Mitochondrion 11 (2011) 722–728

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Evaluation of basic mitochondrial functions using rat tissue homogenates

Alena Pecinová, Zdeněk Drahota, Hana Nůsková, Petr Pecina, Josef Houštěk *

Institute of Physiology and Center for Applied Genomics, Department of Bioenergetics, Academy of Sciences of the Czech Republic, 14220 Prague 4, Czech Republic

ARTICLE INFO

Article history:
Received 15 November 2010
received in revised form 17 May 2011
accepted 25 May 2011
Available online 2 June 2011

Keywords:
Isolated mitochondria
Tissue homogenates
oxidative phosphorylation
Respiratory control
Mitochondrial membrane potential

ABSTRACT

The primary attempt in diagnostic and experimental studies of numerous pathological states associated with mitochondrial dysfunction is a precise evaluation of changes in function, content and structure of mitochondrial OXPHOS system. The analysis of rat heart, liver, brain and kidney by oxygraphy, enzyme activities, membrane potential, and BN/SDS-PAGE western blotting demonstrated that tissue homogenates can substitute for isolated mitochondria, providing comparable qualitative mitochondrial parameters. The use of homogenate avoids the loss of the majority of mitochondria during their isolation. Only 50–100 mg of the tissue is required for the complex OXPHOS analysis, *i.e.* five times less as compared with isolated mitochondria.

1. Introduction

A large number of pathological states associated with mitochondrial dysfunction led to an increasing interest in the analysis of mitochondrial functions in different tissues. In addition to mitochondrial oxidative phosphorylation (OXPHOS) disorders, primary or secondary changes of mitochondria are found in a broad range of human mono- and polygenic diseases that are studied in patient bioptic samples as well as in derived animal models. The primary attempt of both the diagnostic and experimental studies is a precise evaluation of possible changes in the mitochondrial function, content, and structure.

Besides the classical approach using isolated mitochondria, different attempts have been made to analyze the mitochondrial function in the whole tissue. An attractive approach appeared to be the analysis of saponin-permeabilized muscle fibers that allowed for oxygraphic measurements of respiratory chain function using minute amounts of skeletal and heart muscle (Kuznetsov et al., 2004; Saks et al., 1998; Sperl et al., 1997). The method was also successfully adopted to some other tissues (Kuznetsov et al., 2002), but the main limitation remained — the lengthy preparation of muscle fibers that is difficult to be standardized and used for a larger number of samples. Another approach, neglected for years and revitalized recently by Kondrashova is the use of whole tissue homogenates (Kondrashova et al., 2001; Kondrashova et al., 2009), which can be quickly and reproducibly prepared from small amounts of tissue.

While these studies were mainly focused on preservation of mitochondrial filaments, we attempted to test whether gently prepared whole tissue homogenates can be used instead of isolated mitochondria to assess energetic functions as well as the quantity and subunit composition of OXPHOS system in different tissues. We performed a detailed study using high resolution oxygraphy, TPP^+ measurement of mitochondrial membrane potential, SDS-PAGE/WB quantification of respiratory chain enzymes as well as two dimensional BN/SDS-PAGE/WB analyses of native OXPHOS complexes and their subunit composition. The results of all these studies clearly demonstrated that the analysis of homogenates allows for a highly sensitive determination of mitochondrial functions, comparable with those obtained with isolated mitochondria. The small amounts of the tissue needed make this approach broadly applicable to the above stated diagnostic and experimental studies.

2. Materials and methods

2.1. Animals and tissues

Male Wistar rats (Bio-Test Konarovice, Czech Republic) with body weight of 180–220 g were housed at 23 °C and kept for 12 h:12 h light/dark period with free access to standard laboratory chow and water. Rats were starved overnight before experiments. Animals were killed by decapitation in CO_2 narcosis. All animal studies were approved by the animal care committee of the Institute of Physiology and fulfilled NIH guidelines for the human use of animal subjects.

Abbreviations: OXPHOS, oxidative phosphorylation; COX, cytochrome *c* oxidase; CS, citrate synthase; WB, western blot; BN-PAGE, blue native polyacrylamide gel electrophoresis; RCI, respiratory control index; DDM, n-dodecyl β-D-maltoside.

* Corresponding author at: Department of Bioenergetics, Institute of Physiology and Center for Applied Genomics, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic. Tel.: +420 24106 2434; fax: +420 24106 2149.
E-mail addresses: alena.pecinova@biomed.cas.cz (A. Pecinová), drahota@biomed.cas.cz (Z. Drahota), nuskova@biomed.cas.cz (H. Nůsková), petr.pecina@biomed.cas.cz (P. Pecina), houstek@biomed.cas.cz (J. Houštěk).

1567-7249/$ – see front matter © 2011 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2011.05.006

2.2. Homogenates and isolated mitochondria

Preparation of homogenates and all steps of isolation of mitochondria (Fig. 1) were carried out on ice or at 4 °C. Protein concentration was determined by Bradford method (Bradford, 1976) using BSA as standard. 10% homogenate was centrifuged at low speed and the sedimented nuclear fraction (nuclei) was collected. The supernatant was centrifuged at high speed, the resulting postmitochondrial supernatant (PMS) was collected and sedimented mitochondria were washed. From each tissue, fresh samples of homogenate and isolated mitochondria were used for respiration and TPP^+ measurements, aliquots of all samples were stored at −80 °C for other analyses.

Heart mitochondria were isolated in H medium (0.25 M sucrose, 10 mM Tris–HCl, 2 mM EDTA, pH 7.4) using a modified protocol of Pallotti and Lenaz (2007). Briefly, the minced heart tissue was homogenized with teflon-glass homogenizer (clearance 0.25 mm, 7 strokes, 750 rpm), filtered through a polyamide screen (mesh 56 μm), and centrifuged as follows: low speed — 600 *g*, 10 min; high speed — 10,000 *g*, 10 min. The final pellet was resuspended in the H medium.

Liver and kidney mitochondria were isolated in L medium (0.32 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4) according to (Fernandez-Vizarra et al., 2002; Fernandez-Vizarra et al., 2010) with minor modifications. The tissue was homogenized in a teflon-glass homogenizer (clearance 0.15 mm, 10 strokes, 750 rpm), homogenate was filtered and centrifuged: low speed — 800 *g*, 10 min; high speed — 8000 *g*, 10 min. The final pellet was resuspended in the L medium. In addition, different isolation media including sucrose medium (0.25 M sucrose, 10 mM Tris–HCl, 2 mM EDTA, pH 7.4), mannitol medium (0.22 M D-mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.2), and sorbitol medium (25 mM sucrose, 75 mM sorbitol, 0.1 M KCl, 10 mM KH_2PO_4, 10 mM Tris–HCl, 0.05 mM EDTA 5 mM $MgCl_2$, 1 mg/ml BSA, pH 7.4) were used. We did not observe any differences in the mitochondrial respiratory parameters and RCI among the homogenates or mitochondria prepared in different media.

Brain mitochondria were isolated as previously described (Mracek et al., 2009). In short, the minced rat brain was homogenized in a tight teflon-glass homogenizer (clearance 0.15 mm, 20 strokes by hand) in B medium (0.21 M D-mannitol, 70 mM sucrose, 5 mM Tris–HCl, 1 mM EDTA, pH 7.4) and homogenate was centrifuged for 5 min at 600 *g*. The supernatant was centrifuged for 10 min at 15,000 *g*, and the pellet (free mitochondria and synaptosomes) was resuspended in B medium and treated with digitonin (final concentration 0.013%, w/v) for 2 min. Mitochondria were washed twice (12,000 *g*, 10 min) and resuspended with the B medium.

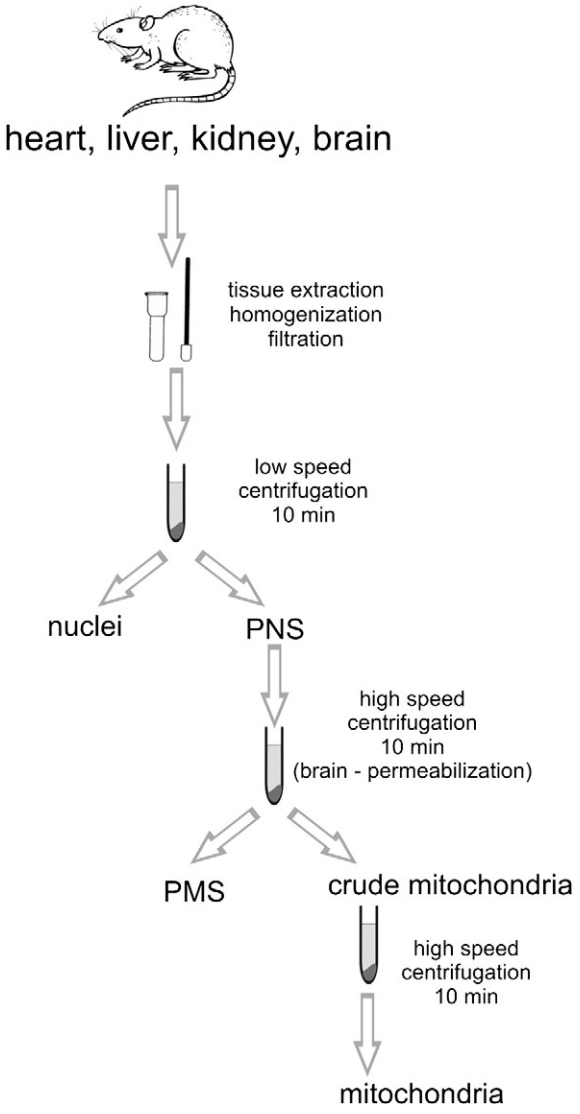

Fig. 1. Schematic representation of the mitochondrial isolation. PNS — postnuclear supernatant, and PMS — postmitochondrial supernatant.

2.3. Enzyme activities

Activities of cytochrome *c* oxidase (COX) (Wharton and Tzagoloff, 1967) and citrate synthase (CS) (Srere, 1969) were determined spectrophotometrically at 30 °C essentially as described. The samples from different tissue fractions were solubilized with 0.03% n-dodecyl β-D-maltoside.

CS activity was determined in a medium containing 0.1 M Tris–HCl, 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 50 μM acetyl coenzyme A, pH 8.1. The reaction was started by adding 0.5 mM oxaloacetate, followed by monitoring changes at 412 nm for 1 min. The data were corrected for the absorbance change without oxaloacetate. CS activities were expressed as nmol/min/mg protein using molar absorption coefficient $\varepsilon_{412} = 13.6$/mM/cm.

COX activity was measured in a medium containing 40 mM K-Pi, 0.1% BSA, pH 7.0. The reaction was started with 30 μM reduced cytochrome *c* and its oxidation was monitored at 550 nm for 40 s. COX activities were expressed as μmol/min/mg protein using molar absorption coefficient $\varepsilon_{550} = 19.6$/mM/cm. Cytochrome *c* solution (5 mM) was reduced by sodium dithionite. The salt was removed by gel filtration through Sephadex G-25 column.

2.4. High resolution oxygraphy

Oxygen consumption was measured at 30 °C as described before (Pecina et al., 2003) using Oxygraph-2k (Oroboros, Austria). The homogenate (0.1–0.3 mg/ml) or the isolated mitochondria (0.05–0.1 mg/ml) were suspended in 2 ml of KCl medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM K-Pi, 2.5 mM malate, 0.5 mg/ml BSA, pH 7.4). For measurements, 10 mM glutamate, 1.5 mM ADP, 20 μM cytochrome *c*, 2 μM oligomycin, and 1 μM antimycin A were used. 0.1 μM FCCP additions were titrated to achieve maximal respiration. When using brain homogenate, digitonin (0.1 g/g of protein) was added to the measuring chamber for permeabilization of the synaptosomes. The oxygen consumption was expressed in pmol oxygen/s/mg protein. RCI was calculated as ratio of state 3-ADP/state 4-oligomycin rates using 10 mM glutamate, 2.5 mM malate, 1.5 mM ADP, and 2 μM oligomycin.

2.5. Analysis of the mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi_m$) was measured with TPP^+-selective electrode as described in (Labajova et al., 2006), using