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Enzymatic assays for probing mitochondrial apoptosis

Zhenyu Wang^{1,2}, Claire Nicolas^{1,2}, Rodolphe Fischmeister^{1,2} and Catherine Brenner^{1,2}

¹INSERM UMR-S 769, LabEx LERMIT, Châtenay-Malabry, France;

²Université de Paris-Sud, Faculté de Pharmacie, Châtenay-Malabry, France

Correspondence to:

Dr. Catherine Brenner, INSERM UMR-S 769, Université de
Paris-Sud, LabEx LERMIT, 5 rue Baptiste Clément,
Châtenay-Malabry, France;

E-mail: catherine.brenner-jan@u-psud.fr

Summary

Isolated mitochondria are an invaluable analytical tool to probe mitochondrial function and evaluate apoptosis induction via the so-called mitochondrial pathway. Irrespective of their tissue origin (e.g. heart, liver, muscle, brain), these organelles participate actively to cell and life decision by producing energy for cell metabolism, but also by undergoing a lethal and irreversible mitochondrial membrane permeabilization (MMP) in stress and pathological conditions. MMP consequences consist, at least in part, in loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), matrix swelling, arrest of respiration and ATP production, and cytochrome *c* release from the intermembrane space to the cytosol. These parameters can be evaluated *in vitro* via several miniaturized assays, which have tremendous applications in the field of pharmacology, toxicology, diagnosis as well as drug discovery.

Key words: apoptosis, cytochrome *c*, fluorescence, heart, inner transmembrane potential, mitochondria.

1 Introduction

During mitochondrial apoptosis, mitochondrial membrane permeabilization (MMP) has been previously defined as an irreversible process that leads to the cell death (1-3). Thus, MMP influences functional and morphological features of mitochondria depending on the nature, the intensity and the duration of the pro-apoptotic signal. Main stimuli of MMP are ions such as calcium (Ca^{2+}), reactive oxygen species (ROS) (e.g. anion superoxide, hydrogen peroxide), proteins (e.g. BAX, BID) and lipids and target the inner membrane, the outer membrane or both. Then, MMP results in loss of transmembrane potential ($\Delta\Psi_m$), matrix swelling, arrest of respiration and ATP production, ROS and cytochrome *c* release from the intermembrane space to the cytosol (4-6). In some tissues such as the heart, abnormal mitochondrial fusion and fission can accompany mitochondrial apoptosis, but its contribution as cause vs a consequence remains to be defined. Some of MMP parameters can be easily measured *in vitro* via several miniaturized assays to gain insights into pro-apoptotic mitochondrial alterations.

Here, we described the methods for (1) the isolation of fresh mitochondria from rat heart by a procedure adapted to the myofibrillar structure of the tissue, (2) the subsequent quantification of $\Delta\Psi_m$ by a fluorescent probe Rhodamine 123 (7) and the colloidosmotic matrix swelling by spectrophotometry (8), (3) the oxygen consumption by the phosphorescent probe Mito-ID® (9), and (4) the release of intermembrane space proteins, such as cytochrome *c* by western-blot (10). These assays are demonstrated to be useful analytical tools as well as high throughput screening tools implemented on technological platforms. Hence, they could have broad applications in the field of pharmacology, toxicology, and diagnosis as well as drug discovery.

2 Materials

All chemicals need to be of the highest purity available. All the reagents are from Sigma unless indicated otherwise. Ultrapure water is systematically used.

2.1 Isolation buffers

1. Buffer H: weigh 102.8g sucrose, 1.146g 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES), 76mg ethylene glycol tetraacetic acid (EGTA). Add water to a volume of 900mL. Mix and adjust pH to 7.2 with potassium hydroxide (KOH), add water to a volume of 1 L. Mix and aliquot into 50mL tubes and store at -20°C.
2. Buffer H+ Bovine serum albumin (BSA): weigh 102.8g sucrose, 1.146g TES, 76mg EGTA, 1g BSA. Add water to a volume of 950mL. Mix and adjust pH to 7.2 with KOH. Complete to 1L with water. Aliquot into 50mL and store at -20°C.

2.2 Mitochondria membrane potential and swelling

1. Buffer S: weigh 68.46g sucrose, 2.09g 3-(N-morpholino)propanesulfonic acid (MOPS), 3.8mg EGTA, 82μL Phosphoric acid (H₃PO₄). Add water to a volume of 950mL. Mix and adjust pH to 7.4 with KOH. Complete to 1 L with water. Aliquot into 50mL tubes and store at -20°C.
2. Succinate stock solution: weigh 1.35g succinate; adjust the volume to 10mL with water to get a concentration of 500mM. Aliquot and store at -20°C.
3. Rotenone stock solution: dissolve 6.99mg of rotenone in 10mL of absolute ethanol to a concentration of 2mM. Aliquot and store at -20°C. (see **Note 1**)
4. Rhodamine 123 stock solution: weigh 3.81mg rhodamine 123 (Invitrogen), make up to 10mL with absolute ethanol to get a concentration of 1mM. Store at -20°C. (see **Note 2**)

2.3 Oxygen consumption

1. Measurement buffer: 250mM sucrose, 15mM Potassium chloride (KCl), 1mM EGTA, 5mM Magnesium chloride (MgCl₂), 30mM Potassium phosphate dibasic (K₂HPO₄), pH 7.4
2. Reconstitute Mito-ID® Extracellular O₂ Sensor kit (Enzo Life Sciences, Inc.) in 1mL of nuclease-free water or media to obtain a 1μM stock solution. Mix gently. Aliquot into 100μL in tubes and store at -20°C. (see **Note 3**)

3. Succinate stock solution: use the same as for membrane potential and swelling measurement.
4. Glutamate stock solution: weigh 0.94g glutamate, add water to 10mL to a concentration of 500mM. Aliquot and store at -20°C.
5. Malate stock solution: weigh 0.45g malate, add water to 10mL to obtain a concentration of 500mM. Aliquot and store at -20°C.
6. ADP stock solution: weigh 0.7g ADP, make up to 10mL with water to a concentration of 165mM. Aliquot and store at -20°C.

2.4 SDS Polyacrylamide Gel

1. Loading buffer (5×): Tris 300mM, Glycerol 50%, sodium dodecylsulfate (SDS) 12.5%, dithiothreitol (DTT) 50mM, BromoPhenol Blue 0.05%, pH 6.8.
2. 4-15% precast gel (BioRad Mini- PROTEAN TGX)
3. Migration buffer (10×): weigh 30g Tris Base, 144g Glycine, 10g SDS. Add water to a volume of 950mL. Mix and adjust pH to 8.3. Complete to 1 L with water.
4. DTT (10×): weigh 1.54g DTT, make up to 10mL with water. Aliquot and store at -20°C.

2. 5 Immunoblot

1. Membrane (Trans-Blot Turbo Transfer Pack Bio-Rad)
2. Trans-blot Turbo transfer system (Bio-Rad)
3. Phosphate buffered saline (PBS)-Tween (10×): weigh 624mg Sodium dihydrogen phosphate monohydrate (NaH_2PO_4) 2.98g Na_2HPO_4 - Sodium phosphate dibasic (Na_2HPO_4), 204.5g Sodium chloride (NaCl), 12.5mL Tween 20, make up to 2.5L with water.
4. Antibody anti-cytochrome *c* (BD Pharmingen)
5. Ultra-sensitive enhanced chemiluminescent (ECL) substrate, e.g. Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific)
6. Gel imaging system, e.g. Chemidoc (BioRad)

3. Methods

3.1 Rat heart isolated mitochondria

1. Kill the rat after injection of pentobarbital and excise the heart rapidly and place it in cold buffer H. (see **Note 4**)
2. Rinse the heart free of blood by using ice-cold buffer H.
3. Cut the heart into pieces in 10mL of Buffer H with a scalpel blade and transfer them into a 50mL falcon tube.
4. Grind the heart with the Polytron 2-3 times rapidly. (see **Note 5**)
5. Use the Potter to homogenize the mix.
6. Transfer the homogenate to a 15mL Falcon tube and add 5mL of Buffer H with BSA to fill the tube to 15mL.
7. Centrifuge at 500 g for 10 min at 4°C. (see **Note 6**)
8. Collect the supernatant by eliminating the maximum of lipids and discard the pellet. (see **Note 7**) Centrifuge at 3,000 g for 10 min at 4°C, 2 times (see **Note 8**).
9. Discard the supernatant and gently resuspend the pellet containing mitochondria in 200μL Buffer H without BSA. (see **Note 9**)
10. Determine protein concentration in an aliquot by the BCA method following manufacturer's instruction using bovine serum albumin as standard. Keep the mitochondria on ice until use. (see **Note 10**)

3.2 Mitochondrial transmembrane potential ($\Delta\Psi_m$) and swelling

1. Prepare buffer S with final concentration of 5mM succinate and 2μM rotenone.
2. Prepare compounds (calcium as control of pro-apoptotic inducer or compound to be tested) in 4× concentrations with buffer S. (see **Note 11**)
3. Add 50μL of compounds or buffer S (control) per well of microtiter plate 96 wells black with transparent and flat bottom.
4. Add 50μL of buffer S per well.
5. Dilute freshly isolated cardiac mitochondria at 0.25 μg/μL with buffer S and add rhodamine 123 to obtain a final concentration of 1μM. Add 100μL of mitochondria in each well. (see **Note 12**)

6. Shake the plate for 5s and then start to measure for 60min with 2 parameters at room temperature. For membrane potential, measure the fluorescence at $\lambda_{ex} = 485\text{nm}$ and $\lambda_{em} = 535\text{nm}$. For swelling, measure absorbance at 540nm.

3.3 Oxygen consumption

1. Warm up a microtiter plate 96 wells black flat bottom to 30°C.
2. For compound testing, prepare compounds at 10 $\times$ and add 20 μL in the well. (see **Note 8**)
3. Dilute the respiratory substrate (glutamate/malate for complex I or succinate for complex II) with measurement buffer and add 20 μL of this solution to obtain concentrations of 5mM (succinate) or 12.5/12.5 mM (glutamate/malate).
4. Dilute mitochondria suspension to 0.5 $\mu\text{g}/\mu\text{L}$ with measurement buffer and add 100 μL to each well. (see **Note13**)
5. Dilute ADP with measurement buffer and add 20 μL to obtain a final concentration of 1.65mM.
6. Add 30 μL of measurement buffer to each well.
7. Add 10 μL Mito-ID® Extracellular O₂ Sensor probe in each well. (see **Note 14**)
8. Using a syringe dispenser, quickly add 100 μL of oil to each well. (see **Note 15**)
9. Insert the plate into a fluorescence plate reader pre-warmed to 30°C. Measure the probe signal for 30-60 min using excitation and emission wavelengths of 380nm and 650nm respectively. (see **Note 16**)

3.4 Cytochrome *c* release

1. Use the same reaction system as membrane potential to increase the volume by 75 times. The final volume is 15mL (see **Note 12**). Incubate mitochondria with the compounds or buffer (control) for 30min.
2. Centrifuge at 9,000 *g* for 15min at 4°C.
3. Collect supernatant in acetone-compatible tube.
4. Add four times the sample volume of cold acetone to the tube. (see **Note 17**)
5. Vortex the tube and incubate overnight at -20°C.

6. Centrifuge at 15,000 *g* for 15 min at 4°C.
7. Decant and properly collect of the supernatant. (see **Note 18**)
8. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 min.
9. Add 20μL of water and vortex thoroughly to dissolve protein pellet.
10. Take the same volume to measure cytochrome *c* by SDS-PAGE and immunoblot.

3.5 SDS-PAGE and western blot

1. Add 14μL of cytochrome *c* samples, 4μL of loading buffer (5×), 2μL of DTT (10×) into an eppendorf tube, mix and heat for 5min at 95°C.
2. Load the samples in the precast gel.
3. Migrate for 15min at 300V.
4. Place the membrane and bottom stack on the cassette base, place the gel on top of membrane, roll the assembled sandwich to expel trapped air bubbles. Close and lock the cassette lid. Transfer for 3min at 2.5V.
5. Block the membrane with 5% of milk in PBS-Tween.
6. Incubate membrane with diluted anti-cytochrome *c* primary antibody in 5% w/v milk PBS-Tween at 4°C with gentle shaking, overnight.
7. Wash the membrane with PBS-Tween for 6×5min.
8. Incubate the membrane with Horseradish Peroxidase-Conjugated secondary antibody for 1h at room temperature.
9. Wash the membrane with PBS-Tween for 6×5min.
10. Incubate the membrane with an ultra-sensitive enhanced chemiluminescent substrate for 5min.
11. Take images with a gel imaging system.

3.5 Statistical analysis

Data can be analyzed using Student's *t*-test and two-way ANOVA for comparisons of mean values to the different treatments tested. Results are presented as the mean ±

standard deviation (SD) of replicates experiments. Significance: *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$. Significance is not shown in the figures for clarity reasons.

Notes

Note 1: Rotenone in organic solvents decomposes and is oxidized upon exposure to light and air. It is imperative to protect the stock solution from direct light by using an aluminium foil. Rotenone is highly toxic: avoid skin contact and inhalation.

Note 2: Rhodamine 123 is light sensitive, so keep it in aluminium foil and avoid exposure to light for long time.

Note 3: Protect the stock solution from direct light by using an aluminium foil. Avoid repeated freeze-thaw cycles.

Note 4: All the steps must be performed on ice.

Note 5: Pre-cool the glassware and homogenizer with pestle in an ice-bath 5 min before starting the procedure. Homogenization as well as the following steps must be carried out on ice to minimize the activation of proteases and phospholipases.

Note 6: Turn on the centrifuge and preset it 4°C.

Note 7: White layer on the top of the tube.

Note 8: Avoid the formation of bubbles during the resuspension process. At the last time, use buffer H without BSA.

Note 9: Do not dilute the mitochondria with buffer as mitochondria retain their functionality for a longer time when kept concentrated, minimizing exposure to oxygen.

Note 10: Mitochondria are ready to be used in experiments; use the preparation within 1–3 h for better functional responses.

Note 11: If the compound is not soluble in water, it can be dissolved in DMSO or other solvent and then diluted with buffer S. It is better to use less concentration of organic solvent. And it is important to use the same concentration of organic solvent in the control well.

Note 12: In order to use less time, it is better to use multi-channel pipette to do this step.

Note13: If the rate of oxygen consumption is too fast, we can dilute the mitochondria to 0.25µg/µL.

Note 14: shake the plate for 10s.

Note 15: Pre-warm mineral oil to 30°C on a water bath.

Note 16: Adjust the gain for an optimal reading of a positive control, and use the same parameters for a series of experiment.

Note 17: Pre-cold (-20°C) acetone, a volume four times that of the protein samples to be precipitated.

Note 18: Be careful to not dislodge the protein pellet.

Fig.1. Co-evaluation of Ca^{2+} effect on the mitochondrial inner transmembrane membrane potential ($\Delta\Psi_m$) and matrix volume of cardiac mitochondria in microtiter plates. (A). Loss of $\Delta\Psi_m$ was simultaneously measured in real time following addition of increasing Ca^{2+} concentrations or without Ca^{2+} (Control) by fluorescence. (B). Mitochondrial swelling induced by different concentrations of Ca^{2+} was concomitantly monitored by absorbance.

Fig.2. Assessment of ADP-stimulated mitochondrial respiration with Mito-ID® Extracellular O_2 Sensor Kit. A: Oxygen consumption is driven by succinate (Control) and is inhibited by antimycin A (AA). B: Oxygen consumption is driven by glutamate/malate (Control) and treated with blocker rotenone (ROT).

Fig.3 Immunoblot of cytochrome *c* release from cardiac mitochondria treated with 10 μ M Ca²⁺ or not (Control). The same volume of sample has been separated and electro-transferred according to (11) and immunorevealed using chemiluminescence.

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