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ORIGINAL ARTICLE

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Mitochondrial transcription factor A and respiratory complex IV increase in response to exercise training in humans

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Abstract Protein levels of mitochondrial transcription factor A (Tfam) and nuclear- and mitochondrial-encoded subunits of respiratory chain complex IV (COX I and COX IV) as well as citrate synthase activity were analysed in muscle biopsy samples of vastus lateralis in six healthy male subjects before and after 4 weeks of one-legged cycle training. One leg was trained with restricted blood flow. The other leg was trained with the same power profile but with non-restricted blood flow. Tfam, COX I and COX IV levels all increased with training, with no differences observed between the legs. The training-induced increase in citrate synthase activity was greater in the leg trained with restricted blood flow. These findings indicate that changed expression of Tfam protein could be one mechanism of exercise-induced mitochondrial biogenesis. The increases of COX I and COX IV indicate a concurrent increase of nuclear- and mitochondrial-encoded subunits of respiratory enzyme complex IV at the protein level in skeletal muscle in response to increased muscle activity. In this study, it was not possible to demonstrate that the greater energy disturbance induced by reduced blood flow further stimulates the expression of mitochondrial proteins, even though it did cause a greater enhancement of citrate synthase activity in concordance with earlier studies.

Keywords Ischemia · Mitochondrial biogenesis · Mitochondrial transcription factor A (Tfam) · Protein expression · Skeletal muscle · Tfam

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Introduction

The adaptation of human and animal skeletal muscle to endurance training is well characterized and includes increases in the number and size of mitochondria, as well as in the activity of enzymes controlling oxidative metabolism. Endurance-training programmes lead to the greatest increases in mitochondrial enzyme activity and mitochondrial biogenesis in humans [2, 20].

The process of mitochondrial biogenesis is complex and requires gene products from both the mitochondrial and the nuclear genomes. For example, complex IV in the respiratory chain, named cytochrome oxidase (COX), consists of 13 polypeptide subunits of which subunits I–III are encoded in the mitochondrial genome and subunits IV–XIII are encoded in the nucleus. Even though the quantitative role of the mitochondrion's own genome and expression machinery is relatively small, it is nevertheless crucial for the function and assembly of the respiratory chain (see [21, 28]). It has, furthermore, been suggested that any change in mitochondrial gene expression occurs as a secondary event that depends on the availability of nuclear gene products [30]. However, the signals coordinating nuclear and mitochondrial gene expression and the pathways regulating mitochondrial transcription and replication are not completely understood.

One transcription factor most likely involved in the coordination and regulation of mitochondrial proteins is mitochondrial transcription factor A (Tfam). It is a 25-kDa nuclear-encoded DNA-binding protein able to translocate into the mitochondria and, together with RNA polymerase, it binds to the mitochondrial promoters (see [21, 28]). Tfam is crucial for the efficient transcription and replication of mitochondrial-encoded genes in vitro and in hereditary mitochondrial disease in humans [4, 6, 8, 13, 18]. A knockout mouse model has further illustrated the great importance of Tfam for both mitochondrial biogenesis and embryonic development through its regulation of the number of mtDNA copies in vivo [14]. Recently it was also shown that Tfam increases at both mRNA and protein levels in response to electrical

stimulation in rat skeletal muscle in combination with increased expression of mitochondrial-encoded transcripts [9]. To our knowledge, there are no data on the expression of Tfam in human tissues in response to physical activity or any other stimulus known to induce mitochondrial biogenesis. Furthermore, a disturbance in cell energy status caused by an impairment of the mitochondrial energy supply to the rat heart by creatine depletion increased both the mitochondrial density and the protein level of Tfam [29]. A disturbed cell energy status, for example reduced ATP/ADP ratio and creatine phosphate levels, has been suggested to be one stimulus for mitochondrial biogenesis in skeletal muscle. For example, exercise training during reduced oxygen delivery increases citrate synthase activity, a marker of mitochondrial volume fraction, more than training under normal exercise conditions [23, 24]. Moreover, patients with unilateral arteriosclerotic leg disease have been reported to have increased citrate synthase activity [12].

Accordingly, the main aims of the present study were to test the following hypotheses: (1) that 4 weeks of endurance training increase the protein levels of Tfam; (2) that this increase occurs concurrently with increased protein levels of COX I and COX IV and citrate synthase activity. Additionally, we wanted to investigate whether the responses to these factors are additive following exercise training performed in conditions of enhanced metabolic stress induced by reduced leg blood flow, compared to exercise under normal flow conditions.

Materials and methods

Subjects

Six healthy male subjects participated in the study. Their range ages, heights and mass were 20–26 years, 174–182 cm and 74–83 kg, respectively. Because of the strenuous nature of the training program, some familiarity with physical training was considered important, whereas recruiting subjects that were too well trained was avoided as this might diminish the possibility of detecting significant training effects. The subjects were carefully informed about the procedures and the nature of the training program before consenting to participate. The study was approved by The Ethics Committee of Karolinska Institutet.

Exercise model

A method first described by Eiken and Bjurstedt [7] was employed to induce restricted blood flow during exercise. Local application of external pressure over the working leg was used to reduce exercise blood flow in a controlled fashion. The subject was positioned supine in the opening of a large pressure chamber with both legs inside the chamber and one leg strapped to a pad on the lower leg. The pad was connected, via a metallic bar, to a crank-arm of an electrically braked cycle ergometer with locked flywheel, the centre of rotation being at the level of the heart. The chamber opening was sealed off at the level of the crotch by a rubber diaphragm with holes and self-sealing sleeves for the legs. Shoulder supports were used to prevent craniad displacement of the body as the chamber pressure was increased. For exercise under restricted blood flow, the chamber pressure acting on the exercising leg was elevated to 50 mmHg above atmospheric pressure. Compared to exercise under normal atmospheric pressure, this has been shown to reduce leg blood flow during one-legged cycle exercise by 15–20%, reduce 10–12 units of oxygen saturation and cause a greater depletion of ATP in the exercising leg and release of lactate [23]. Exercise under non-restricted blood flow conditions was performed using the same experimental arrangements but with normal atmospheric pressure.

Exercise protocol

One-leg training was performed four times a week during a 4-week period, giving a total of 15 training sessions. One leg trained under restricted blood flow condition (R-leg), while the other leg trained with non-restricted blood flow (NR-leg). The subjects were randomized into two groups, one group trained their right leg and the other group their left leg during restricted blood flow. Each training session started with 45 min of training under restricted blood flow conditions. The subjects were instructed to cycle at a constant pedalling rate of 60 rpm at the highest tolerable workload for 45 min, taking in to account that the whole 45-min session must be completed. After 10 min of rest, the same workload protocol was performed by the other leg, but with normal atmospheric pressure. Accordingly, the two legs developed the same power and amount of work in each session. The Borg rate (1–10 scale) target during ischemic training were 9–10 during the last 5 min in all training sessions. The ischemic training was invariably experienced as extremely strenuous with periods of ischemic muscle pain occurring frequently during the training session, whereas training of the contralateral NR-leg was experienced as very light.

Performance test

After familiarization with the experimental model, one-legged step-wise incremental exercise tests were performed during the week before the training period and the week after cessation of the training period. Each leg was tested during non-restricted blood flow conditions. The subject was instructed to maintain a pedalling rate of 60 rpm, starting with 4 min at 20 W. The work load was then increased by 10 W every minute until the prescribed pedalling rate could no longer be maintained. When the pedalling rate fell below 55 rpm for more than 5 s, the time and peak load were recorded and the experiment terminated.

Muscle biopsy procedure

Biopsy samples were obtained from the vastus lateralis muscle of both legs before the start of the first training session and 24 h after the last training session under local anaesthesia using the percutaneous needle technique [1]. Biopsy samples were immediately frozen in isopentane cooled in liquid nitrogen and subsequently stored at –80°C until further analysis.

Citrate synthase activity

For enzyme activity determination, 10-µm-serial cryostat sections were freeze-dried and clearly visible areas of blood, fat or connective tissue were removed with the aid of a dissection microscope. After homogenization in ice-cold 0.1 M phosphate buffer (pH 7.7) with 0.05% BSA (dilution 1 mg dry muscle in 500 µl buffer) citrate synthase activity was analysed by a fluorometric method according to the principles of Lowry and Passonneau [16] as described by Lin et al. [15].

Muscle lysate preparation

Muscle samples were homogenized in ice-cold homogenization buffer (20 µl/mg frozen tissue) as previously described [27]. This buffer contains 20 mM HEPES, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM glycerol-2-phosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100,

5 mM EGTA, 20 µg/ml leupeptin, 50 µg/ml aprotinin and 40 µg/ml PMSF. Homogenates were rotated for 60 min at +4°C and thereafter centrifuged at 15,000 *g* at +4°C for 10 min. After centrifugation, the supernatant was removed and protein concentration was determined according to Bradford (Bio Rad Protein Kit, Richmond, Calif., USA). The samples were mixed with 4× Laemmli buffer pH 6.8 (8% SDS, 40% glycerol, 250 mM Tris-HCl and bromphenol blue) and boiled for 4 min before loading onto the gel for analysis.

Western blot

The muscle homogenates were separated using 15% SDS-PAGE and transferred to nitrocellulose membranes. After transfer, membranes were briefly washed in Tris-buffered saline with 0.1% Tween-20 (TBST) and thereafter stained with Ponceau S to provide visual evidence that uniform loading and the electrophoretic transfer of proteins had been achieved. Membranes were incubated in 5% dry non-fat milk for 60 min at room temperature and then probed with an excess of primary antibody (COX I and COX IV, Molecular Probes, Leiden, The Netherlands; Tfam kind gift from N.-G. Larsson, Karolinska Institutet, Stockholm) in 5% dry non-fat milk over night at +4°C. After being washed in TBST, bound antibodies were detected with peroxidase-linked secondary antibody at room temperature for 60 min and thereafter washed in TBST. Bound antibodies were detected using LumiGLO Chemiluminescent Substrate (BioLabs, Beverly, Mass., USA) and quantified densitometrically. The densitometric signals were defined in arbitrary units. The relationship between the amount of protein and detected signals of Tfam, COX-I and COX-IV was tested by loading increasing amounts of protein. Monoclonal anti-alpha sarcomeric actin (42 kDa) was used as a control for differences in loading (Sigma, Saint Louis, Mo., USA).

Statistical analysis

The overall training-induced changes as well as the differences between conditions, e.g. interaction, for Tfam, COX I and COX IV were analysed by a non-parametric method (Wilcoxon matched pairs test). Absolute changes in time to fatigue, peak load and citrate synthase activity were statistically analysed using ANOVA with two dependent factors: (1) training condition and (2) time. $P<0.05$ were considered statistically significant. Relative changes in citrate synthase activity were analysed by Student's paired *t*-test.

Results

Performance

The work performed during one training session increased by 100% during the training period (Fig. 1). Prior to training, there was no significant difference between the two legs in time to fatigue or in peak load (R-leg 18±1.9 min and 163±21 W; NR-leg 17±1.6 min and 158±15 W, respectively). Independently of the training condition, the relative increase following 4 weeks of exercise training was 13% in time to fatigue and 9% in peak load ($P<0.01$). No significant differences in training-induced changes were observed between the R-leg compared to the NR-leg, in absolute or relative values (R-leg 1.6±1.6 min and 16.7±16.3 W; NR-leg 1.3±0.9 min and 13.3±10.3 W, respectively).

Citrate synthase activity

Prior to the training period, there was no significant difference between legs in basal citrate synthase activity

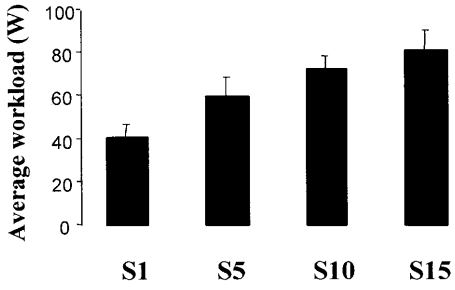


Fig. 1 Average workload during the training program. Average one-legged workload (W) during training sessions (S) 1, 5, 10 and 15. Training was conducted for 4 weeks (four sessions per week, 45 min per session). One leg exercised under restricted blood flow condition (*R-leg*) and the contralateral leg under non-restricted blood flow condition (*NR-leg*) but with identical workload

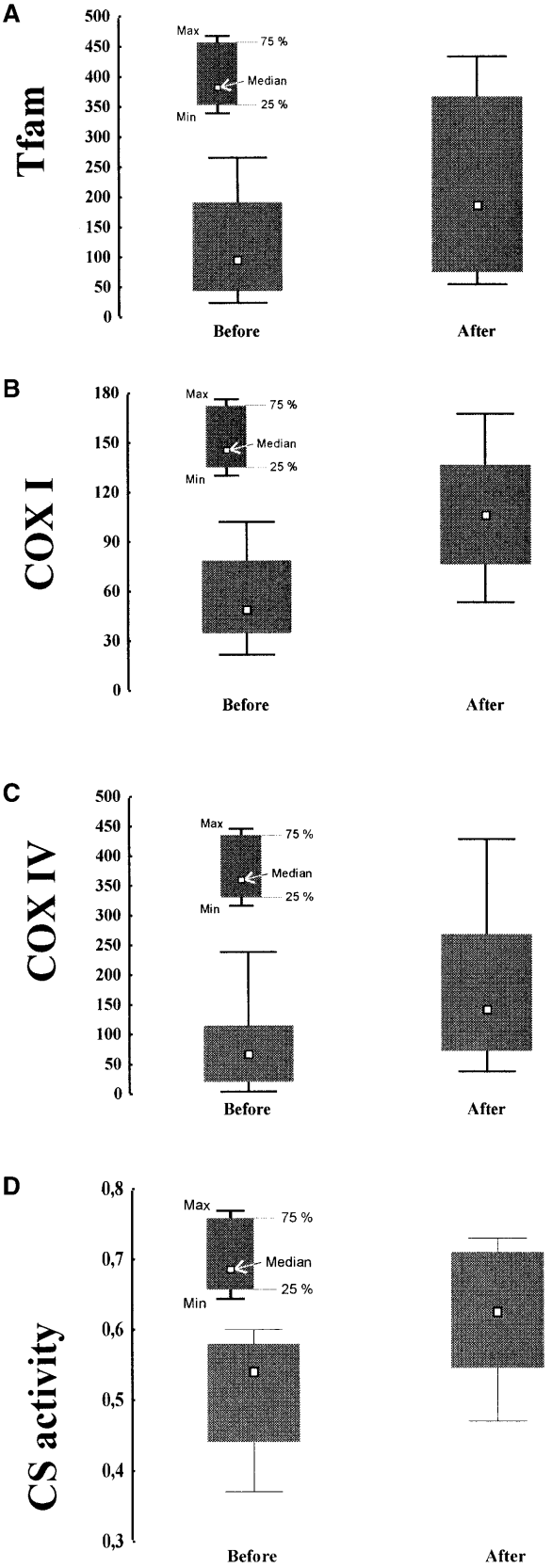
(R-leg 0.53±0.07 µkat/mg wet muscle; NR-leg 0.52± 0.09 µkat/mg wet muscle). Independently of the training condition, the relative increase in citrate synthase activity following 4 weeks of exercise training was 24% ($P<0.05$). No significant differences in training-induced changes in citrate synthase activity were found in absolute values between the R-leg and NR-leg (R-leg 0.10±0.15 µkat/mg wet muscle; NR-leg 0.06±0.11 µkat/mg wet muscle) ($P<0.08$) but the individual relative increase was significantly greater in the R-leg (R-leg 34±17%; NR-leg 13±22%, $P<0.05$).

Protein levels of Tfam, COX I and COX IV

Prior to the training period, there was no significant difference between the legs in terms of the basal protein expression of Tfam, COX I and COX IV. The median relative increase in protein levels for Tfam, COX I and COX IV after 4 weeks of exercise training were 92%, 98% and 154%, respectively ($P<0.05$ for all, Figs. 2A–C, 3). No significant differences in training-induced changes were found between the R-leg and the NR-leg in either absolute or relative values.

Discussion

Increased activity of "oxidative enzymes" (enzymes in the Krebs cycle and respiratory chain) and mitochondrial mass are well-known metabolic and structural changes that occur with a longer period of increased muscle activity (see [2, 20]). Observations from in vitro, knockout models and in pathophysiological processes such as hereditary mitochondrial diseases imply that the mitochondrial transcription factor Tfam plays a critical role in regulating the transcription and replication of the mitochondrial genome [4, 6, 8, 13, 14, 18]. The current study is the first to report increased protein levels of Tfam in human tissue in response to a stimulus known to induce mitochondrial biogenesis, i.e. endurance training. Furthermore, this is the first observation of a training-



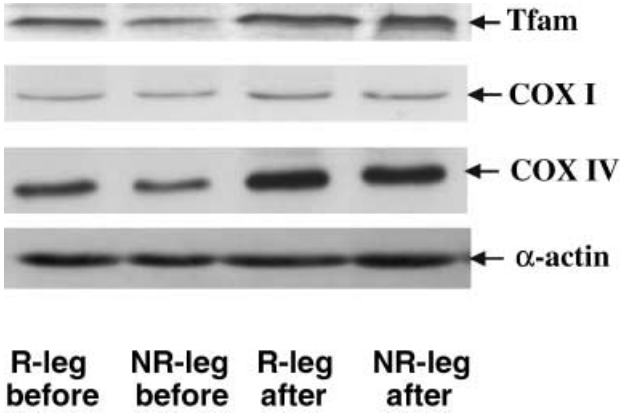


Fig. 3 Western blots. Western blots showing one typical subject for Tfam, COX I and COX IV and α-actin (loading control) in the restricted (*R-leg*) and non-restricted leg (*NR-leg*) before and after 4 weeks of one-legged training

induced increase at the protein level of any factor in human skeletal muscle shown to have a crucial role in the regulation of mitochondrial transcription and/or replication.

Training under restricted and non-restricted blood flow conditions increased endurance performance, as shown by increased time to fatigue and peak load. Furthermore, the mitochondrial oxidative capacity increased in the trained muscles as indicated by increased protein levels of COX I and COX IV subunits and increased citrate synthase activity. The training-induced increase of COX I (mitochondrial-encoded) is suggested to be mediated by Tfam-induced mitochondrial genome activation, since efficient transcription of the mitochondrial genome is only accomplished when Tfam and mitochondrial RNA polymerase act together in binding to the mtDNA promoter [8]. Thus, the exercise-induced increases in Tfam and COX I indicate that altered protein expression of Tfam could be one mechanism of exercise-induced mitochondrial biogenesis in humans. Observations from our laboratory of decreased protein levels of Tfam 3 months after the cessation of a 4-week one-legged training period (unpublished data) are in line with such an assumption. The data from Gordon et al. [9] further supports such a hypothesis. In their study, increased Tfam mRNA expression and protein expression were observed in combination with the increased expression of mitochondrial transcripts in rat skeletal muscle in response to electrical stimulation. The concurrent increase in COX I and COX IV in the present study also supports an earlier hypothesis that

Fig. 2A–D Changes in protein expression. Protein levels (arbitrary units) before and after 4 weeks of one-legged cycle training in six subjects. **A** Mitochondrial transcription factor A (Tfam), **B** mitochondrial-encoded subunit I of cytochrome c oxidase (*COX I*), **C** nuclear-encoded subunit IV of cytochrome c oxidase (COX IV), and **D** citatrate synthase activity before and after 4 weeks of one-legged cycle training in six subjects. *Box plots* show minimum, maximum, 25%, 75% percentiles and median values as indicated by the schematic box plot in the *upper left corner* in each figure

changes in mitochondrial protein levels are related to each other in skeletal muscle in response to increased muscle activity. Similar findings of a concurrent increase of mRNA for different mitochondrial enzyme subunits in response to increased muscle activity have earlier been described for animal species and humans [11, 19].

Previous studies have shown that exercise performed under restricted blood flow conditions induces a lower oxygen tension and a greater energy disturbance in exercising muscle compared to exercise under non-restricted blood flow conditions [23]. In the present study, 4 weeks of endurance training under such reduced oxygen delivery conditions did not further increase the protein levels of Tfam, COX I or COX IV in the R-leg compared to the NR-leg. The lack of a further increase may indicate that additional stimuli were not "activated" enough by the flow restriction compared to exercise training under nonrestricted blood flow conditions. One cannot exclude the possibility of a crossover effect between the legs (e.g. neural or humoral) that may have influenced any "locally obtained" differences in the two training conditions. However, the greater relative increase in citrate synthase activity in the R-leg indicates an ischemically induced difference in "stimulus strength". One explanation for the difference between the legs in changes of mitochondrial protein and citratate synthase activity could be that the immunoblot technique for analysing protein levels is less sensitive than the citrate synthase activity assays, and therefore less able to detect possible protein differences between the two exercise conditions. However, even though oxidative enzyme activity and the amount of mitochondrial protein have been regarded by some researchers as strictly related to each other [3, 20], Soussi et al. [22] observed both a quantitative and a qualitative change in the enzyme activity of cytochrome c oxidase in response to exercise training. It should also be noted that the idea of lower oxygen tension being coupled to higher mitochondrial oxidative capacity as a stimulus in skeletal muscle is not unopposed [5, 10].

The present data do not demonstrate which molecular mechanisms lead to increased Tfam gene expression. The nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) are possible candidates, since they have binding sites in the promoter region of the Tfam gene and are important in the regulation of Tfam gene expression in vitro [26]. In addition, the binding sites for NRF-1 and NRF-2 have also been recognized in the promoter region of several nuclear-encoded mitochondrial proteins. One example is the NRF-1 binding site in the promoter region of COX IV [25]. Thus, the observation that both Tfam and COX IV increase in the current study suggests the involvement of NRF-1 gene activation in the response to exercise. Further support for such a mechanism comes from Murakami et al. [17], who showed increased NRF-1 mRNA levels after one bout of exercise in rats.

In conclusion, the findings in the present study indicate that increased expression of Tfam could be one mechanism of exercise-induced mitochondrial biogenesis in humans. The training-induced increases of the mitochondrial-encoded COX I and nuclear-encoded COX IV proteins support earlier findings that mitochondrial protein levels are related to each other in skeletal muscle in response to increased muscle activity. The hypothesis that the energy disturbance evoked by restricted blood flow increases the mitochondrial protein to a greater extent was not supported.

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