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A simple method for isolating rat brain mitochondria with high metabolic activity: Effects of EDTA and EGTA

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ABSTRACT

Isolated mitochondria are widely used in metabolic and oxidative stress studies for neurodegenerative diseases. In the present work, the influence of EGTA and EDTA has been tested on a sucrose-based differential centrifugation protocol in order to establish the optimal concentrations to be used in this process. Our results showed alterations in both active and resting respiration, which were dependent on both the addition of EDTA or EGTA to the isolation buffer and the chelator concentration used. However, the addition of chelator to the isolation medium does not modify the mitochondria structure as assessed by both distribution of biological markers and electron micrography in the final pellet. Our results endorse this protocol as the method of choice for metabolic and oxidative stress experiments with fresh isolated rat brain mitochondria.

1. Introduction

Mitochondria isolation has recently been revealed as one of the main tools for the study of the bioenergetics of neuronal disorders. Post-mortem studies (Schapira, 2008) have shown that neuronal cells affected in certain disorders such as Parkinson's disease, Friedrich's ataxia, and amyotrophic lateral sclerosis present alterations in the electron transport system (ETS), markers of oxidative stress, and fluctuation in metabolic parameters. To evaluate these parameters, the study of neurodegenerative disorders requires a precise isolation of mitochondria, preserving metabolic activity and an intact membranous system, both related to the activity of the ETS and maintenance of the membrane potential. Nowadays, we know that free calcium ions during isolation process are one of the most influential factors on, the quality of the mitochondria preparation. For this reason, EGTA and EDTA are commonly added to the isolation media used.

Isolation methods to obtain mitochondria from neuronal tissue have been widely described in the literature (Whittaker, 1967; Booth and Clark, 1978; Berman and Hastings, 1999; Lesnefsky and Hoppel, 2006; Frezza et al., 2007; Fernández-Vizarra et al., 2010; Sims and Anderson, 2008). The main difference to previously reported protocols resides in the composition of the isolation buffer and in the application of isopycnic or differential centrifugation. Indeed, these parameters strongly influence the bioenergetic parameters and consequently make it difficult to compare different studies. Classical procedures for organelle isolation are commonly based on the formation of a density gradient with polymeric compounds such as Ficoll or Percoll (Clark and Nicklas, 1970; Cohen et al., 1997). These compounds are essentially non-osmotic, with relatively low densities and high viscosities, but they require a high-speed centrifugation and long isolation times to achieve an efficient gradient. Furthermore, sucrose gradients exhibit high osmotic activity and consequently remove water from membrane-bound particles, thus causing damage to membranous organelles, even though its gradients are formed at a relative low speed of centrifugation. Conversely, sucrose-based differential centrifugation maintains the osmotic pressure highly controlled, but fails in isolating particles with similar sedimentation coefficients.

This present study addresses the ability of chelators to modify structural and functional parameters of our mitochondrial

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population. In order to test mitochondria functionality we performed a respiratory assay to determine the respiratory oxygen consumption rates and the respiratory control ratio (RCR).

2. Materials and methods

2.1. Chemicals

Male Sprague-Dawley rats (200–250 g) were provided by the breeder of the University of Santiago the Compostela. Animals were housed under controlled temperature and 12 h light–dark cycle over 4 days to facilitate their acclimatization before the start of the experiments and according to the European Community Council Directive of 24 November 1986 (86/609/EEC). Albumin bovine serum essentially fatty acid free, adenosine 5′-diphosphate sodium salt (ADP), ethylenediamine-tetraacetic acid dipotassium salt dehydrate (EDTA), ethylene glycol-bis(β-aminoethyleter)-N,N,N′,N′-tetraacetic acid (EGTA), glutamate, 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES), magnesium chloride hexahydrate, malic acid disodium salt, D-mannitol, oligomycin, potassium chloride, potassium dihydrogen phosphate, rotenone and D(+)-sucrose were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Isolation procedure

The here reported method for the isolation of enriched mitochondria is based on previous studies (Iglesias-González et al., 2012), with a series of modifications introduced to maximize both yield and purity for its use in experiments focused on the study of bioenergetics and oxidative stress generated by mitochondria obtained from a neuronal source. Rats were under food deprivation overnight to preserve metabolic levels at basal rates and stunned with carbon dioxide prior to decapitation. After sacrifice, the brain was quickly removed and immersed in an ice-cold isolation buffer (IB) containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES and 1 mg/mL fatty acid free BSA (pH 7.4, isotonized with KOH). Six groups were made up and IB supplied with EGTA or EDTA at concentrations within the range previously reported in the literature (1–3 mM). Forebrains were dissected with a transection at the level of the two colliculli and meninges carefully removed. The resulting tissue was weighed and cut in small pieces for subsequent manual homogenization in a Dounce-type glass homogenizer (pestle clearance: 0.089–0.165 mm and 0.025–0.076 mm) using IB (1/10, w/v) supplemented with the corresponding chelator. The homogenates resulting from two rats were combined, followed by distribution in plexiglass tubes and then centrifuged at 600 × *g* for 10 min (JA-20 fixed angle rotor, set at maximum acceleration and low deceleration, Beckman Instruments, Palo Alto, CA, USA). The supernatant obtained was reserved in a chilled recipient and the pellet resuspended in 10 mL of buffer and centrifuged as earlier described to recover the fast-sedimented mitochondria. The obtained supernatants were pooled together and centrifuged in four tubes at 12,000 × *g* for 8 min. The pellets were resuspended in buffer, distributed into two tubes each containing a final volume of 10 mL, and centrifuged at 12,000 × *g* for 10 min. The pellet obtained exhibits some layers that differ in consistency and colour with an upper white fluffy layer, containing myelin, synaptosomes, and lipids. The brown mitochondrial pellets collected, after discarding the synaptosomal layer, were resuspended in 10 mL of buffer and centrifuged in two tubes at 12,000 × *g* for 10 min. The resulting mitochondria pellet was resuspended in a final volume of 150 μL IB without chelators. Then, the mitochondrial pellet was homogenated using a Dounce-type (pestle clearance: 0.071–0.119 mm and 0.02–0.056 mm), stocked in pre-cooled Eppendorf tubes and, maintained into an ice-bath until the respiratory assay was carried out. This procedure yields 8–10 mg of protein per rat brain, as measured by the method of Bradford. Mitochondria prepared in this way were active for 3–4 h, as determined by the maintaining value obtained for respiratory control ratio. All the isolation procedure was carried out at 4 °C in order to maintain the temperature under control throughout the process.

2.3. Respiratory assay

The respiratory assays were determined as previously described by our group for high-resolution respirometry (Iglesias-González et al., 2012). Active respiratory state (State 3) was assessed with a NADH-linked substrate (glutamate 2.5 mM and malate 1.25 mM) and ADP (250 μM). Also, to avoid the hydrolytic activity of ATPases, resting state (State 4_0) was achieved adding oligomycin (2 μg/mL).

2.4. Enzymatic markers

The activities of lactate dehydrogenase (LDH), cytochrome c oxidase (CytCOx) and citrate synthase (CS) were determined using spectrophotometric techniques previously described by other authors (Bergmeyer, 1962; Clark et al., 1997; Sampson and Alleyne, 2001). LDH was used as a marker of cytosolic contamination by comparing its specific activity in the initial homogenate with that found in the final pellet. To evaluate the integrity of the mitochondrial membrane, CytCOx activity was used as a marker for the internal membrane and CS activity was assessed as the principal hallmark for mitochondrial matrix.

2.5. Electron microscopy

Mitochondria samples were centrifuged for 30 s at 13,000 rpm, fixed by immersion for 45 min in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.3), postfixed with 2% OsO_4 in the same buffer, dehydrated, embedded in Spurr's epoxy resin, and sectioned. Sections were stained with uranyl acetate and lead citrate and examined in a Zeiss 902 electron microscope (Carl Zeiss, Oberkochen, Germany) at 80 kV accelerating voltage and film magnification of 20,000× or 12,000×.

2.6. Statistical analysis

Data were expressed as mean ± SE. Statistical differences between means were tested using one-way ANOVA ($p < 0.05$) followed by Holm–Sidak post hoc ($p < 0.05$). All the means were obtained from three independent experiments with three replicas per experiment.

3. Results

The purity of the isolated mitochondrial fraction and its morphology were analyzed using molecular markers (Table 1) and

Table 1
Biochemical markers for assessing the integrity and purity of mitocondrial fractions. LDH (nmol/min/mg protein) was used to evaluate cytosol contamination; CytCOX (nmol/min/mg protein) and CS (μmol/min/mg protein) were measured to assess both the integrity of the membranous system and purity of mitochondrial fraction purposes. Data are represented as mean ± SE obtained from three independent experiments.

	Specific activity		Relative activity
	Homogenate	Mitochondria fraction	
LDH	30 ± 0.6	12 ± 0.3	0.4
CytcOx	33 ± 8.3	86 ± 13	2.6
CS	0.33 ± 0.02	2.6 ± 0.01	7.9

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