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Isolation of functional mitochondria from rat kidney and skeletal muscle without manual homogenization

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Abstract

Isolation of functional and intact mitochondria from solid tissue is crucial for studies that focus on the elucidation of normal mitochondrial physiology and/or mitochondrial dysfunction in conditions such as aging, diabetes and cancer. There is growing recognition of the importance of mitochondria as both targets for drug development and as off-target mediators of drug side effects. Unfortunately, mitochondrial isolation from tissue is generally carried out using homogenizer-based methods that require extensive operator experience to obtain reproducible, high-quality preparations. These methods limit dissemination, impede scale-up, and contribute to difficulties in reproducing experimental results over time and across laboratories. Here we describe semi-automated methods to disrupt tissue, using kidney and muscle mitochondria preparations as exemplars. These methods utilize either a Barocycler, or The PCT Shredder, or both. The PCT Shredder is a mechanical grinder that quickly breaks up tissue without significant risk of over-homogenization. Mitochondria isolated using The PCT Shredder are shown to be comparable to controls. The Barocycler generates controlled pressure pulses that can be adjusted to lyse cells and release organelles. The mitochondria subjected to pressure cycling-mediated tissue disruption are shown to retain functionality, enabling combinations of The PCT Shredder and Barocycler to be used to purify mitochondrial preparations.

Keywords

Mitochondria; Barocycler; Hydrostatic Pressure; Kidney; Muscle; Drug discovery; Toxicology

Introduction

The most physiologically relevant evaluation of mitochondrial function should be performed *in situ* or *in vivo*. Unfortunately, under such conditions, it is difficult to attribute observations to primary mitochondrial physiology in the context of secondary effects related to the complexity of systems such as intact cells, tissues or animals. Accordingly, there are

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many experiments that can only be conducted on purified mitochondrial preparations. Similarly, many of the questions most relevant to drug development can, theoretically, be effectively and inexpensively brought to scale in isolated preparations as opposed to intact animals or cell cultures. Therefore, in order to better understand mitochondrial function and the interrelation of mitochondrial dysfunction with various diseases, experiments often need to focus on functional isolated mitochondria rather than on intact cells, tissues or animals [1].

Several traditional methods for isolation of functional mitochondria are widely used. However, these current protocols, which typically rely on Dounce, Potter-Elvehjem or rotor-stator shearing homogenizers for initial tissue disruption [2–4], suffer from several critical drawbacks, of which the most pronounced are the demand for highly skilled personnel and the hands-on nature of the initial tissue homogenization. The homogenization procedure is a crucial step that can significantly impact the quality and yield of the resulting mitochondria. For isolation of muscle mitochondria, for example, the most commonly used method is manual homogenization in a Potter-Elvehjem tissue grinder. This physical homogenization is assumed to occur by two types of liquid shearing; one due to pestle rotation and the other due to the up-and-down pestle strokes. Generally, both forces need to be precisely controlled to minimize damage to the mitochondria [5]. This requires slow pestle strokes and consistent pestle rotation. During this manual homogenization, it is difficult to know when sufficient tissue disruption has occurred because large fragments of tissue may still be present, even when significant cell disruption has occurred. In addition, if disruption is carried out long enough for thorough tissue disaggregation, the resulting mitochondria are likely to be severely damaged due to over-homogenization. For this reason, a more hands-off method, i.e., one that is less reliant on the experience and skill of the user, is expected to yield more consistent and reproducible results.

Recently, several authors have used nitrogen cavitation for isolation of mitochondria from cultured cells [6–8]. This method is based on sample pressurization (at ~1500 psi) and the subsequent formation of gas bubbles when the sample is rapidly decompressed. While this method has several drawbacks, including the need to clean the pressure chamber between each sample, the results clearly demonstrate that high quality mitochondria preparations can be obtained from pressurized, rather than homogenized, samples.

Although mitochondria are more difficult to isolate from fibrous tissue, such as muscle, than from softer tissues such as liver, mitochondrial function and dysfunction in muscle tissue have been studied extensively in recent years. The methods for isolation of muscle mitochondria have changed very little over the past three decades [4,9,10], even though a better and more consistent method for isolation of intact muscle mitochondria would greatly benefit this area of research. An instrument-based tissue disruption method, capable of processing multiple samples under essentially identical conditions, is expected to lead to more consistent results, and substantial savings of time and resources. Muscle tissue disruption in The PCT Shredder (or The Shredder SG3, both from Pressure BioSciences, Inc) requires approximately ten seconds per sample and can be performed by an operator with minimal training and experience.

Here we present methods for extraction and assay of rat skeletal muscle mitochondria that do not rely upon a manual, user-regulated homogenization step. In addition, several parameters of the new method were also tested with kidney mitochondria to demonstrate that the methods are likely to be broadly applicable to mitochondria from other tissues. The results confirm that mitochondria extracted using pressure cycling, or The PCT Shredder, or a combination of both methods, are intact and functionally active, and can be used for downstream applications that require high quality, enriched mitochondria preparations.

Materials and Methods

Animals

Male, specific pathogen free, Fischer 344 × Brown Norway F1 (FBNF1) rats were obtained from Harlan (South Easton, MA) at 7–9 weeks of age. Tissues were taken from animals on other study protocols so as to avoid unnecessary animal use. Animals were housed in pairs and maintained in a room with constant humidity and a 12 hour light-dark cycle, and fed *ad libitum*. Animals were sacrificed by rapid decapitation after 8 weeks (at 4 months of age). Following decapitation, both kidneys, or one hind limb gastrocnemius muscle, were quickly removed and placed in ice-cold isolation buffer. All subsequent steps of homogenization and centrifugation were carried out at 4°C.

Since we can only work up at most two samples/day (given animal availability), all studies were done on different days in samples from different animals, unless noted (see Table 1).

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), except where indicated otherwise.

Isolation of mitochondria from kidney tissue

The procedure was based on the protocol for isolation of liver mitochondria used in our group [11]. To cool the tissue rapidly and preserve mitochondrial function, adult rat kidneys (~2 g total) were rapidly dissected and placed into semi-frozen slush of N1 buffer, composed of 250 mM sucrose, 1 mM EGTA, 10 mM HEPES, (final pH adjusted to 7.4 with KOH), supplemented with 0.5% BSA (essentially fatty acid-free). In some experiments (specifically those denoted as “*original protocol*” in Table 3), the kidneys were thoroughly minced with scissors in N1 buffer to wash away blood, the capsule and medulla were removed and the minced tissue was split evenly into three FT-500 PULSE Tubes (Pressure BioSciences, Inc., South Easton, MA). In the *simplified protocol*, each kidney was cut into ~6 pieces in N1 buffer and the capsule and medulla were removed. The intact tissue pieces were placed directly into 3 FT-500 PULSE Tubes (~0.6–0.7 g tissue per tube). For all samples, the PULSE Tube rams were gently inserted and pushed up until the tissue was just compacted between the ram and the lysis disk, forcing extra blood and buffer out through the lysis disk holes. This liquid was withdrawn and discarded to reduce the amount of blood carry-over into the sample. The ram was then pushed up further, forcing the tissue through the holes of the lysis disk. Fresh N1 buffer, 1 ml per PULSE Tube, was added to each sample bringing the volume up to 1.4 ml. In some experiments reported only in the supplemental material, N1 was supplemented with 5% dextran in the pressure cycling step, because preliminary experiments had suggested that the quality of mitochondria extracted from rat liver by pressure cycling could be improved by the addition of 5% dextran. However, initial results indicated that kidney mitochondria pressure cycled in the presence of 5% dextran showed only a borderline improvement in RCR and ADP/O ratio compared to those extracted at the same pressure without dextran (see supplemental material). Therefore, dextran was omitted from all subsequent kidney mitochondria preparations. In Table 2, equivalent data generated with and without dextran were pooled.

Pressure cycling was performed at 4°C for 5, 10 or 20 cycles in an NEP 2320 Barocycler (Pressure BioSciences, Inc.). Each cycle consisted of 20 seconds at high pressure (10,000 or 20,000 psi) and 5 seconds at atmospheric pressure.

After pressure cycling, the three tissue homogenates were pooled and transferred to round bottom, 32.5 ml centrifuge tubes (e.g., Beckman 355642). PULSE Tubes were rinsed with 1

ml each of fresh N1, the rinses were combined with the homogenate adjusted to approximately 20 mL and centrifuged in a fixed angle rotor at $1000 \times g$ for 8 minutes to pellet intact cells, nuclei and large debris. The supernatant was transferred to a clean round bottom tube and centrifuged at $14,000 \times g$ for 8 minutes at 4°C to pellet the mitochondria-enriched fraction. This pellet was resuspended in a 0.5 ml aliquot of fresh N1 and gently homogenized using 5–10 strokes in a 1 or 2 ml glass homogenizer with a Teflon pestle. The round bottom centrifuge tube and homogenizer were rinsed with a second 1 ml aliquot of fresh N1 and the two aliquots were combined. The pooled mitochondrial suspension (~1.5 ml total) was transferred to a 1.5 ml microfuge tube. Mitochondria were centrifuged at $14,000 \times g$ and the supernatant was discarded. In order to wash away the BSA from the N1 buffer, the pellet was resuspended in 0.5 ml N2 (250 mM sucrose, 10 mM HEPES, pH 7.4) using the plastic pestle provided with the microfuge tube. In some experiments, (specifically those denoted as “original protocol” in Table 3) the suspension was transferred to a 1 or 2 ml glass homogenizer, gently homogenized using 5–10 strokes and then transferred to a new tube. In the simplified protocol, this homogenization step was omitted. The first tube and the homogenizer were rinsed with a second 1 ml aliquot of fresh N2 and the rinse was added to the mitochondrial suspension. The sample was then centrifuged again at $14,000 \times g$ and the supernatant was discarded. To this final pellet, 30 μl of N2 was added and the pellet was gently and thoroughly homogenized with the plastic pestle. The final volume of the kidney mitochondria-enriched fraction was ~100–250 μl .

For preparation of kidney mitochondria by mechanical disruption with The PCT Shredder (Pressure BioSciences, Inc.), the metal shredder base was pre-chilled by placing it into a -20°C freezer 10–30 minutes prior to the start of tissue collection. Each kidney was cut into ~6 pieces in N1 buffer and the capsule and medulla were removed. The intact tissue pieces were placed directly into 3 FT 500-S Shredder PULSE Tubes (Figure 1) (0.6–0.7 g tissue per tube) and the rams were gently inserted and pushed up until the tissue was just compacted between the ram and the lysis disk. Extra blood and buffer was withdrawn as described above. N1 buffer (0.5 ml per tube) was added and the samples were shredded for 10 seconds with the pre-cooled PCT Shredder. After shredding, the homogenate was transferred to a Beckman round bottom centrifuge tube. The Shredder tubes were rinsed with additional N1, the rinses were combined with the homogenate and centrifuged at $1000 \times g$ for 8 minutes at 4°C . All subsequent steps were performed as described above for the simplified kidney mitochondria isolation protocol.

For preparation of control kidney mitochondria, minced kidney tissue was homogenized in 3 ml of N1 buffer in a glass homogenizer with a Teflon pestle using 8–10 strokes. The homogenate was transferred to a round bottom centrifuge tube. The homogenizer was rinsed with 3 ml of fresh N1, the rinse was combined with the homogenate and centrifuged at $1000 \times g$ for 8 minutes at 4°C . All subsequent steps were performed as described above.

Isolation of mitochondria from skeletal muscle

Methodology for extraction of mitochondria from skeletal muscle was based loosely on the simplified protocol that was established for kidney mitochondria. The gastrocnemius muscle was rapidly excised and placed into semi-frozen slush of Muscle Mitochondria Isolation Buffer (MMIB; 120 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 20 mM HEPES, pH adjusted to 7.4 with KOH, as described in [12]) in order to rapidly cool the tissue. All subsequent steps were performed at 4°C . The tissue was weighed (1.5–1.8 g each), roughly minced with scissors in MMIB and large pieces of tendon were removed. The tissue was further minced while incubating for 5 minutes in 0.25 mg/ml Nagarse (bacterial proteinase type XXIV) diluted in MMIB. After incubation, the Nagarse solution was gently poured off and the tissue was washed twice in ice-cold MMIB.

For isolation of muscle mitochondria by PCT Shredder with pressure cycling, the minced tissue was split into 8 FT 500-S Shredder PULSE Tubes. Each tissue aliquot was shredded for 10 seconds with a prechilled PCT Shredder in 0.5 ml MMIB. After shredding, the volume in each tube was brought up to 1.4 ml with additional MMIB and the samples were subjected to pressure cycling at 4°C. Pressure cycling was performed at 10,000, 20,000 or 30,000 psi for 5 or 10 cycles. Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

After pressure cycling, the tissue homogenates were pooled and transferred to round bottom centrifuge tubes. Each PULSE Tube was rinsed with 1 ml of fresh MMIB, the rinses were combined with the homogenate and centrifuged at $1000 \times g$ for 8 minutes at 4°C in a fixed angle rotor to pellet intact cells, nuclei and large debris. The supernatant, except for the small amount of milky suspension floating just above the pellet, was transferred to a fresh round bottom tube and centrifuged at $14,000 \times g$ for 8 minutes at 4°C to pellet the mitochondria-enriched fraction. In some experiments, the speed was reduced to 7,500 or $10,000 \times g$ as noted in the Results section (see Tables 6 and 7).

After centrifugation, the mitochondrial pellet consisted of two layers; a darker bottom layer that contained the intact mitochondria, and a pale top layer. To separate the layers, the bottom of the tube was gently tapped on the bench several times, causing the top layer to slide down the side of the tube while the lower layer remained attached. The pale material was then gently aspirated and discarded (note that in several control experiments this pale material was saved and subjected to further analyses to confirm that it did not contain significant amounts of intact mitochondria). The remaining dark pellet was resuspended in 0.5 ml of fresh MMIB and gently homogenized using 5–10 strokes in a 1 or 2 ml glass homogenizer with a Teflon pestle. The round bottom centrifuge tube and homogenizer were rinsed with a second 1 ml aliquot of fresh MMIB and the two aliquots were combined. The pooled mitochondrial suspension (~1.5 ml total) was transferred to a 1.5 ml microcentrifuge tube with matching pestle (G-biosciences, Maryland Heights, MO). Mitochondria were centrifuged at $14,000 \times g$ and the supernatant was discarded. The pellet was resuspended with 30 μ l fresh MMIB and carefully homogenized with the plastic pestle to form a uniform suspension. The final volume of the mitochondria-enriched fraction was ~70–120 μ L.

For preparation of muscle mitochondria by The PCT Shredder alone, samples were processed in the same way as above, but were not subjected to pressure cycling.

For preparation of control muscle mitochondria, muscle tissue was harvested, weighed, minced and incubated in Nagarse as described above. Minced muscle tissue was manually homogenized in ~10 ml ice-cold MMIB in a glass homogenizer with a Teflon pestle. To minimize damage to the mitochondria, the homogenate was poured off after ~5 strokes. Fresh MMIB (~5ml) was added to the remaining intact tissue which was then further homogenized. The homogenates were combined in a round bottom centrifuge tube, and the homogenizer was rinsed with 5 ml of fresh MMIB. The rinse was combined with the homogenate and centrifuged at $1000 \times g$ for 8 minutes at 4°C. All subsequent steps were performed as described above.

Electron Microscopy

For electron microscopy 10 μ l aliquots of the final mitochondria suspension were fixed in 100 μ l of glutaraldehyde fixative. Fixed mitochondria were processed for electron microscopy at the Harvard Medical School EM facility.

Mitochondria Respiration Assays

Mitochondrial respiration was measured using an Oroboros Oxygraph-2k system which allowed simultaneous measurement of two different mitochondrial samples in adjacent thermostatic chambers. This system, equipped with two Clark-type electrodes, is suitable for precise measurement of respiration in isolated mitochondria.

Isolated kidney mitochondria were incubated in respiration buffer composed of 130 mM sucrose, 5 mM HEPES, 5 mM KH_2PO_4 , 50 μM EGTA, 5 mM MgCl_2 , pH 7.2 [13]. Respiration assays consisted of adding either respiratory complex I substrate (5 mM glutamate/5 mM malate) or respiratory complex II substrate (5 mM succinate) prior to addition of mitochondria.

Optimization of respiration buffer for muscle mitochondria is described in the Results section. Isolated skeletal muscle mitochondria were incubated in optimized respiration buffer composed of 10 mM KH_2PO_4 , 25 μM EGTA, 125 mM KCl, 10 mM NaCl, 25 mM HEPES, adjusted to pH 7.2 with KOH (based on [14], with multiple modifications, see below). Respiration assays consisted of adding either respiratory complex I substrate (5 mM glutamate/5 mM malate) or respiratory complex II substrate (5 mM succinate/2 μM Rotenone) prior to addition of mitochondria. The respiration buffer was supplemented with 1 mM MgCl_2 for use with respiratory complex II substrate (see results).

Evaluation of the rates of respiration at state 2 (V_2 per Estabrook [15], not per Chance and Williams, [16]), state 3 (V_3), state 4 (V_4) and fully uncoupled respiration (V_u) was done through sequential additions of respiratory substrate, 100 μM ADP and 0.1 μM of FCCP, respectively. ADP/O and respiratory control ratios (RCR) were determined either as the ratio V_3/V_4 (RCR 3/4) or as the ratio V_3/V_2 (RCR 3/2).

Multiparameter kinetic assessment of mitochondrial physiological parameters isolated from skeletal muscle

Simultaneous measurements of mitochondrial swelling, Ca^{2+} fluxes, change in membrane potential ($\Delta\phi_m$), and changes of pyridine nucleotide oxidation (NAD(P)H) were performed at a mitochondria concentration of 100 μg protein/ml using a multichannel fluorescent setup as previously described; [17]. Mitochondria were energized with 5 mM succinate and challenged with a series of bolus additions of Ca^{2+} (15 μM each). Assays were carried out in buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM KH_2PO_4 , and 2.5 μM EDTA adjusted to pH 7.4 with KOH. A decrease in autofluorescence at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 360/440$ nm was used to detect changes in NAD(P)H oxidation. Ca^{2+} fluxes were detected using Ca-Green 5N (Invitrogen, Carlsbad Ca.), a non-permeable fluorescent dye, while tetramethylrhodamine methyl ester (TMRM) (Invitrogen) was used in self-quenching mode to detect $\Delta\phi_m$. Mitochondrial swelling was detected as a decrease in light scattering at excitation and emission wavelengths of 587 nm measured at an angle of 90° . Calcium retention capacity (CRC) was determined as the amount of exogenously added Ca^{2+} required to induce mitochondrial swelling and decline of other functions, e.g., mitochondrial depolarization, Ca^{2+} release and oxidation of pyridine nucleotides. Under these conditions, mitochondrial swelling was essentially complete, based on subsequent alamethicin exposure which increased swelling by <15% (data not shown). The mPT involvement in Ca^{2+} -induced mitochondrial swelling was assessed in the presence of cyclosporin A.

Two dimensional gel electrophoresis

Recovery of total mitochondrial protein was measured by BCA assay.

For muscle mitochondria 2D gels, desalting prior to isoelectric focusing (IEF) was required due to the high salt concentration present in MMIB. Desalting was performed by diluting 30 μ L of each sample in 500 μ L ProteoSolve-IEF (Pressure BioSciences, Inc.) supplemented with 50 mM DTT, and loading onto Amicon Ultra centrifugal filters with 3 kDa molecular weight cutoff (EMD-Millipore, Billerica, MA). Filters were centrifuged for 30 minutes at $14,000 \times g$. Three more 500 μ L volumes of ProteoSolve-IEF/50 mM DTT were passed through the column under the same conditions. After desalting, protein concentration was measured by Bradford assay (Bio-Rad Laboratories, Inc. Hercules, CA). Kidney mitochondria suspension in N2 buffer was diluted directly in ProteoSolve-IEF with 50 mM DTT without additional cleanup.

For all 2D gels, 200 μ g of mitochondrial suspension was loaded per gel. First dimension separation was carried out on the computer-controlled IsoelectriQ2 IEF apparatus (Proteome Systems, Ltd. Sydney, AU) using 11cm, pH 3–10 ReadyStrip IPG strips (Bio-Rad Laboratories, Inc. Hercules, CA). Separation was programmed with current limited to 50 μ A per strip in two steps: twelve hours on a concave voltage ramp set to start at 100V and end at 1000V, and 8 hours at 1000V. The strips were removed when 90kVh was reached. Second dimension was performed on Criterion Dodeca system (Bio-Rad Laboratories), equipped with the Thermo-EC 570–90 power supply at constant current of 60mA/gel for 2h. Gels were fixed and stained with colloidal Coomassie blue [18] using Bloo Moose™ stain kit (KeraFAST LLC. Winston-Salem, NC).

Western blotting

Antibodies

Anti-actin mouse monoclonal antibody (Chemicon mAb 1501) was purchased from EMD-Millipore (Billerica, MA). Polyclonal rabbit Anti-VDAC/porin (ab15895), anti-HSP-60 (ab53109), anti-prohibitin (ab28172), and anti-calnexin (ab3516) were all purchased from Abcam (Cambridge, MA).

For SDS-PAGE and Western blotting, 10 μ g of mitochondria-enriched preparation or whole tissue lysate was diluted in 1x SDS-PAGE sample buffer with 50 mM DTT and loaded onto 10% or 8–16% tris-glycine gels (Bio-Rad). For Western blotting, proteins were transferred to Immobilon-P membrane (EMD-Millipore) and blocked with 5% non-fat milk in tris-buffered saline with 0.05% Tween-20. Primary antibodies were diluted in blocking solution. Blots were incubated with 1° antibody either for 4 hours at room temperature or overnight at 4°C. Detection was carried out using HRP-coupled secondary antibodies and enhanced chemiluminescence (ECL Advance kit from GE, Piscataway, NJ).

Results

Effect of pressure cycling on muscle and kidney mitochondria

Prior to optimizing PCT Shredder and Barocycler-based protocols for isolation of mitochondria, we tested whether exposure of muscle mitochondria to high hydrostatic pressure cycling was detrimental to mitochondrial function. Muscle mitochondria were prepared using the standard Potter-Elvehjem homogenizer method. The mitochondrial pellet was resuspended in 1.5 ml of the isolation buffer and split into 2 aliquots. One aliquot was transferred to an FT 500-ND diskless pulse tube and subjected to pressure cycling at 10,000 psi for 5 cycles. The control aliquot was kept on ice. After pressure treatment, both aliquots were centrifuged and the mitochondrial pellets were resuspended in 30 μ L of MMIB buffer. The results (Table 1) from matched controls (n=2), indicate that exposure to pressure cycling at 10,000 psi is compatible with normal respiration kinetics as measured by

respiratory control ratio (RCR) and ADP/O ratio of isolated mitochondria. While the N is small, we note that this result is consistently observed, as will be seen below.

The effect of pressure cycling on kidney mitochondria respiration was also assayed. Mitochondria were extracted from fresh rat kidney tissue using either the standard manual homogenizer method, or PCT Shredder alone, or by pressure cycling alone. The respiratory control ratios (measured using complex I substrate) clearly show that pressure cycling at 10,000 psi has no measurable effect on kidney mitochondria respiration, compared to the control samples or the samples extracted using the PCT Shredder alone (Table 2). Kidney mitochondria extracted using 20,000 psi demonstrate only a slight reduction in both RCR 3/2 and RCR 3/4, suggesting that this pressure may also be compatible with isolation of intact mitochondria.

Simplification of tissue pre-processing for isolation of mitochondria

The initial step in mitochondrial isolation, rinsing to remove excess blood and mincing to facilitate homogenization, is inherently problematic since it occurs at a time when the mitochondria are at their most vulnerable due to temperature and surrounding milieu, e.g., the presence of tissue-derived proteases and divalent cations. Initially, we considered whether this step, common to essentially all mitochondrial isolation protocols, would also be required for clean/high quality mitochondria preparations by our methods. Since tissue processing in the PULSE Tube includes a mechanical disruption step when the tissue is forced through the holes of the lysis disk (either with or without the PCT Shredder, depending on the tissue type), we tested whether this quick and easy step could be substituted for the manual mincing. We used kidney tissue for these tests because it is much less fibrous than muscle, and can be forced through the holes in the PULSE Tube lysis disk, effectively mincing the tissue with one quick push of the ram. This enabled us to investigate this issue, PULSE Tube-facilitated mincing, without complications associated with the protease treatment typically used for skeletal muscle (mechanical homogenization of muscle tissue releases primarily subsarcolemmal mitochondria -- treatment with the protease Nagarse is required for efficient extraction of the intermyofibrillar mitochondria fraction [10,19]. Therefore, to extract both subpopulations of muscle mitochondria, muscle samples were always minced in the enzyme prior to further homogenization). We also note that kidney, in contrast to skeletal muscle, has a significant amount of blood in the organ, enabling us to use kidney to examine whether this PULSE Tube-facilitated mincing is compatible with effective removal of blood from the sample.

In addition to extensive tissue mincing, our original kidney protocol for mitochondria extraction included two wash steps at which the mitochondria-enriched fraction was resuspended and washed in a glass homogenizer with a Teflon pestle. Since additional steps increase processing time and sample loss due to multiple transfers, the second of the two homogenizer washes was omitted in the simplified kidney protocol.

The respiratory control ratios of the kidney mitochondria prepared by the original protocol were compared to those obtained by the simplified protocol (Table 3). The results indicate that the simplified protocol yields mitochondria that are very similar to the ones obtained with the more involved original protocol and support the use of the PULSE Tube lysis disk, in place of manual mincing, for tissue disruption of relatively soft tissues such as kidney.

In addition, since the respiration of stable mitochondria preparations should change little over the course of several hours, while unstable damaged mitochondria should exhibit a significant drop in function, the kidney mitochondria prepared by pressure cycling at 10,000 psi using either the original or the simplified protocol, were assayed for stability. Initial respiration readings were performed within ~2 hours of mitochondria isolation, and second

readings were performed after the preps had been stored on ice for an additional 3–4 hours. The results (see supplemental material) indicate that kidney mitochondria isolated by pressure cycling at 10,000 psi using either the original or the simplified protocols are equally stable over the time course studied.

Respiration buffer formulation for skeletal muscle mitochondria respiration assay

Having confirmed that pressure cycling does not disrupt the function or stability of kidney mitochondria, we then proceeded to extend this methodology and apply it to extraction of functional mitochondria from skeletal muscle. Since many different formulations of respiration buffer for rat muscle mitochondria have been reported in the literature [20–22] we first wanted to determine what respiration buffer formulation gave us the best RCR values for skeletal muscle mitochondria, before proceeding to optimization of the extraction methodology. This was done so as to best highlight mitochondrial functional status. While it was impractical to exhaustively test and compare all the different buffer formulations, we began testing certain components of the respiration buffer using the formulation described by Skalska et al. [22] as the starting point (1 mM KH_2PO_4 , 50 μM EDTA, 1 mM MgCl_2 , 125 mM KCl, 10 mM NaCl, 25 mM HEPES, pH 7.2). A pilot study indicated that the presence of MgCl_2 , while necessary for respiration with complex II substrate (succinate), was detrimental to respiration with complex I substrate (glutamate+malate). This pilot involved splitting a single sample of purified muscle mitochondria into two aliquots and assaying one half in buffer containing 1 mM MgCl_2 , and the other half in the same buffer without MgCl_2 . Higher RCRs, especially in RCR 3/4 (>6 vs <4), were obtained when MgCl_2 was omitted from the respiration buffer. At the same time, the efficiency of both mitochondrial samples as judged by ADP/O ratio was comparable, indicating that in both cases mitochondria were similarly intact. This was not investigated further, and we continued to omit MgCl_2 from complex I respiration experiments, as have others [21,23,24]. In addition, muscle mitochondria were assayed with glutamate+malate in respiration buffer containing 1 mM or 10 mM KH_2PO_4 . Increasing the KH_2PO_4 concentration from 1 mM to 10 mM significantly increased both RCR 3/2 and RCR 3/4, as shown in Table 4. Therefore, for subsequent muscle mitochondria assays, 10 mM KH_2PO_4 was included in the respiration buffer.

The effect of chelating agent choice and concentration on muscle mitochondria respiration was also tested. While the effect of chelator did not have as pronounced an effect as KH_2PO_4 and MgCl_2 concentration, it was determined that switching from 50 μM EDTA to 25 μM EGTA was slightly beneficial (data not shown). The final muscle mitochondria respiration buffer formulation was briefly compared to the initial recipe [14] and was shown to result in improvement of RCR with respiratory complex I substrate (Table 5). Again, this was not systematically or extensively examined, as this was not the focus of the current study.

Effect of centrifugation speed on yield and quality of skeletal muscle mitochondria

To further examine the experimental parameters that yield the highest quality mitochondria preparations, we next investigated whether altering the centrifugation speed used to sediment the mitochondria could be used to increase either yield or purity. Accordingly, skeletal muscle mitochondria were initially prepared using three spin speeds for the first high speed spin. The data in Table 6 suggest that the lower yield in PCT-isolated mitochondria is not generally associated with more debris/fragmented mitochondria (which would have been predicted to be disproportionately isolated with the $14,000 \times g$ spin). Since there was no clear gain in protein recovery at higher g-force (Table 6), respiration was next compared using samples prepared by PCT at 10,000 psi and centrifuged at $7,500 \times g$ or $14,000 \times g$ to determine whether the slower spin resulted in better mitochondria preparations

as a result of fewer contaminants (Table 7). The RCR values (RCR 3/2 and RCR 3/4) indicated that muscle mitochondria pelleted at $14,000 \times g$ were as good as, or slightly better than, those prepared using $7,500 \times g$. Thus, Table 7 shows that the subpopulations of mitochondrial isolated are proportionally equivalent in the two methods. Therefore, for all subsequent experiments, samples were centrifuged at $14,000 \times g$.

Effect of PCT Shredder tissue disruption and pressure cycling-enhanced extraction on the yield and quality of skeletal muscle mitochondria

To assess the effect of pressure cycling on the respiration of skeletal muscle mitochondria, muscle tissue was disrupted for 10 seconds in The PCT Shredder. The crude homogenate was either used directly for mitochondria isolation by differential centrifugation (Shredder alone), or was subjected to further extraction by pressure cycling for five cycles at 10,000, 20,000 or 30,000 psi to release additional mitochondria from cells. Mitochondrial yield and respiration were then assayed and compared to control samples extracted using a standard glass homogenizer with a Teflon pestle (Figure 2). Protein assay of the mitochondria-enriched pellet demonstrated that disruption with the PCT Shredder followed by pressure cycling showed a tendency to release more mitochondria than PCT Shredder alone, and that this yield approached that obtained using the more traditional manual homogenizer method (Figure 2C). The results also suggested that little additional yield is recovered at higher pressures. Similar experiments using kidney mitochondria also indicated that, at 10,000 psi, increasing the PCT parameters from five to ten or twenty cycles did not significantly improve yield (see supplemental material).

Respiration assays of muscle mitochondria extracted with and without pressure cycling confirm that mitochondria exposed to hydrostatic pressure of 10,000 psi exhibit normal respiration kinetics with both complex I and complex II substrates (Figure 2A and 2B). As expected, pressure cycling at 30,000 psi results in damaged mitochondria, as indicated by low RCR values (Figure 2A). It is likely that such high pressure damages the mitochondria both by protein denaturation and by disruption, possibly fragmentation, of the mitochondria membrane. However, mitochondria exposed to 20,000 psi appear almost as healthy as controls, indicating that the range of pressures compatible with viable mitochondria is quite wide (Figure 2A).

Mitochondria isolated using either the conventional homogenizer method, or the PCT Shredder with pressure cycling at 10,000 psi, were compared to determine whether pressure cycling affects the ability of muscle mitochondria to respond to Ca^{2+} overload. Skeletal muscle mitochondria prepared by the two methods appear comparable in their membrane potential (Figure 3, panel A), ability to retain exogenously added calcium (as assessed by CRC) (Figure 3, panel B and Table 8), redox level of pyridine nucleotides (Figure 3, panel C) and Ca^{2+} -induced swelling rate/amplitude (Figure 3, panel D). The mitochondria were energized with succinate (+/- rotenone). The involvement of mitochondrial permeability transition (mPT) in Ca^{2+} -induced mitochondrial dysfunction was assessed in the presence of cyclosporine A (CsA). As shown in Table 8, the presence of rotenone in the incubation buffer significantly (more than twice) increased CRC in skeletal muscle mitochondria. The observed increase in the Ca^{2+} retention capacity in the presence of succinate+rotenone is a feature of isolated skeletal muscle mitochondria that has been observed previously [25]. Altogether, the data suggest that skeletal muscle mitochondria isolated by the PCT Shredder/pressure cycling technique are functionally active, respond to and sequester a Ca^{2+} load, and eventually undergo a calcium-overload associated with calcium release. MPT involvement seems relatively minimal (due to slight effect of CsA, but this is peripheral to our interest in this report and was not investigated in detail).

Morphology and purity of mitochondria extracted using the PCT Shredder and pressure cycling

Morphology of muscle mitochondria prepared using the PCT Shredder with or without pressure cycling and of kidney mitochondria prepared by pressure cycling, was next assessed by transmission electron microscopy. The micrographs (Figure 4) confirm that the mitochondria extracted from skeletal muscle by the control homogenizer method are comparable to those extracted using the PCT Shredder alone, or with subsequent pressure cycling at 10,000 psi. The mitochondria in all groups appear generally intact and exhibit a range of morphologies qualitatively similar to control samples. Furthermore, the micrographs show that the purity of the PCT Shredder and Shredder/pressure cycling muscle mitochondria samples is comparable to controls and that all three methods result in samples that contain a small proportion of non-mitochondria organelles and fragments (Figure 4A–F). Micrographs of kidney mitochondria prepared by pressure cycling at 10,000 psi also indicate high purity and intact morphology of the mitochondria prepared by this method (Figure 4G,H).

Purity of the skeletal muscle and kidney mitochondria preparations was next assessed using proteomic approaches. Total protein profiles of mitochondria samples prepared by the Shredder/pressure cycling method were compared to control samples prepared by manual homogenizer, using 2D SDS-PAGE (Figure 5). Muscle mitochondria prepared by the two methods were essentially indistinguishable. Spot densitometry image analysis carried out on the kidney mitochondria 2D gels using REDFIN 3 software (Ludesi AB, Lund, Sweden) also confirmed that there was no significant difference in protein pattern between samples isolated by the different methods.

Western blotting confirmed that mitochondrial preparations from both skeletal muscle and kidney tissues were enriched in markers for all three mitochondrial compartments (Figure 6). Voltage dependent anion channel (VDAC/porin), an outer mitochondrial membrane marker; prohibitin, a marker for the inner mitochondrial membrane; and HSP-60, a soluble protein localized to the mitochondrial inner matrix space, are all present in the mitochondrial preparations. The presence of all three proteins in the mitochondria-enriched samples agrees with the functional studies and indicates that the mitochondria in the preparation are intact. SDS-PAGE gels demonstrate that the protein profiles of control mitochondria samples isolated by homogenizer are essentially the same as the test samples prepared using the PCT Shredder with pressure cycling for muscle and pressure cycling alone for kidney tissue. This protein pattern in the mitochondria enriched preparations is very different from that seen with whole tissue lysate which is not enriched in mitochondria. In addition, western blotting confirms that muscle whole tissue lysate has very low levels of mitochondrial markers, but does contain markers of other organelles (see supplemental material).

Discussion

Extraction of high-quality functional mitochondria from various tissues is required for studies that focus on mitochondrial function and dysfunction, as well as the role of mitochondria as both targets for drug development and as off-target mediators of drug side effects [26]. New robust and reproducible methods for the isolation of intact and functional mitochondria from a wide array of tissues can contribute to areas of research such as cytotoxicity assays, discovery of potential mitochondria-targeted therapeutic agents, the elucidation of signaling pathways, and in some cases, the diagnosis of certain disorders, as well as mitochondrial proteomics, lipidomics and metabolomics. The study of mitochondrial phospholipids is also an emerging field that is likely to lead to greater understanding of mitochondrial function in different tissues [27]. Studies of tissue mitochondria have demonstrated that the mitochondrial proteome is a dynamic system regulated by both the

nucleus and the mitochondrion itself. For this reason, the mitochondrial proteomes of different tissues, and even of the same tissue in different disease states, may vary significantly [28], highlighting the need for convenient mitochondria isolation methods that can be applied to various tissues.

Mitochondrial studies that originate from different laboratories can show disparate, and sometimes contradictory, findings. These inconsistent results are likely due, at least in part, to differences in the quality of mitochondria as a result of different isolation procedures ([29]) or the experience of the operators. Here we present simplified methods for extraction of muscle tissue mitochondria. Several parameters of the new method were also tested in kidney mitochondria to demonstrate that the methods are likely to be generally applicable to mitochondria from other tissues. The key steps when isolating mitochondria from any tissue are usually the same: (i) Tissue disruption using either mechanical methods alone, or a combination of chemical and mechanical methods and (ii) subsequent differential centrifugation to remove debris and large cellular organelles in order to enrich for mitochondria. Despite the importance of the tissue disruption step, there are few techniques available to circumvent the many known drawbacks of homogenizer-based mechanical tissue disruption for mitochondrial isolation. Therefore, current methods require extensive operator involvement in the tissue mincing and homogenization steps. The homogenization process is especially labor intensive when the tissue is fibrous like muscle. The PCT Shredder PULSE Tube simplifies the tissue disruption process. Once the shredded tissue homogenate is forced through the holes of the lysis disk, further grinding is unnecessary. In this way, the PCT Shredder provides both more rapid homogenization than is possible with the Dounce or Potter-Elvehjem homogenizers, and prevents over-homogenization, such as can easily occur with rotor/stator-type homogenizers, which can lead to rapid loss of mitochondrial integrity.

Regardless of how tissue homogenization is performed, it is, at its core a stochastic process and does not guarantee even distribution of mechanical force to the entire sample. In contrast, sample disruption by pressure cycling is a non-stochastic process in which pressure is propagated uniformly throughout the sample and does not depend on any statistical probability of a given cell coming in contact with any other cell or homogenizing surface. Identical, non-shearing disruptive physical forces are exerted upon every cell in the sample, resulting in well-controlled and reproducible cell lysis. This method has been shown to be relatively gentle and has already been used to isolate mitochondria from cell culture ([30]). Furthermore, pressure cycling in the Barocycler is likely to be more robust than conventional extraction techniques in inexperienced hands because the process is automated and programmable, relying less on operator experience and giving the user fine-tunable control of cell disruption for extraction of intact and functional mitochondria with less expected person-to-person and prep-to-prep variability.

Our results demonstrate that tissue disruption with the PCT Shredder, either alone or in conjunction with pressure cycling, can be used to extract intact and functional mitochondria from tissues such as rat skeletal muscle and kidney. The potential to add pressure cycling to isolation techniques may be useful for sample cleanup and preparation for biochemical analysis. Pressure cycling at 10,000 psi for five cycles at 4°C, does not result in any measurable muscle or kidney mitochondria dysfunction, as assessed by respiration assays. Pressure cycling at 20,000 psi can lead to slight reduction in RCR values in kidney mitochondria (Table 2) but appears to have almost no negative impact on muscle mitochondria (Figure 2), indicating that these tissue mitochondria can withstand pressures in the 10,000 to 20,000 psi range. Although it was found that pressure cycling at 10,000 psi was sufficient to extract skeletal muscle mitochondria, it is likely that extraction from different tissues may require different pressure cycling parameters. The observation that five

cycles at 20,000 psi, still yields functional mitochondria, suggests that this method can be used to extract high quality, intact mitochondria even from samples that may require harsher conditions for cell lysis.

Conclusions

The data presented here confirm that mitochondria extracted using pressure cycling, or The PCT Shredder, or a combination of both methods, are intact and functionally active, and can be used for downstream applications that require high quality, enriched functional mitochondria preparations. These mitochondria exhibit respiration and calcium handling properties that are comparable to controls isolated using standard methods performed by skilled operators. Muscle tissue disruption with The PCT Shredder, with or without subsequent pressure cycling, is far less dependent on operator experience than traditional manual homogenizer-based methods. In addition, since pressure cycling in the Barocycler is a programmable and hands-off procedure, it too facilitates reproducible, user-independent mitochondria extraction from tissues. The new methods require little hands-on tissue homogenization, can be easily learned by a novice, and are expected to lead to results that are more consistent and reproducible from lab to lab. Because it is less reliant on operator skill, the technique is also more amenable to scale-up. Furthermore, for applications where highly purified mitochondria are required, the mitochondria-enriched samples generated as described above can be subjected to density gradient centrifugation, or other downstream purification techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FT 500-S Shredder PULSE Tube for use with The PCT Shredder or The Shredder SG3 from Pressure BioSciences

Tissue disruption in the Shredder tube requires approximately ten seconds per sample. Once the homogenate is forced through the holes of the lysis disk, further grinding is unnecessary.

Figure 2. Effect of PCT Shredder and pressure cycling on skeletal muscle mitochondrial yield and respiratory parameters

Mitochondria were extracted using the PCT Shredder with or without pressure cycling at 10,000, 20,000 or 30,000 psi. Mitochondrial yield and respiration were assayed and compared to control samples (homogenizer) **A.** Respiration assays with complex I substrate (Homogenizer, n=5; Shredder alone, n=5; Shredder with pressure cycling at 10,000 psi, n=7; Shredder with pressure cycling at 20,000 psi, n=3; Shredder with pressure cycling at 30,000psi, n=2). **B.** Respiration assays with complex II substrate (Shredder with pressure cycling at 10,000 psi, n=7; homogenizer n=6). **C.** Yield of mitochondria (homogenizer, n=15; Shredder alone, n=19; Shredder with pressure cycling at 10,000psi, n=18; Shredder with pressure cycling at 20,000psi, n=3; Shredder with pressure cycling at 30,000psi, n=2).

Figure 3. Effect of PCT Shredder and pressure cycling on skeletal muscle mitochondria response to Ca^{2+} overload

Skeletal muscle mitochondria isolated by homogenizer (black trace) and by PCT Shredder with pressure cycling at 10,000psi (blue trace) demonstrate similar behavior in response to Ca^{2+} overload. The Ca^{2+} -induced response was monitored by simultaneous four channel recording. Changes in membrane potential were monitored using TMRM. Ca^{2+} -fluxes were monitored with the Ca^{2+} -sensitive dye, CaGreen 5N, which increases its fluorescence yield upon complex formation with free extramitochondrial Ca^{2+} . Oxidation of mitochondrial pyridine nucleotides was recorded as decrease of autofluorescence yield of mitochondrial NADH and NADPH. Mitochondrial swelling was detected as a decrease of light scattering.

Figure 4. Transmission electron micrographs of mitochondria extracted from rat skeletal muscle and kidney

No significant differences in morphology or purity of mitochondria enriched pellet was observed in muscle samples prepared by standard homogenization (A, D), PCT Shredder alone (B, E) or PCT Shredder followed by pressure cycling at 10,000 psi (C, F). A–C: 4000x direct magnification, scale bars = 500nm. D–E: 20,000x direct magnification, scale bars = 100nm. Kidney mitochondria (G, H) prepared by pressure cycling at 10,000 psi also exhibit intact morphology. G: 3000x direct magnification. H: 25,000x direct magnification.

Figure 5. Protein profiling of mitochondria prepared by pressure cycling

Total protein profiles of kidney mitochondria prepared by pressure cycling (10,000 psi) or muscle mitochondria prepared by PCT Shredder with pressure cycling at 10,000 psi were compared to control samples. The proteins were separated by 2D PAGE. Kidney mitochondria gels were compared by spot densitometry. No significant difference in protein expression between samples isolated by the different methods was observed.

Figure 6. SDS-PAGE and Western blotting of mitochondria-enriched fractions from skeletal muscle and kidney

Mitochondrial preparations from skeletal muscle and kidney and are enriched in markers for all three mitochondrial compartments (outer membrane VDAC, inner membrane prohibitin and matrix HSP-60). SDS-PAGE gels demonstrate tissue specific protein patterns of mitochondria prepared from muscle and kidney. The protein profiles of control mitochondrial samples isolated by homogenizer are essentially the same as the test samples prepared using the PCT Shredder with pressure cycling for muscle or pressure cycling alone for kidney tissue.

Table 1

Effect of pressure cycling on muscle mitochondria respiratory parameters

		PCT	Control
RCR 3/2	Sample 1	6.67	6.74
	Sample 2	6.54	6.42
RCR 3/4	Sample 1	4.30	4.28
	Sample 2	4.17	4.28
ADP/O	Sample 1	2.26	2.23
	Sample 2	2.38	2.31

Respiration was measured using complex I substrate (5mM glutamate/malate) in 1 mM KH_2PO_4 , 50 μM EDTA, 1 mM MgCl_2 , 125 mM KCl, 10 mM NaCl, 25 mM HEPES, pH 7.2 [14]. As noted in Methods, “State 2” respiration in this report follows the Estabrook, not Chance definition (i.e., respiration in the presence of excess substrate but no ADP prior to the addition of ADP-initiated State 3 respiration).

Table 2

Effect of pressure cycling or The PCT Shredder on kidney mitochondria respiratory parameters. Data presented as mean $\pm$ SD.

	PCT Shredder (n=5)	10,000 psi (n=15)	20,000 psi (n=7)	Homogenizer control (n=6)
RCR 3/2 ($\pm$ SD)	6.3 $\pm$ 0.54	6.3 $\pm$ 0.54	5.8 $\pm$ 0.27	6.4 $\pm$ 0.39
RCR 3/4 ($\pm$ SD)	3.7 $\pm$ 0.23	3.7 $\pm$ 0.46	3.6 $\pm$ 0.30	3.8 $\pm$ 0.28
ADP/O ($\pm$ SD)	2.6 $\pm$ 0.18	2.8 $\pm$ 0.27	2.8 $\pm$ 0.32	2.6 $\pm$ 0.03

Table 3

Respiratory control ratios of kidney mitochondria prepared using either the longer original protocol, or the shorter simplified method. Respiration was measured using complex 1 substrate. Data are presented as mean $\pm$ SD.

Method	RCR 3/2 $\pm$ SD	RCR 3/4 $\pm$ SD
Original (n=4)	6.6 $\pm$ 0.15	4.1 $\pm$ 0.62
Simplified (n=8)	6.5 $\pm$ 0.55	3.8 $\pm$ 0.55

Table 4

Effect of KH_2PO_4 concentration on muscle mitochondria respiratory parameters in the presence of glutamate-malate. Data are presented as mean $\pm$ SD.

	1 mM PO_4 (n=3)	10 mM PO_4 (n=3)
RCR 3/2 $\pm$ SD	7.60 $\pm$ 1.76	11.04 $\pm$ 1.03
RCR 3/4 $\pm$ SD	4.38 $\pm$ 0.17	7.35 $\pm$ 0.44
ADP/O $\pm$ SD	2.25 $\pm$ 0.05	2.52 $\pm$ 0.25

Table 5

Optimized respiration buffer for rat skeletal muscle mitochondria. Data presented as mean $\pm$ SD.

	25mM HEPES, 1mM KH ₂ PO ₄ , 50 μ M EDTA, 1mM MgCl ₂ , 125mM KCl, 10mM NaCl, pH 7.2 (n=3)	25mM HEPES, 10mM KH ₂ PO ₄ , 25 μ M EGTA, 0mM MgCl ₂ , 125mM KCl, 10mM NaCl, pH 7.2 (n=2)
RCR 3/2 $\pm$ SD	7.60 $\pm$ 1.76	11.40 $\pm$ 0.05
RCR 3/4 $\pm$ SD	4.38 $\pm$ 0.17	10.05 $\pm$ 0.42
ADP/O $\pm$ SD	2.25 $\pm$ 0.05	2.35 $\pm$ 0.01

Table 6

The influence of g-force on the yield of skeletal muscle mitochondrial protein. Skeletal muscle mitochondria extracted at 10,000 psi for 5 cycles were prepared using $7,500 \times g$, $10,000 \times g$ or $14,000 \times g$ for the first high speed spin. Subsequent spins were all conducted at $14,000 \times g$. Yield is presented as mg of mitochondria enriched pellet recovered per gram starting tissue mass (mean $\pm$ SD).

	7,500 $\times g$ (n=3)	10,000 $\times g$ (n=4)	14,000 $\times g$ (n=18)
Yield	1.7 $\pm$ 0.06	1.7 $\pm$ 0.20	1.8 $\pm$ 0.49

Table 7

The influence of g-force on respiration of skeletal muscle mitochondria. Skeletal muscle mitochondria extracted at 10,000 psi for 5 cycles were prepared using $14,000 \times g$ or $7,500 \times g$ for the first high speed spin. Subsequent spins were all conducted at $14,000 \times g$. Respiration was measured using complex I substrate (mean $\pm$ SD).

	$7,500 \times g$ (n=3)	$14,000 \times g$ (n=7)
RCR 3/2 ($\pm$ SD)	8.6 ± 0.50	10.3 ± 1.0
RCR 3/4 ($\pm$ SD)	8.5 ± 1.20	8.6 ± 0.74
ADP/O ($\pm$ SD)	2.2 ± 0.01	2.4 ± 0.14

Table 8

Calcium retention capacity of mitochondria isolated from skeletal muscle (SM) using homogenizer (Homog) or The PCT Shredder with pressure cycling at 10,000 psi (Shredder). Mitochondria were energized with either succinate alone (5mM), or succinate with 1 μ M rotenone, or succinate with rotenone and 1 μ M cyclosporin A (CsA). Calcium retention capacity (nmol/mg of protein) was measured by calculating total amount of Ca²⁺ used to induce mPT. Values are mean $\pm$ SE for n=4–6. For comparison, values obtained from rat liver mitochondria (RLM) prepared using a conventional homogenizer method are also shown.

Mitochondria	Succinate	Succinate+Rotenone	Succinate+Rotenone+CsA
SM (Homog)	500 $\pm$ 80	1030 $\pm$ 80	1100 $\pm$ 90
SM (Shredder)	460 $\pm$ 90	950 $\pm$ 70	1050 $\pm$ 100
RLM	235	240	800