycin and 0.2% fatty acid-free BSA. Respiration was supported by pyruvate (5 mM) + malate (2 mM). Respiring mitochondria were subjected to incremental additions of oleate to final concentrations of 40, 80 and 120 μM, corresponding to calculated free oleate concentrations of 21, 239 and 8365 nM respectively. This was followed by two subsequent additions of GDP (final concentrations 1 and 2 mM). Finally, 10 μM of cyclosporin A (CsA) was added to the reaction mixture. Values are means $\pm$ S.E.M. ($n = 8$); $^*P < 0.05$ compared with previous condition.

In order to evaluate the quality of the mitochondrial preparation and to elucidate the effects of endurance training on the degree of coupling of oxidative phosphorylation, isolated muscle mitochondria were subjected to a State 4–3–4 respiratory experiment. A representative oxygraphic trace of mitochondrial respiration is shown in Figure 1. Before the training period, the respiratory rates in States 3 and 4 were 65.7 ± 3.3 and 7.7 ± 0.6 mmol of $O_2 \cdot min^{-1} \cdot$ units of CS^{-1} respectively, and these were not changed significantly by training. RCRs were 8.8 ± 0.7 and 10.0 ± 0.4 before and after the training programme respectively (not significantly different). The high RCR values indicate that functionally intact mitochondria, with well preserved coupling between oxygen consumption and ATP production, were obtained by the isolation procedure.

The effects of oleate and GDP on proton-leak-dependent respiration in mitochondria isolated from samples obtained before and after the endurance training period are shown in Figure 2. Addition of oleate increased UCR in isolated muscle mitochondria. The effect of oleate increased with increasing concentration, but the relationship was not linear. The data provide an indication of saturation of the uncoupling effect with increasing concentrations of oleate. Subsequent additions of GDP and cyclosporin A to the reaction mixture failed to attenuate UCR induced by oleate. An interesting finding was that the relative increase in respiration rate induced by oleate was significantly higher post- compared with pre-training at all oleate concentrations used ($P < 0.05$). The increase in respiration due to oleate was about doubled in mitochondria from muscle

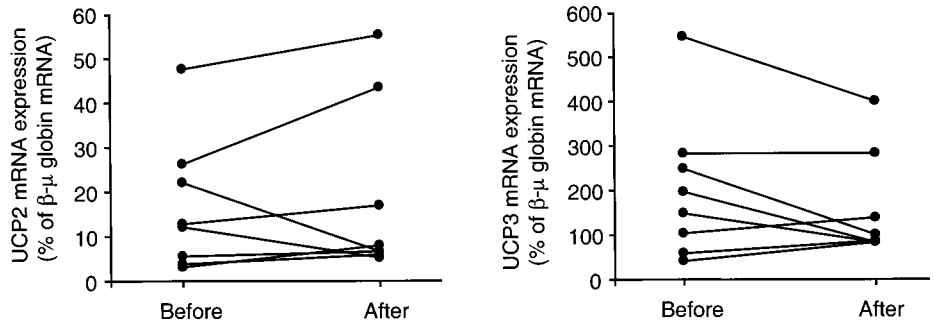

Figure 3 Individual levels of UCP2 and UCP3 mRNAs in human skeletal muscle

UCP2 and UCP3 mRNA levels were measured in human muscle samples obtained before and after a 6-week endurance training period. Results are reported as a percentage of β-microglobin (β-μ globin) mRNA. No statistically significant differences between means were detected (16.6 $\pm$ 5.3% and 18.5 $\pm$ 7.0% before and after training period respectively for UCP2 mRNA; 202.4 $\pm$ 57.5% and 156.6 $\pm$ 42.3% before and after training period respectively for UCP3 mRNA).

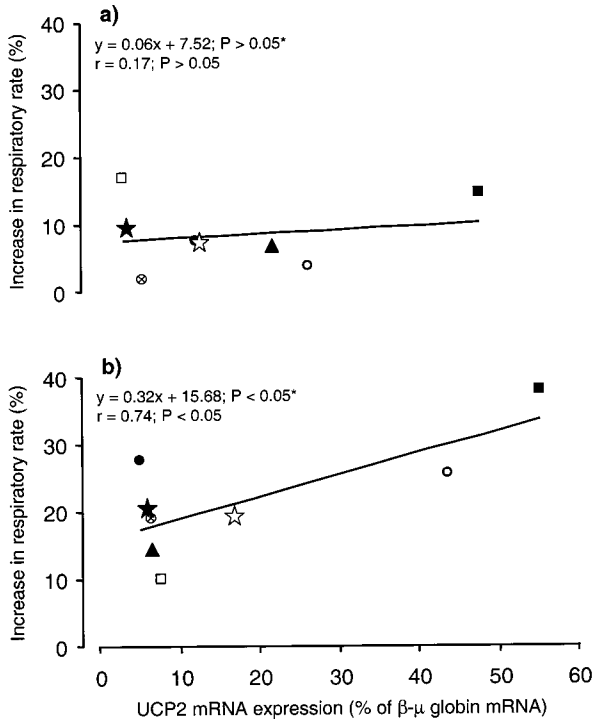

Figure 4 Relationship between UCP2 mRNA expression and the relative increase in the proton-leak-dependent oxygen consumption rate of isolated human skeletal muscle mitochondria induced by oleate

Assay conditions of mitochondrial respiration were identical to those described for Figure 2. Data show the increase in respiration rate induced by a free oleate concentration of 21 nM before (**a**) and after (**b**) the 6-week endurance training period. Abbreviation: β-μ globin, β-microglobin. *Test of the regression slope against $\beta = 0$.

samples taken after the training period compared with the effect in mitochondria from pre-training samples.

The levels of UCP2 and UCP3 mRNAs, before training, were 16.6 $\pm$ 5.3 and 202.4 $\pm$ 57.5% respectively of those of β-microglobin, and levels were not affected by the training period. Individual data are shown in Figure 3. Expression of both UCP2 and UCP3 showed considerable variation among the subjects. However, within the subjects variation was small. No correlation was observed between UCP2 or UCP3 mRNA levels and body mass index (22.6 $\pm$ 1.2; range 19.1–28.0 kg/cm^2), age (26 $\pm$ 2; range 20–37 years), or training status expressed as $V\!O_2$peak, muscle CS activity or lactate threshold. After the training period a significant correlation was observed between the UCP2 mRNA level and the relative increase in respiratory rate induced by the addition of a low (21 nM) (Figure 4) or a high (8365 nM) concentration of free oleate ($r = 0.74$, $P < 0.05$ and $r = 0.82$, $P < 0.05$ respectively). This correlation did not reach statistical significance for an intermediate concentration (239 nM) of free oleate ($r = 0.66$, $P = 0.08$). No correlation between UCP2 expression and the oleate-mediated increase in mitochondrial oxygen consumption was observed before the training period. The oleate-mediated increase in respiratory rate did not correlate with UCP3 mRNA expression either before or after the training programme.

DISCUSSION

The present results demonstrate that UCR in isolated human skeletal muscle mitochondria is increased by oleate. This effect may be mediated by membrane proteins and/or by an unspecific interaction of oleate with the phospholipid bilayer. However, previous studies with pure phospholipid liposomes as well as with proteoliposomes prepared with purified protein extracts of liver mitochondria have demonstrated that NEFA concentrations within the micro- and milli-molar range are required to maintain non-protein-mediated unspecific proton leak [31–33]. The low concentration of oleate (within the nanomolar range) required to elicit an increase in leak-dependent respiration in the present study suggests that this effect is due to the interaction of oleate with native membrane proteins, rather than unspecific interference with the phospholipid bilayer. The observed non-linear dose–response relationship, exhibiting saturation kinetics, supports the conclusion that the oleate-induced proton leak was not unspecific.

The observed NEFA-induced proton leak may theoretically be mediated by specific mitochondrial proteins such as UCP2 and UCP3 [6–9,12,14], ADP/ATP translocase [33,34], ATP synthase [35] or non-specific cyclosporin A-sensitive permeability transition pores [36]. In order to minimize a potential interaction of NEFA with ADP/ATP translocase and ATP synthase, the experiments were carried out in the presence of saturating concentrations of atractyloside and oligomycin, which inhibit

NEFA-mediated proton leakage through these mitochondrial components [33,35]. The possibility that the NEFA-mediated increase in mitochondrial respiration was a consequence of a drop in membrane potential caused by opening of the permeability transition pores was ruled out by the addition of cyclosporin A. The positive correlation observed between the NEFA-mediated increase in respiratory rate and UCP2 mRNA expression after the training period provides indirect evidence that UCP2 may account for at least a part of the NEFA-induced increase in proton-leak-dependent oxygen consumption in isolated human skeletal muscle mitochondria. However, because no relationship was found between the UCP2 mRNA level and proton leakage before training, and since UCP2 mRNA expression was unaffected by the training programme, the role of UCP2 in NEFA-induced uncoupling remains unclear. Definitive studies require the determination of UCP2 and UCP3 protein expression in parallel with measurements of leak-dependent mitochondrial oxygen utilization; however, the antibodies that are currently available do not permit this type of analysis.

The expression of UCP2 and UCP3 mRNAs in skeletal muscle was not affected by the endurance training period. Our data are in agreement with earlier observations in a cross-sectional study by Schrauwen and collaborators [10], where the muscle levels of UCP2 and UCP3 mRNAs expressed relative to β-actin were not significantly different between trained and untrained human subjects. In rodents, skeletal muscle UCP3 [22,24] and UCP2 [22] mRNAs are increased 3 h after exercise, but have returned to basal levels [22,24] or are reduced [21] after 24 h. Taken together, these results from animal studies indicate that each exercise bout elicits a transient increase in UCP2 and UCP3 mRNA expression within a few hours after exercise. This increase seems to recede within 24 h. The time course of UCP2 and UCP3 mRNA expression after acute exercise has not been studied in human skeletal muscle. However, if it is similar to that in animal muscle, a potential increase in the levels of UCP2 and UCP3 mRNAs may already have been dissipated at the point when muscle biopsies were obtained in the present study (i.e. 48 h after the last exercise bout). Despite the lack of changes in UCP2 and UCP3 mRNA levels observed in the present study, the possibility cannot be excluded that transient increases in UCP2 and UCP3 mRNA expression following each exercise bout during the training period may cause augmented expression of UCP2 and UCP3 proteins in skeletal muscle.

We also demonstrate that endurance training enhances the sensitivity of leak-dependent respiration to NEFA, as shown by the larger relative increase in leak-dependent respiration in human skeletal muscle at the same oleate concentration in samples obtained after compared with before training. Increased sensitivity of leak-dependent respiration to NEFA has been noted previously in isolated skeletal muscle mitochondria from cold-acclimated ducklings [37], and has been associated with increased non-shivering thermogenesis in skeletal muscle [38]. By analogy, it is possible that the training-induced increase in the sensitivity to NEFA of leak-dependent oxygen consumption observed in the present study may result in elevated resting oxygen utilization and increased heat production in the skeletal muscle of endurance-trained subjects as compared with untrained controls (assuming a similar resting concentration of NEFA in muscle cells).

Recently it has been demonstrated that the NEFA content in human myocytes decreases during exercise [39]. The observed increased sensitivity of proton leakage to NEFA in trained muscle would lead to a greater inhibition of proton leakage and, thus, a larger rise in the coupling efficiency of oxidative phosphorylation in response to the decline in the NEFA concentration during exercise. This would allow a more rapid switching from proton leak to ATP production when energy demand increases. Furthermore, the transition to a higher ATP production rate could be achieved with smaller changes in oxygen consumption and cytosolic free ADP concentrations. Thus enhanced sensitivity of proton leakage to NEFA in trained muscle will increase sensitivity and decrease response time to changes in ATP utilization in the cell. This will reduce metabolic perturbations and increase the potential for regulation of aerobic energy production. The increase in sensitivity of mitochondrial proton leakage to NEFA may represent a new type of adaptation strategy in skeletal muscle subjected to chronic exercise.

Studies on the effects of endurance training on the antioxidative defence system in humans have demonstrated that training-induced augmentation of aerobic power in skeletal muscle is not accompanied by up-regulation of mitochondria and whole-muscle antioxidative capacity (antioxidative enzyme activities; glutathione status) [30,40]. Since mitochondria represent an important source of ROS, this may lead to a mismatch between oxidative and antioxidative potential, and a shift towards a pro-oxidative state. It is possible that training-induced up-regulation of the sensitivity of mitochondrial proton leakage to NEFA, resulting in increased uncoupling, may prevent an increase in ROS generation in trained muscle by decreasing the mitochondrial membrane potential, thus increasing the electron transport rate through the respiratory chain. This would minimize ROS production by the electron transport chain, which is known to be a function of mitochondrial membrane potential and electron flux [5].

It is well known that a conformational change induced by binding of purine nucleotides to the nucleotide-binding domain inhibits the transport activity of UCP1. Since UCP2 and the long form of UCP3 also have a purine-nucleotide-binding domain, analogous to that observed in UCP1 and ADP/ATP translocase, it was suggested that these proteins would be regulated by nucleotides [9,15,20,41]. In the present study, addition of 1 or 2 mM GDP could not abolish the NEFA-induced increase in leak-dependent respiration. These results are in line with previous data by Monemdjou and co-workers [41] on isolated brown fat mitochondria from UCP1-deficient mice, showing that the proton-leak-dependent respiration was insensitive to 1 mM GDP. Negre-Salvayre and co-workers [20] reported that pre-incubation of mitochondria from UCP2-expressing rat cells with GDP induced a rise in the mitochondrial membrane potential. However, these results are difficult to interpret, since ADP/ATP translocase was not inhibited in the experiments.

In summary, the present study demonstrates that proton-leak-dependent oxygen consumption in isolated human skeletal muscle mitochondria is increased in the presence of low concentrations of oleate. The sensitivity of leak-dependent respiration to oleate was significantly augmented by a 6-week endurance training period. It is suggested that this adaptation may enhance the potential for metabolic regulation, increase the whole-body basal metabolic rate and prevent excess formation of ROS by the mitochondrial electron transport chain in trained muscle. The muscle content of UCP2 and UCP3 mRNAs was unaffected by training. Although a positive correlation between the NEFA-mediated increase in mitochondrial oxygen consumption and UCP2 mRNA expression was observed after the training period, no such relationship was found before training. Furthermore, the oleate-induced uncoupling was insensitive to GDP. Therefore the role of UCP2 and UCP3 in NEFA-induced uncoupling remains unclear. Direct measurements of UCP in parallel with determination of mitochondrial leak-dependent oxygen consumption are needed to finally clarify this point.

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