Intact and Functional Mitochondria from Solid Tissue: Effective Semi-Automated Extraction Using Gentle Mechanical Homogenization and Pressure Cycling Technology

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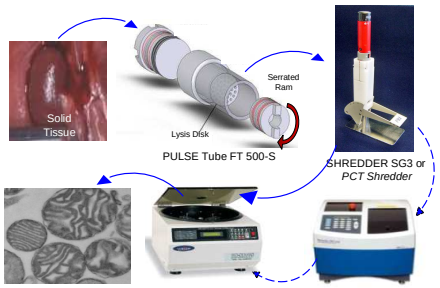
Introduction

Impaired mitochondrial function has been linked to many diseases, such as stroke, heart disease, cancer, Type II diabetes and Parkinson's disease. Due to the generation of reactive oxygen and nitrogen species, mitochondria are a major cause of oxidative stress in aging. In order to gain a better understanding of the contributions and interrelations of mitochondrial dysfunction with various diseases, as well as their role in normal development, experiments often need to focus on purified or enriched preparations of mitochondria rather than on intact tissues or whole organisms. Proteomic and metabolomic profiling of mitochondria from various tissues have the potential to provide important insights into mitochondrial function and dysfunction, and may also address fundamental questions of cellular energetics and oxidative stress. As potential drug targets, high quality functional mitochondrial isolates are also important for drug screening studies [1].

Extraction of mitochondria from whole tissues is typically performed by homogenization using labor-intensive manual disruption methods [2] that require extensive operator experience, and often result in damage to fragile organelles and high sample-to-sample variability. Here we describe a semi-automated method that uses a novel gentle mechanical homogenizer (The PCT Shredder, or The Shredder SG3) followed by hydrostatic pressure cycling to release mitochondria from rat tissues with minimal hands-on time (Figure 1).

Pressure Cycling Technology (PCT) destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel. At pressures in the 10,000 - 20,000 psi range, PCT can be used to gently lyse cells and release intact organelles, such as mitochondria. This method has been shown to be relatively gentle, and it has already been used to isolate mitochondria from cell culture [3].

Figure 1. Mitochondria Isolation Workflow



Methods

Muscle mitochondria-enriched fractions were prepared from fresh rat gastrocnemius muscle using the PBI Mitochondria Isolation Kit: Rat Muscle as described by the manufacturer. Samples were either processed for mitochondria purification directly after shredding ("Shredder alone"), or were subjected to pressure cycling for additional mitochondria extraction ("Shredder with PCT"). PCT was performed at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C. Functional mitochondria were extracted from kidney tissue by pressure cycling at 10,000 psi for 5 cycles, followed by differential centrifugation.

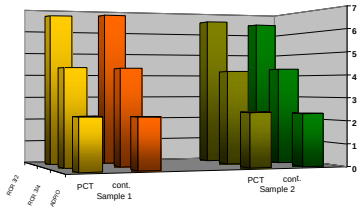
Lung mitochondria-enriched fractions were prepared from frozen/thawed rat lung tissue as described in the PBI Mitochondria Isolation Kit: Rat Lung. The samples were either pressurized at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C, or were not subjected to pressurization ("Shredder alone").

Control samples were homogenized gently by hand using a Potter-Elvehjem homogenizer. All subsequent steps were carried out as described above. In order to generate consistent controls and eliminate user-to-user variability, all control samples produced by manual homogenization were prepared by the same experienced individual.

Mitochondrial respiration was measured using an Oroboros Oxygraph-2k system.

Results

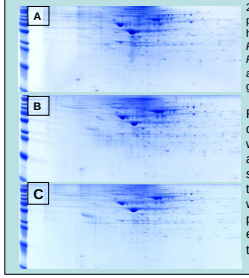
Figure 2. Mitochondria Function Before and After Pressure Cycling



Skeletal muscle mitochondria were isolated using a standard homogenizer protocol. Half of each sample was subjected to pressure cycling at 10,000 psi (PCT) while the control half was kept on ice (cont). No significant difference in RCR or ADP/O ratio was observed.

Results: The data confirm that five pressure cycles at 10,000 psi do not measurably impact mitochondrial respiration, thus supporting the use of PCT in mitochondria isolation protocols.

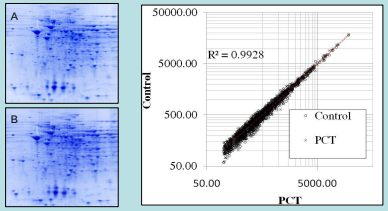
Figure 3. Total Protein Profile of Rat Lung Mitochondria



2D PAGE of Mitochondria prepared using the control homogenizer method (A); *The PCT Shredder* alone (B); or *The PCT Shredder* followed by PCT at 10,000 psi (C) (125 µg per gel).

Result: No significant differences in protein pattern was observed on 2D gels in any of the samples, further supporting the hypothesis that the Shredder, with or without PCT, can be used to prepare a mitochondria-enriched fraction from tissues

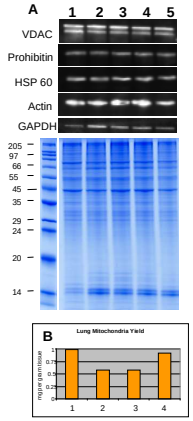
Figure 4. Total Protein Profile of Rat Kidney Mitochondria



Kidney Mitochondria were isolated by homogenizer (A) or by the PCT method at 10,000 psi (B). Protein load per gel is normalized to 200 µg. Matched spot intensities were plotted against each other. The two methods produce nearly identical protein patterns on 2D-PAGE.

Result: As in Figure 3, these data indicate that PCT can be used to isolate a mitochondria-enriched fraction from tissue, that is comparable to what is obtained using a standard manual homogenizer protocol.

Figure 5. Rat Lung Mitochondria Preparations.



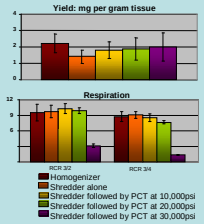
A. Western blots were probed with antibodies to VDAC (outer mitochondria membrane), Prohibitin (inner mitochondria membrane), HSP60 (mitochondrial matrix), actin and GAPDH (cytosolic protein). The presence of GAPDH in all the lung mitochondria preparations is likely due to carryover of cytoplasmic material and could be further reduced by additional washing of the mitochondrial pellet. The presence of actin in the mitochondrial pellet is expected, as it has been reported to be associated with mitochondria [4]. SDS-PAGE protein load was adjusted to 8 µg per lane.

Samples as follows:
1. Homogenizer control
2. Shredder alone
3. Shredder with PCT at 10,000psi for 5 cycles
4. Shredder with PCT at 20,000psi for 5 cycles
5. Shredder with PCT at 20,000psi for 15 cycles

B. Mitochondria were prepared as above. Protein yield was measured by Bradford assay and normalized to mass of starting material.

Result: Western blots of mitochondria-enriched fractions from rat lung tissue confirm that the preparations are enriched for protein markers for all three mitochondrial compartments. Protein assays of the mitochondria-enriched preparations demonstrate that sample processing by PCT after initial Shredder disruption, releases more mitochondria than shredding alone. The yield of lung mitochondria was significantly increased when shredding was followed by PCT.

Figure 6. Yield and Respiration of Skeletal Muscle Mitochondria.



Mitochondria were extracted using a standard homogenizer method or *The PCT Shredder* with or without subsequent pressure cycling at 10,000, 20,000 or 30,000 psi.

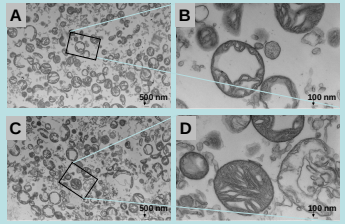
Upper Panel: Protein yield was measured by Bradford assay and normalized to mass of starting material. (Control, n=15; Shredder alone, n=19; 10,000psi, n=18; 20,000psi, n=3; 30,000psi, n=2).

Lower panel: Respiratory control ratios (RCRs) of mitochondria prepared with and without PCT. (Control, n=5; Shredder alone, n=5; 10,000 psi, n=7; 20,000 psi, n=3; 30,000 psi, n=2).

Result: More mitochondria are released by PCT after disruption with *The PCT Shredder* than by *The PCT Shredder* alone, with yields that approach the control method. While a pressure of 20,000 psi resulted in optimal mitochondria yield from frozen/thawed lung tissue (Figure 5B), for isolation of muscle mitochondria, a pressure of 10,000 psi is sufficient to increase yield and little additional yield is recovered at higher pressures.

Respiration assays confirm that muscle mitochondria exposed to hydrostatic pressure of 10,000 psi for five cycles, exhibit normal respiration kinetics. As expected, mitochondria exposed to 30,000 psi exhibit very low RCR values, indicating significant disruption of the organelles. However, the mitochondria exposed to 20,000 psi appear almost as robust as controls, indicating that the range of pressures compatible with isolation of functional mitochondria, is quite wide.

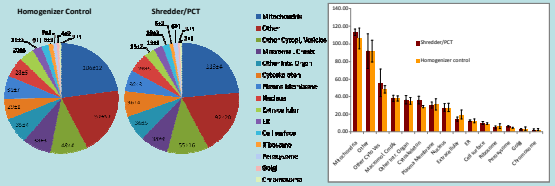
Figure 7. Electron Micrographs of Muscle Mitochondria Preparations



A, B: Muscle mitochondria, homogenizer control. **C, D**: Muscle mitochondria, Shredder with PCT at 10,000 psi, 5 cycles

Result: Electron micrographs demonstrate the comparable morphologies of mitochondria extracted using the control manual method and those isolated using pressure cycling at 10,000 psi, further supporting the conclusion that the new method results in isolation of intact mitochondria.

Figure 8. Mass Spectrometry Analysis of Mitochondria from Rat Skeletal Muscle



Proteomic analysis of mitochondria prepared by conventional homogenizer and Shredder/PCT at 10,000 psi (n=3 individual preps per method, 1-3 technical replicates per prep). Trypsin digests of mitochondria-enriched samples were subjected to nanoLC-MS/MS analysis on an LTQ-Orbitrap. Average of 7 runs for each method (Error bars represent standard deviation). Only proteins with an identification confidence level of ≥90% were included.

Result: Using GO terms protein localization data analysis (STRAP 1.0 [5]), these data confirm that the samples are highly enriched in mitochondrial proteins. In addition, the similarity of protein distribution between samples prepared by both methods supports the conclusion that PCT can be used for effective and reproducible preparation of mitochondria-enriched fractions for proteomic analyses. Further analysis is under way to determine if there are subtle differences in protein composition or quantity between methods.

Conclusions

We demonstrate convenient extraction of intact mitochondria from solid tissues, using two new PBI Mitochondria Isolation kits. Both kits utilize the Shredder for initial tissue disruption, followed by pressure cycling-enhanced cell lysis to release intact mitochondria. Intact and functional mitochondria were also isolated from freshly harvested rat kidneys, using a similar PCT-based protocol.

Traditional manual methods for isolation of intact mitochondria from solid tissues rely heavily on operator experience and skill. Considerable training is required in order to avoid common mistakes, such as tissue over-homogenization, that can result in damaged mitochondria and highly variable results. The new method described here allows for reproducible and convenient isolation of intact mitochondria. During initial tissue shredding, the design of the FT 500-S PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment. This simple design significantly reduces the likelihood of sample over-homogenization. In addition, we demonstrate that overall yield of mitochondria can be increased if initial shredder processing is followed by a brief PCT treatment at 10,000 psi (for muscle mitochondria) or 20,000 psi (for lung mitochondria). Since PCT-based cell lysis is conducted under controlled thermodynamic conditions (time, temperature and pressure), it is expected to yield more reproducible results than manual methods. Our data demonstrate that mitochondria extracted by the PCT sample preparation system are intact, functional, and exhibit an overall protein pattern comparable to controls isolated using a conventional manual method [6].

References

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