THE PANCREAPEDIA
Exocrine Pancreas Knowledge Base
www.pancreapedia.org

METHODS PAGE

Isolation of pancreatic mitochondria and measurement of their functional parameters

Irina V. Odinokova,[1,2] Natalia Shalbuyeva,[1] Anna S. Gukovskaya,[1] Olga A. Mareninova.[1]

[1]Veterans Affairs Greater Los Angeles Healthcare System and Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles, CA 90073, USA; [2]Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia.

e-mail: odinokova@rambler.ru; nshalb@yahoo.com; agukovsk@ucla.edu; olgam@ucla.edu

Version 1.0, June 21, 2011 [DOI: 10.3998/panc.2011.25]

Abbreviations

CCCP	carbonyl cyanide m-chlorophenylhydrazone
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid;
ER	endoplasmic reticulum
$\Delta\Psi m$	mitochondrial membrane potential
RCR	respiration control ratio
TPP^+	tetraphenyl phosphonium ion;
TMRM	tetramethylrhodamine methyl ester

Data obtained during the last decade by our group and others indicate that mitochondria play an important role in exocrine pancreas diseases (2-4,6,8,9,11, 15,16). Mitochondrial damage occurs in various animal and ex-vivo models of pancreatitis. Manifestations of mitochondrial dysfunction in pancreatitis are loss of mitochondrial membrane potential ($\Delta\Psi m$), release into the cytosol of the apoptosis mediator cytochrome c, decrease in cellular ATP leading ultimately to acinar cell death through apoptosis and necrosis (3,6,8-10). Our recent data (Mareninova et al., 2011, submitted) show that preventing mitochondrial dysfunction improves many parameters of experimental pancreatitis and ameliorates the disease severity. On the other hand, promoting mitochondrial dysfunction in pancreatic cancer diminishes the resistance of cancer cells to apoptosis, and thus represents a potential therapeutic approach (11). Thus, studies with isolated mitochondria can be a useful tool in understanding the underlying mechanisms of mitochondrial dysfunction in pancreatitis and pancreatic cancer. Several methods are available for the isolation of mitochondria from animal tissue and all of them are based on tissue disruption in an appropriate medium and subsequent differential centrifugation of homogenate (1,14,18). This chapter presents

detailed protocol developed by our group for isolation of pancreatic mitochondria and assessment of their functional parameters.

1. Isolation of pancreatic mitochondria

1. 1. Materials

1. Isolation buffer: 250 mM sucrose, 1 mM EGTA, 10 mM Tris, adjusted pH to 7.4 with 1N HCl. On the day of experiment add 0.5% BSA (Sigma, Cat. A6003; Essentialy Fatty Acid Free) and 0.25 mg/ml soybean trypsin inhibitor (Worthington Biochemical Corp, NJ). **(**Note 1**)**

2. Mitochondria storage buffer: 250 mM sucrose, 10 mM Tris, adjusted to pH 7.4 with 1 N HCl.

3. Stirrer Motor with Electric Speed Controller tissue-homogenizing system with Teflon Pestle (<0.3-mm clearance, 40-ml working volume; Cole-Parmer Instrument Inc., IL).

4. Refrigerated high-speed centrifuge with a fixed-angle rotor, such as Sorvall with SS-34 rotor.

1. 2. Methods

1. Harvest rat or mouse pancreata, place immediately in ice-cold PBS, trim of fat and connective tissue, and wash with ice-cold isolation buffer to remove blood contamination. All operations should be done on ice, using ice-cold buffers and instruments. Isolated mitochondria should be kept on ice at all times. Mitochondria maintain their functional activity for 4-5 h.

2. Place pancreas in 5 ml of isolation buffer, mince tissue into small pieces with scissors and homogenize using 5 to 6 up-and-down strokes at 600 rpm. The tissue/buffer ratio should be 1:40 (vol/vol).

3. Centrifuge the homogenate for 10 min at 850 x g, 4^{o}C. Discard pellet, containing nuclei and unbroken cells. Centrifuge supernatant for 15 min at 6500 x g, 4^{o}C, to obtain mitochondrial pellet. Discard supernatant.

4. Resuspend pellet in the isolation buffer (1:40, vol/vol) and spin for 15 min at 6500 x g, 4^{o}C. This will remove broken mitochondria and reduce ER membranes.

5. Add 0.2 - 0.3 ml of mitochondria storage buffer to the pellet, re-suspend mitochondria by passing through a 200-μl pipet tip, transfer suspension into eppendorf tube

6. Determine the protein concentration using the Bradford protein assay (Bio-Rad Laboratories Inc., CA) with bovine serum as a standard.

2. Measurement of the respiration control ratio (RCR) of mitochondria

2.1. Materials

1. Buffer for mitochondrial function measurements (assay buffer): 250 mM sucrose, 22 mM KCl, 22 mM triethanolamine (pH 7.4), 3 mM $MgCl_2$, 5 mM KH_2PO_4. The major constituents in the assay buffer are an osmoticum, pH buffer and phosphate. Triethanolamine maintains pH and also neutralizes fatty acids, and solubilises oils and other ingredients that are not completely soluble in water.

2. Stock solutions: 1 M potassium succinate, 50 mM ADP, 1 mM CCCP.

3. 10- and 50-μl Hamilton syringes (Sigma).

4. Standard Oxygraph system (Hansatech Instruments, Norfolk, England) equipped with a Clark-type electrode disc, with stirring.

2.2. Methods

1. Add an aliquot of mitochondria suspension (final protein concentration 1 mg/ml) to the oxygraph system chamber containing assay buffer (1ml) supplemented with 10 mM potassium succinate.

2. Monitor oxygen consumption rate for 2 min, then add 150 μM ADP.

3. Add 1 μM CCCP to stimulate oxygen consumption.

4. Calculate the respiration control ratio (RCR) as described below and in the figure 1. When mitochondria are incubated in the presence of substrate, addition of ADP causes a sudden burst of oxygen uptake as the ADP is converted into ATP. The actively respiring state is referred to as "state 3" respiration, while the slower rate after all the ADP has been phosphorylated to form ATP is referred to as "state 4". State 4 respiration is usually faster than the original rate before the first addition of ADP because some ATP is broken down by ATPase activities contaminating the preparation, and the resulting ADP is then re-phosphorylated by the intact mitochondria. The ratio [state 3 rate] : [state 4 rate] is called the respiratory control ratio (RCR) and indicates the tightness of the coupling between respiration and phosphorylation. Addition of CCCP allows to determine the rate of uncoupled respiration that represents the maximal activity of the mitochondrial respiratory chain. The measurement of RCR for pancreatic mitochondria is illustrated in the figure 1. RCR value 3.6 indicates that mitochondria are functional with tight coupling between respiration and phosphorylation. RCR < than 3.0 indicates that mitochondria are damaged, uncoupled and should not be used for the experiments.

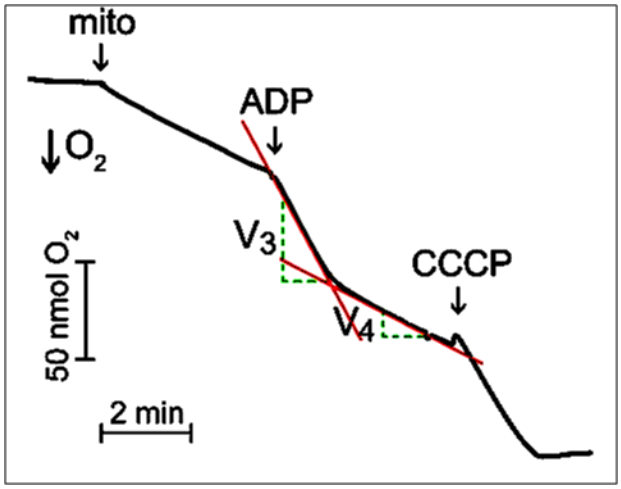

Figure 1. RCR measurement. Oxygen consumption rate was measured in suspension of isolated pancreatic mitochondria with Clark electrode. Arrows indicate additions of mitochondria (mito), 150uM ADP, and 1uM CCCP. RCR calculated as V3/V4 = 3.6.

3. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

3.1. Materials

1. All the materials are the same as described under 2.1 (1-3).

2. Thermostated 1.5 ml glass or plastic chamber.

3. Thermostat.

4. Magnetic stirrer, stir-bars.

5. TPP^+-sensitive electrode (for example, from Microelectrodes Inc., Bedford NH) or custom-made according to (5). (Note 3)

6. Commercially available reference electrode connected to an amplifier and chart recorder (World Precision Instruments Inc., Sarasota, Fl).

7. Dual-wavelength recording spectrofluorometer (for example, Shimadzu RF-1501) with 543 nm excitation and 578 nm emission with thermostated cuvette holder and stirring.

8. Stock solutions: 0.5mM TPP^+, 0.1 mM TMRM (Molecular Probes, Eugene, OR).

3.2. Methods

3.2.1. $\Delta\Psi m$ measurement with TPP^+-sensitive electrode

An ion selective tetraphenylphosphonium (TPP^+)-electrode is commonly applied to measure $\Delta\Psi m$. TPP^+ accumulates in the mitochondrial matrix as a function of the mitochondrial membrane potential. The signal, which is monitored by TPP^+-electrode, is linearly dependent on the logarithm of free [TPP^+]. TPP^+-electrode is calibrated by sequential additions of known amounts of TPP^+Cl^- before addition of mitochondria as illustrated in the figure 2. Isolated pancreatic mitochondria with RCR >3.5 accumulate ~ 1.5 µM TPP^+. The calculation of $\Delta\Psi m$ mV values from measurements with TPP^+-electrode is a difficult and far from settled topic, and usually is not performed.

1. Add assay buffer supplemented with 10 mM potassium succinate to the thermostated 1 ml glass or plastic chamber.

2. Add 1 µM TPP^+.

3. Add an aliquot of mitochondrial suspension (final protein concentration 1 mg/ml).

4. Start recording changes in $\Delta\Psi m$.

5. To cancel $\Delta\Psi m$ add 1µM CCCP in the end of each experiment.

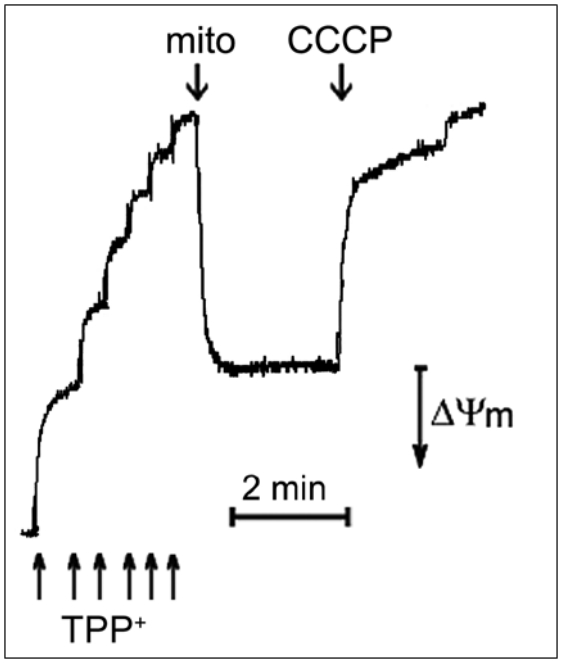

Figure 2. **Measurement of pancreatic mitochondria membrane potential with TPP^+-sensitive electrode.** Arrows indicate additions of mitochondria (mito), 0.03 µM TPP^+Cl^- (TPP^+) and 1 µM CCCP. TPP^+-sensitive electrode measures the level of TPP^+ in extramitochondrial medium. An increase in $\Delta\Psi m$ leads to accumulation TPP^+ within mitochondria and its decrease in extramitochondrial medium. Thus, decrease in TPP^+ values indicates an increase in $\Delta\Psi m$, and vice versa. TPP^+-sensitive electrode response was calibrated by 6 sequential additions of 0.03 µM TPP^+. Pancreatic mitochondria accumulated ~1.5 µM TPP^+.

3.2.2. ΔΨm using a spectrofluorometer with ΔΨm-sensitive fluorescent probe TMRM

Measurements of ΔΨm with low and high TMRM are based on different principles (5; 17). The low concentration mode relies on the Nernst distribution of the positively charged, fluorescent indicator between cytosol and mitochondria. Depolarization of mitochondria should result in redistribution of the indicator from the mitochondria to the cytosol, causing a decrease of mitochondrial fluorescence, whereas increase in ΔΨm results in an increase in TMRM fluorescence. Differently, when loaded at high concentrations, TMRM further concentrates in the negatively charged mitochondria forming aggregates with low fluorescence manifest in fluorescence quenching. Depolarization of the mitochondria results in release of the indicator from mitochondria to the cytosol, dequench of the indicator, and therefore an overall increase of the fluorescence. The measurement of pancreatic ΔΨm using dequench mode is illustrated in the figure 3. Of note, for pancreatic mitochondria high concentration dequench mode (≥ 0.5 μM TMRM) is much more sensitive than the low concentration mode to the changes in ΔΨm (17).

1. Add assay buffer supplemented with 10 mM potassium succinate to the thermostated 1 ml glass or plastic chamber.

2. Add an aliquot of mitochondrial suspension (final protein concentration 1 mg/ml).

3. Add 0.5 μM TMRM and monitor changes in the fluorescence (See Note below).

4. To cancel ΔΨm add 1μM CCCP in the end of each experiment.

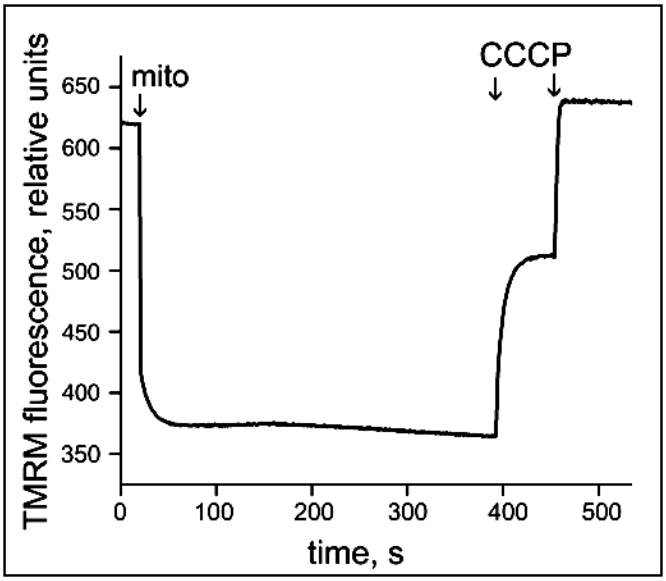

Figure 3. Measurement of pancreatic mitochondria membrane potential with ΔΨm-sensitive fluorescence dye TMRM using dequench mode. Arrows indicate additions of mitochondria (mito), and 1μM CCCP. TMRM accumulates within mitochondria along the membrane potential resulting in a fluorescence quench manifest in a decrease in the fluorescence intensity. CCCP cancels ΔΨm resulting in the distribution of TMRM to cytosol, and fluorescence dequench, which manifests in an increase in fluorescence intensity.

Notes

Note 1: Important ingredients in the isolation medium are major osmoticum (i.e., sucrose), buffer to keep the pH above 7.2 but below 8.0, and BSA. Sucrose was shown to preserve the chemical and morphological integrity of the mitochondria better than high potassium or sodium (12). BSA protects the mitochondria against injurious action of fatty acids during their isolation. Pancreatic mitochondria are very sensitive to Ca^{2+}-induced damage; therefore the presence of Ca^{2+}-chelator (EGTA) is necessary during pancreatic mitochondria isolation.

Note 2: Assessment of the purity of isolated pancreatic mitochondria with electron microscopy and immunoblot analysis indicate that in addition to mitochondria the preparation contains some ER and very few zymogen granules. Further purification of mitochondria preparations could be achieved with Percoll gradient centrifugation (7; 13), although it decreases the yield by 30-50%. In particular, Percoll purification procedure removes most of the ER (measured by the immunoblot of ER markers). Importantly, we found that functional parameters of pancreatic mitochondria, in particular, respiratory control, membrane potential and effects of Ca^{2+} were the same in crude and purified mitochondria preparations. Of note, mitochondrial assay buffer does not include ATP, and therefore ER is not functional. We additionally confirmed that mitochondrial characteristics are the same in the presence and absence of thapsigargin, an inhibitor of ER Ca^{2+}-ATPase.

Note 3: Be careful with handling TPP^+-electrode, it is fragile. At the end of each experiment, wash TPP^+-electrode with distilled water, then with 100 mM KCl solution, rinse with distilled water again and dry thoroughly. Between the experiments (1-3 days), keep TPP^+-electrode dry at room temperature. For longer storage, keep TPP^+-electrode in 1 mM of TPP^+ solution at room temperature (wash well with distilled water before use). TPP^+ electrode stored under proper conditions is stable for at least one year.

Acknowledgements

This work was supported by the US Veterans Administration Merit Review (A.S.G.) and the NIH grants R01DK59936 (A.S.G), the American Gastroenterological Association Foundation Designated Research Scholar Award in Pancreatitis (O.A.M.), the Southern California Research Center for Alcoholic Liver and Pancreatic Diseases (S.J.P. and A.S.G.), R01AA19730 (I.G. and O.A.M.) and the Russian Federation BR grant 09-04-00739 (I.V.O.).

References

1. **Frezza C, Cipolat S and Scorrano L.** Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts. Nature Protocols 2: 287-295, 2007. PMID: 17406588
2. **Gerasimenko J, Ferdek P, Fischer L, Gukovskaya AS and Pandol SJ.** Inhibitors of Bcl-2 protein family deplete ER Ca2+ stores in pancreatic acinar cells. Pflugers Arch 460: 891-900, 2010. PMID: 20617337
3. **Gukovskaya AS, Gukovsky I, Jung Y, Mouria M and Pandol SJ.** Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. Roles in cell injury processes of pancreatitis. J Biol Chem 277: 22595-22604, 2002. PMID: 11964411
4. **Gukovskaya AS and Gukovsky I.** Which Way to Die: the Regulations of Acinar Cell Death in Pancreatitis by Mitochondria, Calcium, and Reactive Oxygen Species. Gastroenterology 2011. PMID: 21524653

5. **Kamo N, Muratsugu M, Hongoh R and Kobatake Y.** Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 49: 105-121, 1979. PMID: 490631
6. **Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I and Gukovskaya AS.** Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. J Biol Chem 281: 3370-3381, 2006. PMID: 16339139
7. **Moreno-Sanchez R, Rodriguez-Enriquez S, Cuellar A and Corona N.** Modulation of 2-Oxoglutarate Dehydrogenase and Oxidative-Phosphorylation by Ca2+ in Pancreas and Adrenal-Cortex Mitochondria. Archives of Biochemistry and Biophysics 319: 432-444, 1995. PMID: 7786025
8. **Mukherjee R, Criddle DN, Gukovskaya A, Pandol S, Petersen OH and Sutton R.** Mitochondrial injury in pancreatitis. Cell Calcium 44: 14-23, 2008. PMID: 18207570
9. **Odinokova IV, Sung KF, Mareninova OA, Hermann K, Evtodienko Y, Andreyev A, Gukovsky I and Gukovskaya AS.** Mechanisms regulating cytochrome c release in pancreatic mitochondria. Gut 58: 431-442, 2009. PMID: 18596195
10. **Odinokova IV, Sung KF, Mareninova OA, Hermann K, Gukovsky I and Gukovskaya AS.** Mitochondrial mechanisms of death responses in pancreatitis. J Gastroenterol Hepatol 23 Suppl 1: S25-S30, 2008. PMID: 18336659
11. **Ohno I, Eibl G, Odinokova I, Edderkaoui M, Damoiseaux RD, Yazbec M, Abrol R, Goddard WA, III, Yokosuka O, Pandol SJ and Gukovskaya AS.** Rottlerin stimulates apoptosis in pancreatic cancer cells through interactions with proteins of the Bcl-2 family. Am J Physiol Gastrointest Liver Physiol 298: G63-G73, 2010. PMID: 19762431
12. **Siess EA.** Influence of Isolation Media on the Preservation of Mitochondrial Functions. Hoppe-Seylers Zeitschrift fur Physiologische Chemie 364: 279-289, 1983. PMID: 6862377
13. **Sims NR and Anderson MF.** Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nature Protocols 3: 1228-1239, 2008. PMID: 18600228
14. **Stahl WL, Smith JC, Napolitano LM and Basford RE.** Brain Mitochondria .1. Isolation of Bovine Brain Mitochondria. Journal of Cell Biology 19: 293-&, 1963. PMID: 14090748
15. **Sung KF, Odinokova IV, Mareninova OA, Rakonczay Z, Jr., Hegyi P, Pandol SJ, Gukovsky I and Gukovskaya AS.** Prosurvival Bcl-2 proteins stabilize pancreatic mitochondria and protect against necrosis in experimental pancreatitis. Exp Cell Res 315: 1975-1989, 2009. PMID: 19331832
16. **Vaquero EC, Edderkaoui M, Nam KJ, Gukovsky I, Pandol SJ and Gukovskaya AS.** Extracellular matrix proteins protect pancreatic cancer cells from death via mitochondrial and nonmitochondrial pathways. Gastroenterology 125: 1188-1202, 2003. PMID: 14517801
17. **Voronina SG, Barrow SL, Gerasimenko OV, Petersen OH and Tepikin AV.** Effects of secretagogues and bile acids on mitochondrial membrane potential of pancreatic acinar cells: comparison of different modes of evaluating DeltaPsim. J Biol Chem 279: 27327-27338, 2004. PMID: 15084611
18. **Wilson JS, Korsten MA and Lieber CS.** The isolation and properties of mitochondria from rat pancreas. Biochem Biophys Res Commun 121: 545-551, 1984. PMID: 6145416