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Extraocular muscle mitochondria have a lower respiratory capacity: evidence for intrinsic differences in mitochondrial composition and function

Samir P. Patel[1,2], **Jorge L. Gamboa**[2], **Colleen A. McMullen**[2], **Alexander Rabchevsky**[1,2], and **Francisco H. Andrade**[2,*]

[1]*Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington KY 40536*

[2]*Department of Physiology, University of Kentucky, Lexington KY 40536*

Abstract

Purpose: The constant activity of the extraocular muscles is supported by abundant mitochondria. These organelles could enhance energy production by increasing the content of respiratory complexes. Therefore, we tested the hypothesis that extraocular muscle mitochondria respire faster than those from limb muscles because of higher content of respiratory complexes.

Methods: Inner mitochondrial membrane density was determined by stereological analysis of triceps surae (a limb muscle) and extraocular muscles of adult male Sprague-Dawley rats. We measured respiration rates of isolated mitochondria using a Clark-type electrode. The activity of respiratory complexes I, II and IV was determined by spectrophotometry. The content of respiratory complexes was estimated by western blot.

Results: State 3, 4 and 5 respiration rates in extraocular muscle mitochondria were 40-60% less than in limb muscle mitochondria. Extraocular muscle inner mitochondrial membrane density was similar to other skeletal muscles. Activity of complexes I and IV was less in extraocular muscle mitochondria (~50% the activity in triceps), but their content was ~15-30% higher. There was no difference in complex II content or activity, or complex III content. Finally, complex V was less abundant in extraocular muscle mitochondria.

Conclusions: Our results demonstrate that extraocular muscle mitochondria respire at slower rates than mitochondria from limb muscles, despite similar mitochondrial ultrastructure. Instead, we found differences in the activity (I, IV) and content (I, IV, V) of electron transport chain complexes. The discrepancy between activity and content of some complexes is suggestive of alternative subunit isoform expression in the extraocular muscles compared to limb muscles.

INTRODUCTION

The voluntary and reflexive eye movements initiated by the ocular motor system rely on the fast and constant activity of the extraocular muscles. These small skeletal muscles have one of the highest mitochondrial contents of mammalian skeletal muscles [1]. This has been considered to reflect the metabolic demands imposed by their activation patterns. Recently, we showed that mitochondria serve also as Ca^{2+} sinks and influence the kinetics of cytosolic free Ca^{2+} concentration during the activation of extraocular muscle fibers, increasing the dynamic response range for this muscle group [2].

*Correspondence: MS508 UKMC 800 Rose Street Lexington KY 40536-0298 USA E-mail: paco.andrade@uky.edu Fax: (859) 323-1070

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Aerobic capacity is typically measured by mitochondrial volume density (% of muscle fiber occupied by mitochondria). In general, mitochondrial volume density is well matched to the metabolic needs of skeletal muscle and it scales almost linearly with maximal oxygen uptake amongst muscles and across mammalian species [3, 4]. In other words, the consensus is that changes in the oxidative (aerobic) capacity of mammalian skeletal muscles are met by corresponding increases or decreases in mitochondrial volume density. Since the mitochondrial content and the activity of respiratory complexes and enzymes of mitochondrial metabolic pathways change in parallel, enzymatic activities are used as indices of mitochondrial content and aerobic capacity. Highly aerobic muscle groups in mammals have abundant capillaries and elevated mitochondrial volume density [5, 6]. That is clearly not the case in some extremely aerobic non-mammalian skeletal muscles where the increase in oxidative capacity is achieved by combining high mitochondrial content with modified mitochondrial ultrastructure and composition. For example, the mitochondrial cristae in hummingbird flight muscles are packed more tightly, increasing their ability to use oxygen by more than 50% over the maximal limit in mammalian muscle mitochondria [7].

Given that the extraocular muscles have arguably the highest mitochondrial content of all mammalian skeletal muscles, it seemed likely their mitochondrial density would be high enough to require changes in their composition and/or function in order to maximize aerobic capacity without restricting contractile performance. We anticipated that, in order to minimize the mitochondrial volume needed to sustain the high activation patterns of the extraocular muscles, mitochondrial respiratory capacity would be enhanced by increasing protein content. Therefore, the purpose of this study was to test the hypothesis that extraocular muscle mitochondria respire at faster rates than those from limb muscles because of a higher content of respiratory complexes. First, we measured the *in vitro* respiration rates of mitochondria isolated from extraocular and limb muscles. Then, we determined whether the activities and relative content of mitochondrial respiratory complexes correlated with differences in respiration between mitochondria isolated from these two muscle groups.

MATERIALS AND METHODS

Animals

Use of experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Kentucky and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male Sprague Dawley rats (300-350 g, Harlan, Indianapolis, IN) were anesthetized with ketamine hydrochloride/xylazine hydrochloride (100 mg/8 mg per kg body weight injected i.p.). For electron microscopy, rats were perfused transcardially with phosphate-buffered saline (pH 7.4), and then with 2% paraformaldehyde-4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and 130 mM NaCl. For biochemistry, rats were killed by exsanguination following a medial thoracotomy and then extraocular muscles (8 rectus and 2 superior oblique muscles pooled per animal) and mid-belly samples of triceps surae (a mixed fiber type limb muscle) were dissected and used immediately to isolate mitochondria.

Morphometry

Perfusion-fixed extraocular muscle samples were post-fixed in 1% osmium tetroxide, stained en bloc in uranyl acetate, dehydrated in a methanol series and propylene oxide, and embedded in epoxy resin. Thin (70 nm) sections were stained with uranyl acetate and lead citrate, and photographed with a Philips Tecnai 12 transmission electron microscope. Four extraocular muscles from two rats were studied. In each muscle, 5 fibers were selected randomly; then 4 mitochondria per fiber were imaged at a magnification of 120,000x. The surface density of inner mitochondrial membranes (cristae) was determined from digital image files using a

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standard point-counting method (144-point square-grid) and assuming randomly oriented structures [8, 9].

Mitochondrial isolation

Upon dissection, muscles were placed in 4 ml of ice-cold isolation buffer with 1mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin or BSA, 20 mM HEPES, 1 mM EGTA and pH adjusted to 7.2 with KOH). Nagarse or trypsin (0.25 mg/ml of isolation buffer) was added and the tissue was minced with scissors and homogenized in ice with motor-driven Potter-Elvehjem homogenizer. A protease inhibitor cocktail (P8340 from Sigma, 40 μl per 4 ml of homogenate) was added to the homogenates with trypsin. The homogenates were centrifuged at 600 *g* for 5 min at 4°C; the supernatant was decanted and centrifuged at 5,000 *g* for 10 min at 4°C. The resulting mitochondrial pellets were re-suspended in isolation buffer at a concentration of ~ 12-15 mg/ml and stored on ice until subsequent use. We confirmed that this method resulted in a pure mitochondrial preparation by following the disappearance of transaminase (cytosolic enzyme, monoclonal antibody from the Developmental Studies Hybridoma Bank, University of Iowa) and enrichment of mitochondrial proteins from initial homogenate to final mitochondrial pellet in western blots. Figure 1 shows that transaminase was present in the cytosolic fraction (lanes labeled "**c**") in both muscles and it disappeared in the mitochondrial pellet (lanes labeled "**m**"). In contrast, complexes IV and V went from barely detectable in the cytosolic fraction to abundant in the mitochondrial pellet.

Mitochondrial respiration

Mitochondrial respiration was measured with a miniature Clark-type electrode (Hansatech Instruments, Norfolk, England) in a sealed, thermostatically controlled chamber at 37°C as described previously [10]. Briefly, mitochondria were added to the chamber containing respiration buffer (215 mM mannitol, 75 mM sucrose, 2 mM $MgCl_2$, 2.5 mM inorganic phosphates, 0.1% BSA, 20 mM HEPES, pH 7.2) to yield a protein concentration of ~200-300 μg of mitochondrial protein/ml in 250 μl (final volume). Measurement of respiration was started with the addition of pyruvate (5 mM) and malate (2.5 mM) and designated as state 2 respiration, followed by the addition of 0.75 μM ADP (state 3 respiration) [11, 12]. Oligomycin (1μM, an ATP synthase inhibitor) was added to induce state 4 respiration, a measure of mitochondrial uncoupling activity [11-13]. State 5 respiration was induced with the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 μM) to get pyruvate+malate-driven (complex I) maximum uncoupled electron transport. The complex I inhibitor rotenone (0.8 μM) was then added to the buffer, followed by succinate (10 mM) to allow for quantification of complex II-driven maximum electron transport (state 5-succinate). The individual respiration states were calculated from the oxygen consumption slopes as nanomoles of oxygen consumed/min/mg of mitochondrial protein. The respiratory control ratio (RCR) was calculated by dividing the slope of the response of isolated mitochondria to state 3 respiration (presence of ADP) by slope of the response to oligomycin-induced state 4 respiration.

Activity of mitochondrial respiratory complexes

The activities of selected respiratory complexes were measured with the Multi-detection Microplate Reader (Bio-Tek Instruments, INC, Winooski, VT, USA) and normalized to mg of mitochondrial protein. First, mitochondria were diluted in 10 mM phosphate buffer to a concentration of 1 mg/ml and freeze-thawed and sonicated three times. Complex I activity was measured as the rotenone-sensitive decrease in NADH absorption at 340 nm with ubiquinone-1 as the final acceptor, as previously described [14], with slight modifications. The assay was performed in 25 mM KPO_4 buffer (pH 7.2) containing mitochondrial protein (6 μg), 5 mM $MgCl_2$, 1 mM KCN, 1 mg/ml BSA, and 150 μM NADH. The reaction was preincubated for 2

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minutes at 30°C, and initiated by addition of ubiquinone-1 (50 μM). NADH fluorescence was monitored (340 nm excitation, >450 nm emission) over time. The assay was also performed in the presence of rotenone (10 μM) to determine the rotenone-insensitive activity. The rotenone-sensitive complex I activity was calculated by subtracting the rotenone-insensitive activity from the total activity.

The complex II (succinate dehydrogenase) assay was performed in 100 mM KPO_4 buffer, 20 mM succinate, 10 μM EDTA, 0.01 % Triton, 1 μg/100 μl coenzyme Q_2 containing mitochondrial protein (6 μg) at 30°C. The reaction was initiated by the addition of 0.07% 2,6-dichloroindophenol and the rate of reduction of coenzyme Q by succinate was determined by following the reduction of 2,6-ichloroindophenol at 600 nm [15].

Complex IV (cytochrome c oxidase) assay was carried out in 10 mM KPO_4 buffer and 50 μM reduced cytochrome c. The reaction was initiated by addition of 6 μg of mitochondrial protein. The rate of oxidation of cytochrome c was measured at 30°C by following decrease in absorbance of reduced cytochrome c at 550 nm [16, 17].

Content of mitochondrial respiratory complexes

We used western blots to compare the differences in the content of representative subunits of the respiratory complexes between mitochondria isolated from extraocular muscle and triceps surae. Only mitochondria isolated with trypsin were used because nagarse is not compatible with the sample buffer used for SDS-polyacrylamide gel electrophoresis and leads to almost complete proteolysis (not shown and [18]). Mitochondrial proteins (15 μg of protein per lane) were resolved electrophoretically in 10-20% SDS-polyacrylamide gels (Bio-Rad, Hercules CA) and transferred to polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica MA). Equivalent protein loading was confirmed by total protein stain with Ponceau S. This strategy avoids the biases potentially introduced by the use of “housekeeping” proteins as loading controls [19, 20]. Membranes were blocked for one hour at room temperature and then incubated overnight with mouse monoclonal antibodies (Invitrogen, Carlsbad CA) against the following subunits of mitochondrial respiratory complexes: α subcomplex of complex I (39 kDa), iron-sulfur protein of complex II (30 kDa), core I of complex III (51.6 kDa), subunit VIb of complex IV (10 kDa) and subunit α of complex V (F_1F_0-ATPase subunit a, 59.6 kDa). After washing the membranes with phosphate-buffered saline and 0.1% Tween, they were incubated for 1 hr with Alexa Fluor 680-conjugated goat anti-mouse secondary antibody (1:7500, Invitrogen) and then washed again with PBS and 0.1% Tween. Membranes were finally rinsed with PBS and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE). Density of resulting bands was quantified using NIH Image J software [21].

Data analysis

All results are presented as means ± standard error of *n* observations, unless otherwise noted. Mitochondrial volume density, respiration and enzymatic activities were compared with Student's *t* tests. The significance level for rejection of the null hypothesis was set at $P \leq 0.05$ for all comparisons.

RESULTS

Mitochondrial bioenergetics

The first step in this study was to compare the oxygen consumption by mitochondria isolated from triceps surae and extraocular muscles. Typical oxygen consumption traces for mitochondria isolated from triceps and extraocular muscles are shown in figure 2: compared to triceps, oxygen consumption was slower in mitochondria from extraocular muscles

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throughout most of the protocol. The respiration rates were calculated in terms of nanomoles of oxygen consumed per minute per mg of mitochondrial protein. We started the study using nagarse to isolate muscle mitochondria. To check that the enzyme was not responsible for the observed differences, we also measured oxygen consumption rates in mitochondria isolated with trypsin. The RCRs after isolation with nagarse or trypsin were very similar and in the range consistent with organelle integrity: 7.4±0.3 and 7.8±0.4 in extraocular muscle, 9.1±0.3 and 8.7±0.4 in triceps surae, nagarse and trypsin respectively. Therefore, data from both enzymes were pooled. Significant differences were noticed between mitochondria from triceps and extraocular muscles: the respiration states 3, 4, 5 and 5+succinate in mitochondria from extraocular muscles were only 45.6±1.8, 55.2±3.7, 38.1±3.0 and 57.6±4.8% of the rates measured in mitochondria from triceps (figure 3). No significant difference was noted for state 2 respiration in mitochondria from extraocular muscle (84.3±11.5% of state 2 in mitochondria from triceps). Importantly, RCR was high for mitochondrial preparations from both muscles: 8.9±0.2 for triceps and 7.5±0.2 for extraocular muscle. While the difference was statistically significant ($P < 0.001$), both values are in the range that demonstrates mitochondrial integrity following the isolation procedure.

Mitochondrial morphology

The decreased in oxygen consumption rates in extraocular muscle mitochondria could be potentially due to smaller inner mitochondrial membrane (cristae) surface area in these muscles. As exemplified in figure 4 (panels **A** and **B**), the extraocular muscles have one of the highest mitochondrial volume densities (% of muscle fiber occupied by mitochondria) of mammalian skeletal muscles [1, 2]. The mitochondria in extraocular muscle appear to have a typical internal architecture with densely packed cristae (inner mitochondrial membrane, figure 4D). The estimate of inner mitochondrial membrane surface density (surface area per volume of mitochondria) was 40.9±9.7 m^2 cm^{-3} for extraocular muscle mitochondria, well within the range reported for a variety of mammalian skeletal muscles [4, 8].

Content and activity of respiratory complexes

Given the major differences in the capacity of extraocular muscle mitochondria to consume oxygen, we measured the isolated activities of some of the respiratory complexes (figure 5). The activity of complex I (NADH oxidoreductase) in mitochondria from extraocular muscles was only 55.1±4.5% of the activity measured in triceps ($P < 0.001$). A similar difference was found for complex IV (cytochrome c oxidase): its activity in extraocular muscle mitochondria was 47.9±4.9% of the level obtained in triceps ($P < 0.001$). That was not the case for complex II (succinate dehydrogenase) since we did not detect a significant difference in its activity between the mitochondria from the two muscles: extraocular muscles had 88.6±7.8% of the activity measured triceps mitochondria.

To follow up on the enzymatic activity measurements, we estimated respiratory complex content by immunodetection of individual subunits for each complex. Surprisingly, we observed differences in respiratory complex content that did not correspond with the enzymatic activity (figure 6). The content of complexes I and IV was significantly higher in extraocular muscle mitochondria ($P < 0.05$): 116±4.1 and 132.9±7.3% of the content measured in triceps mitochondria, respectively ($P < 0.05$). In contrast, the content of complex V in extraocular muscle mitochondria was only 42.6±6.2% of triceps mitochondria. Finally, the content of complex II and III was not significantly different in extraocular muscle mitochondria, 79.2±2.5 and 103.1±5.2% of triceps, respectively.

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DISCUSSION

Much to our surprise, the results do not support our original hypothesis that the extraocular muscle mitochondria have faster respiratory rates than mitochondria from limb muscles. Instead, the opposite conclusion seems more likely: mitochondria from extraocular muscles have lower content or lower activity of some enzyme complexes of the electron transport system, causing them to respire at slower rates. These findings were not explained by differences in the ultrastructure of extraocular muscle mitochondria: the surface area of their inner membrane was comparable to values reported for other skeletal muscle [8]. Furthermore, the differences were not generalized or systematic: complex II content and activity, and complex III content were similar in mitochondria from triceps surae and extraocular muscle. Complexes I and IV gave the most puzzling results: their activities were lower but their content was higher in the extraocular muscle mitochondria. These are multimeric protein complexes and differential expression of isoforms of some subunits has been described in skeletal muscle and other tissues [22]. Therefore, the content of some electron transport chain complexes (I, IV and V) and the subunit composition of some others (I and IV) may not be the same in the extraocular muscles compared to limb muscles. We and others have already reported evidence of divergence between extraocular and limb muscles in the relative importance of major metabolic pathways, including an alternative mitochondrial biogenesis program in the extraocular muscles [23-26]. The present study demonstrates that metabolic divergence between extraocular and limb muscles includes major differences in the composition and basic function of their respective mitochondrial populations.

Mitochondrial bioenergetics in extraocular muscle

The main finding in our study is that the respiration rates of extraocular muscle mitochondria are substantially less than those in mitochondria from limb muscles. This applies particularly to faster substrate- and ADP-driven (state 3) and uncoupled (state 5) respiration rates that are normalized to mitochondrial protein (figures 2 and 3), indicating that the activity or content of one or more of the respiratory complexes is lower in the mitochondria from extraocular muscles. Our findings confirmed this notion: the lower *activities* of complex I and IV and lower complex V *content* in extraocular muscle mitochondria compared to triceps surae mitochondria were fully consistent with the observed differences in respiration rates (figures 5 and 6). To complicate matters, we detected significantly higher *content* of complexes I and IV in extraocular muscle mitochondria. Since there is no evidence of differences in mitochondrial complex subunit stoichiometry between tissues, we propose that differences in the complex subunit isoforms expressed in extraocular and limb muscle mitochondria will explain the apparent discrepancy between the activity and content of complexes I and IV. Others have reported that mitochondrial composition can vary due to tissue-specific reliance on certain mitochondrial pathways and differential expression of respiratory complex subunit isoforms [27]. For example, cytochrome c oxidase (complex IV) subunits IV, Via, VIIa and VIII are known to have tissue-specific expression in mammalian mitochondria [27-29]. One gene for each subunit isoform is transcribed only in striated muscle (H isoform) and the other (L isoform) is present in most tissues thus far examined, including at low levels in heart and muscle [30-33]. The molecular diversity observed for subunit VIa isoforms was proposed to be responsible for tissue-specific regulation of the efficiency of energy transduction in cytochrome c oxidase of heart/muscle mitochondria [34]. Moreover, isoforms of the nuclear encoded subunits of cytochrome c oxidase affect the activity of the whole complex and are regulated by environmental and developmental signals and probably allow tissues to adjust their energy production to different energy demands [35]

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Matching metabolic capacity to contractile function

The primary role of mitochondria is to generate ATP. While our results demonstrate that extraocular muscle mitochondria have a lower intrinsic respiratory capacity as mitochondria from limb muscles, we do not know if this is enough to limit oxygen consumption *in vivo*. There is evidence of greater blood flow (per muscle mass) to the extraocular muscles [36, 37]. However, we are not aware of any data on maximal oxygen uptake (VO_{2max}) by the extraocular muscles *in situ* that would permit the comparison to other skeletal muscles; this is an issue that will require a novel technical approach. Moreover, the content of respiratory complexes is just one parameter behind tissue variations in mitochondrial respiration, and some argue that it is not particularly relevant for metabolic control [38]. Under experimental conditions, mitochondrial respiration in skeletal muscle and heart is regulated at the level of the respiratory chain while in liver, kidney and brain, it is controlled mainly at the phosphorylation level by ATP synthase (complex V) and phosphate carrier [38, 39]. That may not be the case *in vivo*, where different parameters such as cellular steady state, the energy demand and the energy supply of the tissue may also regulate mitochondrial respiration [39]. For the extraocular muscles, allosteric regulation of respiratory complexes may combine with changing metabolite concentrations to maintain mitochondrial respiration closer to its theoretical maximum [38, 40]. For example, a mechanism to enhance energy production in extraocular muscle is mitochondrial calcium influx during contractile activity [2]. Calcium influx into mitochondria coordinates ATP demand by the contractile apparatus with ATP supply by aerobic metabolism [41]. For the extraocular muscles, rapid mitochondrial calcium uptake during contractions would couple metabolic supply to demand. Increases in mitochondrial calcium stimulate the activity of enzyme systems that exert strong control on substrate oxidation: pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase and glycerol 3-phosphate dehydrogenase [42]. The combined activity of these enzymes maintains a high $NADH/NAD^+$ ratio and increases the driving force for oxidative phosphorylation. ATP synthase (complex V) and adenine nucleotide translocator may also be activated by calcium [43].

Metabolic integration in the extraocular muscles

High mitochondrial content is one of the hallmarks of the extraocular muscles (*e.g.*, Figure 4 and [44]). Recent evidence is beginning to put this fact into the more global perspective of extraocular muscle aerobic metabolism. First, glycogen content is low and the glycogenolysis pathway seems to be downregulated in the extraocular muscles [23, 24]. Second, creatine kinase activity and content, including the mitochondrial isoform present in striated muscles (sarcomeric creatine kinase), are significantly lower in the extraocular muscles [25, 45]. Moreover, inhibition of creatine kinase activity does not change the fatigue resistance of the extraocular muscles *in vitro*, indicating that phosphocreatine may be a less important temporal and spatial ATP buffer in these muscles [45]. In other words, mitochondrial ATP production may be sufficiently high and close to cellular sinks as to obviate the need for energy buffers in the form of phosphocreatine or glycogen. Third, the extraocular muscles can use lactate as an oxidizable substrate due to the presence of a lactate dehydrogenase isoform that catalyzes the conversion of lactate to pyruvate that then goes to Krebs cycle [46]. These facts indicate that mitochondrial metabolism is the predominant source of ATP in the extraocular muscles. In this context, the results from this study demonstrate that these mitochondria are intrinsically different and their control may depend on processes particular to the extraocular muscles.

Finally, an intriguing possibility is that extraocular muscle mitochondria could optimize their respiratory capacity by reducing their proton leak, the inverse of the mechanism of thermogenesis in brown fat. Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, has been implicated in the regulation of energy balance and body temperature [47]. Brown fat can increase its thermogenic activity by decreasing the efficiency of coupling between respiration and ATP production with uncoupling protein 1

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(UCP1, increases the magnitude of the proton leak), leading to the generation of heat [48]. Its homologues UCP2 and UCP3 are expressed skeletal muscle (and other tissues) and may regulate energy metabolism [47, 49, 50]. Oligomycin-induced state 4 respiration is a measure of mitochondrial uncoupling activity and it was significantly lower in extraocular muscle mitochondria (figure 3). Thus, the extraocular muscles may have a *lower* content of these proteins than other skeletal muscles, allowing for more efficient use of the generated proton gradients. This is an intriguing possibility that we are actively pursuing.

Conclusions and future directions

Our results clearly demonstrate that the respiratory capacity of extraocular muscle mitochondria is significantly less than that of limb muscle mitochondria, due to a combination of decreased respiratory complex activity and content. In addition, these data add weight to the concept that the metabolic organization of the extraocular muscles is unique. A very interesting question that is not addressed by this study is why the extraocular muscles need to maintain such a high mitochondrial volume density. In other words, what functions do the extraocular muscles gain by accumulating mitochondria with a built-in handicap?

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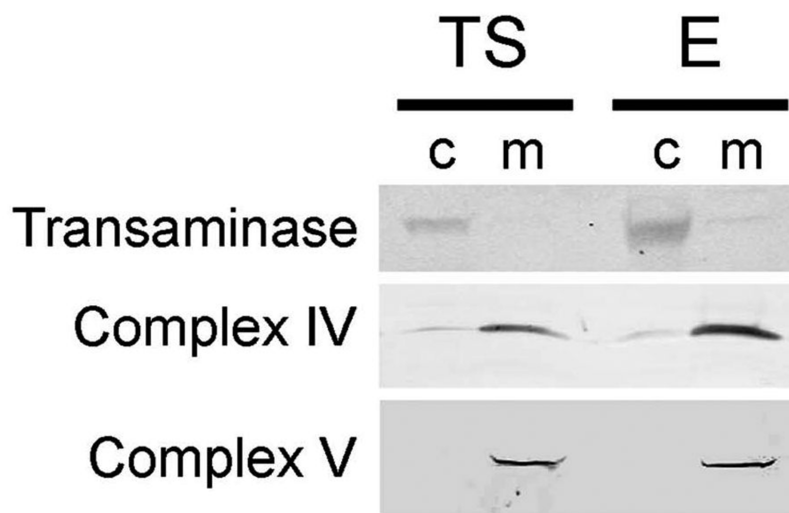

Figure 1. Mitochondrial isolation minimizes cytosolic contamination

Cytoplasmic (**c**) and mitochondrial (**m**) fractions from triceps surae (**TS**) and extraocular muscles (**E**) were evaluated by western blots of transaminase (cytosolic protein), complex IV (subunit Vb) and complex V (a subunit). Transaminase is present in the cytosolic fractions in triceps and extraocular muscle and is absent in the mitochondrial fractions. The signals for complexes IV and V are faint in the cytosolic fractions, but become strongly positive in the mitochondrial preparations.

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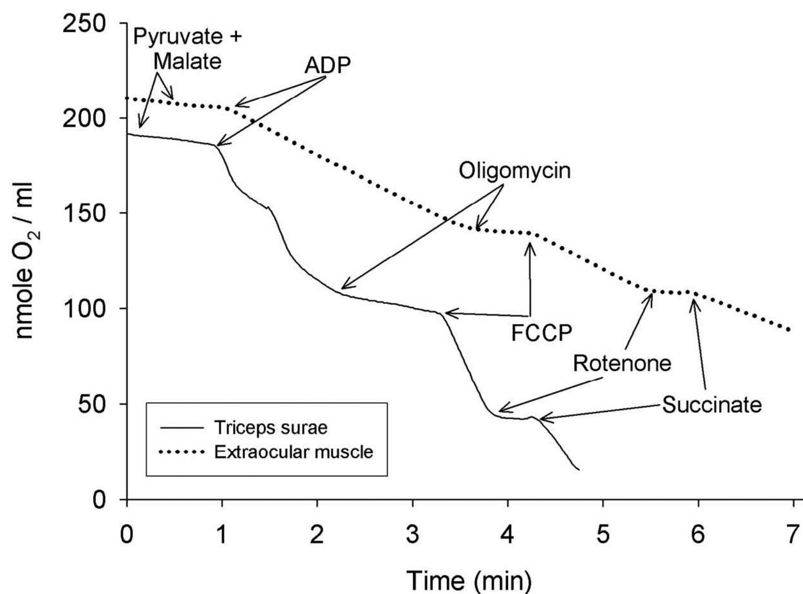

Figure 2. Reduced oxygen consumption in mitochondria from extraocular muscles

Representative tracings of oxygen consumption by mitochondria isolated from rat triceps surae (solid line) and extraocular muscles (dashed line). Respiration was initiated by addition of the substrates pyruvate + malate (state 2 respiration), followed by ADP (state 3 respiration). Oligomycin was then added to determine state 4 respiration. The mitochondrial uncoupler FCCP was added to measure complex I-driven maximum respiration (state 5). Finally, rotenone (complex I inhibitor) and succinate were added to determine state 5-succinate respiration.

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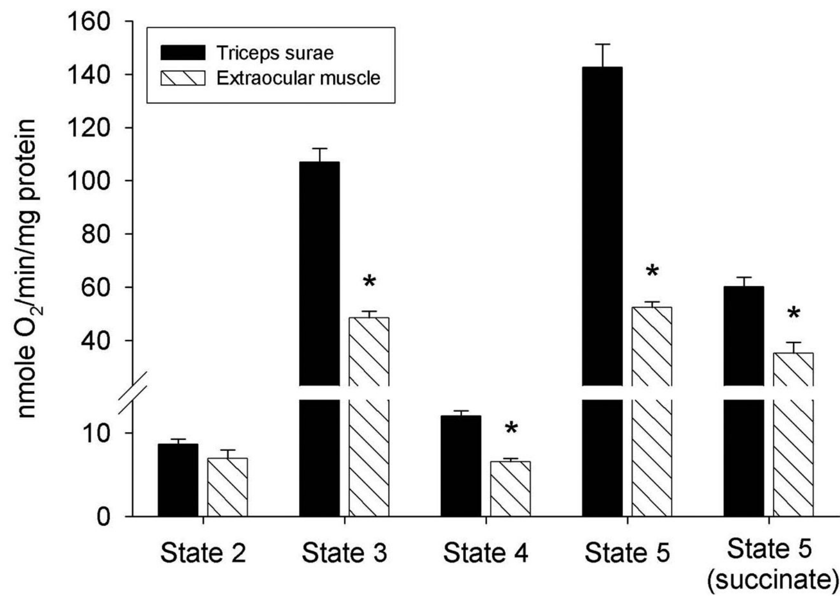

Figure 3. Respiration rates are lower in mitochondria from extraocular muscles

Respiration rates of mitochondria isolated from triceps surae (black bars) and extraocular muscles (hatched bars). Respiration was calculated as nanomoles of oxygen consumed/min/mg protein. All respiration rates except state 2 were significantly lower in mitochondria from extraocular muscle compared to mitochondria from triceps (* $P < 0.001$ for all comparisons). Data are means ± SEM, n = 10 rats.

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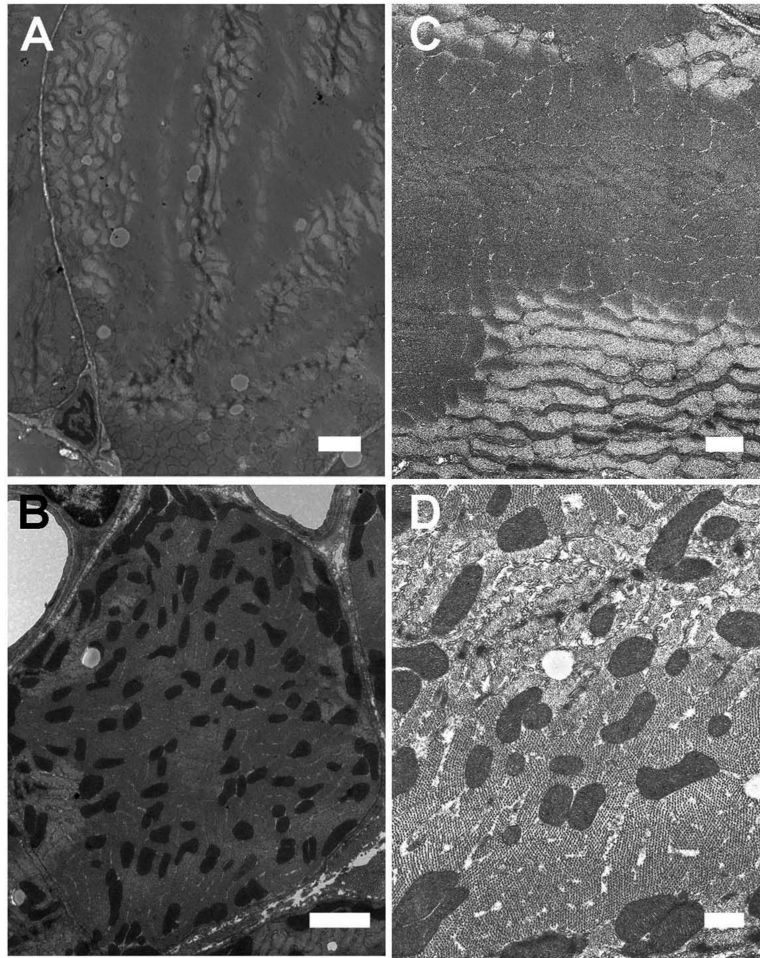

Figure 4. Electron microscopy of triceps and extraocular muscle mitochondria

(**A** – **C**) Mitochondria in triceps surae muscle fibers showing scattered mitochondria, some clustered close to the sarcolemma (bottom of panel **A**); scale bars = 2 μm in **A** and 1 μm in **C**. (**B** – **D**) Mitochondria in extraocular muscle fibers showing numerous mitochondria throughout the sarcoplasm; scale bars = 2 μm in **B** and 0.5 μm in **D**.

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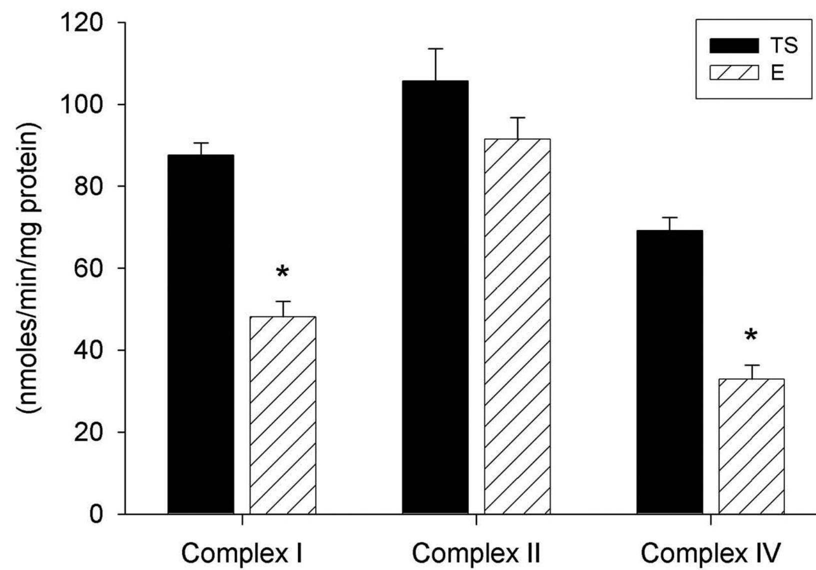

Figure 5. Lower activities of respiratory complexes in extraocular muscle mitochondria
Enzymatic activities of complexes I, II and IV in mitochondria from triceps surae (**TS**) and extraocular muscles (**E**). The activities of complexes I and IV were low in the mitochondria from extraocular muscle compared to triceps. Bars represent means ± SEM, n=6 samples/group. * $p<0.05$ compared to triceps.

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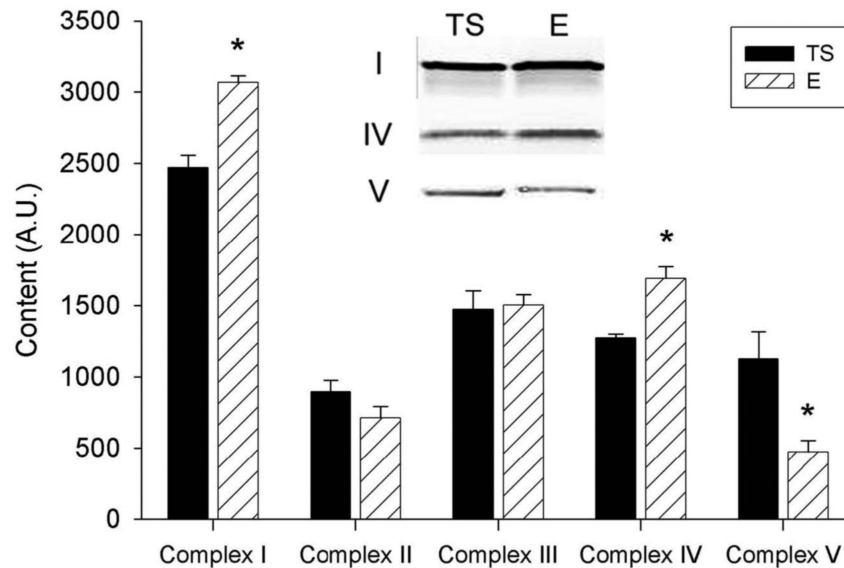

Figure 6. Differences in the content of respiratory complexes in extraocular muscle mitochondria
Mean relative content of the 5 mitochondrial respiratory complexes in triceps surae (**TS**) and extraocular muscle (**E**); n = 6 samples/group. Complexes I and IV were significantly more abundant in mitochondria from extraocular muscles compared to triceps ($P < 0.05$). Complex V content was lower in extraocular muscle mitochondria ($P < 0.05$). The content of complexes II and III was not significantly different between mitochondria from triceps and extraocular muscles. (***Inset***) Representative western blots showing the relative abundance of respiratory complexes (rows marked I – V) in mitochondrial samples from triceps surae (lanes labeled “TS”) and extraocular muscle (lanes labeled “E”).