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EFFECT OF 5-WEEK MODERATE INTENSITY ENDURANCE TRAINING ON THE OXIDATIVE STRESS, MUSCLE SPECIFIC UNCOUPLING PROTEIN (UCP3) AND SUPEROXIDE DISMUTASE (SOD2) CONTENTS IN *VASTUS LATERALIS* OF YOUNG, HEALTHY MEN

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In the present study fifteen male subjects (age: 22.7±0.5 years; BMI: 23.5±0.6 kg · m^{-2}; VO_{2max} 46.0±1.0 mL · kg^{-1}·min^{-1}) performed 5 week moderate intensity endurance training. The training resulted in a significant increase in maximal oxygen uptake (VO_{2max}) (P=0.048) and power output reached at VO_{2max} (P=0.0001). No effect of training on the uncoupling protein 3 (UCP3) content in the *vastus lateralis* was found (P>0.05). The improvement of physical capacity was accompanied by no changes in cytochrome-*c* and cytochrome-*c* oxidase contents in the *vastus lateralis* (P>0.05). However, the training resulted in an increase (P=0.02) in mitochondrial manganese superoxide dismutase (SOD2) content in this muscle. Moreover, a significant decrease (P=0.028) in plasma basal isoprostanes concentration $[F_2isoprostanes]_{pl}$ accompanied by a clear tendency to lower (P=0.08) gluthatione disulfide concentration $[GSSG]_{pl}$ and tendency to higher (P=0.08) total antioxidant capacity (TAC) was observed after the training. We have concluded that as little as 5 weeks of moderate intensity endurance training is potent to improve physical capacity and antioxidant protection in humans. Surprisingly, these effects occur before any measurable changes in UCP3 protein content. We postulate that the training-induced improvement in the antioxidant protection at the muscle level is due to an increase in SOD2 content and that therefore, the role of UCP3 in the enhancement of physical capacity and antioxidant protection, at least in the early stage of training, is rather questionable.

Key words: *endurance training, oxidative stress, superoxide dismutase, uncoupling protein 3*

INTRODUCTION

It is well established that regular endurance activity enhances not only exercise tolerance (1), but exerts also beneficial effects among others on cardiovascular function, glucose and lipid metabolism (2). It is also known that each muscular activity is accompanied by reactive oxygen species (ROS) and reactive nitrogen species (RNS) production (3). The excessive ROS production during strenuous exercise decreases muscle force, accelerates fatigue (4) and without adequate antioxidant defence leads to oxidative stress and damage of the cells (5, 6). On the other hand, ROS are also signalling molecules, which exert regulatory effects in the human body not only by an enhancement of the antioxidant protection but also by the influence on the metabolic pathways related to glucose transport, calcium metabolism, ATPases activities (3, 7). It should be mentioned that exercise-induced ROS production, through the activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), plays an important role in the mitochondrial biogenesis (8) - the main endurance training-induced adaptation at skeletal muscle level (9) leading to the enhancement of the metabolic stability and exercise tolerance (10).

It is well known that in endurance-trained individuals systemic oxidative stress is lower (11, 12), while muscle antioxidant enzymes activities are higher when compared to the untrained subjects (13). The endurance training-induced increase in the antioxidant protection in skeletal muscles is mainly dedicated to the up-regulation of the antioxidant enzymes content/activity such as mitochondrial superoxide dismutase (SOD2), glutathione peroxidase (GPX) and γ-glutamylcysteine synthetase (GCS) *via* ROS-dependent signalling pathways (6, 14).

It is suggested by some authors that skeletal muscle specific uncoupling protein (UCP) - UCP3, which belongs to the family of the inner mitochondrial membrane anion carriers is involved in the antioxidant system of muscle cell by participating in the activated proton leak (15). In the *in vitro* studies it has been shown that UCP3 mediated proton leak to the mitochondria matrix, bypassing ATP-synthase leading to a small decrease in mitochondrial electrochemical gradient (mild uncoupling) is

responsible for the enhancement of respiration rate and *via* negative feedback loop for the lowering of ROS production in the electron transport chain ETC (16). Recently, it has been demonstrated that an increase in ROS production during single bout of exercise is accompanied by an increase in expression of UCP3 in the presence of no changes in SOD2 content (17). It is well known, that the regular endurance exercise increases the skeletal muscle antioxidant enzymes activity/contents after several weeks of training (18). Therefore, in the early stage of training before the changes in antioxidant enzyme content/activity an increase in UCP3 activity/content might play a protective role in view of an increased ROS production due to regular exercise. It has been demonstrated indeed that endurance training in its early stage has an effect on the UCP3 protein content. However, the results concerning the effect of endurance training on the UCP3 content at least in the early stage of training (up to few weeks) are ambiguous, since a decrease in the UCP3 contents (19, 20) as well as an increase in this protein (21, 22) has been reported. These discrepancies are more likely due to different sensitivities to training of various subjects (23). Based on the cross-sectional study it is evident that long term endurance training (years of training) decreases UCP3 protein content (24). What seems to be important from the practical point of view is the role of UCP3 in the antioxidant status of muscle cell in humans undertaking moderate physical endurance training.

We have hypothesised that 5-week moderate intensity endurance training, which is frequently recommended for the improvement of the cardiorespiratory and metabolic status in untrained people, is potent to enhance both physical capacity and muscle antioxidant protection. Therefore, in the present study we have examined the effect of the moderate intensity training on the physical capacity, plasma biomarkers of the oxidative stress as well as on the UCP3 and SOD2 levels in the *m. vastus lateralis* in young, healthy previously untrained men.

SUBJECTS AND METHODS

Subjects

Fifteen untrained, but physically active male volunteers (mean ±S.E.M: age 22.7±0.5 years; body mass (BM) 76.4±2.3 kg; body mass index (BMI) 23.5±0.6 kg·m^{-2}; VO_{2max} 46.0±1.0 mL·kg^{-1}·min^{-1}) took part in this study. Subjects were asked to avoid heavy exercise the day before and on the day of the test as well as to avoid caffeinated and alcoholic beverages 24 hours before exercise. All subjects were aware of the aims of the study and gave informed written consent. The study protocol was approved by the Local Ethical Committee and was performed in accordance with the Declaration of Helsinki.

Endurance training programme

The subjects underwent a 5-week endurance training programme. Training was performed on cycloergometers Monark 874 E at pedaling rates amounting to 60 rev·min^{-1}. The programme included four training sessions per week. Two various training protocols were applied: (a) continuous endurance cycling – performed at the power output (PO) corresponding to 90% of oxygen consumption measured at previously determined lactate threshold (LT) (*i.e.* 90% VO_2 at LT) lasting 40 minutes and (b) intermittent endurance cycling composed of 6 minutes cycling without resistance (unloaded cycling) followed by 3 minutes cycling at the power output corresponding to 50% Δ, repeated 4 times. The power output corresponding to 50% Δ was calculated for each subject as follows: 50% Δ = PO at LT + 0.5×(PO at VO_{2max}–PO at LT) (5). Continuous endurance cycling training was performed on Tuesdays and Fridays, whereas intermittent endurance training on Mondays and Thursdays. On Wednesdays, Saturdays and Sundays no training was applied. During the five-week training the subjects performed 20.8±0.6 training sessions lasting in total 13.9±0.4 hours. The training workload applied was predominantly of moderate intensity since 85% of the training workloads (expressed in time) were performed below the lactate threshold and only 15% above LT (at 50 % Δ see above). For details see Majerczak *et al.* (26).

Exercise protocol

The incremental exercise test was performed on the cycloergometer Ergo-Line GmbH & Co KG 800s (Bitz, Germany) twice: three days before and three days after completing a 5-week endurance training. Before the test, a 6-min resting period was allowed to determine the cardio-respiratory parameters at rest. The exercise test was performed at pedaling rates of 60 rev·min^{-1} started at power output 30 W, followed by a gradual increase of power output by 30 W every 3 minutes and it was continued until exhaustion (27).

Gas exchange variables

Gas exchange variables were measured continuously breath by breath using the Oxycon Champion, Mijnhardt BV (Bunnik, The Netherlands), starting from the 6th minute prior to exercise until the test was stopped. Before and after each test, gas analysers were calibrated with certificated calibration gases as previously described by Zoladz *et al.* (28).

Blood sampling

Blood samples were taken about 15 minutes prior to the onset of the incremental exercise using an Abbot Int-Catheter, Ireland (18G/1.2×45 mm) connected to an extension set using a "T" Adapter SL Abbot, Ireland (the tube 10 cm in length) inserted into the antecubital vein. Immediately before blood sampling, 1 mL of blood volume was drawn in order to eliminate blood from the catheter and the T-set. Blood samples for measurements of free 3,5,3'-triiodo-L-thyronine [fT_3], total antioxidant capacity (TAC), thiol groups concentration [Thiols], glutathione [GSH], glutathione disulfide [GSSG] concentrations, 8-epi-prostaglandin $F_{2\alpha}$ concentration [F_2isoprostanes] were taken at rest in the morning hours 7:30-8:00 a.m. in fasting state, before and after five weeks of endurance training. Blood samples for plasma lactate measurements [La^-] were taken in a fed state prior to the exercise test, at the end of each step of the incremental exercise (the last 15 seconds before increase of power output) and at the moment of ending the exercise protocol.

Plasma lactate measurements $[La^-]_{pl}$

The samples for $[La^-]_{pl}$ measurements (0.5 mL each) were placed in 1.8 mL Eppendorf tubes containing 1 mg ammonium oxalate and 5 mg sodium fluoride and mixed for about 20 seconds and then centrifuged. The blood plasma (about 200 μL) was stored at -40°C for further analysis of $[La^-]_{pl}$ using an automatic analyser Vitros 250 Dry Chemistry System, Kodak (Rochester, NY, USA). Lactate threshold in this study was defined as the highest power output above which $[La^-]_{pl}$ showed a sustained increase of more than 0.5 mmol·L^{-1} step^{-1} (28).

Serum free 3,5,3'-triiodo-L-thyronine $[fT_3]_s$

Blood for $[fT_3]_s$ was collected into plain tubes and left to clot for a minimum of 30 minutes at room temperature and then

centrifuged at 4000 rev·min^{-1} for 5 min. Samples were stored at minus 80°C until further analysis. $[fT_3]_s$ was determined using electrochemiluminescence immunoassay (ECLIA) using automatic analyzer Elecsys 2010 (Roche Diagnostics). The clinical reference range of $[fT_3]_s$ for healthy men was 2.80-6.80 pmol·L^{-1}.

Measurements of plasma TAC, $[Thiols]_{pl}$, $[GSH]_{pl}$, $[GSSG]_{pl}$, $[F_2isoprostanes]_{pl}$

The blood samples for TAC, [Thiols], [GSH], [GSSG] and [F_2isoprostanes] determination were collected in tubes containing EDTA, then centrifuged at 3000 rev·min^{-1} for 10 min at 4°C and stored at - 40°C for further analysis.

Estimation of total antioxidant capacity

Total antioxidant capacity of blood plasma was estimated by $ABTS^{\bullet+}$ decolorization method (29) modified for a microplate reader. $ABTS^{\bullet+}$ solution was diluted to give absorbance at 405 nm of 1. Then 2 µL of serum, diluted 1:1 with PBS, was added to 200 µL of $ABTS^{\bullet+}$ solution in a 90-well microplate. After 1 min, decrease in absorbance was measured at 405 nm. TAC was expressed in Trolox equivalents.

Estimation of thiol groups

The concentration of free thiol groups in plasma was assayed by the monobromobimane method (30). Briefly, plasma samples were diluted 2× in phosphate-buffered saline, monobromobimane (a thiol-specific reagent which generates a fluorescent product upon reaction with thiol groups) was added to a final concentration of 100 µmol · L^{-1} and the initial rate of reaction was measured by following the rate of increase in fluorescence (excitation wavelength 380 nm, emission wavelength 490 nm) using a Fluoroskan Ascent FL (Thermo Labsystems) kinetic fluorimeter. A calibration curve was prepared using reduced glutathione as standard and results were expressed as absolute thiol group concentration values.

Determination of concentration of reduced and oxidized glutathione in plasma

The concentrations of reduced glutathione $[GSH]_{pl}$ and oxidized glutathione $[GSSG]_{pl}$ in plasma were assayed by the *o*-phthalaldehyde method (31). Fluorescence was measured using a Fluoroskan Ascent FL (Thermo Labsystems) fluorimeter (excitation wavelength 355 nm, emission wavelength 430 nm). Authentic GSH and GSSG were used for preparation of a calibration curve.

Estimation of lipid peroxidation

8-epi-prostaglandin $F_{2\alpha}$ concentration in plasma samples ($[F_2isoprostanes]_{pl}$) was assayed by BIOXYTECH® 8-Isoprostane Assay Kit *Oxis*Research™ according to manufacturer's instructions following the extraction procedure.

Muscle biopsy

Muscle biopsy samples were taken from the *vastus lateralis m. quadricipitis femoris* 15 cm above the upper margin of patella, under local anaesthesia (1% lidocaine), using 2 mm ϕ biopsy needle (Pro-Mag ™ I 2.2, MDTECH). Specimens were frozen and stored in liquid nitrogen until further analyses. Muscle biopsy was taken twice *i.e.* before and after 5 weeks of endurance training. The post-training muscle biopsies were taken about 24±2 hours after the last training session.

Muscle biopsies were used for measurements of myosin heavy chain (MyHC) composition, UCP3mRNA, *UCP3* protein, cytochrome-*c* and cytochrome-*c* oxidase proteins in fifteen subjects. Due to the shortage of muscle samples SOD2 content was determined only in eleven subjects.

RNA extraction, RT-PCR and real-time quantitative PCR

Total RNA from deep frozen human muscle tissue was isolated using the NucleoSpin RNA II kit (Macherey-Nagel) following DNase I treatment according to the manufacturer's instruction. RNA concentration was determined using Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen) according to the manual and the cDNA was synthesized using 1 µg of total RNA, oligo-dT primer and ImProm-II Reverse Transcriptase (Promega) according to the manufacturer's instruction. The cDNA quality was verified in a PCR reaction, carried out with UCP3 specific primers (forward: 5'ATGAGCTTCGCCTCCATCC3' and reverse: 5'CTGGCGATGGTTCTGTAGG3'; size of product was 248 bp) as described by Russell *et al.* (32). Real-time quantitative PCR reactions were performed using the Assay-on-Demand Gene Expression Product (Applied Biosystems). For *UCP3* gene the probe Hs00243297_m1 was used, as control the *β-actin* probe (Hs99999903_m1) and synthesized cDNA. Reactions were run on a real-time PCR iCycler device (Bio-Rad, Hercules, CA, USA) with the 3.0a software version. The relative changes of UCP3 expression was calculated as described previously Krugluger *et al.* (33). For statistical analysis the mean values from two independent real time PCR experiments, each with new synthesized cDNA were used. The results were presented in arbitrary units (AU).

Muscle proteins analysis
Antibodies

The following primary antibodies were used [1] rabbit anti-UCP3 against amino acid residues 254-267 of human UCP3 (U 7757, Sigma Saint Louis, Missouri, USA), which shows no cross-reactivity with UCP1 and UCP2 (Product Information); [2] mouse monoclonal anti-cytochrome-*c* antibody (556433, BD PharMingen); [3] mouse monoclonal anti-cytochrome oxidase subunit I antibody (A6403, Molecular Probes); [4] mouse monoclonal anti-manganese superoxide dismutase (SOD2) antibody raised against full length SOD-2 of human origin (sc-58426, Santa Cruz Biotechnology).

Muscle protein extraction

Muscle biopsies (8-28 mg) were ultrasonicated (UP 50H sonicator, Dr. Hielscher GmbH) with 150-200 µL of buffer consisting of 62.5 mmol · L^{-1} Tris pH 6.8, 10% glycerol, 2.5% SDS. After centrifugation supernatants were assayed for protein content using the Bicinchoninic Acid Protein Assay Reagent (Sigma) and Bovine Serum Albumin as standard. The remaining supernatants were treated with 2-mercaptoethanol at a final concentration of 2.5%.

Immunoprecipitation

In case of SOD2 protein extracts were suspended in the immune precipitation buffer, the composition of which was described previously (34). Samples containing 25 µg of protein were incubated for 3 h at 4°C on the shaking platform with 5 µL of anti-SOD2 antibody. Afterwards 5 µL of protein A-agarose was added to each sample and samples were incubated overnight at 4°C. Complexes were washed three times with radioimmune precipitation buffer and resulting pellet was resuspended in 25 µL of Western blot sample buffer.

SDS-PAGE and Western blotting

For myosin heavy chains analysis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4% stacking and 6% separating gels containing 37.5% glycerol as previously described (35). For UCP3, cytochrome-*c* (cyt-*c*) and cytochrome oxidase subunit I (COX I) analyses SDS-PAGE was carried out with 4.5% stacking and 12.5% separating gels. Lanes were loaded with 8 μg of protein in case of UCP3 and cyt-*c* analyses and 4 μg of protein for COX I analysis. Samples transferred onto immobilon-P transfer membranes (Millipore Corporation, Bedford, USA) were blocked with 10% non-fat dry milk in TBS, 0.1% Tween 20 (dilution buffer) and incubated with primary anti-UCP3 antibody diluted 1:1000 in dilution buffer, anti-cyt-*c* antibody at 1:500, or antibody against COX I diluted 1:1000. The secondary antibody - donkey anti-rabbit or goat anti-mouse IgG alkaline phosphatase conjugate (31345 and 31323, Pierce Chemical Co., Rockford, IL, USA) diluted 1:2000 were visualized with BCIP/NBT substrate. The relative amounts of proteins were assayed using CCD camera (Fotodyne Incorporated) and Gel Pro Analyzer software. The optical density of each sample was related to that of a standard (human muscle extract of known concentration) run on the same gel and presented in arbitrary units (AU).

In case of SOD2 analysis protein samples were run in 12% SDS-PAGE, transferred onto PVDF membrane (BioRad, USA) and probed with anti-SOD2 antibody diluted 1:1000 following by an appropriate HRP conjugated secondary antibody diluted 1:1000. Detection of membrane bound proteins was performed using BM Chemiluminescence Blotting Substance (Boehringer Mannheim, Germany).

Statistics

The results are expressed as means and standard errors (x±S.E.M). Statistical significance was tested using Wilcoxon-signed-rank test (for paired samples). Non-asymptotic, exact, two-sided P-values are presented. Significance was set at $P<0.05$. The statistics was done using the statistical packet StatXact 6.1 (Cytel software Corporation, Cambridge, MA, USA) and STATISTICA 8.0 (StatSoft, Tulusa USA).

RESULTS

Characteristics of studied group of subjects before and after the endurance training

Characteristics of studied group of subjects (n=15) is presented in *Table 1*. In the group of fifteen subjects the 5 week endurance training resulted in a higher (about 4%) maximal oxygen uptake (VO_{2max}), (P=0.048) and in the significantly higher (about 8%, P=0.0001) power output reached at VO_{2max} (PO at VO_{2max}).

Moreover, after the 5 weeks of endurance training a significant decrease in the net oxygen cost of generating PO reached at VO_{2max} (VO_{2net}/PO at VO_{2max}) was observed (P=0.002, *Fig. 1A*). A significantly higher (P=0.008) plasma lactate concentration at VO_{2max} ($[La^-]_{pl}$ at VO_{2max}) after endurance training was found. No significant changes in body mass (BM), power output at lactate threshold (PO at LT), myosin heavy chain type 2 isoform (MyHC 2) percentage in the *vastus lateralis* muscle after endurance training were observed (P>0.05, *Table 1*). Moreover, a significantly lower $[fT_3]_s$ ($P<10^{-4}$, *Fig. 1B*) after the training has been found.

Training-induced changes in UCP3 mRNA and mitochondrial protein expression (UCP3, cytochrome-c, cytochrome-c oxidase subunit I) in the vastus lateralis muscle in the studied group of subjects

In the studied group of subjects (n=14) no significant changes in *UCP3* mRNA levels (1.486±0.235 *vs.* 1.182±0.195 AU, respectively for the pre-training and post-training values, P=0.27) as well as no significant changes in UCP3 protein content (n=15) (P=0.60, *Fig. 1C*) after training were found. Moreover, no significant changes in cyt-*c* protein content (P=0.79), COX I (P=0.28) and no significant changes in UCP3 relative to cyt-*c* protein content (P=0.68) after endurance training were noticed.

Training-induced changes in the oxidative stress (n=15)

A significantly lower $[F_2isoprostanes]_{pl}$ after the endurance training was found (P=0.028, *Table 2*). A tendency to lower $[GSSG]_{pl}$ after training (P=0.08) and a tendency to higher TAC (P=0.08) were observed (*Table 2*). There were no significant changes in $[Thiols]_{pl}$ and $[GSH]_{pl}$ after the training (*Table 2*). Moreover, no significant changes in $[GSH]_{pl}$ to $[GSSG]_{pl}$ ratio after the training was noticed (P=0.35).

Training-induced changes in manganese superoxide dismutase (SOD2) in the vastus lateralis (n=11)

In the group of the eleven subjects from the studied group a significant increase in SOD2 content in the *vastus lateralis* muscle after the training has been found (P=0.02, *Fig. 2A*), despite no significant changes in MyHC composition (P=0.76). This was accompanied by a significant decrease (by 10% in this group of subjects) in $[F_2isoprostanes]_{pl}$ (P=0.005, *Fig. 2B*).

Table 1. Characteristics of studied group of subjects (n=15) before (pre-training) and after (post-training) the endurance training.

	n=15		
	Pre - training	**Post - training**	**P - value**
BM, kg	76.4±2.3	75.8±2.2	0.20
MyHC 2, %	61.4±2.1	61.8±2.3	0.67
PO at LT, W	114±9	122±8	0.36
VO_{2max}, $mL \cdot min^{-1}$	3498±86	3601±86	0.048
VO_{2max}, $mL \cdot kg^{-1} \cdot min^{-1}$	46.0±1.0	47.8±1.1	0.03
PO at VO_{2max}, W	256±6	277±6	0.0001
$[La^-]_{pl}$ at VO_{2max}, $mmol \cdot L^{-1}$	10.1±0.6	11.7±0.8	0.008

Values are means ±S.E.M. BM, body mass; MyHC 2, myosin heavy chain type 2 in *vastus lateralis* muscle; PO at LT, power output reached at lactate threshold; VO_{2max}, maximal oxygen uptake; PO at VO_{2max}, power output reached at VO_{2max}; $[La^-]_{pl}$ at VO_{2max}, plasma lactate concentration determined at VO_{2max}. Non-asymptotic, exact, 2-sided P-values (Wilcoxon-signed-rank test) are given.

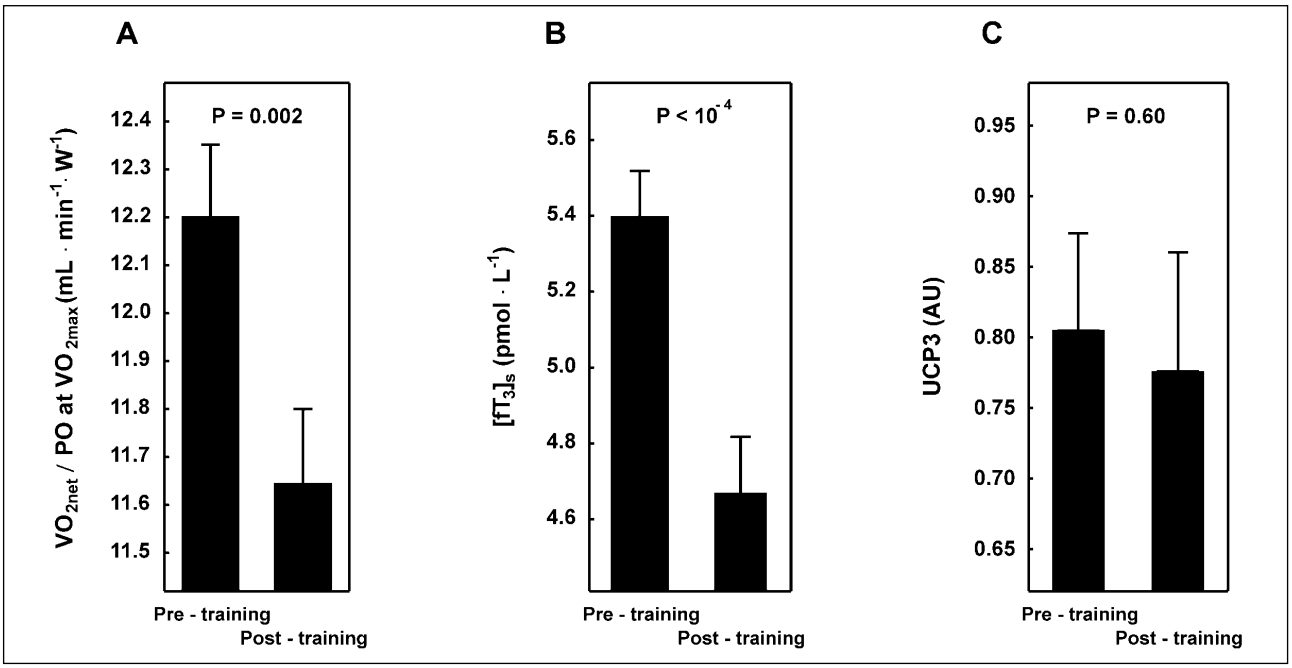

Fig. 1. The endurance training-induced changes in the net oxygen uptake to end-exercise power output ratio (VO_{2net}/PO at VO_{2max}, panel A), serum free 3,5,3'-triiodo-L-thyronine ($[fT_3]_s$, panel B) and uncoupling protein 3 content in *the vastus lateralis* (UCP3, panel C) in the group of fifteen subjects. Data presented as mean value ±S.E.M.

Table 2. Plasma total antioxidant capacity, thiol groups concentration, F_2isoprostanes concentration, glutathione as well as glutathione disulfide concentrations determined at rest after overnight fasting in studied group of subjects (n=15) before (pre-training) and after (post-training) the endurance training.

	n=15		
	Pre - training	**Post - training**	**P - value**
TAC, Trolox equivalents, $\mu mol \cdot L^{-1}$	647±8	727±30	0.08
Thiols, $\mu mol \cdot L^{-1}$	65.8±2.1	68.2±1.8	0.23
$[F_2isoprostanes]_{pl}$, $nmol \cdot L^{-1}$	0.720±0.031	0.617±0.023	0.028
$[GSH]_{pl}$, $\mu mol \cdot L^{-1}$	5.70±0.14	5.58±0.12	0.41
$[GSSG]_{pl}$, $\mu mol \cdot L^{-1}$	1.32±0.04	1.26±0.04	0.08

Values are means ±S.E.M. TAC, plasma total antioxidant capacity; $[Thiols]_{pl}$, plasma thiol groups concentration; $[F_2isoprostanes]_{pl}$, plasma F_2isoprostanes concentration; $[GSH]_{pl}$, plasma glutathione concentration; $[GSSG]_{pl}$, plasma glutathione disulfide concentration. Non-asymptotic, exact, 2-sided P-values (Wilcoxon-signed-rank test) are given.

Similarly to the whole group of subjects (n=15) in this group of subjects (n=11) no significant changes in UCP3 (P=0.83), cyt-*c* (P=0.97), COX I (P=0.41) contents in the *vastus lateralis* has been observed after the training.

DISCUSSION

The present study shows that as little as five weeks of endurance training of moderate intensity in previously untrained subjects resulted in a significant increase in physical capacity expressed by an increase in PO at VO_{2max} (P=0.0001) and in maximal oxygen uptake (P=0.048). Moreover, a decrease in the net oxygen cost of PO reached at VO_{2max} has been found after the training (P=0.002, *Fig. 1A*). It should be noted that a training-induced enhancement of the exercise tolerance was obtained without changes in mitochondrial marker proteins *i.e.* cytochrome-*c* and cytochrome-*c* oxidase contents (P>0.05) as well as with no changes in UCP3 content in the *vastus lateralis* (P=0.60, *Fig. 1C*). The increase in VO_{2max} in the absence of an increase in mitochondrial proteins in the presents study is most likely due to the training-induced enhancement of oxygen delivery to the working muscle, considered as the main factor determining VO_{2max} in humans (36). It should be mentioned, that we have recently demonstrated, on the same group of subjects, that this training resulted in a significant systemic release of prostacycline during exercise (37). The exercise-induced increase in systemic release of prostacycline, which was shown to be correlated with VO_{2max} (38), can improve oxygen delivery and VO_{2max} in humans in several ways (for discussion see 37).

It has been postulated that the training-induced decrease in UCP3 protein content may have a beneficial effect on muscle energetics by an increase of the efficiency of the energy production in the oxidative phosphorylation process through the decrease in the UCP3-mediated proton leak and an increase in mitochondrial coupling (19, 39). Moreover, it has been reported that thyroid hormone (T_3) is involved in the energy metabolism by exerting its effects on mitochondrial energy coupling (40) among others through the influence on the UCP3 expression (41) and UCP3 activity (42). However, in our study, no effect of

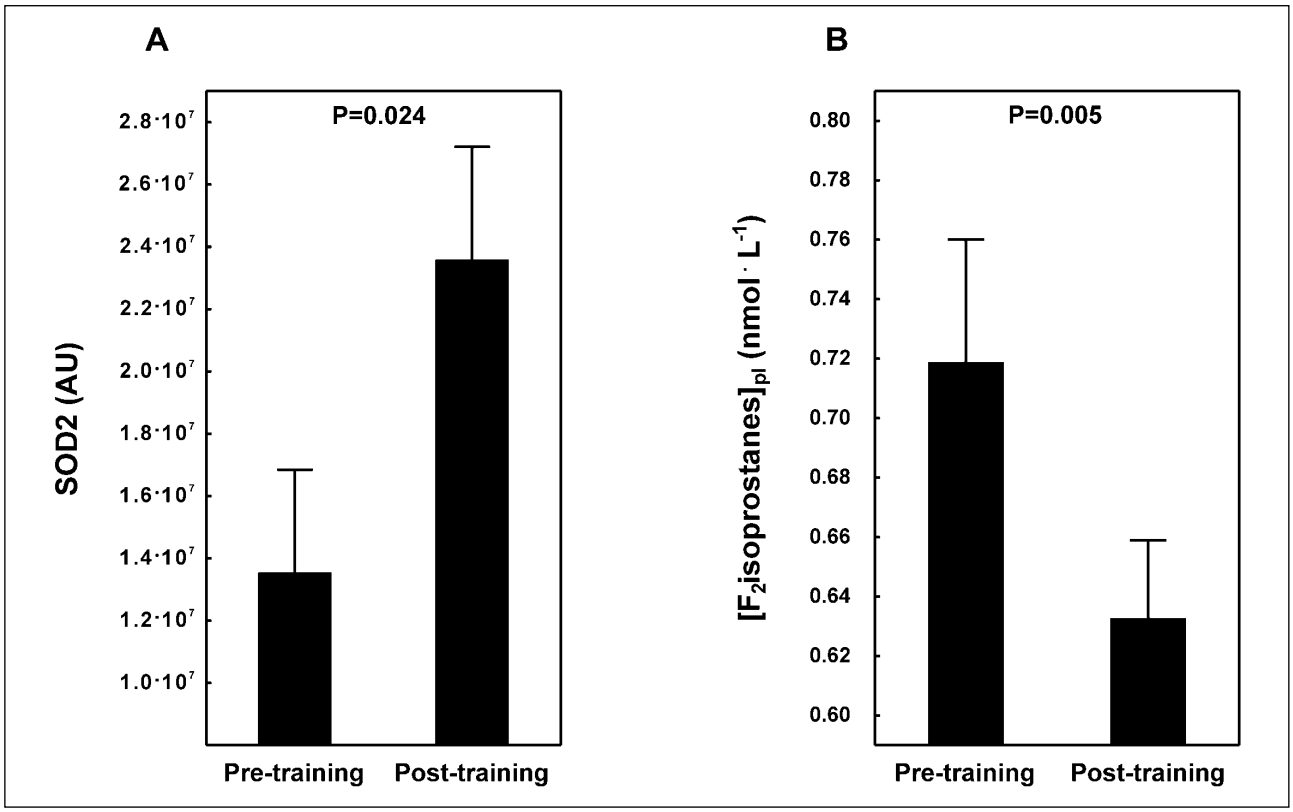

Fig. 2. The endurance training-induced changes in manganese superoxide dismutase (SOD2) content in *the vastus lateralis* (panel A) and in the basal plasma isoprostanes concentration $[F_2\text{isoprostanes}]_{pl}$ (panel B) in the group of eleven subjects. Data presented as mean value ±S.E.M.

training on the *UCP3* mRNA (P=0.27) and UCP3 protein content (P=0.60, *Fig. 1C*) has been found despite a significant decrease in the thyroid hormone concentration ($P<10^{-4}$, *Fig. 1B*). Probably longer period of training is necessary to induce the changes in UCP3 protein content and mitochondrial coupling as in the case of the endurance-trained athletes, who possess indeed significantly lower UCP3 protein content (24) and higher mitochondrial coupling (43) in their muscles than the untrained individuals.

The most important findings of the present study are that the endurance training of moderate intensity lasting five weeks resulted in a significant decrease in the basal isoprostanes concentration (P=0.028, *Table 2*), considered as one of the most reliable biomarkers of oxidative stress and lipid peroxidation *in vivo* (44) in the presence of no changes in the UCP3 protein content (P=0.60, *Fig. 1C*). Moreover, a clear tendency to lower plasma oxidized glutathione concentration $[GSSG]_{pl}$ (P=0.08, *Table 2*) and to a higher total antioxidant capacity (P=0.08, *Table 2*), a parameter describing the sum of the antioxidants present in plasma (45) has been found. It is well known that physical exercise enhances the ROS and RNS production and that chronic physical activity increases antioxidant protection and decreases oxidative stress (14). Based on the literature data several weeks of training is needed to see a decrease in the oxidative stress and an enhancement of the antioxidant protection in humans (46, 47, 48, for review see 18).

It has been suggested that in the early stage of training UCP3 might play a role in the antioxidant protection of muscle cell before an increase in antioxidant enzyme content (49). It should be mentioned, however that the physiological role of the UCP3 and its significance in the antioxidant protection *in vivo* is under debate (50). Firstly, the amount of UCP3 in the inner mitochondrial membrane is low when compared to the UCP1 content in brown adipose tissue mitochondria (below 1% of all inner mitochondrial membrane proteins contents *vs.* 5% in case of UCP1) (16). Hence, it is argued that *in vivo* UCP3 and UCP2 can not catalyze a sufficiently high proton leak to allow for the degree of uncoupling needed to play an antioxidant role (50). Secondly, in contrary to the *in vitro* studies in which the relationship between UCP3 and oxidative stress has been demonstrated (15) *in vivo* no convincing data are present (50). As suggested, UCP3 might have an antioxidant function *in vivo* selectively when protonmotive force is high *i.e.* in resting condition (low ATP demand) (16) or when activity of ETC enzyme complexes is inhibited, such as during hypoxia (51). In our study, a training-induced lowering of the basal oxidative stress as reflected by a lowering of the $[F_2\text{isopostanes}]_{pl}$ and by a decrease in $[GSSG]_{pl}$ (*Table 2*), was accompanied by no significant changes in the UCP3 protein content (*Fig. 1C*). Moreover, we have observed a training-induced reduction in basal free T_3 concentration (*Fig. 1B*). Concerning the role of T_3 in the mitochondrial coupling (40, 42) our results (*Fig. 1B*) might rather suggest a T_3-dependent lowering of the UCP3 mediated proton leak, an increase in protonmotive force and an enhancement of the mitochondrial coupling in the basal condition after the training. Therefore, based on our results the role of UCP3 in the antioxidant protection in the early stage of training seems to be questioned.

Despite no significant changes in the UCP3 protein content (*Fig. 1C*) in eleven subjects from the entire group (n=15) we have observed a significant increase (P=0.02, *Fig. 2A*) in mitochondrial manganese superoxide dismutase (SOD2) content after the training. This training-induced increase in SOD2 content was accompanied by a significant decrease in

$[F_2isoprostanes]_{pl}$ (P=0.005, *Fig. 2B*) as well as by no changes (P>0.05) in ETC proteins contents (see Results) in this group (n=11). Therefore, a lowering of the basal systemic oxidative stress in the early stage of the moderate intensity endurance training might result from an increase in the content of SOD2 in the *vastus lateralis*, since it has been demonstrated that SOD2 is critical in the antioxidant defence and that its antioxidant effect is strongly dependent on its expression level (52). The importance of SOD2 in the antioxidant protection might be demonstrated by the fact the homozygous null mutation in the *Sod2*$^{-/-}$ *gene* is lethal (53), whereas homozygous mutation in other genes coding for the major antioxidant enzymes *e.g. Sod1, Sod3, GPX* appear largely normal except for an increased sensitivity to certain types of oxidative stress (54-56). Moreover, it is well known that from the group of antioxidant enzymes, superoxide dismutase is the most sensitive to the endurance training (14).

The results of our study concerning the effect of short-term endurance training on the SOD2 content (*Fig. 2A*) and the influence of this training programme on the typical marker protein of mitochondrial biogenesis (cyt-*c* and COX I) are in contrast to the results obtained by others for the groups of untrained subjects (46, 47). No effects of few weeks of endurance training on the muscle antioxidant enzyme activities/contents (SOD1, SOD2, GPX) were observed despite a training-induced enhancement of the citrate synthase activity - a marker of mitochondrial biogenesis (46, 47). In the study by Devries *et al.* (48) despite a significant reduction in the systemic oxidative stress (a decrease in urinary 8-isoprostanes and 8-hydroksy-2-deoxyguanosine) no lowering of the muscle oxidative stress (protein carbonyls and 4-hydroxynonenal) and no significant changes in the content of muscle antioxidant enzymes (SOD1, SOD2, catalase) in lean and obese women have been found after 12 weeks of endurance training. It should be noticed however, that in the present study, opposite to the above studies mentioned (46-48) we have applied a moderate intensity training programme, performed mostly at intensity below the LT (see Methods). The main part of training in the present study corresponded to about 45% of VO_{2max}, whereas in the other cited studies the applied training intensity was much higher. For example in the study by Tiidus *et al.* (46) the 8-week endurance training consisted of 30 minutes biking session, three times per week at the workload corresponding to 70% of VO_{2max}. Similarly, in the study by Tonkonogi *et al.* (47), the 6 week endurance training consisted of 30 min cycling, performed at intensity corresponding to 70% of VO_{2max} four times per week. In study by Devries *et al.* (48) endurance training started with two biking session at 50% of VO_{2peak} and increased to three 60 min biking sessions per week at 65% of VO_{2peak}. From our experience (57) in case of untrained subjects exercise intensity above 50% of VO_{2max} exceed the LT and should be considered according to Whipp (58) not as moderate but as heavy exercise intensity.

Recently, it has been demonstrated that the moderate intensity exercise, in which small increase in ROS production is observed (3) exerts a beneficial effects on the antioxidant protection (59). As proposed by some authors (6, 59, 60) the contradictory signalling function of ROS (cellular adaptation *vs.* damage of the cell) might be explained on the basis of the hormesis theory (60) according to which a low dose of substance is stimulatory and a high dose of this substance is inhibitory. Hence, low level of ROS production like during moderate intensity exercise (59) influence signalling pathways and can induce adaptive responses that protect against a subsequent stronger stress (60). Therefore, we postulate that the training-induced increase in the muscle SOD2 content, as demonstrated in our study was due to up-regulation of ROS-dependent signalling pathways in response to the applied moderate intensity training programme generating rather small amount of ROS, when compared to a heavy exercise intensity used in the above mentioned studies (46-48).

It should be mentioned that a significant decrease in the systemic oxidative stress (*Table 2*) might result from training-induced decrease in the oxidative stress outside the skeletal muscle. One should take into consideration the training-induced decrease in ROS production in adipose tissue as a potential factor explaining our results since a positive relationship between adipose tissue mass and oxidative stress has been demonstrated (61). However, in our group of subjects no significant effect of training on the body mass and body mass index has been observed (*Table 1*) despite a training-induced decrease in the oxidative stress (*Table 2*). Moreover, our study indicates that a training-induced decrease in the systemic oxidative stress (*Table 2*) is most likely due to the protective effect of a training-induced increase in SOD2 content in the working muscles.

We have concluded that as little as 5 weeks of moderate intensity endurance training is potent to improve physical capacity and antioxidant protection in previously untrained men. Surprisingly, these effects occur before any measurable changes in UCP3 protein content. We postulate that this training-induced improvement in the antioxidant protection at the muscle level is due to an increase in SOD2 content. Moreover, in the light of this study the involvement of UCP3 in the enhancement of physical capacity and antioxidant protection, at least in the early stage of training is rather questionable, as jugged by no changes in the muscle UCP3 content after training.

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