Analytical Biochemistry 444 (2014) 25–31

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Preservation of mitochondrial functional integrity in mitochondria isolated from small cryopreserved mouse brain areas

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ARTICLE INFO

Article history:
Received 6 May 2013
Received in revised form 19 August 2013
Accepted 30 August 2013
Available online 7 September 2013

Keywords:
Brain mitochondria
Cryopreservation
Mitochondria isolation
Mitochondrial oxidative phosphorylation

ABSTRACT

Studies of mitochondrial bioenergetics in brain pathophysiology are often precluded by the need to isolate mitochondria immediately after tissue dissection from a large number of brain biopsies for comparative studies. Here we present a procedure of cryopreservation of small brain areas from which mitochondrial enriched fractions (crude mitochondria) with high oxidative phosphorylation efficiency can be isolated. Small mouse brain areas were frozen and stored in a solution containing glycerol as cryoprotectant. Crude mitochondria were isolated by differential centrifugation from both cryopreserved and freshly explanted brain samples and were compared with respect to their ability to generate membrane potential and produce ATP. Intactness of outer and inner mitochondrial membranes was verified by polarographic ascorbate and cytochrome *c* tests and spectrophotometric assay of citrate synthase activity. Preservation of structural integrity and oxidative phosphorylation efficiency was successfully obtained in crude mitochondria isolated from different areas of cryopreserved mouse brain samples. Long-term cryopreservation of small brain areas from which intact and phosphorylating mitochondria can be isolated for the study of mitochondrial bioenergetics will significantly expand the study of mitochondrial defects in neurological pathologies, allowing large comparative studies and favoring interlaboratory and interdisciplinary analyses.

Mitochondrial energy metabolism is crucial for providing the cell with the fuel needed to perform a host of biological processes; likewise, dysregulation of mitochondrial energy metabolism is directly involved in the pathogenic mechanisms of a plethora of pathological states [1,2].

Mitochondrial defects are usually analyzed in frozen biopsy samples using histochemical, enzymatic, or molecular biology methods. Although the maximal activities of key mitochondrial enzymes, such as citrate synthase and cytochrome *c* oxidase (COX),[1] have been widely used as indicative of mitochondrial oxidative capacity [3–5], it is unlikely that measurement of individual enzyme activities can accurately reflect the overall function of an organelle as complex as the mitochondrion. Thus, a critical requirement is to implement more direct analyses of mitochondrial function by measuring their ability to synthesize ATP via oxidative phosphorylation, which provides more complete information and, in addition, represents a relevant screening procedure to check potential mitochondrial bioenergetic dysfunctions.

Studies on mitochondrial function are conventionally performed in mitochondria isolated from fresh tissues or cultured cells because they yield tightly coupled mitochondria; on the contrary, mitochondria from frozen tissues are inevitably poorly intact and coupled. Thus, the major limitation of investigating mitochondrial function is that it must be performed immediately after tissue explants, with this being not always feasible due to both time and technical constraints. This limitation reduces the possibility to perform basic mitochondrial studies on brain bioenergetics and to analyze mitochondrial defects in various neurological diseases. Therefore, there is a compelling need to obtain functional mitochondria from long-term preserved tissues. Moreover, because a limited number of centers perform functional analysis of mitochondria, tissue cryopreservation is also crucial to ship samples to distant laboratories for additional assays.

Previous studies demonstrated preservation of mitochondria inside cryopreserved sperm, cardiac, and skeletal muscle fibers [6–8] as well as cryopreservation of isolated brain mitochondria [9].

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[1] *Abbreviations used:* COX, cytochrome *c* oxidase; ROT, rotenone; AA, antimycin A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BSA, bovine serum albumin; ASC, ascorbate; cyt *c*, cytochrome *c*; MYXO, myxothiazole; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; CN^-, cyanide; SUCC, succinate; CoA, coenzyme A; DTNB, dithionitrobenzoic acid; TNB, thionitrobenzoic acid; $\Delta\psi$, mitochondrial membrane potential; GLU, glutamate; MAL, malate; ATP ds, ATP detecting system; HK, hexokinase; G6P-DH, glucose 6-phosphate dehydrogenase; Ap5A, diadenosine pentaphosphate; DMSO, dimethyl sulfoxide.

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http://dx.doi.org/10.1016/j.ab.2013.08.030

Here we demonstrate, for the first time, that mouse brain mitochondria can be isolated from cryopreserved frozen small brain biopsies, maintaining their intactness and an optimal efficiency of oxidative phosphorylation process comparable to that of mitochondria isolated from fresh mouse brain tissues.

Materials and methods

Chemicals

All reagents and enzymes were obtained from Sigma (St. Louis, MO, USA) with the exception of Triton X-100, Hepes, and Tris (Baker). All chemicals were of the purest grade available and were used as Tris salts at pH 7.0–7.4 adjusted with Tris or HCl. Rotenone (ROT), antimycin A (AA), mixothiazol, oligomycin, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were dissolved in ethanol.

Animal experimental model and brain tissue dissection

Female mice of an outbred CD-1 Swiss-derived strain (ICR) were purchased from a commercial breeder (Harlan, Italy). On arrival, the animals were housed in an air-conditioned room (temperature = 21 ± 1 °C, relative humidity = 60 ± 10%, lights on from 20.00 to 08.00 h). Animals were housed in groups of 2 or 3 individuals in polycarbonate transparent cages (33 × 13 × 14 cm) with a metal top and sawdust bedding and were provided with a complete pellet diet ad libitum (Altromin, Germany) and tap water.

At approximately 4 months of age, animals were sacrificed and their brains were dissected. Either entire (right or left) hemispheres or isolated brain regions (cortex, hippocampus, striatum, and cerebellum) were carefully dissected. Brain samples were immediately processed for mitochondria isolation (fresh samples), frozen in dry ice (frozen samples), or cryopreserved as described below.

Cryopreservation of different regions of brain samples

For cryopreservation of brain tissue, dissections were carried out in ice-cold solution consisting of 50 mM K-Mes (pH 7.1), 3 mM K_2HPO_4, 9.5 mM $MgCl_2$, and 3 mM ATP. Brain samples were then transferred into a cryotube containing 500 µl of the same solution plus 20% glycerol and 10 mg/ml fatty acid-free bovine serum albumin (BSA). Samples were then frozen in liquid isopentane to achieve a rapid and uniform freezing and were maintained at −30 to −35 °C. Subsequently all samples were stored at −80 °C or stored in dry ice and shipped.

For mitochondria isolation, tubes containing cryopreserved tissues were placed on ice, and when the cryopreservation solution was almost completely thawed, brain tissues were immediately transferred and washed in ice-cold respiratory medium consisting of 210 mM mannitol, 70 mM sucrose, 3 mM $MgCl_2$, 20 mM Tris–HCl, and 5 mM KH_2PO_4/K_2HPO_4 (pH 7.4) plus 2 mg/ml BSA. Mitochondria were then isolated as described below.

Mitochondria preparation

Mitochondria were isolated from fresh, frozen, or cryopreserved mouse brain samples, essentially as described in Ref. [10] with some modifications. All procedures were carried out at 4 °C. Briefly, brain samples were homogenized (5 strokes at 500 rpm) in sucrose medium consisting of 0.25 M sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM Tris–HCl (pH 7.4) at a ratio of 10–20 µl/mg tissue. The homogenate was centrifuged at 5500*g* for 3 min, and the supernatant obtained was subsequently centrifuged at 8500*g* for 10 min. The pellet was suspended in 1 ml of sucrose medium and centrifuged again at 8500*g* for 10 min. This step was repeated twice to obtain a mitochondrial enriched fraction (namely crude mitochondria) suspended in 0.1–0.2 ml of respiratory medium and used for mitochondrial functional measurements.

Analyses of outer and inner mitochondrial membrane integrity

The outer membrane integrity in crude mitochondria isolated from different cryopreserved brain areas was checked by (i) ascorbate (ASC) test [11] and (ii) cytochrome *c* (cyt *c*) test [12]. The ASC test was carried out monitoring ASC oxidation, which occurs only in case of disruption of the outer mitochondrial membrane because ASC cannot permeate it [13]. ASC oxidation was checked polarographically by measuring oxygen consumption after the addition of 5 mM ASC to crude brain mitochondria (0.25–0.5 mg protein) incubated at 37 °C in 1 ml of respiratory medium in the presence of 3 µM ROT, 2 µM AA, and 3 µM myxothiazole (MYXO), followed by the addition of 0.2 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and 1 mM cyanide (CN^-). The percentage of integrity of outer membrane was calculated as $1 - (vo{-}TMPD/vo{+}TMPD) \times 100$, where vo = the rate calculated as tangent to the initial part of the progress curve. Cyt *c* test was carried out monitoring the rate of oxygen consumption under state 3 of respiration, that is, in the presence of ROT (3 µM), succinate (SUCC, 5 mM), and ADP (1 mM) after the addition of 10 µM cyt *c* to isolated mitochondria (0.25 mg protein) [12]. Oxygen consumption measurements were carried out at 37 °C in 1 ml of respiratory medium (pH 7.4) by using a Gilson 5/6 oxygraph equipped with a Clark electrode.

Mitochondrial inner membrane integrity was assessed by measuring citrate synthase activity in isolated mitochondria (0.04 mg protein) before (vo−TX-100) and after (vo+TX-100) membrane disruption by the detergent Triton X-100 (0.2%). The percentage of integrity of inner membrane was calculated as $1 - (vo{-}TX\text{-}100/vo{+}TX\text{-}100) \times 100$. Measurements of citrate synthase activity were carried out at 37 °C in 0.8 ml of assay medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, and 20 mM Hepes/Tris (pH 8.2) in the presence of saturating concentrations of acetyl-coenzyme A (CoA) (0.3 mM), oxaloacetate (0.5 mM), and dithionitrobenzoic acid (DNTB, 0.2 mM), following the increase in absorbance at 412 nm due to thionitrobenzoic acid (TNB) formation, as in Ref. [12], by using a Jasco V-550 ultraviolet/visible (UV/VIS) spectrophotometer.

Measurement of mitochondrial membrane potential generation

Mitochondrial membrane potential ($\Delta\psi$) generation was measured by monitoring fluorescence changes of the probe safranin O at 520-nm excitation and 570-nm emission wavelengths, essentially as described in Ref. [14]. Accumulation of safranin O in mitochondria is driven by membrane potential and subsequently results in a decrease of fluorescence. Depolarization results in the release of safranin O from mitochondria and a subsequent increase of fluorescence. Isolated mouse brain mitochondria (1 mg protein) were incubated at 37 °C in 2 ml of medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, and 20 mM Hepes–Tris (pH 7.0) plus 10 µM safranin O, and $\Delta\psi$ generation was monitored by adding 5 mM SUCC plus 3 µM ROT or glutamate (GLU) plus malate (MAL) (5 mM each) in the presence of 0.5 mM ADP. $\Delta\psi$ generation was abolished by adding the uncoupler FCCP (1.25 µM). Changes of safranin O fluorescence were recorded by using a PerkinElmer LS50 spectrofluorimeter.