Skeletal muscle oxidative function *in vivo* and *ex vivo* in athletes with marked hypertrophy from resistance training

Desy Salvadego[1], Rossana Domenis[1], Stefano Lazzer[1], Simone Porcelli[1-2], Jörn Rittweger[3-4], Giovanna Rizzo[2], Irene Mavelli[1], Bostjan Šimunič[5], Rado Pišot[5], Bruno Grassi[1-2]

[1] Department of Medical and Biological Sciences, University of Udine, Udine, Italy

[2] Institute of Bioimaging and Molecular Physiology, National Research Council, Milan, Italy

[3] Institute of Aerospace Medicine, German Aerospace Center, Cologne, Germany

[4] Institute for Biomedical Research into Human Movement and Health, Manchester Metropolitan University, Manchester, United Kingdom

[5] Institute for Kinesiology Research, Science and Research Center, University of Primorska, Koper, Slovenia

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Corresponding author:

Bruno Grassi, MD PhD

Dipartimento di Scienze Mediche e Biologiche

Piazzale M. Kolbe 4

I – 33100 Udine

Italy

Tel. +39-0432-494335, fax +39-0432-494301

E-mail: bruno.grassi@uniud.it

Abstract

Oxidative function during exercise was evaluated in 11 young athletes with marked skeletal muscle hypertrophy induced by long-term resistance training (RTA, body mass 102.6±7.3 kg, mean±SD) and 11 controls (CTRL, body mass 77.8±6.0). Pulmonary O_2 uptake ($\dot{V}O_2$) and vastus lateralis muscle fractional O_2 extraction (by near-infrared spectroscopy) were determined during an incremental cycle ergometer (CE) and one-leg knee-extension (KE) exercise. Mitochondrial respiration was evaluated *ex vivo* by high-resolution respirometry in permeabilized vastus lateralis fibers obtained by biopsy. Quadriceps femoris muscle cross sectional area, volume (determined by magnetic resonance imaging) and strength were greater in RTA *vs.* CTRL (by ~40%, ~33% and ~20%, respectively). $\dot{V}O_2$peak during CE was higher in RTA *vs.* CTRL (4.05±0.64 L min^{-1} *vs.* 3.56±0.30); no difference between groups was observed during KE. The O_2 cost of CE exercise was not different between groups. When divided per muscle mass (for CE) or quadriceps muscle mass (for KE) $\dot{V}O_2$peak was lower (by 15-20%) in RTA *vs.* CTRL. Vastus lateralis fractional O_2 extraction was lower in RTA *vs.* CTRL at all work rates, both during CE and KE. RTA had higher ADP-stimulated mitochondrial respiration (56.7±23.7 pmolO_2·s^{-1}·mg^{-1} ww) *vs.* CTRL (35.7±10.2), and a tighter coupling of oxidative phosphorylation. In RTA the greater muscle mass and maximal force, and the enhanced mitochondrial respiration seem to compensate for the hypertrophy-induced impaired peripheral O_2 diffusion. The net results are an enhanced whole body oxidative function at peak exercise, and unchanged efficiency and O_2 cost at submaximal exercise, despite a much greater body mass.

Key words: skeletal muscle hypertrophy; mitochondrial respiration; oxidative metabolism during exercise.

Introduction

Resistance training programs have been developed with the aim of improving variables of muscle function such as strength, power, speed, local muscular endurance, coordination, and flexibility (21). Resistance training is now considered an important part of training and rehabilitation programs for healthy subjects and for various types of patients, such as cardiac patients (45), patients with pulmonary diseases (10), patients undergoing prolonged bed rest periods (2), or elderly subjects (28). In these populations, the combination of resistance training with the more conventional endurance exercise improves the patients' outcomes and quality of life (45).

An increase in the cross sectional area of skeletal muscle fibers and a shift of fiber type distribution towards type 2 fibers are typical adaptations induced by resistance training; these adaptations enhance the muscle force-generating potential (12) but could represent an impairment to skeletal muscle oxidative metabolism. On the other hand, muscles with higher maximal force would need to recruit a lower number of motor units, and therefore more oxidative (and more efficient) muscle fibers (20, 26). According to other authors strength training may increase skeletal muscle efficiency (4) and enhance skeletal muscle "metabolic stability" (50). Other studies reported, after resistance training, unchanged values of maximal O_2 uptake (6), as well as unchanged (19) or lower (42, 43) mitochondrial volume density, oxidative enzyme activity, and capillary density in the hypertrophic muscles. Thus, the specific effects of resistance training, with the related changes in muscle phenotype, on oxidative metabolism appear difficult to reconcile in a unifying *scenario*. The aim of the present study was to determine whether increases in muscle mass induced by chronic resistance training are associated, in humans, with alterations in skeletal muscle oxidative function and aerobic performance. Experiments were carried out on a group of resistance-trained athletes (RTA), in whom muscle adaptations to resistance exercise are expected to be particularly marked. An integrative approach was applied by analyzing oxidative metabolism

at different levels, spanning from pulmonary gas exchange to skeletal muscle function and mitochondrial respiration. Oxidative function was assessed *in vivo* during incremental cycle ergometer (CE) exercise and dynamic knee extension (KE) exercise with one leg (3). During KE the recruitment of a relatively small muscle mass, *i.e.* the *quadriceps femoris* of one leg, significantly reduces constraints to oxidative function deriving from cardiovascular O_2 delivery, thereby allowing a more direct assessment of quadriceps muscle oxidative capacity *in vivo*. The intrinsic properties of mitochondria were assessed *ex vivo,* in permeabilized muscle fibers obtained by biopsy, by high-resolution respirometry (36).

We hypothesized, in RTA *vs.* control subjects (CTRL), an impaired skeletal muscle oxidative function *in vivo* and an impaired mitochondrial respiratory function *ex vivo*.

Materials and Methods

Subjects

We evaluated eleven male RTA (age 25.4 ± 6.1 [mean ± SD] years; body mass (BM) 102.6 ± 7.3 kg; stature 1.84 ± 0.04 m; body mass index (BMI) 30.1 ± 2.6 $kg \cdot m^{-2}$), who had been following for at least 5 years resistance training programs specifically designed to increase quadriceps muscle strength and power, and eleven physically active CTRL (age 25.4 ± 3.8 years; BM 77.8 ± 6.0 kg; height 1.81 ± 0.05 m; BMI 23.8 ± 2.2 $kg \cdot m^{-2}$). A thigh circumference above 60 cm (with thigh fat thickness below 10 mm) was taken as a criterion for including subjects in the RTA group. A diary of the training regimen and physical activity, focused on the 8 weeks preceding the evaluation, was collected. RTA practiced sport activities such as American football (tackle position), track and field (throwing), and weightlifting for an average of 487 ± 204 minutes per week. Most of them took part at national-level competitions and championships. In their training sessions RTA included standard protocols of resistance exercise targeting all major upper body and lower body

muscle groups. The RTA training regimen could be categorized as high-intensity and low-volume resistance exercise training (21). The RTA subjects also practiced aerobic exercise such as running and cycling for about 127 ± 150 minutes per week. CTRL practiced running and cycling for about 153 ± 133 minutes/wk and sport activities such as tennis, handball or gym activities for 102 ± 143 minutes/wk, respectively, essentially for recreational purposes. They did not follow any resistance training program or any other specific training schedule.

Careful medical and pharmacological histories were collected. RTA and CTRL were not affected by any disease and were not taking any drug at the time of the study. RTA did not report any drug misuse to support their training. Participants were informed about the aims and methods of the investigation and gave their written informed consent. The experiments were carried out at the Valdoltra Orthopaedic Hospital of Ankaran, Slovenia. All procedures conformed to the declaration of Helsinki (2000) and were approved by the Slovenian National Medical Ethics Committee.

All tests were conducted under close medical supervision and following standard safety procedures, and the subjects were continuously monitored by 12-lead electrocardiography (ECG).

Exercise protocols

Incremental exercise protocols were carried out by utilizing a mechanically braked cycle (CE) ergometer (Monark Ergomedic 839E), and a custom-built knee extension (KE) ergometer (modified Monark cycle ergometer), as originally described by Andersen et al. (3). The exercise protocols were conducted in random order during different experimental sessions separated by a 24h recovery period.

During CE subjects performed an initial 4 min pedalling at 80 W, thereafter 20 W increments were imposed every minute until voluntary exhaustion. Pedalling frequency was kept at ~60 rpm, as imposed by a metronome. During KE subjects were constrained on an adjustable seat by a

126 safety belt, which anchored the angle of the hip at ~90°. Subjects pushed on a padded bar attached
127 to a lever arm connected to the crank of the cycle ergometer, and allowing a knee extension
128 between ~90 to ~170 degrees. This type of exercise confines muscle contractile activity to the
129 *quadriceps femoris* muscle of one leg, which is activated during the extension phase. The return of
130 the leg to the starting position is brought about passively by the momentum of the flywheel of the
131 ergometer (see 37 for details). Before data collection, each subject was familiarized with the setup
132 environment and the exercise protocol by short preliminary practice runs. After an initial 3 min of
133 unloaded KE exercise, an incremental test was performed. Work rate increments were imposed
134 every minute, in order to allow the subjects to reach exhaustion in ~10 min. Work rate was applied
135 by adjusting the tension of a strap around the ergometer flywheel, as in a mechanically braked
136 cycle ergometer. Throughout the test the active KE and passive knee flexion cycle was carried out
137 ~40 times per minute, as imposed by a metronome. During each cycle (total duration 1.5 s) KE
138 lasted ~1 s. In other words muscle contraction corresponded to ~65% of the duty cycle.
139 All the exercises were conducted up to the limit of tolerance. The latter was defined as the
140 inability to maintain the imposed work rate at the required frequency, despite vigorous
141 encouragement by the operators. Mean values of cardiovascular, ventilatory, gas exchange and
142 muscle oxygenation variables (see below) were calculated during the last 20 seconds of each work
143 rate; values obtained during the exhausting work rate were considered "peak" values.
144
145 **Measurements**
146 *Anthropometry.* Fat-free mass (FFM) and total skeletal muscle mass were assessed by bioelectric
147 impedance analysis (BIA) performed by a tetrapolar device (Human IM, Dietosystem, Italy) in
148 accordance with the conventional standard technique (25).
149 *Anatomical CSA, muscle volumes and muscle force.* Anatomical cross sectional area (CSA) of
150 right thigh muscles and of the right quadriceps were measured from turbo spin-echo, T1-weighted,

Magnetic Resonance Images (MRI) obtained with a 1.5 Tesla (Magnetom Avanto; Siemens Medical Solution, Erlangen, Germany). On each MRI slice, contours corresponding to the thigh muscles and quadriceps were delineated by an expert of MRI imaging, using the image processing tools available in the commercial software package AMIRA (v 4.1, Mercury Computer System Inc., Chelmsford, MA, USA). The volumes of thigh muscles and quadriceps were then derived as the geometrical volumes included in these contours.

Before the incremental tests, subjects performed two maximal voluntary isometric contractions (MVC) of the knee extensors of the right leg at a 110° knee angle. Force was measured by an electrical transducer (TSD121C, BIOPAC Systems, Inc., USA) implemented on a custom-built chair for isometric contractions of knee extensor and flexor muscle groups. Force was sampled at a frequency of 1 kHz (MP100, BIOPAC Systems).

Measurements during the incremental tests. Time to exhaustion was taken as an index of performance. Pulmonary ventilation ($\dot{V}E$), tidal volume (VT), respiratory frequency (fR), O_2 uptake ($\dot{V}O_2$) and CO_2 output ($\dot{V}CO_2$) were determined on a breath-by-breath basis by means of a metabolic unit (Quark b^2, Cosmed, Italy). Expiratory flow measurements were performed by a turbine flow meter calibrated before each experiment by a 3 L syringe at three different flow rates. Calibration of O_2 and CO_2 analyzers was performed before each experiment by utilizing gas mixtures of known composition. The gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. The gas exchange threshold (GET) was determined by the V-slope method (5). All the data related to GET were expressed as a percentage of $\dot{V}O_2$ peak.

During KE a biphasic pattern in the $\dot{V}O_2$ *vs.* work rate relationship was observed (see Richardson et al., 34). During CE $\dot{V}O_2$ and $\dot{V}O_2$ peak values were expressed as $L \cdot min^{-1}$ and normalized per unit of whole body muscle mass ($mL \cdot min^{-1} \cdot kg^{-1}$), whereas during KE $\dot{V}O_2$ and $\dot{V}O_2$ peak values were expressed as $L \cdot min^{-1}$ and normalized as $mL \cdot min^{-1} \cdot 100g^{-1}$ of quadriceps muscle mass (QM).

175 The latter was calculated on the basis of quadriceps muscle volume (see above), after assuming a
176 standard value for skeletal muscle density of 1.060 $kg \cdot L^{-1}$ (27).
177 Heart rate (HR) was determined by ECG. Stroke volume (SV) was estimated beat-by-beat by
178 impedence cardiography (Physio Flow, Manatec, Paris, France) (33). The accuracy of this device
179 has been previously evaluated during incremental exercise in healthy subjects against the direct
180 Fick method (33); in that study, the correlation coefficient between the two methods was $r = 0.946$
181 ($P<0.01$), and the mean difference was equal to -2.78 ± 12.33% (2 SD). Overall, the accuracy of
182 this method can be considered "acceptable" for the purpose of the study, that is to demonstrate
183 that the cardiac involvement during KE was not "maximal" also at peak exercise (as confirmed
184 also from HR measurements). Cardiac output ($\dot{Q}$) was calculated as HR·SV. Cardiac index (CI;
185 $\dot{Q}$/body surface) was also determined in order to take into account the difference in body size
186 between RTA and CTRL.
187 Oxygenation changes in *vastus lateralis* muscle were evaluated by near-infrared spectroscopy
188 (NIRS) (7, 13). Reliability of tissue oxygenation indices obtained by NIRS, evaluated by the
189 intraclass correlation coefficient for repeated measurements on the same subject during different
190 days, was found to be very high for skeletal muscle (41). NIRS measurements in muscle tissue
191 have been shown to be well correlated with local venous O_2 saturation (48). A portable NIR
192 continuous-wave photometer (PortaMon, Artinis, The Netherlands) was utilized. Specific details
193 on the method can be found in recent papers by our group (32, 37). The instrument measures
194 micromolar (μM) changes in oxygenated haemoglobin (Hb) + myoglobin (Mb) concentrations
195 (Δ[oxy(Hb+Mb)]), and in deoxygenated [Hb + Mb] (Δ[deoxy(Hb+Mb)]), with respect to an initial
196 value arbitrarily set equal to zero and obtained during the resting condition preceding the test.
197 Δ[deoxy(Hb+Mb)] is relatively insensitive to changes in blood volume and has been considered an
198 estimate of skeletal muscle fractional O_2 extraction (ratio between O_2 consumption and O_2
199 delivery) (15, 18). A "physiological calibration" of Δ[deoxy(Hb+Mb)] values was performed by

obtaining a transient ischemia of the limb after the exercise period: data obtained during exercise were expressed as a percentage of the values of maximal muscle deoxygenation obtained by pressure cuff inflation (at 300-350 mm Hg), carried out at the inguinal crease of the thigh for a few minutes, until Δ[deoxy(Hb+Mb)] increase reached a plateau (18). Δ[deoxy(Hb+Mb)] kinetics during the incremental tests were fitted by a sigmoid function, as proposed by Ferreira et al. (14).

Skeletal muscle biopsy and high-resolution respirometry. Muscle samples were obtained from the *vastus lateralis* muscle of the left limb by percutaneous biopsy which was taken, for all subjects, 5 h after the last incremental test. Biopsy was done after anesthesia of the skin, the subcutaneous fat tissue and the muscle fascia with 2 mL of lidocaine (2%). A small skin incision was then made to penetrate skin and fascia, and the tissue sample was harvested with a purpose-built rongeur (Zepf Instruments, Tuttlingen, Germany). The muscle samples were divided into two portions. One portion (~10 mg wet weight) was immediately frozen in liquid nitrogen and stored at –80°C until enzymatic determination of citrate synthase (CS) activity. The other portion (~10 mg wet weight) was used to measure mitochondrial respiration *ex vivo* and was immediately placed in an ice-cold relaxing solution containing: EGTA-calcium buffer (10 mM) (free Ca^{2+} concentration 100 nmol L^{-1}), imidazole (20 mM), taurine (20mM), K^+/4 morpholinoethanesulfonic acid (K-MES; 50 mM), dithiothreitol (DTT; 0.5 mM), $MgCl_2$ (6.56 mM), ATP (5.77 mM), phosphocreatine (15 mM), pH 7.1.

The fiber bundles were separated with sharp-ended needles, leaving only small areas of contact, and were incubated in 5 mL of the above solution (4°C) containing 50 µg mL^{-1} saponin for 30 min with continuous gentle stirring, in order to ensure complete permeabilization. After being rinsed twice for 10 min in a respiration medium (MiR05, Oroboros, Innsbruck, Austria; 0.5 mM EGTA, 60 mM potassium lactobionate, 3 mM $MgCl_2$ $6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 1 g/L BSA, pH 7.1), permeabilized fibers were measured for wet

weight and immediately transferred into a respirometer (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) for the analysis.

Mitochondrial respiratory function was evaluated by measuring O_2 consumption polarographically by high-resolution respirometry (17, 29). Data were digitally recorded using DatLab4 software (Oroboros Instruments). The instrumentation allows for O_2 consumption measurements with small amounts of sample in closed respiration chambers containing 3 mL of air-saturated respiration medium at 30°C; 3-5 mg of muscle fibers were used for the analysis. Standardized instrumental and chemical calibrations were performed to correct for back-diffusion of O_2 into the chamber from the various components, leak from the exterior, O_2 consumption by the chemical medium, and sensor O_2 consumption (29). The O_2 concentration in the chamber was maintained between 250 and 400 μM to avoid O_2 limitation of respiration. Intermittent reoxygenation steps were performed during the experiments by adding a 200 mM hydrogen peroxide solution into the medium containing catalase (29). A substrate-uncoupler-inhibitor-titration protocol with a substrate combination that matches physiological intracellular conditions was applied (17). Non phosphorylating resting mitochondrial respiration was measured in the presence of malate (4 mM), glutamate (10 mM), and succinate (10 mM), without ADP, so that O_2 consumption was mainly driven by the back leakage of protons through the inner mitochondrial membrane ("leak" respiration). ADP-stimulated mitochondrial respiration ("state 3" respiration) was measured after the subsequent addition of ADP (2.5 mM) as phosphate acceptor with malate, glutamate and succinate as substrates. The addition of cytochrome *c* (10 μM) had no additive effects on respiration, with minor increases of ~2%, thereby confirming the integrity of the outer mitochondrial membrane. We also examined electron transport system capacity by stepwise addition of the chemical uncoupler protonophore carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) to optimum concentration (1.25 μM). Rotenone (1 μM) and antimycin A (2.5 μM) were added to inhibit complexes I and III, providing a measure of

residual O_2 consumption, indicative of non-mitochondrial O_2 consumption. Mitochondrial respiration was then corrected for O_2 flux due to the residual O_2 consumption. The respiratory acceptor control ratio (RCR), taken as an index of coupling between electron transfer and phosphorylation, was obtained by dividing state 3 respiration by leak respiration.

CS activity was determined in frozen fiber bundles (~5 mg wet weight), which were homogenized in buffer (2,5% w/v) containing 250 mM sucrose, 40 mM KCl, 2 mM EGTA, 20 mM Tris HCl (pH 7.4) and supplemented with 0,1% Triton X-100. CS activity was assayed according to Srere (40) by spectrophotometric methods (Lambda 14 Spectrometer; Perkin Elmer) and was expressed as micromoles of substrate per minute per gram of total protein content. Protein content was estimated by Bradford assay (8).

Statistical analysis

Results were expressed as means ± standard deviation (SD). Statistical significance of differences between groups (RTA *vs.* CTRL) was checked by two-tailed Student's *t* test for unpaired data. Bonferroni correction was used for multiple comparisons. The level of significance was set at $P<0.05$. Statistical analyses were carried out with software packages (GraphPad Prism 4.0, GraphPad Software Inc.; SPSS, Statistical Package Social Sciences, 13.0.1).

Results

Values of the anatomical cross-sectional area (CSA) of quadriceps muscle, calculated at 50% femur length, are shown in **Figure 1A**. As expected on the basis of the selective inclusion criteria of the study, CSA in RTA was significantly greater (by 37%) than in CTRL. Also the volume of the quadriceps was significantly greater (by 33%) in RTA *vs.* CTRL **(Figure 1B)**. Values of BM, BMI, fat-free mass, skeletal muscle mass, and quadriceps muscle mass are reported in **Table 1**.

For all variables, values in RTA were significantly (by about 30%) greater in RTA *vs.* CTRL. Also the percentage of body fat was slightly but significantly greater in RTA *vs.* CTRL.

The mean force exerted by the knee extensors during MVC was significantly higher (by 18%; P=0.026) in RTA (998 ± 163 N) *vs.* CTRL (811 ± 188 N). Once normalized per unit of CSA, values did not differ between groups (7.9 ± 0.8 $N \cdot cm^{-2}$ in RTA *vs.* 8.7 ± 1.9 $N \cdot cm^{-2}$ in CTRL).

Peak values of the main ventilatory and gas-exchange variables determined at the limit of tolerance during CE and KE are presented in **Table 2**. Ventilatory variables were not different in the two groups, both during CE and KE, with the exception of a higher VT peak in RTA during CE. When expressed in $L \cdot min^{-1}$, $\dot{V}O_2$ peak was higher in RTA *vs.* CTRL during CE; no significant difference was observed during KE. When $\dot{V}O_2$ peak was expressed per unit of body mass or body muscle mass (for CE) and per unit of quadriceps muscle mass (for KE), values were significantly lower (by ~15-20%) in RTA *vs.* CTRL. Peak work rate was slightly but not significantly higher during CE in RTA *vs.* CTRL; time to exhaustion values were not different in the two groups, either during CE or KE. R peak values were higher than 1.1 in both groups during both types of exercise. Peak values of HR, $\dot{Q}$ and CI were similar between RTA and CTRL, either during KE or during CE (Table 2). As expected, in both groups peak values of cardiovascular variables obtained in KE were significantly lower than in CE.

Pulmonary $\dot{V}O_2$ values ($mL\ min^{-1}$) are plotted as a function of work rate in Figure 2, during CE (left panel) and during KE (right panel). To obtain these figures, individual $\dot{V}O_2$ values were grouped for discrete work rate intervals, which were determined in order to have, in each interval, each subject represented by one data point. When the subject had more than one "original" data points in the interval, mean individual values were calculated, both for the x and the y variables, and were taken in consideration to obtain the figure.

As for CE, mean (± SD) values of the intercepts and of the slopes of the individual linear regression lines (shown in the Figure legend) were not significantly different in RTA *vs.* CTRL. The mean slopes of the individual regression lines were 10.6 ± 0.9 mL min^{-1} $watt^{-1}$ in RTA, and 10.3 ± 0.7 mL min^{-1} $watt^{-1}$ in CTRL (no significant difference). Peak $\dot{V}O_2$ and peak work rate values are described in Table 2. As for KE, whereas the mean values of the slopes (submaximal work rates) were not different in the two groups, the intercepts values were higher in RTA. The mean slopes of the individual regression lines were 9.5 ± 1.1 mL min^{-1} $watt^{-1}$ in RTA, and 9.7 ± 1.9 mL min^{-1} $watt^{-1}$ in CTRL (no significant difference). Peak $\dot{V}O_2$ and peak work rate values are described in Table 2. Peak $\dot{V}O_2$ values normalized for muscle mass (for CE) or quadriceps muscle mass (for KE) are shown in Figure 3.

Values of NIRS-obtained Δ[deoxy(Hb+Mb)], which was taken as an estimate of *vastus lateralis* muscle fractional O_2 extraction, are shown in **Figure 4** as a function of work rate, during CE (left panel) and KE (right panel). Work rate intervals were defined according to the principles described above for Figure 2. Both in RTA and CTRL, either during CE or KE, the dynamics of Δ[deoxy(Hb+Mb)] followed a sigmoid pattern, with a tendency to plateau at ~85% of peak work rate. At all work rate levels, values of Δ[deoxy(Hb+Mb)] were significantly lower in RTA *vs.* CTRL, both during CE and KE; peak values were ~30% lower in RTA (*vs.* CTRL) either during CE or KE.

ADP-stimulated mitochondrial respiration (state 3 respiration), fueled by malate, glutamate and succinate, as determined by high-resolution respirometry, was significantly higher (by ~60%) in RTA *vs.* CTRL (**Figure 5A**). CS activity, taken as an estimate of mitochondrial content (24), was similar in the two groups **(Figure 5B)**. Maximal mitochondrial respiration induced by the chemical uncoupler FCCP, reflecting the maximal oxidative capacity of the electron transport

system (ETS), was not significantly different in RTA (68.0 ± 31.4 $pmolO_2 \cdot s^{-1} \cdot mg^{-1}$ wet weight) *vs.* CTRL (52.1 ± 22.3). No differences between groups were found in "leak respiration" (**Figure 5C**), reflecting the basal O_2 consumption not coupled to ATP synthesis, thereby suggesting that in RTA and in CTRL the rate of "energy" dissipated by the membrane proton leak was similar. The "respiratory acceptor control ratio" (RCR), that is the ratio between state 3 respiration and leak respiration, was taken as an index of coupling of mitochondrial respiration for a specific substrate supply (malate, glutamate, succinate in this study). A significantly higher RCR was observed in RTA *vs.* CTRL (**Figure 5D**), suggesting a tighter coupling between oxidation and phosphorylation in RTA.

Discussion

The present study provides an integrative evaluation of oxidative metabolism in a group of young resistance-trained athletes (RTA) characterized by a marked skeletal muscle hypertrophy. Different variables of oxidative function were evaluated, spanning from pulmonary gas exchange and skeletal muscle function *in vivo* to mitochondrial respiration *ex vivo* in permeabilized fibers. We hypothesized, in RTA *vs.* control subjects (CTRL), an impaired oxidative function *in vivo* and an impaired mitochondrial respiratory function *ex vivo*. The obtained results did not confirm these hypotheses.

Whole body peak oxidative function was enhanced in RTA *vs.* CTRL. Figure 2 (left panel), in which pulmonary $\dot{V}O_2$ data are expressed in L min^{-1} shows that during CE $\dot{V}O_2$ peak was indeed *higher* (by ~15%) in RTA vs. CTRL, in association with a slightly higher (by ~10%) peak work rate. Moreover, despite the significantly greater body mass, during CE no impairment of oxidative function was seen in RTA at submaximal work rates: the efficiency and the "O_2 cost" of exercise

(as indicated by the slopes and the intercepts of the $\dot{V}O_2$ *vs.* work rate relationships) were indeed the same in the two groups. This finding is of interest, also in comparison to what is usually seen in another population characterized by a significantly greater body mass, such as obese patients, in which the $\dot{V}O_2$ *vs.* work rate relationship is shifted upward compared to that seen in normal controls (22), indicating a higher O_2 cost of CE exercise deriving from the excess in body mass. The situation was in part different for KE (right panel of Figure 2): the slopes of the $\dot{V}O_2$ *vs.* submaximal work rates relationships were again similar in the two groups, but the intercept was higher in RTA (suggesting a higher O_2 cost for KE exercise), and $\dot{V}O_2$ peak and peak work rate values were only slightly higher in RTA. No clear-cut explanation can be given for the higher O_2 cost of exercise observed in RTA during KE, but not during CE. Cardiovascular constraints cannot be hypothesized, since they were by definition much lower in KE *vs.* CE. It can be hypothesized that the work of accessory muscles (see Richardson et al., 34) during KE was overall higher in RTA *vs.* CTRL. We indeed measured pulmonary $\dot{V}O_2$ and not $\dot{V}O_2$ across the leg, and as a consequence of this we cannot discriminate between $\dot{V}O_2$ attributable to the legs and $\dot{V}O_2$ attributable to the rest of the body.

On the other hand, after we normalized $\dot{V}O_2$ peak per whole body muscle mass (for CE) or quadriceps muscle mass (for KE) values were significantly lower (by ~15-20%) in RTA *vs.* CTRL (see Figure 3). This finding could be interpreted from two different perspectives: despite a lower $\dot{V}O_2$ peak per unit muscle mass, RTA reached slightly higher (during CE) or similar (during KE) peak work rate levels than CTRL, suggesting a higher muscle/metabolic efficiency (see below). On the other hand, it should also be recognized that $\dot{V}O_2$ peak/ muscle mass was substantially lower in RTA, suggesting a substantial impairment of peak oxidative function *in vivo* when normalized for the markedly greater muscle mass. Or, to put it in other words, in RTA (*vs.* CTRL) the increase in muscle mass was more pronounced than the increase in maximal oxidative power.

How to interpret all these findings in a reasonably coherent *scenario*? The answer may come both from some literature data and from data obtained in the present study.

First of all, strength training may have positive effects on aerobic performance and muscle oxidative metabolism. In the present study RTA had greater maximal isometric force *vs.* CTRL. For the same force (and power) output, muscles with higher maximal force would need to recruit a lower number of motor units, and therefore more oxidative (and more efficient) muscle fibers (20, 26). Previous literature data confirm that strength training may increase skeletal muscle efficiency (4) and enhance skeletal muscle "metabolic stability" (50). This would also be in agreement with our high-resolution respirometry data, suggesting an enhanced mitochondrial respiratory function in RTA (see below).

On the other hand, the marked muscle hypertrophy *per se* could impair the peripheral diffusion of O_2, if it is not accompanied by a proportional increase in muscle capillary supply. In the present study quadriceps muscle mass and cross sectional area were ~35% greater in RTA than in CTRL. It is generally assumed that the increase in muscle mass and size obtained by resistance training is mainly determined by hypertrophic processes involving single muscle fibers (12). According to Aagaard et al. (1), in the hypertrophic muscle the increase of CSA of individual fibers is more pronounced (by ~6%) to that observed for the muscle as a whole, as a consequence of changes in the pennation angle of fibers within the muscle. Thus, CSA of individual fibers in the RTA of the present study could have been 40-45% greater than that of CTRL, possibly representing an important impediment to muscle O_2 diffusing capacity (see below). Muscle capillary supply was not determined in the present study, and literature data are not univocal. According to some authors skeletal muscle hypertrophy induced by strength training could determine a capillary "dilution" in the tissue (43). A lower microvascular surface area available for gas exchange and/or an increased diffusion distance from the capillary to mitochondria would impair peripheral O_2

399 diffusion (46). In the present study we did not determine muscle capillarity, or peripheral O_2
400 diffusion; the latter could be determined by the invasive method developed in Dr. Wagner's
401 laboratory over the years (see *e.g.* 46). These approaches could be attempted in future studies. In
402 the hypertrophic skeletal muscles also an altered matching between O_2 delivery and O_2 utilization
403 within the muscles could lead to the lower muscle O_2 availability.
404 Evidence in favor of this phenomenon is provided, in the present study, by the observation in RTA
405 of lower fractional O_2 extraction values, both at submaximal and at peak work rates, both during
406 CE and KE (Figure 4). As nicely discussed by Poole et al. (31), it would be an oversimplification
407 to interpret fractional O_2 extraction simply as a result of "muscle factors"; indeed, this variable
408 may be affected by a combination of interrelated factors responsible for perfusive O_2 transport to
409 and within active muscles and diffusive O_2 transport within fibers. In the present study, however,
410 the lower O_2 extraction cannot be accounted by cardiovascular O_2 delivery (the impaired O_2
411 extraction was described both during CE and KE, in which, by definition, cardiovascular
412 constraints are reduced or eliminated), nor by an impaired mitochondrial respiratory function,
413 which was enhanced, at least in *ex-vivo* conditions, in RTA *vs.* CTRL. Thus, the "bottleneck" of
414 oxidative metabolism in RTA would reside "downstream" of bulk cardiovascular O_2 delivery, but
415 "upstream" of intracellular oxidative metabolism. The main impairment could then reside in the
416 diffusing capacity of the muscle for O_2 and/or in the intramuscular matching between O_2 delivery
417 and O_2 utilization, which could be altered as a consequence of the marked muscle hypertrophy.
418 By measures of high resolution respirometry carried out in permeabilized skeletal muscle fibers
419 (experimental conditions of unlimiting O_2 availability), we indeed observed higher values of
420 ADP-stimulated mitochondrial respiration in RTA *vs.* CTRL, associated with a higher degree of
421 coupling between oxidation and phosphorylation. These observations suggest an enhanced *ex vivo*
422 respiratory function in RTA. These data were obtained in the presence of a similar CS activity in
423 the two groups. Although the method has some limitations (see Leek et al., 24), the activity of the

enzyme is frequently utilized to estimate mitochondrial content. Thus, in the markedly hypertrophic skeletal muscles of RTA mitochondrial biogenesis occurred proportionally to contractile protein synthesis. The finding appears consistent with studies on molecular signaling showing that the mammalian target of rapamycin (mTOR) kinase, which is known to be activated by resistance training and to be involved in the signaling pathway of protein synthesis and muscle growth, also regulates the expression of mitochondrial genes and may have a critical regulatory role on mitochondrial biogenesis and function (38, 47). State 3 mitochondrial respiration was higher in RTA *vs*. CTRL, whereas no differences were described between the two groups in terms of leak respiration. These data suggest a tighter coupling of mitochondrial respiration and an improved efficiency of the phosphorylation system in RTA. Similar findings have been observed by other authors after long-term exercise training (44, 51), in association with a more efficient control of mitochondrial respiration by the creatine kinase system. These adaptations are expected to limit perturbations of cellular homeostasis during exercise and to delay the increased reliance on substrate level phosphorylation for ATP regeneration, thus preserving exercise tolerance (51), and are likely associated with the concept of muscle "metabolic stability" (49).

Which could be the cause(s) responsible for the improved mitochondrial respiratory function *ex vivo* in RTA? Apart from the direct effects of training, it can be hypothesized that the impaired peripheral O_2 diffusing capacity and/or the impaired matching between O_2 delivery and O_2 utilization, discussed above, could determine within the exercising hypertrophic muscles areas of relative lack of O_2, that is of hypoxia. An improved coupling of mitochondrial respiration could then result from adaptive responses of mitochondria to repeated hypoxic stimuli. In skeletal muscle, a hypoxia-sensitive signal activation of transcription may occur in the myocellular compartment as a direct consequence of a drop in tissue oxygenation, in order to maintain the homeostasis of PO_2 at the levels considered critical for the function of cell (11). The multi-gene hypoxia-inducible transcription factor (HIF)-1 is recognized as the master transcription regulator

449 of cell hypoxic signaling (39). HIF-1 drives the expression of a variety of genes involved in
450 mitochondrial metabolism in skeletal muscle under hypoxic exposure, among which those
451 controlling the efficiency of cytochrome *c* oxidase enzyme (COX) in the mitochondrial respiratory
452 chain. HIF-1 would coordinate a switch from COX4-1 subunit to COX4-2, thereby increasing the
453 efficiency of electron transfer to O_2 at complex IV, and minimizing electron leakage at complexes
454 I and III. This switch would also enhance the efficiency of ATP synthesis, by increasing proton
455 pumping into the mitochondrial intermembrane space (16). By this way, electron transfer and
456 phosphorylation would result more tightly coupled, and the efficiency of oxidative
457 phosphorylation improved.
458
459 ***Methodological considerations***
460 In the present study the values of ADP-stimulated mitochondrial respiration and maximal
461 uncoupled respiration were in the low-end of values for healthy subject's quadriceps muscle. A
462 wide range of values for mitochondrial respiratory capacity can be found in the literature (17).
463 This could be attributable to the investigated population, to intrinsic metabolic characteristics of
464 tissue samples, and to methodological procedures, such as titration protocol, sequence and
465 combination of administered substrates, [ADP], experimental temperature, wet or dry state of
466 tissue mass utilized for normalization, fresh or frozen tissue samples, etc (17). In any case, the
467 data obtained in the present study are similar to those obtained, after following substantially the
468 same protocol of the present study, in the healthy control subjects of recent studies (9, 23, 30). In
469 the present study, after adding cytochrome c to the measuring chamber, the increase in
470 mitochondrial respiration was very small (~2%). A small increase in respiration after adding
471 cytochrome c is a standard criterion to check the absence of a significant damage of the outer
472 mitochondrial membrane, possibly occurring during the preparation of samples.

473 At first sight a comparison of $\dot{V}O_2$ between the two groups at submaximal work rates may not be
474 warranted since the two groups presumably have different $\dot{V}O_2$ kinetics (slower in the resistance-
475 trained athletes), and as a consequence different rates of adjustment to the work rate increments.
476 However, as elegantly discussed in the recent review by Rossiter (35), during a standard
477 incremental test different time-constants of the $\dot{V}O_2$ kinetics do not affect the slope of the $\dot{V}O_2$
478 *vs.* work rate relationship, but only shift the linear relationship to the right (in case of a slower
479 $\dot{V}O_2$ kinetics) or to the left (in case of a faster $\dot{V}O_2$ kinetics), the "horizontal distance" between
480 these linear relationships being reflected by the difference in time-constants. We did not determine
481 the $\dot{V}O_2$ kinetics in the two groups of subjects of our study. However, if we assume a slower
482 $\dot{V}O_2$ kinetics in the strength-trained group, imagine a difference in time-constants of 15 seconds
483 (this is likely an exaggeration, considering that both groups had a common endurance training
484 background), and assume the same gain in the two groups (as actually observed in this study), the
485 small horizontal shifts of the $\dot{V}O_2$ *vs.* work rate relationships do not change data interpretation:
486 same slopes and intercepts between the two groups during CE, same slopes but higher intercepts
487 in RTA during KE, suggesting a slightly higher O_2 cost of KE exercise in this group.
488
489 ***Conclusions***
490 In a group of young resistance-trained athletes characterized by a marked skeletal muscle
491 hypertrophy, the greater muscle mass and maximal force, and the enhanced mitochondrial
492 respiratory function seem to compensate for the hypertrophy-induced impaired peripheral O_2
493 diffusion. The net results are an enhanced whole body oxidative function at peak exercise, and
494 unchanged efficiency and O_2 cost at submaximal exercise, despite the much greater muscle mass
495 and body mass.
496
497

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636 **Author contributions**

637 The experiments were carried out at the Valdoltra Hospital, Ankaran, Slovenia. B.G., S.L.
638 conceived the study and obtained the financial support. All authors contributed to the design of the
639 experiments. R.P., S.L. and B.S. were responsible for the recruitment of the subjects and for the
640 logistics of the study. Data were collected and analyzed by D.S., R.D., S.P., J.R, G.R. and B.S.
641 D.S. and B.G. interpreted the data and wrote the first draft of the manuscript. All authors
642 contributed to the critical revision of the manuscript and approved its final version.

643

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TABLE 1 Anthropometric and body composition characteristics of subjects.

	RTA	CTRL
Body mass (kg)	102.6 ± 7.3*	77.8 ± 6.0
Stature (m)	1.84 ± 0.04	1.81 ± 0.05
BMI (kg·m^{-2})	30.1 ± 2.6*	23.8 ± 2.2
FFM (kg)	90.4 ± 6.1*	66.8 ± 11.0
Body muscle mass (kg)	46.1 ± 3.1*	35.2 ± 2.8
Quadriceps muscle mass (kg)	3.26 ± 0.44*	2.45 ± 0.26
Body fat (%)	12.2 ± 3.5*	9.9 ± 0.8

Data are expressed as means ± SD. RTA: resistance-trained athletes; CTRL: controls. BMI: body mass index. FFM: fat-free mass.
*: significantly different *vs.* CTRL (P<0.05; unpaired Student's t test).

651

652 **TABLE 2** Peak values of investigated variables during cycle ergometer (CE) and knee-extension
653 (KE) exercise.

	CE		KE	
	RTA	CTRL	RTA	CTRL
$\dot{V}E$ peak ($L \cdot min^{-1}$)	161.6 ± 27.5	145.9 ± 28.3	69.6 ± 18.9	62.0 ± 18.0
VT peak (L)	3.2 ± 0.5*	2.7 ± 0.3	1.8 ± 0.5	1.5 ± 0.5
fR peak ($br \cdot min^{-1}$)	51.3 ± 8.2	55.3 ± 10.0	41.2 ±.7.4	43.8 ± 6.0
$\dot{V}O_2$ peak ($L \cdot min^{-1}$)	4.05 ± 0.64*	3.56 ± 0.30	1.59 ± 0.23	1.50 ± 0.33
$\dot{V}O_2$ peak/BM ($mL \cdot min^{-1} \cdot kg^{-1}$)	39.6 ± 5.3*	45.9 ± 3.0	-	-
$\dot{V}O_2$ peak/muscle mass ($mL \cdot min^{-1} \cdot kg^{-1}$)	87.9 ± 11.7*	101.5 ± 6.7	-	-
$\dot{V}O_2$ peak/QM ($mL \cdot min^{-1} \cdot 100\ g^{-1}$)	-	-	51.5 ± 9.1*	63.7 ± 13.0
R peak	1.15 ± 0.06	1.17 ± 0.04	1.17 ± 0.14	1.13 ± 0.11
GET (% $\dot{V}O_2$ peak)	76.7 ± 3.8	73.8 ± 4.1	78.7 ± 3.6*	72.4 ±.3.9
Work rate peak (W)	314 ± 56	284 ± 15	103 ± 16	98 ± 13
Time to exhaustion (min)	15.7 ± 2.8	14.2 ± 0.7	14.3 ± 2.2	13.5 ± 1.7
HR peak ($b \cdot min^{-1}$)	184 ± 8	186 ± 11	142 ± 13	146 ± 11
$\dot{Q}$ peak ($L \cdot min^{-1}$)	29.1 ± 3.9	27.2 ± 2.8	18.9 ± 4.5	17.9 ± 3.2
CI peak ($L \cdot min^{-1} \cdot m^{-2}$)	12.8 ± 1.6	13.7 ± 1.4	8.3 ± 1.9	9.1 ± 1.4

Data are expressed as means ± SD. RTA: resistance-trained athletes; CTRL: controls. $\dot{V}E$: pulmonary ventilation. VT: tidal volume. fR: respiratory frequency $\dot{V}O_2$/BM: oxygen uptake per unit of body mass. $\dot{V}O_2$/muscle mass: oxygen uptake per unit of whole body muscle mass. R: gas exchange ratio. GET: gas exchange threshold. HR: hart rate. $\dot{Q}$: cardiac output. CI: cardiac index. See text for further details.
*: Significantly different *vs.* CTRL ($P<0.05$; unpaired Student's *t* test).

Figure captions

654 **Figure 1 (A)** Anatomical cross-sectional area (CSA) of the quadriceps muscle, obtained by
655 magnetic resonance imaging at 50% femur length, and (B) quadriceps muscle volume in resistance
656 trained athletes (RTA) and control subjects (CTRL). Mean (± SD) values are shown. *: $P<0.05$ *vs.*
657 CTRL. See text for further details.

658

659 **Figure 2** Mean (± SD) $\dot{V}O_2$ values during CE **(A)** and KE **(B)** are plotted as a function of mean
660 (± SD) work rate (Watt) for resistance-trained athletes (RTA) and control subjects (CTRL).
661 Individual $\dot{V}O_2$ values were grouped for discrete work rate intervals. As for CE, the mean (± SD)
662 values of the slopes of the individual regression lines were 10.6±0.9 mL min^{-1} $watt^{-1}$ in RTA and
663 10.3±0.7 in CTRL (n.s.); the mean values of the intercepts were 652±96 mL min^{-1} in RTA and
664 613±83 in CTRL (n.s.). As for KE, the mean values of the slopes of the individual regression lines
665 (submaximal work rates) were 9.5±1.1 mL min^{-1} $watt^{-1}$ in RTA and 9.7±1.9 in CTRL (n.s.); the
666 mean values of the intercepts were 545±71 mL min^{-1} in RTA and 421±81 in CTRL ($P<0.05$). *:
667 $P<0.05$ *vs.* CTRL. See text for further details.

668

669 **Figure 3** Peak $\dot{V}O_2$ values normalized for whole body muscle mass during CE **(A)** or quadriceps
670 muscle mass during KE **(B)**, in resistance-trained athletes (RTA) *vs.* controls (CTRL). *: $P<0.05$
671 *vs.* CTRL.

672

673 **Figure 4** Mean (± SD) values of the NIRS-obtained muscle deoxygenation index
674 (Δ[deoxy(Hb+Mb)]), which estimates *vastus lateralis* fractional O_2 extraction, as a function of
675 work rate, during CE **(A)**, and KE **(B)**, in resistance trained athletes (RTA) *vs.* controls (CTRL).
676 Δ[deoxy(Hb+Mb)] data are expressed as a percentage of values obtained during a transient limb

677 ischemia at the end of the test. The sigmoid functions (see 14) fitting the data are also shown. *:
678 $P<0.05$ *vs.* CTRL (Student's *t* test with Bonferroni correction). See text for further details.
679
680 **Figure 5 (A)** Mean (± SD) values of mitochondrial state 3 respiration (with glutamate, malate, and
681 succinate as substrates) per unit tissue mass (wet weight) measured in permeabilized muscle
682 fibers, and **(B)** citrate synthase activity (in $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein), in resistance trained athletes
683 (RTA) and controls (CTRL). **(C)** Mean (±SD) values of leak respiration (respiratory rate in the
684 presence of substrates without ADP), and **(D)** respiratory acceptor control ratio (RCR, state 3
685 respiration/ leak respiration), denoting the degree of coupling of mitochondrial respiration, in RTA
686 and CTRL. *: $P<0.05$ *vs.* CTRL. See text for further details.

Figure 1

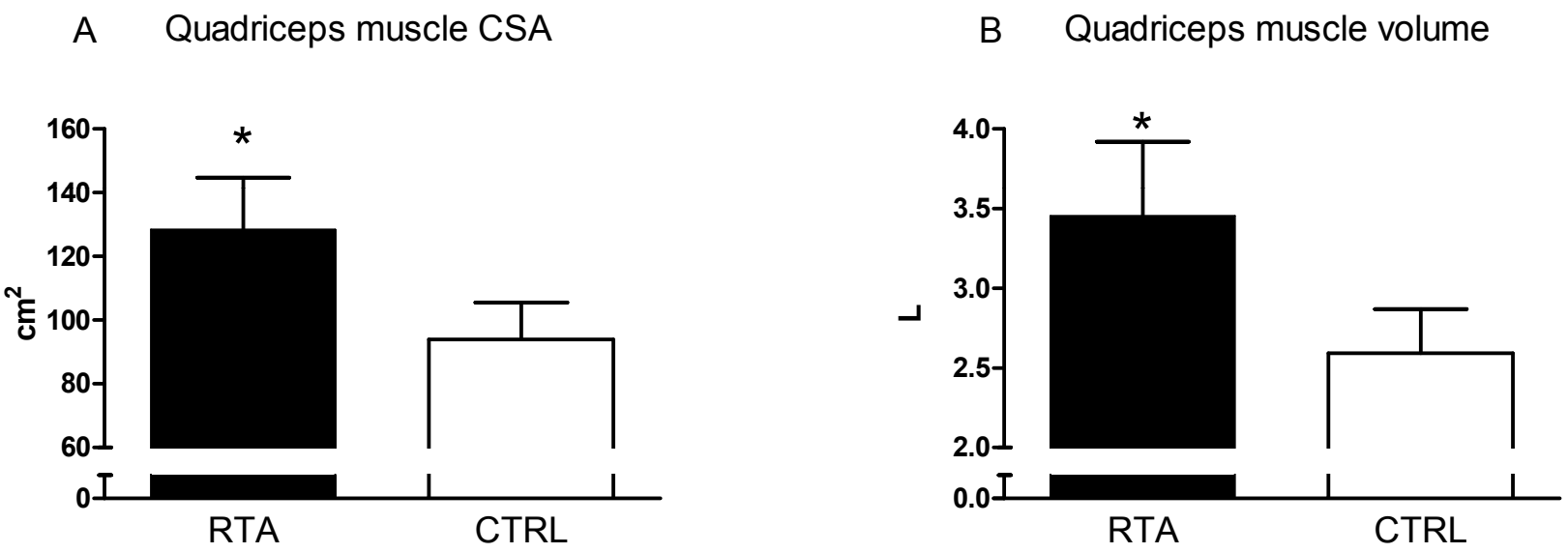

Figure 2

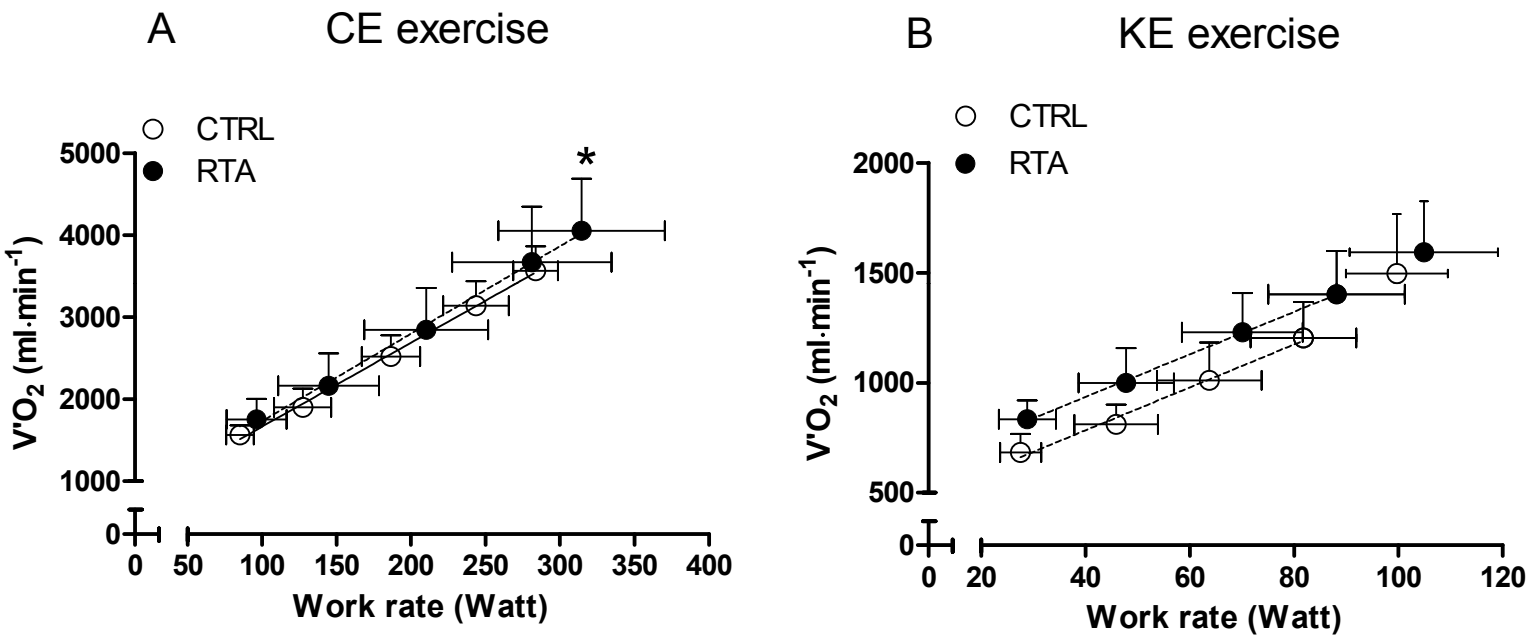

Figure 3

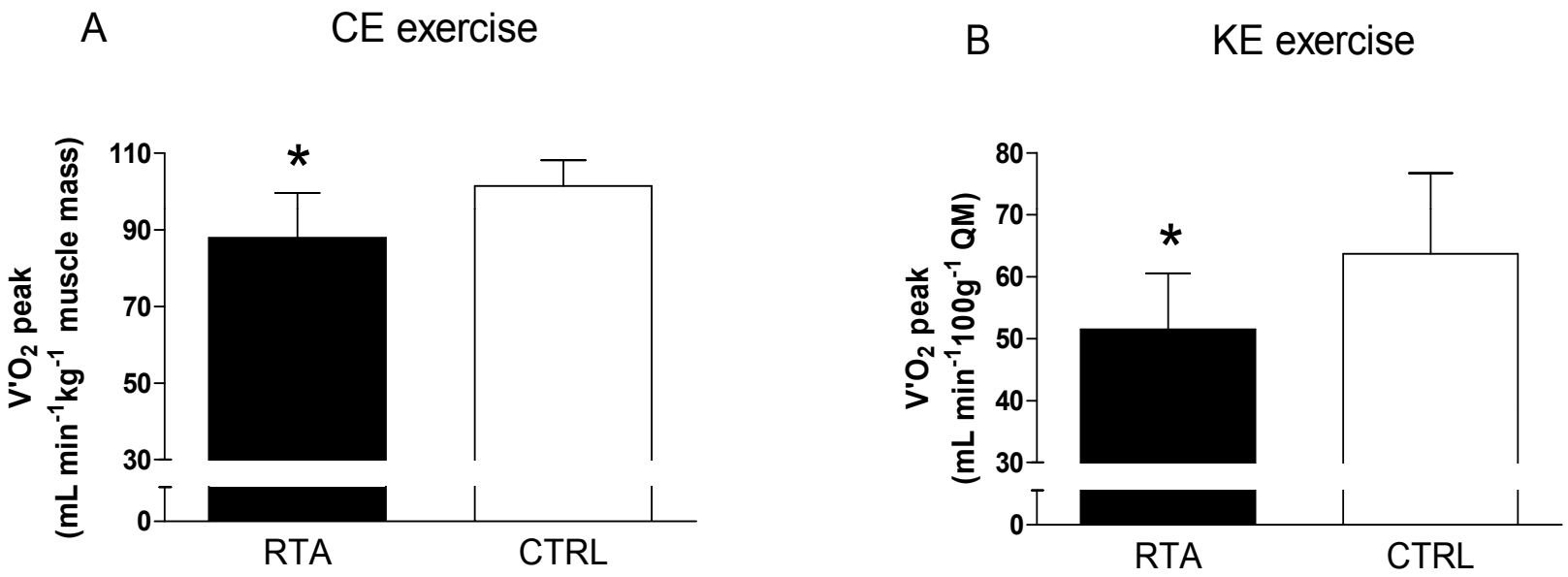

Figure 4

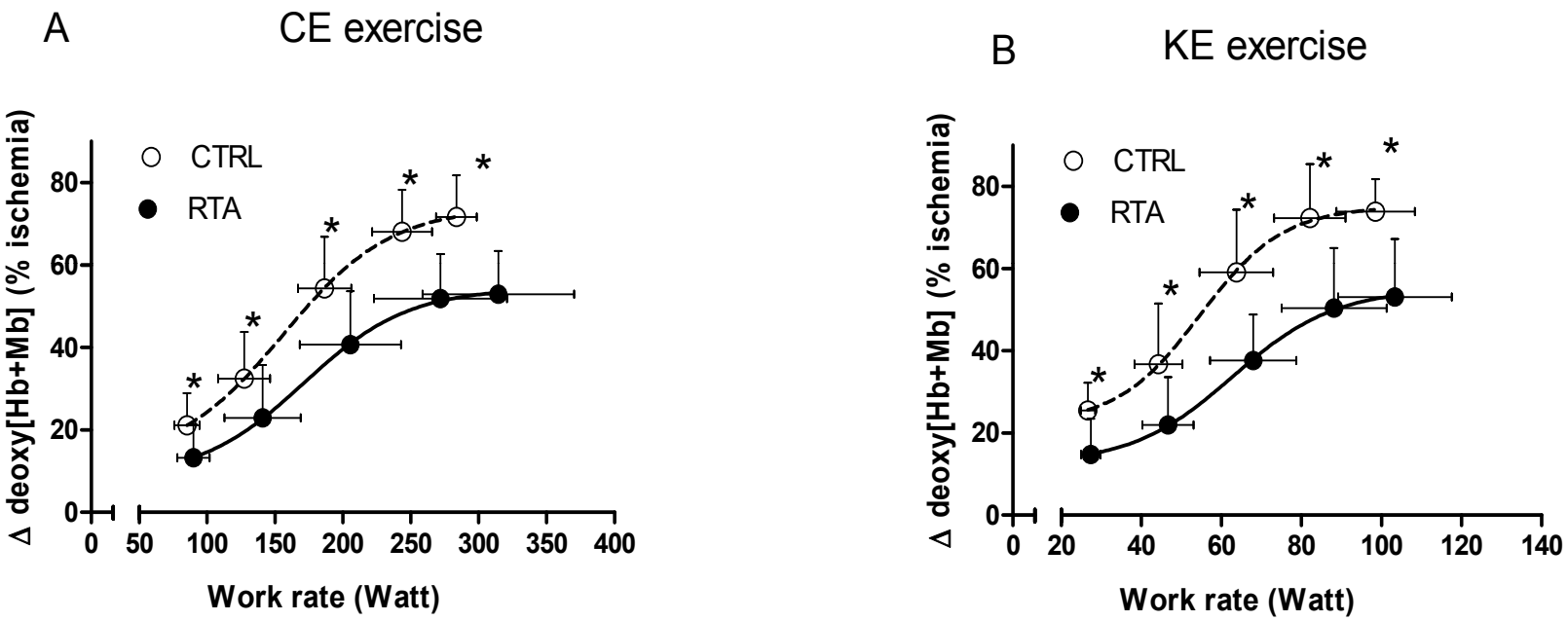

Figure 5

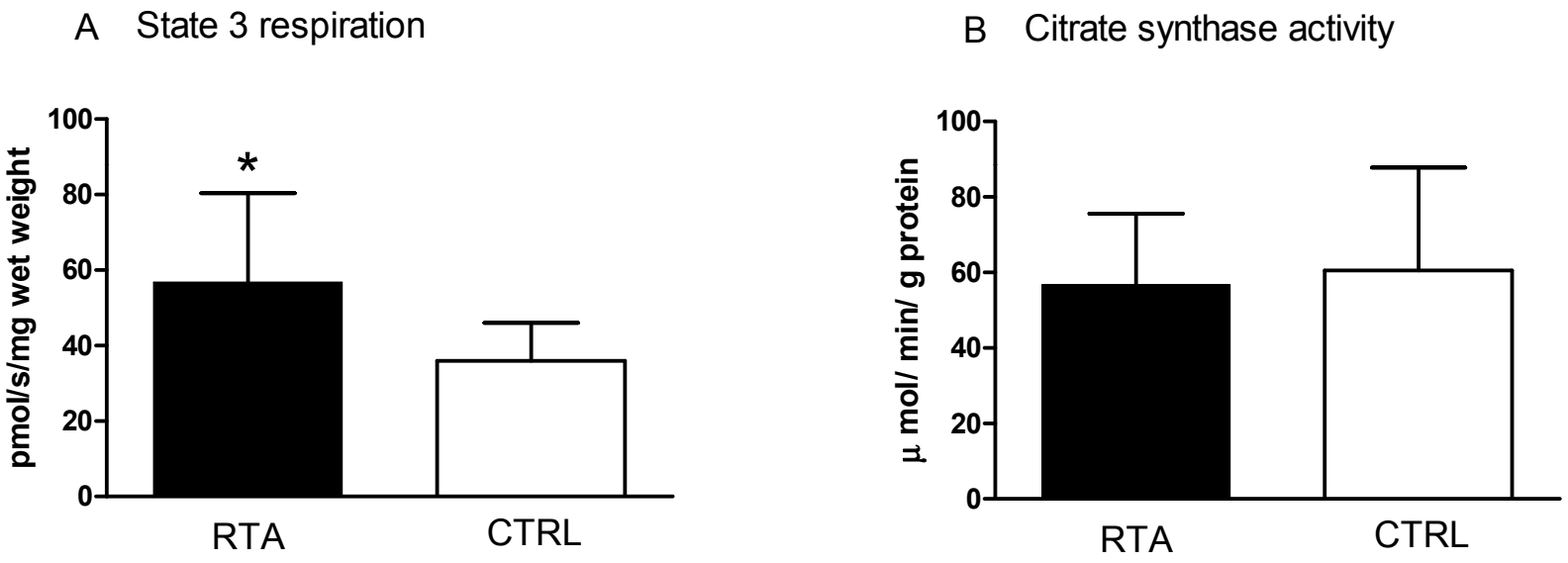

Figure 5