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Isolation of functionally active and highly purified neuronal mitochondria from human cortex

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HIGHLIGHTS

- Combined Percoll gradient centrifugation and anti-TOM22 magnetic bead extraction is used to isolate mitochondria.
- The mitochondria obtained are neuronal (from synaptosome disruption).
- The mitochondria obtained have minimal cytoplasmic contaminants (plasma membrane, peroxisomes, lysosomes, synaptosomes, endoplasmic reticulum).
- The mitochondria are functionally active based on measurements of respiration and protein import.

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ABSTRACT

Background: Functional and structural properties of mitochondria are highly tissue and cell dependent, but isolation of highly purified human neuronal mitochondria is not currently available.
New method: We developed and validated a procedure to isolate purified neuronal mitochondria from brain tissue. The method combines Percoll gradient centrifugation to obtain synaptosomal fraction with nitrogen cavitation mediated synaptosome disruption and extraction of mitochondria using anti mitochondrial outer membrane protein antibodies conjugated to magnetic beads. The final products of isolation are non-synaptosomal mitochondria, which are a mixture of mitochondria isolated from different brain cells (i.e. neurons, astrocytes, oligodendrocytes, microglia) and synaptic mitochondria, which are of neuronal origin. This method is well suited for preparing functional mitochondria from human cortex tissue that is surgically extracted.
Results: The procedure produces mitochondria with minimal cytoplasmic contaminations that are functionally active based on measurements of mitochondrial respiration as well as mitochondrial protein import. The procedure requires approximately four hours for the isolation of human neuronal mitochondria and can also be used to isolate mitochondria from mouse/rat/monkey brains.
Comparison with existing methods and conclusions: This method will allow researchers to study highly enriched neuronal mitochondria without the confounding effect of cellular and organelle contaminants.

1. Introduction

Mitochondria play an important role in neuronal homeostasis by providing energy and participating in numerous signaling pathways (Zhang et al., 2013; Yano et al., 2014; Friedlander, 2003; Hensley and Harris-White, 2015; Wang et al., 2008). Mitochondrial function has been shown to be different in various tissues and cell types (Carafoli and Lehninger, 1971; Zhang et al., 2013; Teng et al., 2004; Zhou et al., 2014). Given that mitochondrial dysfunction

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plays a critical role in neurodegeneration, it is of outmost importance to accurately evaluate highly purified functional neuronal mitochondria (Zhu et al., 2002; Zhang et al., 2008; Kristal et al., 2004). Isolation of highly purified functional neuronal mitochondria has been very challenging due to the heterogeneity of brain tissue. The only method shown to be able to provide neuronal mitochondria involves their extraction from synaptosomes. Synaptosomes are vesicles formed from neuronal processes during mechanical homogenization of brain tissue and encapsulate neuronal mitochondria. It has been shown that synaptosomal mitochondria respond differently to stressors such as Ca^{2+} overload as compared with non-synaptosomal mitochondria (Brown et al., 2006). Our group has recently reported that mitochondrial protein import is impaired exclusively in synaptosomal (or neuronal) mitochondria of presymptomatic R6/2 mice, a mouse model of Huntington's disease (HD) (Yano et al., 2014). It is of interest that primary neurons and cell lines expressing mutant huntingtin demonstrated a mitochondrial protein import defect as well (Yano et al., 2014).

The most robust protocols to isolate synaptosomal mitochondria include a Percoll/Ficoll discontinuous gradient centrifugation step followed by synaptosomal disruption with either digitonin or nitrogen cavitation pressurization (Sims and Anderson, 2008; Kristian, 2010; Hansson et al., 2008; Barksdale et al., 2010). Neither method, however, allows for the elimination of contaminants, including synaptic vesicles, lysosomes, and peroxisomes. Whole synaptosomes are also still found in the final mitochondrial fraction using these methods (Kristian, 2010). To mitigate this obstacle, we used specific anti-mitochondrial outer membrane protein TOM22 antibodies to purify synaptosomal mitochondria without loss of function. These antibodies are conjugated with magnetic microbeads for use in magnetic activated cell sorting (MACS). Using a magnetic field, the microbeads are able to selectively retain mitochondria as they bind to the TOM22 antibodies. Any non-mitochondrial fraction of the sample will therefore be washed out. The exclusive use of the MACS technique was shown to be sufficient to obtain brain mitochondria from a mixture of cell types (neuronal body, glia) but not pure neuronal mitochondria because it requires disruption of the synaptosomes before application of the antibodies. The method described in this report yields highly purified and functional neuronal mitochondria. We successfully characterized the mitochondrial status of human cortical neurons using mitochondria obtained from this protocol. Previous reports described brain mitochondria isolation (Hansson et al., 2011). To our knowledge, this is the first reported protocol to isolate highly pure functional neuronal mitochondria from adult human cortex. This report describes the isolation of pure synaptosomal/neuronal mitochondria. It also yields highly purified non-synaptosomal mitochondria that can be used for functional or structural analyses.

2. Materials

2.1. Reagents

Surgically extracted fresh brain tissue sample.
Sucrose (Sigma, cat. no. S0389)
Mannitol (Sigma, cat. no. M9546)
Percoll, (Sigma, cat. no. 17-0891-01)
Tris base (Sigma, cat. no. T1503)
HEPES (Sigma, cat. no. H3375)
Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA; Sigma, cat. no. E4378)

2.2. Equipment

Motor-driven Teflon Potter Elvehjem homogenizer (Sigma, cat. no. Z403903)
50 mL round bottom transparent centrifuge tubes (Nalgene, cat. no. 3138-0050)
16 mL polycarbonate round bottom centrifuge tubes for the F21-8×50y rotor (18 mm × 100.6 mm; Nalgene, cat. no. 3117-0160)
1.7 mL microcentrifuge tubes (Avant, cat. no. 2925)
Refrigerated hi-speed centrifuge with fixed-angle rotor (Thermo, Sorvall RC-6, F21-8×50y rotor or any compatible hi-speed centrifuge).
Cell disruption vessel (Parr Instrument Company, cat. no. 4639).
Mitochondria Isolation Kit for human tissue, which includes anti-TOM22 microbeads, 10× separation buffer, MACS separation LS Columns, and magnetic Quadro MACS Separator (Miltenyi Biotec, cat. no. 130-094-532).
Nitrogen tank

2.3. Reagent preparation

Isolation Buffer 1 (IB-1): 225 mM sucrose, 75 mM mannitol, 1 mM EGTA, 5 mM HEPES adjusted to pH 7.4 with Tris base.
Isolation Buffer 2 (IB-2): 225 mM sucrose, 75 mM mannitol, 5 mM HEPES adjusted to pH 7.4 with Tris base.
40% Percoll solution: 80 mL of Percoll dissolve in 120 mL of Isolation Buffer 1. Adjust pH to 7.4 with HCl.
24% Percoll solution: 30 mL of 40% Percoll bring to 50 mL with Isolation Buffer 1.
15% Percoll solution: 17.5 mL of 40% Percoll bring to 50 mL with Isolation Buffer 1.
200 mM EGTA (pH 7.4) Dissolve 7.6 g of EGTA in 80 mL of bi-distilled water, adjust pH to 7.4 with KOH, bring the solution to 100 mL with de-ionized water.
Store all the reagents at 4 °C.

3. Procedure

Mitochondria were isolated from different fresh brain tissues including human and mouse. The technique can also be applied to other species (i.e. monkey, rat, cow) (unpublished data). The method has also been successfully used to isolate mitochondria from fresh frozen brain tissue (unpublished data). This procedure will yield non-synaptosomal and synaptosomal (neuronal) mitochondria. The general workflow is shown in Fig. 1.

Isolation of mitochondria from human brain tissue (approx. 3 h)

(a) Put the tissue extracted during surgery in 45–50 mL of ice-cold IB-1 and place the container on ice during transport back to the workbench.
(b) Rinse the tissue of blood by using ice-cold IB-1.
(c) Transfer the washed tissue into a beaker with fresh IB-1.
(d) Mince the tissue into small pieces using scissors to eliminate blood trapped inside the specimen.

Critical step. If trapped blood in the specimen is not removed by proper mincing, the blood products may compromise mitochondrial activity

(e) Transfer the minced tissue to the glass potter.
(f) Homogenize the tissue using 20 slow up and down strokes with a Teflon pestle operated at 400 rpm. Keep the volume of added IB-1 5 to 10 times the estimated volume of the tissue sample.