Isolation of Functional Mitochondria from Whole Rat Brain Using a PBI Shredder and Pressure Cycling Technology (PCT)

Introduction

Isolation of intact mitochondria from human and animal tissue is crucial for studies that focus on the elucidation of their function and dysfunction in conditions such as aging, diabetes and cancer. As potential drug targets, high quality functional mitochondrial isolates are important for drug screening studies [1]. Mitochondria isolation from solid tissue is usually carried out using labor-intensive homogenizer-based methods [2] that require extensive operator experience. Here we describe a semi-automated method to release mitochondria from solid rat brain tissue, using a PBI Shredder and PCT in place of traditional manual homogenization.

The PBI Shredder

The PBI Shredder comes in two configurations; *The PCT Shredder* and *The* Shredder SG3 are both designed to quickly and gently grind solid tissues such as brain and kidney, or even fibrous samples such as plant leaves and muscle tissue. Since shredding is performed in a disposable sample container (Figure 1), the likelihood of cross contamination with other samples is very low. In addition, shredding for 10-20 seconds generates relatively little heat and thus does not increase protein denaturation or damage delicate organelles.

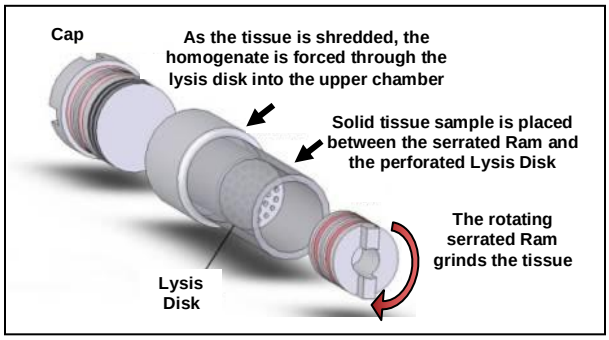

Figure 1. The FT500-S Shredder PULSE Tube. As the tissue is disrupted, the Shredder Ram of the PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment, significantly reducing the risk of sample over-homogenization and mitochondrial damage.

Pressure Cycling Technology (PCT)

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high pressures (up to 45,000 psi [310 MPa, 3000 Atm]). High hydrostatic pressure acts preferentially on the more compressible constituents of the sample, leading to destabilization of molecular interactions, but having little impact on covalent bonds. PCT at lower pressures (e.g. 10,000 - 20,000 psi) can lyse cells and release their intracellular contents, including intact organelles. This method has been shown to be relatively gentle, and it has already been used to isolate intact mitochondria from cell culture [3], as well as from fresh skeletal muscle and kidney tissues [4].

Methods

Mitochondria were prepared from freshly harvested, whole adult rat brain (1 brain per sample, n=4). Tissue was rapidly excised and placed into semi-frozen slush of Brain Mitochondria Isolation Buffer (BMIB) (320mM sucrose, 10mM HEPES (free acid) 0.5mM EGTA, pH adjusted to 7.4 with KOH). To maintain mitochondrial viability, the samples were kept on ice, or at 4˚C, for all subsequent steps.

The tissue was roughly chopped in BMIB to wash away surface blood, split into four FT500-S PULSE Tubes and shredded with 0.5 mL BMIB for 10 seconds. The metal Shredder stand was pre-chilled to help dissipate any heat that might be generated during shredding.

Prior to pressure cycling, the volume of each PULSE Tube was brought up to 1.4 mL with additional BMIB. PCT was performed at 10,000 psi for 5 cycles at 4˚C. Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

Tissue homogenates from the four PULSE Tubes were pooled and centrifuged for 3 minutes at 1300 x g. The supernatant was transferred to a fresh tube and re-centrifuged as above to pellet any residual intact cells, nuclei or other large debris. The supernatant was then centrifuged for 8 minutes at 17,000 x g to pellet the mitochondria-enriched fraction. The pellet was gently suspended in 1.5 mL fresh BMIB using 5-10 strokes in a 1 or 2 mL glass homogenizer with a Teflon pestle, and applied to the top of a Ficoll gradient (Ficoll gradient was generated by layering 5ml of 7.5% Ficoll on top of 5 mL of 10% Ficoll). Gradient centrifugation was performed at 99,000g for 12 minutes in a swinging bucket rotor to separate the brain mitochondria from the myelin fraction and synaptosomal mitochondria. After centrifugation, the bottom pellet was suspended in 1.5 mL BMIB and transferred to an Eppendorf tube. The mitochondria were centrifuged at 14,600g for 8

minutes to wash away Ficoll. After this wash, the mitochondria pellet was suspended in Brain Mitochondria Wash Buffer (BMWB: 320mM sucrose, 10mM HEPES free acid, 20µM EGTA, pH 7.4 with KOH) supplemented with 0.1% BSA and centrifuged at 12,000g for 8 minutes. The final pellet was suspended by gently homogenizing in 20 µL of BMWB (without added BSA) using a small plastic pestle. The final volume of the mitochondria-enriched suspension was ~30-50 µL.

Multiparameter Kinetic Assessment of Brain Mitochondria Physiology

Simultaneous measurements of mitochondrial swelling, Ca^{2+} fluxes, change in membrane potential ($\Delta\varphi_m$), and changes of pyridine nucleotide oxidation (NAD(P)H) were performed using a multichannel fluorescent setup as previously described ([4] and references therein).

Mitochondria (0.15 mg/mL) were incubated with 5 mM glutamate/malate, 150µM ATP, 150µM Mg and were challenged with a series of bolus additions of Ca^{2+}. Alamathicin (Ala) a pore-forming agent, was added at the end of each run to assess complete swelling, Ca^{2+} release, de-energization and oxidation of pyridine nucleotides.

A decrease in autofluorescence at $\lambda_{ex}/\lambda_{em}$ = 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 Ca^{2+}-sensitive, non-permeable fluorescent dye, while tetramethylrhodamine methyl ester (TMRM) (Invitrogen) was used in self-quenching mode to detect changes in mitochondrial membrane potential. Mitochondrial swelling was detected as a decrease in light scattering at excitation and emission wavelengths of 587 nm measured at an angle of 90^0.

Results and Discussion

Here we demonstrate that high quality mitochondria can be extracted from rat brain tissue using The PBI *Shredder* and PCT. Similar results would be achieved using *The* SHREDDER SG3 and PCT. This method is based on previous data that support the use of the Shredder and PCT for isolation of intact mitochondria from cell cultures [3], kidney and skeletal muscle [4], and lung tissue [5].

Total protein concentration of the final mitochondria preparations was 24 ± 7.4 mg/mL (n=4). Previous data generated using muscle tissue, suggest that no improvement in yield is achieved when pressure is increased above 10,000 psi [4]; however it is possible that with brain tissue, higher pressures could be beneficial.

The ability of isolated brain mitochondria to respond to Ca^{2+} is shown in Figure 2. These results confirm that rat brain mitochondria exposed to hydrostatic pressure cycling at 10,000 psi are intact and exhibit a normal response to Ca^{2+} overload.

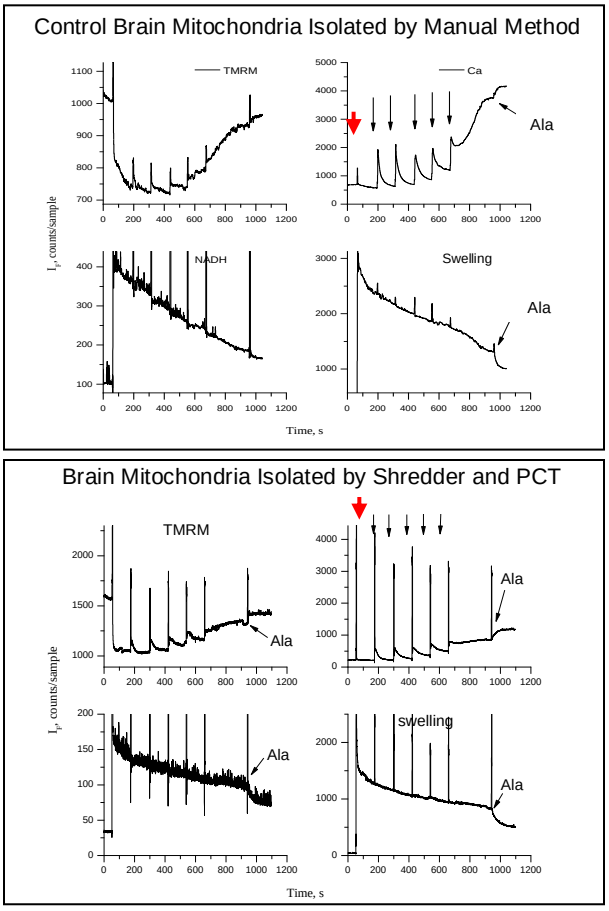

Figure 2. Mitochondria isolated by a conventional technique [6] (top panel) and Shredder/PCT method (bottom panel) demonstrate that mitochondria prepared by both methods are intact as assayed by their ability to respond to exogenous Ca^{2+}. Addition of mitochondria to the sample chamber is indicated by the red arrow. Each Ca^{2+} addition is indicated by a black arrow (note that the scale on the Y-axes is not always the same in the 2 panels). 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 CaGreen 5N, which increases its fluorescence when complexed with free extra-mitochondrial Ca^{2+}. Oxidation of mitochondrial pyridine nucleotides was measured as a decrease in autofluorescence of NADH and NADPH. Swelling was detected as a decrease of light scattering.

Conclusions

Traditional manual methods for isolation of functional mitochondria from tissues rely heavily on operator training, experience and skill. Without considerable training, common mistakes such as tissue over-homogenization can result in damaged mitochondria and highly variable results. Here we describe a convenient method for isolation of intact and functional mitochondria from fresh rat brain tissue using a PBI Shredder and PCT. During 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 but effective design significantly reduces the likelihood of sample over-homogenization. Following the shredding step, brief PCT treatment at 10,000 psi is used extract the intact mitochondria.

The PBI Shredders and PCT are physical tissue disruption/lysis methods that are widely applicable to many sample preparation needs. In the case of mitochondria isolation, the buffers described above can be readily replaced by other mitochondria isolation buffers depending on the needs and preferences of the user. As previously demonstrated with other tissue types, the mitochondria isolated using this convenient method can be used for proteomic as well as functional studies [3, 4]. This method is simple, easy to learn, and eliminates many of the problems of traditional manual mitochondria preparation methods.

References

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