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Isolation of mitochondrial subpopulations from skeletal muscle: optimizing recovery and preserving integrity.

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Abstract

Aim.—The subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria in skeletal muscle appear to have distinct biochemical properties affecting metabolism in health and disease. The isolation of mitochondrial subpopulations has been a long-time challenge while the presence of a continuous mitochondrial reticulum challenges the view of distinctive SSM and IFM bioenergetics. Here, a comprehensive approach is developed to identify the best conditions to separate mitochondrial fractions.

Methods.—The main modifications to the protocol to isolate SSM and IFM from rat skeletal muscle were: a) decreased disperse content and homogenization speed; b) trypsin treatment of SSM fractions; c) recentrifugation of mitochondrial fractions at low speed to remove subcellular components. To identify the conditions preserving mitochondrial function, integrity, and maximizing their recovery, microscopy (light and electron) were used to monitor effectiveness and efficiency in separating mitochondrial subpopulations while respiratory and enzyme activities were employed to evaluate function, recovery and, integrity.

Results.—With the modifications described, the total mitochondrial yield increased with recovery of 80% of mitochondria contained in the original skeletal muscle sample. The difference between SSM and IFM oxidative capacity (10%) with complex-I substrate was significant only

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Conflict of Interest

The authors declare that they have no conflict of interest associated with this manuscript

with a saturated ADP concentration. The inner and outer membrane damage for both subpopulations was less than 1% and 8%, respectively, while the respiratory control ratio was 16.

Conclusion.—Using a multidisciplinary approach, conditions were identified to maximize SSM and IFM recovery while preserving mitochondrial integrity, biochemistry and morphology. High quality and recovery of mitochondrial subpopulations allow study the relationship between these organelles and disease.

Keywords

Light microscopy; Electron microscopy; Oxidative Phosphorylation; Electron Transport Chain; Polarography; Spectroscopy

1. Introduction

After the development of the protocol for isolation of subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria from heart,¹ a similar method was used for isolation of SSM and IFM in skeletal muscle, showing biochemical differences between the two populations similar to those observed in heart muscle.^{2–4} However, the isolation of the entire population or the subpopulations of mitochondria from skeletal muscle has been a challenge. Some isolation issues are related to the unique morphology of skeletal muscle in comparison to other tissues. In particular, there are fewer mitochondria in skeletal muscle than in heart, kidney, or liver,⁵ also disruption of myofibrils and their filaments is more difficult to achieve than that of typical subcellular components in other tissues. Skeletal muscle protocols do not provide high mitochondrial recovery because actin and myosin are not easily separated from these organelles in this tissue. Previous electron microscopy of the skeletal muscle SSM fraction separated from the myofibrillar pellet using the Polytron (Brady L. and Hoppel C., unpublished work) revealed the presence of an assortment of membrane fragments in this fraction.

The isolation of the two subpopulations of skeletal muscle mitochondria is based upon two steps: a) homogenization to disrupt the sarcolemma to release and separate SSM from myofibrils; b) enzymatic treatment to digest myofibrils and release IFM from their incarceration within the contractile elements. An obstacle in the first step, is the separation of SSM from myofibrils without disrupting the integrity of the former. It was reported that a combination of homogenization and use of dispase, a proteolytic enzyme, in the isolation procedure in skeletal muscle of humans and rodents improved the harvesting of SSM.^{6,7} However, electron microscopy was not used to determine mitochondrial purity as previously reported for heart muscle.¹ Because nagarse alters some of the mitochondrial properties related to fatty acid oxidation and transport,^{8,9} trypsin has replaced nagarse for both skeletal^{6,7,10} and heart muscle¹¹, in the second step.

A major issue in isolating the entire mitochondrial population¹² or the two subpopulations^{2–4,13,14} of these organelles from skeletal muscle is the low recovery. Most procedure protocols lead to recovery of 20–30% with only one particular experiment yielding a 50% recovery.¹⁵ This raises the concern as to how a small number of mitochondria can represent the entire sample. An important issue in the isolation of

mitochondria is the damage to the mitochondrial inner and outer membranes. Thus, evaluation of the inner and outer membrane integrity should be systematically determined, which has not been done in previous studies.^{2–4}

In the present work, a comprehensive approach was designed that combines light and electron microscopy and respiration and enzyme activities to identify the conditions a) to decrease dispase content and homogenization speed; b) for the use of trypsin treatment of SSM fractions; c) to remove intruding subcellular components by recentrifugation of mitochondrial fractions at low speed; d) maximize recovery while at the same time preserving integrity of the two populations.

2. Results

The body weight of the Wistar rats at 23 weeks was 600 ± 73 g. Skeletal muscle mitochondrial subpopulations were isolated from *vastus lateralis* and from *gastrocnemius* muscles (2 g wet tissue) using the protocol presented in Figure 1. These muscle groups were used to facilitate the comparison between the results of this work with those obtained by other investigators who used rat quadriceps³ and gastrocnemius² muscles and because the vastus lateralis is typically used in human bioenergetics studies.

2.1 Subcellular fractionation techniques

Morphological examination of *in situ* skeletal muscle cells revealed the typical two populations of mitochondria (Figure 2). The effect of the first enzymatic treatment on the skeletal muscle tissue is shown in the EM micrographs of the dispase pellet (Fig. 3a and 3b). The dispase protease treatment breaks the anchorage elements that keep the myofibers adherent and exposes the sarcolemma without the interposition of fibrous connective tissue. Several experiments were conducted to optimize the amount of dispase (0.09 – 5 mg g⁻¹), homogenizer speed (100 – 2200 rpm), and number of strokes (1 – 5) (Fig. 1S and 2S). As shown in the supplemental data (Fig. 1S–2S), using the same dispase treatment and low homogenizer speed (<200 rpm) led to an incomplete separation of the SSM from myofibrillar pellet while higher homogenizer speed (<900 rpm) and number of strokes (>2) led to a disruption of the interfibrillar region with a consequent mixing of the two populations. The disruption of the muscle fiber appeared more sensitive to the homogenizer speed and number of strokes when the concentration of dispase used to treat the sample was high. The conditions to separate and isolate SSM and IFM were selected to obtain a) the highest state 3 respiration rate and respiratory control ratio (RCR); b) a separation of SSM from the myofibrillar pellet; c) minimal myofibrillar pellet disruption during the first homogenization. To perform this step, light microscopy (Fig. 1S, 2S) of myofibrillar pellet was used to monitor each condition to evaluate the degree of myofibrillar pellet disruption and the presence of residual SSM, while SSM respiration rates were measured polarographically. It appears that the lower the dispase concentration, the higher respiration rate with a saturated concentration of ADP and RCR (Fig. 3S). So our study focused on experimental conditions requiring a low concentration of dispase (<0.36 mg g⁻¹). The optimal range of the homogenizer speed appears to be 700 – 900 rpm for dispase concentrations lower than 0.18 mg g⁻¹ with one stroke. The absence of SSM within the

myofibrillar pellet is shown by EM (Fig. 4a and 4b), while the IFM are still embedded between the myofibrils. Thereafter, the myofibrillar pellet is treated with trypsin to facilitate the release of IFM. After two cycles of homogenization and centrifugation, a pellet with the IFM fraction is obtained. The IFM is re-suspended, homogenized and centrifuged (Fig. 1) to separate the IFM fraction from other subcellular components. The effect of this separation is shown in the EM micrographs of the pellet following trypsin digestion (Fig. 5). This figure shows aggregates of actin filaments with the presence of a few trapped mitochondria.

Both SSM and IFM pellets are resuspended and centrifuged for 5 minutes at low speed to pellet nuclei and myofibrillar fragments. This short centrifugation leads to more efficient mitochondria purification in the subsequent resuspension and centrifugation cycles with CP2 and KME (Fig. 1). EM (Fig. 6a and 6b) shows the purity of the isolated SSM and IFM.

2.2 Mitochondrial content

The yield of the two mitochondrial populations was similar in both vastus lateralis and gastrocnemius (Table 1). For both muscles, the SSM yield is 35% that of the IFM yield. To quantify mitochondrial content in skeletal muscle, citrate synthase (CS) and succinate dehydrogenase (SDH) were used as marker enzymes. No differences in the activities of CS and SDH were observed between the two mitochondrial fractions (Table 1) in both muscle groups. The recovery of the total population of mitochondria based on the inner membrane-bound enzyme (SDH) was approximately 75–83% (Table 1).

2.3 Mitochondrial oxidative phosphorylation

In the presence of glutamate, the results obtained for oxidative phosphorylation of the SSM and IFM isolated from vastus lateralis are similar to those from the gastrocnemius (Table 2). State 3 respiratory rate of SSM with an unsaturated concentration of ADP is similar to that observed for IFM. It should be noted that the SSM pellet was treated with trypsin during the first centrifugation at 7000 *g* to eliminate membranous material. The effect of trypsin on the State 3 respiration rate and RCR was investigated within a concentration range of 0–10 mg g⁻¹. State 3 respiration rate in the presence of trypsin treatment was 20% higher than that in absence of trypsin. Thus, the removal of non-mitochondrial membranes leads to a purer preparation.

State 4 respiration rate in both SSM and IFM was low. Maximal respiratory rate of SSM obtained with a saturated concentration of ADP is significantly lower than that measured for IFM. The uncoupled rate (DNP) for both SSM and IFM was significantly increased (15–20%) compared to that with a saturated concentration of ADP, while oxidative phosphorylation and uncoupling respiratory rates of IFM are approximately 10% higher than those observed for SSM.

2.4 Mitochondrial integrity

The RCR values indicate that SSM and IFM are highly coupled in both muscle groups (Table 2). The ADP/O ratios for SSM and IFM are similar to those reported in the literature.¹⁶ To show the degree of integrity of both populations of mitochondria, the respiration rate in the presence of NADH is less than 1% of that obtained with DOC, indicating highly intact

inner mitochondrial membranes (Table 3). The integrity of the outer membrane of mitochondria also is well preserved, as the respiration rate in the presence of cytochrome *c* is 5–8% of that obtained with DOC. These results clearly indicate that the isolation procedures employed in this study lead to the isolation of very well-coupled subpopulations of mitochondria with intact inner and outer membranes.

2.5 Electron transport chain and cytochrome *c*

The activities of the electron transport chain complexes responsible for electron flux in SSM were not different from those in IFM for both vastus lateralis and gastrocnemius (Table 4). Complex I activity was lower than that of NCR. This difference is most likely methodological as we observe the same phenomena in our previous studies.^{6, 11} The complex V activity in SSM was lower than that observed in IFM for both muscles. Also, in both muscles, the cytochrome assay showed that the content of cytochrome *aa₃*, *b*, and *c_I* in SSM was similar to that measured for IFM (Figure 7). In contrast, cytochrome *c* content of IFM was significantly higher than that observed for SSM. This difference was observed in both muscles.

3. Discussion

This study provides a comprehensive approach for optimizing experimental conditions to isolate large amounts of skeletal muscle mitochondria with emphasis on obtaining separated subsarcolemmal and interfibrillar organelles without compromising integrity and respiration rates. Mitochondrial recovery and the degree of integrity obtained with this protocol are higher than those previously reported for skeletal muscle.^{2–4,6} This isolation protocol revealed that the oxidative capacity of SSM with a complex I substrate is 10% less than that observed for IFM. A combination of specific characteristics of the medium,¹² homogenization,¹⁸ proteolytic enzyme,^{19–21} and differential centrifugation^{22,23} has been used to isolate mitochondria from this tissue focusing on one population using protease.^{24,25} In this work, salt-based medium was used rather than sucrose/mannitol-based because has been shown to be more suitable for isolation of skeletal muscle mitochondria.^{26–28} The focus of the study was on the homogenization and protease conditions to isolate both subpopulations of mitochondria. In dog⁶ muscle study, we used dispase to separate SSM and IFM fractions and trypsin to isolate IFM. Major modifications were: a) reduced amount of dispase used to treat the sample; b) homogenization to separate SSM from myofibrillar pellet was optimized at 750rpm using microscopy; c) SSM fraction was treated with trypsin as the IFM fraction without collagenase type 2; d) intermediate centrifugation at low *g* to separate out the heavy subcellular components. Microscopy was essential to provide tangible evidence of an efficient separation of the two fractions of mitochondria.

3.1 Mitochondrial yield and recovery

The experimental conditions were optimized to enhance the yield of both SSM and IFM. Electron microscopy was essential to optimize the experimental conditions in the first step and to provide evidence of mitochondrial purity as previously reported for heart muscle;¹ this technique was not used for skeletal muscle in which actin and myosin are not easy to separate from mitochondria. Centrifugation at low *g* for both SSM and IFM fractions before

the washing cycles at moderate centrifugation speed allows an efficient removal of heavier subcellular components from mitochondria. Another factor affecting SSM yield is related to the use of dispase, which separates the adherent myofibrils before the first homogenization of the tissue. The SSM yield determined in this work was almost 4.7-fold higher than that obtained in the absence of dispase treatment using Potter-Elvehjem⁴ and 1.4 fold higher than that obtained using the Polytron^{2,3}. Also, the IFM yield was 2.3–3.1 fold higher than that obtained in previous studies.^{2–4} The overall mitochondrial recovery was the highest reported (75–83%) among protocols for isolation of SSM and IFM using a few grams of tissue. For the isolation of all mitochondria in small samples, a specific protocol was developed to obtain a mitochondrial recovery of 40–50% of the homogenate of 25–100mg of skeletal muscle tissue,^{15,21} whereas protocols employing a few grams of tissue yield only 20–30%.^{12,29} For the isolation of SSM and IFM previous protocols obtained a mitochondrial recovery of less than 40%.^{2–4,14}

In the present study, the difference between the mitochondrial recovery obtained with CS and SDH could be related to a leakage of mitochondrial CS. A loss of 12–17% of malate dehydrogenase and citrate synthase from rat skeletal muscle mitochondria has been previously reported.³⁰ In the same work, the loss of CS content, attributed to mitochondrial leakage, was compatible with the intactness of mitochondria membranes. Also, the slight damage of the mitochondrial membrane observed in our study suggested that the CS leakage, if present, does not significantly affect mitochondrial integrity. A significant leakage of soluble enzymes would be associated with swollen mitochondria, low ADP/O, and RCR ratio. Instead, our isolated SSM and IFM have very high respiration rates and RCR with normal ADP/O ratio, i.e., 2.8–2.9, while EM images showed that mitochondria were not swollen.

3.2 Purity and integrity of mitochondria;

Both inner and outer membranes are intact (Table 3), with a low degree of damage compared to that previously reported for both SSM and IFM.^{3,4,6} In particular, the inner membrane damage was approximately 6% for SSM⁶ and 2–6% for IFM^{3,6} while in this work it was less than 1% for both mitochondria populations. The outer membrane damage observed in previous studies on rat skeletal muscle was 10–30%,^{4,6} while in this work it was 5–8%. It should be noted that the protocol used to quantify the intactness of mitochondria membrane varies among all previous studies. Nevertheless, the lower integrity observed in the previous rat studies could be related to the use of nagarse which has been shown to produce a loss of mitochondrial matrix constituent.³¹ Homogenization conditions, i.e., rpm and loose pestle, of the tissue samples and the gentle resuspension of the pellet after each centrifugation are important factors affecting the integrity of mitochondria.

The low state 4 respiration rate in both subpopulations of mitochondria indicates that the isolation procedure did not result in an uncoupling effect. Additionally, the high RCR (>16) for both subpopulations provided further evidence of coupled mitochondria. The RCR is used to evaluate the quality of mitochondria. Although a cutoff number so far has not been identified, it is accepted that the higher the RCR the higher the quality of the mitochondria. In this study, for SSM, RCR was the highest value, i.e., 16, compared to those reported in

literature (5–13);^{2–4} for IFM, it was similar or higher, i.e., 16–26, to those previously reported (7–18).^{2–4} In these studies, RCR values appear to be determined with higher ADP concentration (0.4–0.6mM) than by us (0.2 mM) to determine state 3 respiratory rate.

ADP/O ratio for NADH-linked substrate in both subpopulations of mitochondria (2.8–2.9) was similar to or higher than that reported in other studies.¹⁶ We standardize our ADP as noted in the method section and include AMP. While, the methods of mitochondrial isolation and respiration measurement are affecting this ratio, using standardized ADP is critical. The high value of ADP/O reported in our study is consistent with good integrity of the isolated mitochondria.

3.3 IFM vs. SSM characteristics

In our study, the respiration rate of the two populations was similar in the presence of an unsaturated (0.2mM) concentration of ADP, but a difference was observed with a saturated (2 mM) concentration of ADP (Table 2). Because the mitochondrial respiration rate depends on ADP availability,³² mitochondria were stimulated with unsaturated or saturated ADP. With unsaturated ADP, the absence of a respiration rate difference between SSM and IFM was reported in a rat skeletal muscle study¹⁰ when mitochondria were stimulated with a low ADP concentration [0.085–0.3mM]; for saturated ADP, differences between the two types of mitochondria were observed with an ADP concentration of [0.4–0.6] mM^{2–4,13}. Nevertheless, a systematic comparison with earlier work is not possible because these previous studies did not investigate the effect of ADP concentration.

Under saturated ADP concentration, the lower respiration rate in SSM than that observed in IFM (Tab. 2) is unrelated to the phosphorylation process because the respiration rate difference between the two persists with uncoupling. Also, biochemical properties of the main component of the electron transport chain (Tab. 3) are not responsible for this difference because the activity of complex I, II, and IV, as well as flavin protein domain of complex I- (NFR) and I-III (NCR) of SSM, were similar to those measured for IFM, indicating similar electron transport chain component characteristics in SSM and IFM. The respiration rate difference should not be attributed to effects of trypsin because both mitochondrial fractions were treated with the same trypsin concentration. Similar approaches were successfully used to exclude the possibility that differences between the two populations of mitochondria were due to the isolation procedures.^{33,34} It should be noted that the SSM respiration rate was 90% of that of IFM, while in a previous work in which SSM was not treated with trypsin, SSM respiration rate was 80% of that of IFM. Thus, in our work the trypsin treatment of the SSM fraction appears to enhance the purity of SSM.

To further investigate the difference in maximal oxidation, cytochrome content was determined in both mitochondrial populations. The reduced content of cytochrome *c* and *c₁* found in SSM in comparison to that observed for IFM is consistent with a lower respiration rate of SSM compared to that of IFM. Experimental^{35,36} and computational^{37–39} studies support a strong relationship between cytochrome *c* content and respiratory capacity. In a rat kidney study from our laboratory, a decrease in oxidative phosphorylation was reported to be related to a reduced cytochromes *c* content of mitochondria with intact outer membranes.³⁵

Also, in a rat skeletal muscle study, a twofold increase in the respiratory capacity in white, red, and mixed muscle fibers was attributed to an approximately twofold increase of citrate synthase, cytochrome *c*, and cytochromes *c* oxidase.³⁶ Computational studies confirmed a close relationship among cytochrome *c* reduced and oxidized concentrations and mitochondrial respiration rate.^{37–39}

In addition to its role in facilitating the mobilization of electrons through the electron transport chain, cytochrome *c* also is involved in apoptosis. The presence of a different level of cytochrome *c* in the two populations also was reported in skeletal muscle regarding mitochondrial susceptibility to apoptotic stimuli.^{40,41} A distinctive role of SSM and IFM in promoting apoptotic signals in skeletal muscle has been reported in which IFM have a predominant proapoptotic initiation activity.⁴⁰

It has been proposed that mitochondria form a continuous reticulum.^{42–44} In support of compartmentalized oxidative and phosphorylation processes, a study performed on mouse skeletal muscle reported complex IV and V distribution obtained by analysis of confocal images of muscle fibers,⁴² showed localized differences in the reticulum: distribution of complex IV and V in the mitochondria neighboring blood vessels differed from that of the IFM. In the periphery of the cell, paravascular mitochondria are rich in complex IV whereas the more central portions, i.e., IFM, have a higher amount of complex V. In our work, the activity of complex V is higher in IFM than SSM and consistent with the observation that the complex is more concentrated in the central region of the reticulum. Although the activity of complex IV is similar in the two populations of mitochondria (unlike that observed by Glancy et al.⁴²), the proportions of complex IV and V are different in SSM and IFM and consistent with a different electron transport chain distribution of mitochondria.⁴²

Substrate transport and metabolism differences in mitochondria between peripheral and central fiber regions can coexist even in the presence of a mitochondria reticulum to accommodate morphological changes to conditions such as exercise training^{44–46} and disease.⁴¹ While there is still a debate as to the existence of two subpopulations of mitochondria,^{47–49} it has been shown that the subpopulations have different morphological characteristics^{50,51} and responses to exercise training.^{44–46} It is not clear how the concept of two distinct populations of mitochondria fit into the scheme of a mitochondria reticulum. Reconciliation of these two concepts of skeletal muscle mitochondria is important because these organelles play a key role in the homeostasis of muscle cells, with SSM phenotypes different from that of IFM in healthy and disease states.⁴¹ For instance, there is evidence that in type 2 diabetes the diseases differentially affects the two populations of mitochondria.^{7,9,52} The method presented in this study provides an optimal design for separating the subpopulations of mitochondria in skeletal muscle. This approach may prove complementary to the study of the function and structure of mitochondria reticula.

3.4 Isolated mitochondria vs. permeabilized fibers

The degree of mitochondrial integrity obtained with current protocols has been questioned,³¹ with less disruptive approaches involving permeabilized fibers being favored.⁵³ Other studies reported bioenergetics differences between permeabilized fiber and isolated mitochondria suggesting that the disruption of mitochondrial structure during the isolation

process might alter mitochondria function probably because of a loss of matrix constituents.³¹ Nevertheless, inner and outer mitochondrial membrane integrity was not evaluated in those studies. In a heart study by our group, it was shown that whereas the isolated mitochondria become spheroid their cristae were 77% lamelliform, as were those in the SSM in *in situ* fibers.⁵⁴ In a heart muscle study, the respiratory capacity in isolated mitochondria was the same as that observed in fibers.⁵⁵

3.5 Limitations

Large amounts of tissue, skill and time needed to perform the protocol are obstacles in isolating both populations of mitochondria in comparison to permeabilized fibers. Although muscle fibers provide a simpler approach with a small amount of tissue, the respiration rate reflects both subpopulations of mitochondria. However, this approach cannot determine whether the defect is in both populations or only in one. Bioenergetics in isolated mitochondria does not take into consideration the influence of the cytoskeleton and endoplasmic reticulum as do muscle fibers. The absence of these influences could be advantageous or disadvantageous according to the hypothesis tested.

In this protocol, experimental conditions were optimized for 2 grams of skeletal muscle mass. Nevertheless, the protocol could be scaled down to isolate SSM and IFM from small muscle samples (400mg). Subpopulations of heart mitochondria have been harvested from 400mg.⁵⁶ It should be noted that the use of small amounts of tissue as is the case with needle biopsy allows obtaining a small amount of mitochondria, which limits the number of bioenergetics assays.

4. Conclusion

A comprehensive approach that combines light and electron microscopy, centrifugation separation, polarography, and enzymatic assays is ideal to optimize the experimental conditions used to isolate high quantity and quality of subpopulations of mitochondria to study the relationship between function and structure of skeletal muscle organelles. This may be particularly important in determining the role of specific subpopulations of mitochondria in muscle diseases.

5. Methods

5.1 Materials

Reagents: Dispase (neutral protease) was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Unless otherwise indicated, all other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Buffers: The relaxing buffers, Chappell–Perry (CP) (100 mM KCl, 50 mM MOPS, 5 mM MgSO₄, 1mM ATP) and CP2 (Buffer CP plus 0.2% defatted BSA and 1 mM EGTA), as well as KME (100 mM KCl, 50 mM MOPS and 0.5 mM EGTA, pH 7.4) were prepared for tissue storage, mitochondrial isolation and storage.^{57,58} The respiration buffer (80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH₂PO₄, and 1 mg mL⁻¹ defatted BSA, pH 7.4) was used for mitochondrial oxygen uptake measurements.⁵⁹

5.2 Animal model

The experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the National Research Council⁶⁰ and were approved by the Case Western Reserve University Institutional Animal Care and Use Committee. Wistar colony rats were obtained from Charles River Laboratories, Wilmington, MA. Three pairs of male Wistar (W) rats were housed in the Animal Resource Center facilities of Case Western Reserve University with 12:12-h light-dark cycle and were fed a standard diet chow (Prolab Isopro RMH 3000, LabDiet, St. Louis, MO). The rats were euthanized at 23 wk (n=6) of age.

5.3 Isolation of mitochondria

Skeletal muscle SSM and IFM were isolated from the vastus lateralis and the gastrocnemius using a previously described protocol^{1,6} with several important modifications to improve the mitochondrial isolation procedure (See Figure 1). After fat and connective tissue were removed, 2 g wet skeletal muscle was blotted dry and weighed for each preparation. To separate fibers, the tissue was minced and suspended in cold (4 °C) buffer (CP) (20 ml), then incubated with 0.09 mg (1 U) dispase per gram wet weight tissue (g tissue) in CP buffer with stirring on ice for 10 min. Out of the multiple proteases tested to break the anchorage elements that keep the myofibers adherent, dispase, a neutral dispase, provided the best separation of the fibers, exposing the sarcolemma (Fig.3) rendering it susceptible to disruption. After removing dispase, gentle homogenization releases subsarcolemmal mitochondria, preserving the skinned myofibrils. Subsequently, 20 ml of CP2 buffer g tissue⁻¹ was added to the sample and centrifuged at 7650 g for 10 min. The pellet was resuspended in CP2 buffer (20 mL g tissue⁻¹) and homogenized with a loose-fitting pestle (Potter-Elvehjem) at 750 rpm (Fisher Maxima Overhead Stirrer) with one stroke and immediately centrifuged for 10 min at 580 g. The supernatant was separated from the myofibrillar pellet and treated with trypsin (6 mg/120000 BAEE units g tissue⁻¹) during centrifugation at 7000 g for 20 min to collect the SSM fraction. The SSM pellet was resuspended in CP2 buffer (10 ml g tissue⁻¹) and centrifuged at 630 g for 5 minutes and the supernatant containing the SSM fraction collected. The myofibrillar pellet was resuspended in CP buffer (10 mL g tissue⁻¹), treated with trypsin (6 mg/120000 BAEE units g tissue⁻¹) for 10 minutes, and homogenized at 1600 rpm with three strokes of a tight-fitting pestle (Potter-Elvehjem). An equal volume of CP2 buffer was added to the homogenate, which was centrifuged for 10 min at 11,950 g. The pellet was resuspended in CP2 buffer (10 ml g tissue⁻¹), homogenized at 1600 rpm with three strokes of a tight-fitting pestle and centrifuged at 345 g for 10 min. The supernatant was collected by filtering through two layers of gauze and kept on ice. The pellet was again resuspended in CP2 buffer (10 ml g tissue⁻¹) and homogenized at 1600 rpm with three strokes of a tight-fitting pestle (Potter-Elvehjem), and centrifuged at 345 g for 10 minutes. The supernatant collected by filtering through two layers of gauze was added to the first supernatant with the IFM fraction, then the combined fractions centrifuged at 7000 g for 20 min. The IFM pellet was resuspended in CP2 buffer (10 ml g tissue⁻¹) and centrifuged at 345 g for 5 minutes (trypsin pellet of IFM), and the supernatant containing IFM fraction was collected.

Both SSM and IFM fractions were resuspended first with 10 ml g tissue⁻¹ of CP2 buffer and then with 5 ml g tissue⁻¹ of buffer BSA free (KME) at 7,000 *g* for 20 and 10 minutes, respectively. The KME buffer was used to resuspend the final pellets to a concentration of approximately 30–40 mg mitochondrial protein ml⁻¹. Ice-cold conditions were maintained throughout the SSM and IFM isolation procedure. The Lowry method was used to determine mitochondrial protein concentration.⁶¹

5.4 Oxidative phosphorylation

Oxygen consumption by SSM and IFM was measured with a Clark-type electrode (YSI model 53) in a final volume of 0.5 mL respiration buffer^{57,62} at 30°C in the presence of the substrate, glutamate (20 mM). Glutamate was the oxidative substrate used because it has its own transporter through the inner membrane and in the matrix, it is dehydrogenated by glutamate dehydrogenase yielding NADH, which is then oxidized by complex I of the ETC. Among the complex I substrates it is the only substrate that does not require any further additions. The concentration of glutamate, uncoupler, and ADP refers to the initial concentrations in the chamber. An ADP concentration of 0.1 mM was used to deplete endogenous substrates before glutamate was added and the mitochondria stimulated with 0.2 mM ADP (ADP-stimulated) to measure the State 3 respiration rate.⁶³ After ADP depletion, State 4 respiration was measured (ADP-limited). The mitochondria State 3 and 4 respiration rates were measured twice. To determine maximal oxidative phosphorylation, the respiratory rate also was measured in the presence of a saturated concentration of ADP, 2 mM. Finally, 0.2 mM of the uncoupler dinitrophenol (DNP) was added to the chamber⁶ to measure oxidative capacity of the electron transport chain. Respiratory control ratio (RCR, State 3 divided by State 4) quantifies the control of oxygen consumption by adenosine nucleotide translocator, phosphate transporter, and phosphorylation ('coupling'). The concentration of the ADP was determined with an enzymatic method⁶⁴ and used to calculate the ADP/O ratio (ADP mole added per mole of oxygen atom consumed).

The respiratory rate is reported as nAO min⁻¹ mg⁻¹: nano atoms of oxygen consumed per minute and mg of mitochondrial protein while the conversion factor to express the rate in pmolO₂ s⁻¹ mg⁻¹ is 8.33.

5.5 Preparation of samples, enzymatic assays and recovery

Mitochondrial enzyme activities were measured as previously described.^{59,62,65,66} Briefly, fresh SSM and IFM (1 mg of mitochondrial protein) samples were treated with cholate (10 mg cholate mg⁻¹,⁶⁷) and 25 mM KPi 2mM EDTA⁻¹ buffer to a final volume of 1 mL and supplemented with mammalian protease inhibitor cocktail (10 µL mL⁻¹). The samples were diluted 1:10 for the enzymatic assays. The electron transport chain activity components were determined using biochemical kinetics principles in which donors and acceptors span specific regions of the electron transport chain.^{62,65,66} Assays are based on the oxidation-reduction reactions of Coenzyme Q, oxidation of NADH, formation of 2-nitro-5-benzoic acid, reduction of cytochrome c and dichlorophenol-indophenol which are measured spectrophotometrically at 340, 412, 550, and 600 nm, respectively. Rotenone-sensitive NADH-Q reductase (CI): it measures NADH oxidation coupled to the reduction of coenzyme Q with exogenous decylubiquinone and anitmycin A to inhibit complex III and in

the absence and presence of rotenone (R) to inhibit complex I. Rotenone-sensitive NADH-cytochrome c reductase (NCR) is designed to evaluate the linked activity of complexes I and III with a measure of reduction of cytochrome c, the acceptor for complex III. In this case, the oxidation of NADH by complex I is coupled to the reduction of cytochrome c. The reduction of cytochrome c is quantified in the presence of exogenous oxidized cytochrome c, potassium cyanide to inhibit complex IV, and in the absence and presence of R. NADH-ferricyanide reductase (NFR); it evaluates the activity of the NADH-dehydrogenase, the first three subunits of complex I, including flavin-mononucleotide. The assay measures NADH oxidation coupled to the reduction of ferricyanide ($K_6Fe(CN)_6$) in the presence of sodium azide (NaN_3) to inhibit complex IV. Thenoyltrifluoroacetone-sensitive succinate-Q reductase (CII) assay measures succinate oxidation coupled to the reduction of coenzyme Q and dichlorophenol-indophenol with and without thenoyltrifluoroacetone to inhibit complex II. Thenoyltrifluoroacetone-sensitive succinate-Q reductase + quinone (CII+Q₁) assay is designed to measure total CII activity by supplementing with coenzyme Q₁ with and without thenoyltrifluoroacetone. Anitmycin A-sensitive decylubiquinol-cytochrome c reductase (CIII): CIII activity is determined with measurement of the reduction of cytochrome c coupled to the oxidation of decylubiquinol to decylubiquinone in the presence of oxidized cytochrome c and potassium cyanide with and without anitmycin A. Cytochrome c oxidase (CIV) is determined as the first order rate constant with and without potassium cyanide. Citrate synthase (CS) activity was determined by measurement of the formation of 2-nitro-5-benzoic acid from 5', 5'-dithiobis 2-nitrobenzoic acid coupled to the reaction between acetyl-CoA and oxaloacetate.⁶⁸ Succinate dehydrogenase (SDH) activity was determined by measurement of the reduction of dichlorophenol-indophenol coupled to the oxidation of succinate to fumarate using phenazine ethosulfate as the intermediate acceptor.⁶⁷

ATP synthase activity was measured spectrophotometrically in frozen-thawed mitochondria as oligomycin-sensitive ATPase activity.^{6,69} The mitochondrial cytochrome contents (*aa*₃, *b*, *c*₁, *c*) were determined according to the protocol developed by Williams et al.¹⁷

Mitochondrial yield is the sum of the amount of isolated mitochondrial protein (SSM and IFM) divided by the grams of skeletal muscle mass used giving mg of mitochondrial protein per gram wet weight muscle ($mg\ gww^{-1}$). Mitochondrial recovery provided an assessment of the fraction of mitochondria in skeletal muscle tissue recovered in SSM and IFM. Two mitochondrial marker enzymes were used: CS, a mitochondrial matrix enzyme and SDH, an integral inner membrane protein. Enzymatic activity was determined in the original skeletal muscle homogenate ($mU\ gww^{-1}$)_H, fractions harvested included the isolated SSM ($mU\ mg^{-1}$)_{SSM} and IFM ($mU\ mg^{-1}$)_{IFM}. The mitochondrial recovery (MR) was calculated as follows:

$$MR = 100\% \frac{(mg/gww)_{SSM}(mU/mg)_{SSM} + (mg/gww)_{IFM}(mU/mg)_{IFM}}{(mU/gww)_H}$$

5.6 Mitochondrial integrity

The inner and outer mitochondrial membrane integrity was assessed using a Clark-type electrode (YSI model 53) with 0.25 mg mitochondrial protein in a final volume of 0.5 mL

respiration buffer at 30°C.^{59,62} Following addition of mitochondria, ADP (2 mM), NADH (2.8 mM), and cytochrome *c* (0.032 mM) were added sequentially to the chamber, then deoxycholate (DOC 120 mM of stock solution) was titrated with 1 µL additions until maximal respiration was achieved. Typically, 5–6 µL were required to reach plateau. In intact mitochondria, the inner membrane is impermeable to NADH while the outer membrane is impermeable to cytochrome *c*. Thus, an increase of mitochondrial respiration after the addition of NADH is related to NADH oxidation due to an alteration of the inner mitochondrial membrane;⁷⁰ the increase of mitochondrial respiration after the addition of cytochrome *c* is related to its reduction by NADH-cytochrome *c* reductase and subsequent oxidation by cytochrome oxidase and reflects alteration of the outer mitochondrial membrane. The addition of deoxycholate permeabilizes inner and outer membranes, while the mitochondrial respiration reflects maximal oxidation of the NADH through complex IV.

5.7 Light and Electron Microscopy

Isolated mitochondria were fixed in an equal volume of phosphate-buffered quarter-strength Karnovsky's fixative using a method reported previously.⁷¹ Muscle tissue was fixed by immersion in triple aldehyde/DMSO,⁷² homogenates, pellets, and isolated mitochondria were fixed by immersion in phosphate-buffered quarter-strength Karnovsky's fixative⁷³ and processed according to a method previously reported.⁷¹ Sections 0.7 µm thick were stained with toluidine blue and examined by light microscopy. Thin sections were sequentially stained with acidified uranyl acetate followed by a modification of Sato's triple lead stain⁷⁴ and examined in an FEI Tecnai Spirit (T12) electron microscope with a Gatan US4000 4kx4k CCD.

5.8 Statistical Analysis

Mean and standard deviation were used for statistical analysis of the results. This approach was justified by the normal distribution of the data verified using the Shapiro-Wilk test. The student's paired *t* test with two tails was used to evaluate the null hypothesis of no difference between SSM and IFM variables. A paired test was used because each set of SSM and IFM data was obtained from the same tissue. A difference of $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate

BSA	Bovine serum albumin
CI	Rotenone-sensitive NADH-Q reductase
CII	Thenoyltrifluoroacetone-sensitive succinate-Q reductase
CHH	Anitimycin A-sensitive decylubiquinol-cytochrome c reductase
CIV	Cytochrome c oxidase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
J_{NADH}	Oxygen consumption in presence of NADH
J_{Cytochrome c}	Oxygen consumption in presence of endogenous Cytochrome c
J_{DOC}	Oxygen consumption in presence of deoxycholate DOC
KH₂PO₄	Potassium dihydrogen phosphate
KCl	Potassium chloride
KPi	Potassium phosphate
nAO	nano atoms of oxygen
MOPS	3-(N-morpholino)propanesulfonic acid
NCR	Rotenone-sensitive NADH-cytochrome c reductase
NFR	NADH-ferricyanide reductase

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Figure 1.
Schematic representation of the protocol to isolate subpopulations of mitochondria.
Centrifugation, homogenization and enzymatic reactions are performed at 4°C.

Figure 2. Electron micrograph of a longitudinal section of *vastus lateralis* (Scale line = 1μm). The sarcolemma, subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria are identified.

Figure 3.

Two distinct fragments of a cell found within the skeletal muscle pellet treated with dispase protease (Scale line = 2μm) in panel (a) and (b). The sarcolemma, subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria are identified.

Figure 4.

Two distinct cell fragments found within a myofibrillar pellet after homogenization and centrifugation of disperse pellet (Scale line = 2μm) in panel (a) and (b). The sarcolemma and intermyofibrillar (IFM) mitochondria are identified.

Figure 5.

A fragment of the discarded pellet after resuspension, homogenization and centrifugation of myofibrillar pellet (Scale line = 2μm).

Figure 6. Isolated subsarcolemmal (SSM, a) and interfibrillar mitochondria (IFM, b) fixed and processed as described in the methods section (Scale line = 0.5μm).

Figure 7.

The cytochrome content (aa₃, b, c₁ and c) in isolated skeletal SSM and IFM for vastus lateralis (panel a) and gastrocnemius (panel b) determined with the method of Williams et al.

¹⁷ ‡(p<0.001) (n=6), Mean±SD.

Table 1.

Specific activities of isolated subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria and mitochondrial yield for vastus lateralis and gastrocnemius muscles. Values are reported as means $\pm$ standard deviation (n=6).

	Unit	Skeletal muscle homogenate *	SSM	IFM	p
<i>Vastus Lateralis</i>					
Yield	[mg gww ⁻¹]	-	2.35 $\pm$ 0.4	6.3 $\pm$ 0.4	<10 ⁻⁶
Citrate Synthase	[mU gww ⁻¹]	29800 $\pm$ 3900			
	[mU mg ⁻¹]		1941 $\pm$ 133	2067 $\pm$ 103	0.3
Succinate Dehydrogenase	[mU gww ⁻¹]	3450 $\pm$ 350			
	[mU mg ⁻¹]		309 $\pm$ 50	332 $\pm$ 40	0.3 2
<i>Recovery</i>					
Citrate Synthase	[%]	-	15.4 $\pm$ 2.5	43.3 $\pm$ 3.8	
Succinate Dehydrogenase	[%]		21.3 $\pm$ 5.2	61.8 $\pm$ 6	
<i>Gastrocnemius</i>					
Yield	[mg gww ⁻¹]		2.1 $\pm$ 0.3	6.6 $\pm$.6	<10 ⁻⁵
Citrate Synthase	[mU gww ⁻¹]	28300 $\pm$ 3300			
	[mU mg ⁻¹]		2046 $\pm$ 56	2023 $\pm$ 130	0.3
Succinate Dehydrogenase	[mU gww ⁻¹]	3580 $\pm$ 500			
	[mU mg ⁻¹]		324 $\pm$ 50	346 $\pm$ 42	0.4
<i>Recovery</i>					
Citrate Synthase	[%]	-	13.7 $\pm$ 2.7	48.2 $\pm$ 9.1	
Succinate Dehydrogenase	[%]		16.8 $\pm$ 3.1	59 $\pm$ 8.5	

* The enzymatic activity in skeletal muscle homogenate and subpopulations of mitochondria is normalized to the gram of wet weight of skeletal muscle homogenate (gww) and gram of mitochondria protein (mg), respectively.

Table 2.

Oxidative phosphorylation characteristics of skeletal muscle subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) in vastus lateralis and gastrocnemius muscles. The respiratory rate is related to the mg of mitochondrial protein. Values are reported as means $\pm$ standard deviation (n=6). [†] (P<0.002) significantly different from high ADP.

	Unit	SSM	IFM	P
<i>Vastus Lateralis</i>				
State 3	[nAO min ⁻¹ mg ⁻¹]	352 $\pm$ 28	356 $\pm$ 10	0.7
State 4	[nAO min ⁻¹ mg ⁻¹]	22 $\pm$ 7	23 $\pm$ 5	0.6
RCR	[-]	16 $\pm$ 3	16 $\pm$ 3	0.5
ADP/O	[-]	2.78 $\pm$ 0.15	2.88 $\pm$ 0.11	0.2
High ADP	[nAO min ⁻¹ mg ⁻¹]	435 $\pm$ 27	473 $\pm$ 18	<0.01
Uncoupled	[nAO min ⁻¹ mg ⁻¹]	502 $\pm$ 37 [†]	560 $\pm$ 22 [†]	<0.002
<i>Gastrocnemius</i>				
State 3	[nAO min ⁻¹ mg ⁻¹]	350 $\pm$ 18	357 $\pm$ 12	0.5
State 4	[nAO min ⁻¹ mg ⁻¹]	24 $\pm$ 5	16 $\pm$ 5	<0.005
RCR	[-]	15 $\pm$ 3	26 $\pm$ 9	<0.005
ADP/O	[-]	2.85 $\pm$ 0.16	2.89 $\pm$ 0.07	0.4
High ADP	[nAO min ⁻¹ mg ⁻¹]	417 $\pm$ 30	460 $\pm$ 20	<0.005
Uncoupled	[nAO min ⁻¹ mg ⁻¹]	490 $\pm$ 34 [†]	549 $\pm$ 20 [†]	<0.01

nAO min⁻¹ mg⁻¹: nano atoms of oxygen consumed per minute and mg of mitochondrial protein

RCR: respiratory control ratio;

ADP/O: ADP to oxygen ratio;

High ADP: Saturated concentration of ADP;

DNP: Uncoupling state in presence of dinitrophenol;

Table 3.

Membrane integrity of SSM and IFM. The oxygen consumption rate in presence of NADH (J_{NADH}), cytochrome c ($J_{cytochrome\ c}$) and deoxycholate (J_{DOC}) are reported as means $\pm$ standard deviation (n=6).

	Unit	SSM	IFM
<i>Vastus Lateralis</i>			
J_{NADH}	[nAO min ⁻¹ mg ⁻¹]	1.1 $\pm$ 0.02	1.1 $\pm$ 0.01
$J_{Cytochrome\ c}$	[nAO min ⁻¹ mg ⁻¹]	158 $\pm$ 26	100 $\pm$ 13
J_{DOC}	[nAO min ⁻¹ mg ⁻¹]	1978 $\pm$ 213	1968 $\pm$ 271
$J_{NADH} J_{DOC}^{-1}$	[%]	0.06 $\pm$ 0.003	0.05 $\pm$ 0.01
$J_{Cytochrome\ c} J_{DOC}^{-1}$	[%]	8.1 $\pm$ 1.2	5.2 $\pm$ 1
<i>Gastrocnemius</i>			
J_{NADH}	[nAO min ⁻¹ mg ⁻¹]	1.1 $\pm$ 0.01	1.1 $\pm$ 0.03
$J_{Cytochrome\ c}$	[nAO min ⁻¹ mg ⁻¹]	122 $\pm$ 16	94 $\pm$ 8
J_{DOC}	[nAO min ⁻¹ mg ⁻¹]	1954 $\pm$ 189	2066 $\pm$ 150
$J_{NADH} J_{DOC}^{-1}$	[%]	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01
$J_{Cytochrome\ c} J_{DOC}^{-1}$	[%]	6.4 $\pm$ 0.8	4.6 $\pm$ 0.5

nAO min⁻¹ mg⁻¹: nano atoms of oxygen consumed per minute and mg of mitochondrial protein

Table 4.

Electron Transport Chain Activity: complex I, II, III and IV (CI, CII, CIII and CIV), NADH-cytochrome c reductase (NCR), NADH-ferricyanide reductase (NFR), complex II (CII), succinate-Q reductase + quinone (CII+Q). The biochemical reaction rates are related to the mg of mitochondrial protein. The CV data represent a first order reaction. Values are reported as means $\pm$ standard deviation (n=4).

	Unit	SSM	IFM	<i>p</i>
<i>Vastus Lateralis</i>				
CI	[mU mg ¹]	302 $\pm$ 64	281 $\pm$ 20	0.6
NCR	[mU mg ¹]	6113 $\pm$ 580	6251 $\pm$ 351	0.8
NFR	[mU mg ¹]	2446 $\pm$ 298	2350 $\pm$ 203	0.4
CII	[mU mg ¹]	81 $\pm$ 15	82 $\pm$ 28	0.9
CII+Q	[mU mg ¹]	779 $\pm$ 74	812 $\pm$ 53	0.3
CIII	[mU mg ¹]	7390 $\pm$ 1190	7224 $\pm$ 1053	0.8
CIV	10 ⁻³ [min ⁻¹ mg ¹]	531 $\pm$ 133	576 $\pm$ 97	0.3
CV	[mU mg ¹]	322 $\pm$ 110	682 $\pm$ 327	<0.01
<i>Gastrocnemius</i>				
CI	[mU mg ¹]	262 $\pm$ 31	290 $\pm$ 22	0.4
NCR	[mU mg ¹]	4987 $\pm$ 850	5556 $\pm$ 404	0.5
NFR	[mU mg ¹]	2418 $\pm$ 347	2626 $\pm$ 281	0.5
CII	[mU mg ¹]	74 $\pm$ 10	90 $\pm$ 16	0.5
CII+Q	[mU mg ¹]	527 $\pm$ 19	783 $\pm$ 120	0.3
CIII	[mU mg ¹]	8275 $\pm$ 1423	6840 $\pm$ 1113	0.4
CIV	10 ⁻³ [min ⁻¹ mg ¹]	765 $\pm$ 20	820 $\pm$ 66	0.2
CV	[mU mg ¹]	296 $\pm$ 85	454 $\pm$ 65	<10 ⁻⁴