Original Article

Pig Brain Mitochondria as a Biological Model for Study of Mitochondrial Respiration

(mitochondria / oxidative phosphorylation / respiratory state / respirometry)

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Abstract. Oxidative phosphorylation is a key process of intracellular energy transfer by which mitochondria produce ATP. Isolated mitochondria serve as a biological model for understanding the mitochondrial respiration control, effects of various biologically active substances, and pathophysiology of mitochondrial diseases. The aim of our study was to evaluate pig brain mitochondria as a proper biological model for investigation of activity of the mitochondrial electron transport chain. Oxygen consumption rates of isolated pig brain mitochondria were measured using high-resolution respirometry. Mitochondrial respiration of crude mitochondrial fraction, mitochondria purified in sucrose gradient, and mitochondria purified in Percoll gradient were assayed as a function of storage time. Oxygen flux and various mitochondrial respiratory control ratios were not changed within two days of mitochondria storage on ice. Leak respiration was found higher and Complex I-linked respiration lower in purified mitochondria compared to the crude mitochondrial fraction. Damage to both outer and inner mitochondrial membrane caused by the isolation procedure was the greatest after purification in a sucrose gradient. We confirmed that pig brain mitochondria can serve as a biological model for investigation of mitochondrial respiration. The advantage of this biological model is the stability of respiratory parameters for more than 48 h and the possibility to isolate large amounts of mitochondria from specific brain areas without the need to kill laboratory animals. We suggest the use of high-resolution respirometry of pig brain mitochondria for research of the neuroprotective effects and/or mitochondrial toxicity of new medical drugs.

Introduction

The current approach to studying the pathophysiology of many diseases, including neurodegenerative and mental disorders, involves mitochondrial dysfunctions and drug-induced mitochondrial effects. Mechanisms enabling production of cellular energy in the form of adenosine triphosphate (ATP) and its regulation by substrates, inhibitors, uncouplers, and various biologically active molecules are studied in both isolated mitochondria and intact or permeabilized cells (Gnaiger, 2014). The specific resting metabolic rates of major organs and tissues were suggested to be maximal for the heart, kidneys, brain, and liver (Wang et al., 2010). Cells or mitochondria from these tissues or from muscle cells are preferentially used in research of the role of bioenergetics in both normal and pathological physiological processes.

The respiratory rate of isolated mitochondria can be different from the respiratory rate of mitochondria in intact cells. On the other hand, this approach allows a defined change of the environment in which the mitochondria occur. It is possible to use substrates and inhibitors that do not cross the plasma membrane. With isolated mitochondria, respiratory steady states can be achieved, as defined and implemented by Chance and Williams (1955), or at least respiratory states very close to them.

The advantages of working with isolated mitochondria include: a relatively simple and well understood biological system; no interference from cytosolic fac-

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Abbreviations: ADP – adenosine diphosphate, ANTI – antimycin A, ATP – adenosine triphosphate, CMF – crude mitochondrial fraction, CS – citrate synthase, *CS* – citrate synthase activity, cyt *c* – cytochrome *c*, D – ADP, DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid), ETS – electron transfer system, FCCP – carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, FCR – flux control ratio, *LEAK* – non-phosphorylating resting state of intrinsic uncoupled or dyscoupled respiration, M – malate, MiR05 – mitochondrial respiration medium, O – oligomycin, OXPHOS – oxidative phosphorylation, P – pyruvate, PMP – mitochondria purified in Percoll gradient, PMS – mitochondria purified in sucrose gradient, RCR – mitochondrial respiratory control ratio, ROT – rotenone, *ROX* – residual oxygen consumption, S – succinate.

tors; easy to isolate from many tissues; substrates, inhibitors, and other reagents can be added directly (and enable control over experimental conditions); methods are well established; easy normalization to the protein content or mitochondrial enzyme activity. The disadvantages of working with isolated mitochondria include: lack of cellular content; damage and selection of mitochondria during the isolation; isolation from small or tough tissues can be problematic; the experimenter has to choose appropriate experimental conditions; existing methods often need large amounts of samples (Brand and Nicholls, 2011). It is apparent that utilization of pig brains eliminates or minimizes some conventional disadvantages such as the need for large amounts of samples, problematic isolation from small samples of tissues, and losses during the isolation.

The electron flow to oxygen through a cascade of mitochondrial respiratory complexes can be regulated by addition of specific substrates, inhibitors, and uncouplers. Many of these substances and other mitochondrial metabolites do not permeate freely through the biological membranes and require specific carriers. Thus, there is an abundance of mitochondrial targets that can be affected to regulate the respiration rate (Pesta and Gnaiger, 2012). There is the convergent electron input from Complex I, Complex II, glycerol-3-phosphate dehydrogenase complex, and electron transferring flavoprotein complex into Q-junction followed by cytochrome *c* (cyt *c*)-mediated electron transport from Complex III to Complex IV (Gnaiger, 2014). Thus, the currently used term "electron transport chain" should be understood as an "electron transfer system" (ETS), because processes of electron transfer are not arranged as a chain.

A sensitive measure of ETS functions is formation of ATP and the rate of oxygen consumption. Large changes in mitochondrial respiration are associated with rather small changes in proton-motive force. The rate of mitochondrial oxygen utilization is an accurate measure of the total mitochondrial proton current. These experiments can be designed to obtain information about the substrate transport into the cell, cytoplasmic metabolism, transport into mitochondria, mitochondrial metabolism, electron delivery to the respiratory chain, activities of ETS complexes, ATP synthesis, proton leak, etc. (Brand and Nicholls, 2011).

A straightforward indicator of the function of isolated mitochondria is their absolute respiration rate, normalized to total protein, citrate synthase (CS) activity, or cyt *c* oxidase activity. Mitochondrial respiratory control ratio (RCR) is the unique, the most useful general measure of the function in isolated mitochondria. RCR determined as the (State 3 rate)/(State 4 rate) ratio is a sensitive indicator of mitochondrial dysfunctions (Brand and Nicholls, 2011). A high RCR implies that the mitochondria have a high capacity for substrate oxidation and ATP turnover and low proton leak. Low RCR usually indicates dysfunction. In isolated mitochondria the coupling is partially disturbed, probably as a result of mechanical damage during the isolation of mitochondria.

Biological models for the study of mitochondrial function

Changes in the mitochondrial function, and therefore changes in the cellular energy metabolism, have long been studied in isolated mitochondria and intact or permeabilized cells and tissues. Muscle cells obtained by biopsy (Rasmussen and Rasmussen, 2000), blood elements such as platelets or lymphocytes (Hroudová et al., 2013; Sjövall et al., 2013; Kang et al., 2014), or cultured cells (Petit et al., 2005) are used most commonly for studying the mitochondrial function and dysfunction in humans. Unlike in immunological research, in bioenergetic studies, there is relatively little use of samples from larger mammals such as pig and cattle. Brain or liver mitochondria isolated from rodent tissue are usually used as a biological model; however, biopsies of pig liver have also been used (Kuznetsov et al., 2002). Given the advantage of isolating sufficient amounts of mitochondria from selected brain structures, and limiting the use of animals for experimental purposes, we chose isolated mitochondria from the brain of slaughtered pigs as a biological model for studying the bioenergetic changes induced by administration of various biologically active substances (Hroudová and Fišar, 2012; Fišar et al., 2014; Singh et al., 2015).

High-quality preparation of a mitochondria-enriched fraction from the tissue homogenate may represent an optimum compromise for a variety of respirometric, spectrophotometric and fluorometric studies. Differential centrifugation guarantees removal of whole cells or nuclei and minimizes possible contamination of the crude mitochondrial fraction (CMF) by microsomes, plasma membranes, lysosomes and cytosol. However, synaptosomes and other contaminants are present in CMF (Brunner and Bygrave, 1969; Whittaker, 1969; Wieckowski et al., 2009). Additional purification using gradient centrifugation could reduce the content of contaminants, but it can affect the functionality of purified mitochondria (Fernández-Vizarra et al., 2002).

Oxidative phosphorylation (OXPHOS) is ensured by function of enzymes and respiratory complexes in unbroken mitochondria. Cyt *c* does not pass the intact outer mitochondrial membrane; damage to the outer mitochondrial membrane may result in subsequent loss of cyt *c* and decrease of the respiration rate. A cyt *c* test (stimulation of respiration after addition of cyt *c*) could be applied to evaluate the intactness of the outer mitochondrial membrane in mitochondrial preparations (Kuznetsov et al., 2004). The cyt *c* test was used to evaluate the disturbance of ETS function by both the technique of mitochondria preparation and sample preservation for a prolonged period.

The inner mitochondrial membrane contains key components of OXPHOS; therefore, its integrity is crucial for mitochondrial respiration. CS is an enzyme localized in the mitochondrial matrix that is commonly used as a quantitative enzyme marker for the presence of intact mitochondria (Kuznetsov et al., 2002). Damage

to the inner mitochondrial membrane (membrane permeability) can be evaluated by measurement of changes in specific activity of matrix enzyme CS in the absence or presence of Triton X-100 detergent (Cafè et al., 1994; Niklas et al., 2011).

In our study, we tested the use of mitochondria from pig brains as a model for studying the *in vitro* effects of various biologically active substances on the cellular respiration. The results were analysed of respirometric measurements with mitochondria isolated by three different techniques. To determine the influence of the protocol for isolation of pig brain mitochondria and the effect of the preservation period, we compared Complex I+II-linked respiration of (1) mitochondria isolated by the standard differential centrifugation technique (Whittaker, 1969; Fišar, 2010) referred to as crude mitochondrial fraction (CMF), (2) mitochondria purified in sucrose gradient (PMS) (Whittaker 1969; Pinna et al., 2003), and (3) mitochondria purified in Percoll gradient (PMP) (Graham, 2001). The damage to the outer mitochondrial membrane was evaluated using the cyt *c* test and the damage to the inner mitochondrial membrane was assayed by CS activity, all measured at various times after isolation of mitochondria.

Material and Methods

Media and chemicals

Buffered sucrose (sucrose 0.32 M, HEPES 4 mM, pH 7.4) was used both as isolation medium in mitochondria preparation and as preservation medium. The mitochondrial respiration medium (MiR05) consisted of sucrose 110 mM, K^+-lactobionate 60 mM, taurine 20 mM, $MgCl_2.6H_2O$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA essentially fatty acid free 1 g·l^{-1} and HEPES 20 mM, adjusted to pH 7.1 with KOH (Gnaiger et al., 2000).

Stock solutions of substrates, inhibitors, and uncouplers are listed in Table 1, with the volume added (using Hamilton syringes) to the measuring 2-ml chamber; the corresponding final concentration is calculated. Pyruvate was prepared freshly; other stock solutions were stored at –20 °C. Adenosine diphosphate (ADP) was dissolved in water solution of 0.3 M $MgCl_2$. The chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Pig brain mitochondria preparation

The crude mitochondrial fraction and mitochondria purified in sucrose gradient were prepared as described previously (Whittaker, 1969). Standard protocols were used for purification of mitochondria in sucrose gradient (Pinna et al., 2003) or Percoll gradient (Graham, 2001). In many prior publications, electron microscopy, immunochemical, and proteomic methods were performed to analyse the quality (purity and integrity) of the mitochondrial preparations, including the content of marker mitochondrial, peroxisomal, lysosomal, endoplasmic reticulum, and cytosolic proteins (Almeida and Medina, 1998; Rajapakse et al., 2001; Franko et al., 2013; Schmitt et al., 2013). Contaminants were not quantified in the present study because they do not interfere with the rate of oxygen consumption under our experimental conditions, when mitochondrial respiration is regulated by specific substrates, inhibitors, and uncouplers of OXPHOS.

Crude mitochondrial fraction (CMF)

Pig brains were obtained from a slaughterhouse within 1 h after CO_2 stunning of animals and killing them by bleeding to death. Brains were transported in ice-cold saline; further procedures, all solutions and equipment were kept on ice or cooled to 2–4 °C. The brain cortex was separated without cerebellum, brain stem, and most of the midbrain. The brain cortex was gently homogenized in ten volumes (w/v) of ice-cold buffered sucrose 0.32 M, supplemented with aprotinin 10 μg·ml^{-1}, by means of a glass homogenizer with rotary Teflon piston (10 up and down strokes at 840 rpm). The homogenate was centrifuged at 1,000 *g* for 10 min to remove unbroken cells, nuclei and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions (1,000 g for 10 min). Supernatants were collected and centrifuged at 10,000 *g* for 15 min. The pellet was

Table 1. Respirometry protocol for evaluation of sample quality

Order	Substance	Abbr.	Stock conc. (solvent)	Volume added (μl)	Final concentration
1	Mitochondria	CMF PMS PMP			0.05-0.10 mg·ml^{-1}
2	Malate	M	0.8 M (H_2O)	5	2 mM
3	Pyruvate	P	2 M (H_2O)	5	5 mM
4	ADP	D	0.5 M (H_2O)	4	1 mM
5	Succinate	S	1 M (H_2O)	20	10 mM
6	Cytochrome *c*	cyt *c*	4 mM (H_2O)	5	10 μM
7	Oligomycin	O	4 mg·ml^{-1} (EtOH)	1	2 μg·ml^{-1}
8	FCCP	FCCP	1 mM (EtOH)	titration: 1–4	0.5–2.0 μM
9	Rotenone	ROT	1 mM (EtOH)	1	0.5 μM
10	Antimycin A	ANTI	0.5 mg·ml^{-1} (EtOH)	5	1.25 μg·ml^{-1}

washed twice with buffered sucrose (10,000 *g*, 15 min), resuspended to a protein concentration of 20–40 mg·ml^{-1} (final volume 10–15 ml for the initial 30 g of brain cortex) and stored on ice until the assay.

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Mitochondria purified in sucrose gradient (PMS)

CMF, prepared as described above, was diluted ten times by buffered sucrose 0.32 M and carefully layered (9 : 4, v : v) on cooled sucrose 1.2 M (pH 7.4); e.g. 22.5 ml of diluted CMF per 10 ml sucrose 1.2 M. Following centrifugation at 126,000 *g* for 45 min at 4 °C, purified mitochondria were obtained as a sediment (Pinna et al., 2003). The pellet was washed with buffered sucrose 0.32 M (17,000 *g*, 10 min) to restore isosmotic milieu, resuspended to a protein concentration of 20–40 mg·ml^{-1}, and stored on ice until the assay.

Mitochondria purified in Percoll gradient (PMP)

CMF was resuspended in 15% Percoll solution (diluted ten times by 16.7% Percoll in buffered sucrose 0.32 M). Ten ml each of 40% and 23% Percoll solution in sucrose 0.32 M was layered in a centrifugation tube and 7.5 ml of mitochondrial suspension in 15% Percoll was layered on top. Following centrifugation at 31,000 *g* for 20 min at 4 °C, purified mitochondria were harvested from the band at the lowest interface, diluted with five volumes of ice-cold buffered sucrose 0.32 M, and centrifuged at 17,000 *g* for 10 min (Graham, 2001). Mitochondrial pellet was resuspended in buffered sucrose at about 10–20 mg protein per ml and stored on ice until the assay.

High-resolution respirometry

Mitochondrial respirometry provides indirect quantification of the cellular metabolism rate by measuring the oxygen consumption by mitochondria. From the rate of the oxygen decline in the sample, which is placed in a closed metabolic chamber with an oxygen sensor, the respiratory rate of the mitochondria can be determined.

High-resolution respirometry measures electron transfer through ETS as oxygen consumption in a sample containing mitochondria. The OROBOROS Oxygraph-2k (O2k; OROBOROS INSTRUMENTS Corp., Innsbruck, Austria) was used. Samples in a volume of 2 ml were measured in two glass chambers equipped with Clark polarographic oxygen electrodes. DatLab software (OROBOROS INSTRUMENTS Corp.) enabled us to obtain on-line respiratory rate. Integrated electronic control includes Peltier temperature regulation, stirrer control, and an electronic barometric pressure transducer for air calibration.

Common experimental conditions include physiological temperature 37 °C, stirring at 750 rpm, closed-chamber mode of operation, the oxygen solubility factor of the mitochondrial respiration medium MiR05 0.92, calibration of the polarographic oxygen sensor before each measurement, and periodic measurement of instrumental background oxygen consumption. In our experimental approach, we proceeded mainly from previously published protocols for measuring the respiratory rate of isolated mitochondria (Pesta and Gnaiger, 2012; Gnaiger, 2014). Table 1 summarizes the experimental protocol used for the measurement.

Citrate synthase activity

Citrate synthase (CS; EC 2.3.3.1) activity (*CS*) was determined spectrophotometrically according to Srere et al. (1963) with some modifications (Kojima et al., 1995; Raisch and Elpeleg, 2007). The enzyme activity was assayed by measuring the increase in absorption at 412 nm due to the reaction of coenzyme A (formed from acetyl-CoA and oxaloacetate in the presence of CS) with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB). The reaction mixture consisted of DTNB 0.1 mM, acetyl CoA 0.3 mM, mitochondrial sample 3–7 μg protein·ml^{-1}, and oxaloacetate 0.5 mM, all in MiR05 medium without BSA adjusted to pH 8.1 (Kojima et al., 1995). The assay was performed at 37 °C and started by oxaloacetate; the initial rate of reaction was measured for 3 min.

The optimal procedure for permeabilization of the inner mitochondrial membrane by Triton X-100 was used according to Niklas et al. (2011); mitochondria were incubated at 37 °C for 5 min with 0.05% (v/v) Triton X-100 before measurement of CS activity. The integrity of the inner mitochondrial membrane was measured by CS activity, both in the absence or presence of Triton X-100; the enzyme activity induced by Triton X-100 (in ruptured mitochondria) was taken as reference and set to 100 % (Camilleri et al., 2013).

Data analysis

The oxygen consumption rate (respiration rate) is proportional to the negative slope of oxygen concentration in a closed metabolic chamber with time. Mass-specific oxygen flux (pmol·s^{-1}·mg^{-1}) was calculated as the oxygen consumption rate (in pmol O_2 per 1 sec) per 1 mg of total protein in the sample. Specific activity of CS was expressed as the activity that catalyses formation of 1 μmol of citrate per min per mg of total protein at 37 °C. Mitochondrial marker-specific respiration (pmol O_2 per 1 μmol of citrate) was obtained by normalization of oxygen flux relative to *CS*. The following terms were used to characterize the respiratory states (Brand and Nicholls, 2011; Gnaiger, 2014): *OXPHOS capacity* (mitochondrial respiratory capacity at saturating concentrations of ADP, inorganic phosphate, oxygen, and defined substrate supply; *State 3*), *ETS capacity* (the respiratory electron transfer system capacity of mitochondria in the experimentally induced non-coupled state; *State 3u*; it represents an internal functional mitochondrial marker), residual oxygen consumption (*ROX*, the respiration due to oxidative side reactions in mitochondria incubated with substrates after application of ETS inhibitors), *LEAK* respiration (the respiration af-

ter inhibition of ATP synthase by oligomycin; *State 4o*), respiratory control ratio (*RCR*, a ratio between *State 3* and *State 4o* of respiration), and flux control ratio (*FCR*, respiratory rate expressed relative to *ETS capacity* as a common respiratory reference state).

DatLab software was used for respirometry data acquisition. Statistical analysis was performed using the STATISTICA data analysis software system (StatSoft, Inc., Tulsa, OK). Analysis of variance (ANOVA) and Scheffé or Dunnett post-hoc comparison between means was used.

Results

Isolated mitochondria (CMF, PMS, PMP) were preserved in isolation medium (buffered sucrose 0.32 M) on ice. The effect of the method of preparation and storage period on mitochondrial respiration was tested by high-resolution respirometry (Table 1). Complex I-linked respiration (*CI respiration*) was stimulated by addition of malate 2 mM, pyruvate 5 mM, and ADP 1 mM to the sample in respiration medium MiR05. To determine Complex I+II-linked respiration (*OXPHOS capacity low*), succinate 10 mM was added. Damage to the outer mitochondrial membrane leading to loss of cyt *c* was determined as the increase of Complex I+II-linked respiration rate after addition of cyt *c* 10 μM (*OXPHOS capacity*, *State 3*). *LEAK* respiration (*State 4o*) was measured after inhibition of the phosphorylation system by oligomycin 2 μg·ml^{-1}. *ETS capacity* (*State 3u*) was estimated by titration with uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). *CI inhibition* represents the respiration after Complex I inhibition by rotenone 0.5 μM. Finally, residual oxygen consumption (*ROX*) was measured after addition of antimycin A 1.25 μg·ml^{-1} to be subtracted from all other values. Illustrative OROBOROS Oxygraph-2k runs are shown in Fig. 1 for respiration of isolated pig brain mitochondria (CMF) 1 h after mitochondria isolation (Fig. 1A) and 53 h after mitochondria isolation (Fig. 1B).

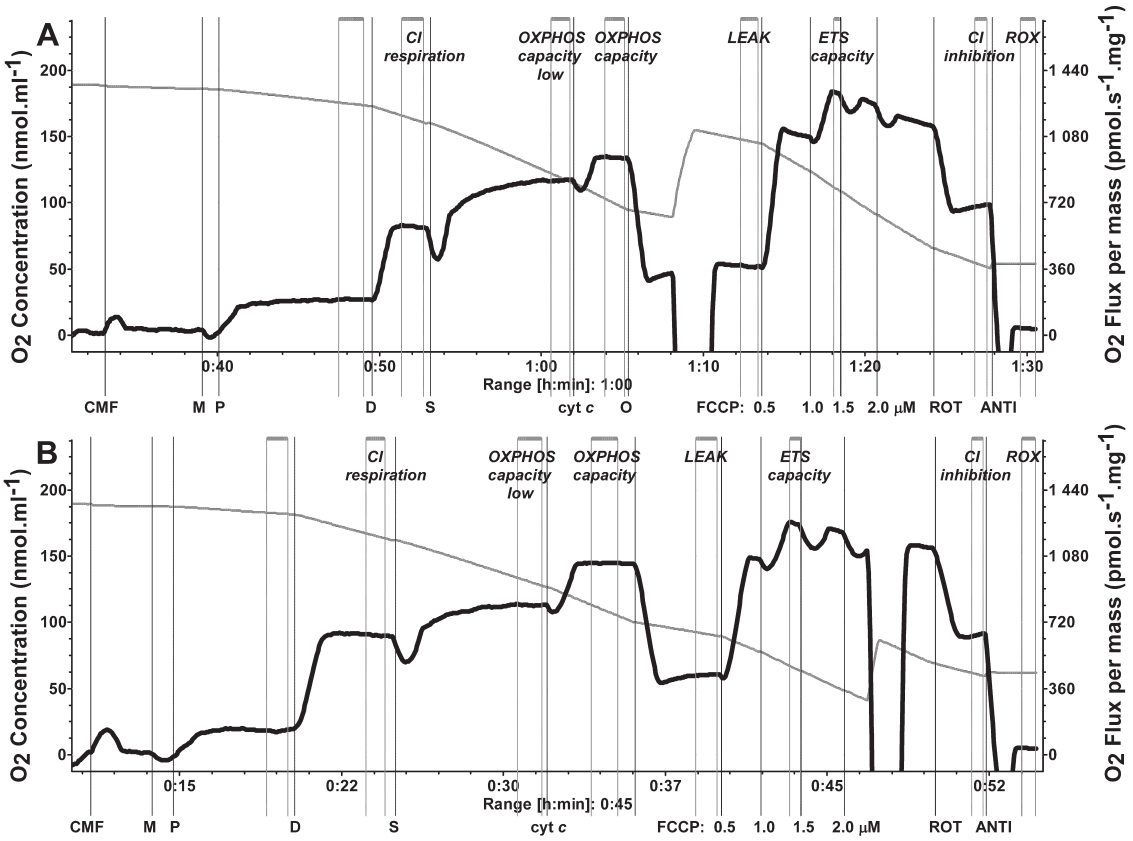

Fig. 1. Illustrative Oroboros Oxygraph-2k runs for respiration of isolated pig brain mitochondria (crude mitochondrial fraction, CMF) at various times after isolation of mitochondria: (**A**) 1 h after mitochondria isolation, (**B**) 53 h after mitochondria isolation. Samples containing mitochondria at a concentration of 0.10 mg protein·ml^{-1} were continuously stirred and incubated at 37 °C in respiration medium MiR05. Complex I-linked respiration (*CI respiration*) was measured at saturating malate (M), pyruvate (P), and adenosine diphosphate (D) concentrations. Complex I+II-linked respiration (*OXPHOS capacity low*) was induced by succinate (S). Damage to the outer mitochondrial membrane leading to loss of cytochrome *c* (cyt *c*) was determined as the increase of respiration rate after addition of cyt *c* (*OXPHOS capacity*). *LEAK* respiration was measured after inhibition of the phosphorylation system by oligomycin (O). *ETS capacity* was estimated by FCCP (uncoupler) titrations. Respiration after Complex I inhibition by rotenone (ROT) was determined (*CI inhibition*) and residual oxygen consumption (*ROX*) was measured after addition of antimycin A (ANTI).
Grey line: oxygen concentration (nmol·ml^{-1}); black line: oxygen flux per mass (pmol·s^{-1}·mg^{-1}).

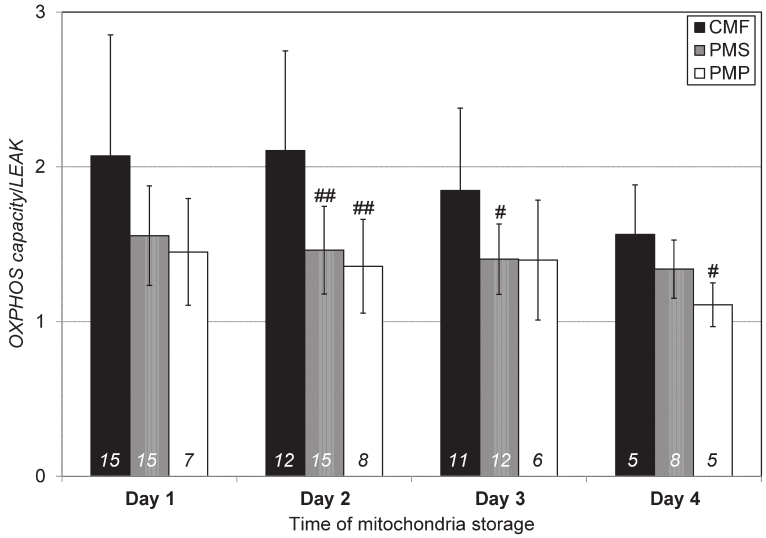

Fig. 2. Respiratory control ratio in crude mitochondrial fraction (CMF), mitochondria purified in sucrose gradient (PMS), and mitochondria purified in Percoll gradient (PMP) during long-time storage of samples. To determine *OXPHOS capacity* (*State 3*) in Complex I+II-linked respiration, malate, pyruvate, ADP, succinate and cytochrome *c* were added to the mitochondria (Table 1). *LEAK* respiration (*State 4o*) was measured after addition of oligomycin. Mean ± standard deviation is shown; the number of experiments is indicated in each column. The differences between means were evaluated using ANOVA and Dunnett or Scheffé post-hoc test. No significant differences were found when comparing respiratory control ratios in the same mitochondrial sample on Day 1 with Day 2, 3, or 4. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, when compared with CMF at the same time point.

Respiratory control ratio

Crude (CMF) and purified (PMS, PMP) mitochondria were repeatedly measured during several days using the same experimental protocol (Table 1). Respiratory control ratio (RCR) was calculated as a ratio between *OXPHOS capacity* (*State 3*) and oligomycin-induced *LEAK* state (*State 4o*) of respiration for Complex I+II-linked respiration. Figure 2 summarizes RCRs in all three mitochondrial samples measured at various time points after isolation of mitochondria. Day 1 represents 1–6 h, Day 2 18–29 h, Day 3 42–53 h, and Day 4 62–74 h after mitochondria isolation. We observed a slight decrease of RCR with time of the sample storage. Nonsignificant negative correlation coefficients between RCR and hours after mitochondria isolation were found for CMF ($r = –0.22$, $P = 0.154$, $N = 43$), PMS ($r = –0.27$, $P = 0.055$, $N = 50$), and PMP ($r = –0.33$, $P = 0.096$, $N = 26$). In accordance with this we did not find any significant change when comparing RCR on the day of mitochondria isolation (Day 1) with RCR determined on Day 2, 3, or 4.

RCR in purified mitochondria (PMS, PMP) was found lower compared to RCR in crude mitochondria (CFM). No significant differences were found between PMS and PMP at the same time point.

Oxygen flux normalized to citrate synthase activity

Specific CS activity was found to be 1252 ± 90 μmol·min^{-1}·mg^{-1} (mean ± SD, N = 9) in CMF, 2090 ± 287 μmol·min^{-1}·mg^{-1} (mean ± SD, N = 8) in PMS, and 1338 ± 177 μmol·min^{-1}·mg^{-1} (mean ± SD, N = 10) in PMP. Oxygen flux normalized relative to *CS* (Table 2) and respiration FCR normalized to *ETS capacity* (Table 3) were used to evaluate both damage to the outer mitochondrial membrane during sample preparation and sample stability for long time periods.

We observed a decrease of Complex I-linked respiration (*CI respiration*) with the time of storage of all three mitochondrial samples (Table 2). Significant negative correlation coefficients were calculated for PMS ($r = –0.47$, $P = 0.001$, $N = 50$) and PMP ($r = –0.56$, $P = 0.004$, $N = 24$), but not for CMF ($r = –0.22$, $P = 0.161$, $N = 43$). Compared to Day 1, a significant decrease occurred in PMS on Days 3 and 4, in PMP on Day 4 only. Changes of other investigated parameters (*OXPHOS capacity*, *ETS capacity*, *LEAK*, *CI inhibition*) related to the time of sample storage were not significantly changed.

Sample preparation significantly affected the investigated parameters of mitochondrial respiration (Table 2). Compared to CMF, *CI respiration* was significantly lower both in PMS (on Days 1, 2, and 3) and PMP (on Days 2 and 4), *OXPHOS capacity low* was lower in PMS (on Days 1 and 2) but not in PMP, the full *OXPHOS capacity* was not different for both PMS and PMP, *LEAK* respiration was increased in PMS (on Days 1 and 2) but not in PMP, *ETS capacity* was significantly decreased in PMP (on Days 1 and 2) but not in PMS, and *CI inhibition* was not different for both PMS and PMP. When comparing PMS and PMP samples, we found significantly higher *OXPHOS capacity* (on Days 1, 2, and 3), *ETS capacity* (on Days 1 and 2), and *CI inhibition* (on Days 1 and 2) in PMS.

Table 2. Mean values of oxygen flux normalized relative to citrate synthase activity at various time points after mitochondria isolation

Sample	Time	*CI respiration/ CS*	N	*OXPHOS capacity low/CS*	N	*OXPHOS capacity/CS*	N	*LEAK/CS*	N	*ETS capacity/ CS*	N	*CI inhibition/ CS*	N
CMF	Day 1	36.4 ± 19.1	15	59.6 ± 15.9	15	79.2 ± 22.5	15	44.4 ± 21.4	15	92.2 ± 22.9	15	57.4 ± 20.6	10
	Day 2	36.5 ± 20.8	12	55.2 ± 18.9	12	78.2 ± 23.9	12	41.3 ± 19.7	12	88.8 ± 23.7	12	56.2 ± 20.2	9
	Day 3	28.4 ± 16.7	11	47.5 ± 16.6	11	72.8 ± 24.6	11	43.5 ± 21.4	11	80.0 ± 25.9	11	54.9 ± 24.6	9
	Day 4	26.1 ± 14.9	5	50.9 ± 18.2	5	80.1 ± 24.0	5	51.1 ± 13.6	5	82.2 ± 22.4	5	73.2 ± 17.9	4
PMS	Day 1	$^{\#}$21.1 ± 7.8	15	$^{\#\#}$43.9 ± 7.3	15	$^{\$}$95.0 ± 17.0	15	$^{\#}$62.4 ± 11.1	15	$^{\$}$91.7 ± 16.6	15	$^{\$}$77.6 ± 15.5	10
	Day 2	$^{\#\#}$16.3 ± 8.6	15	$^{\#}$38.5 ± 13.6	15	$^{\$}$90.2 ± 25.0	15	$^{\#}$64.0 ± 24.0	15	$^{\$}$86.5 ± 22.8	15	$^{\$}$76.3 ± 25.1	12
	Day 3	$^{\#}$**12.1 ± 5.4	12	35.5 ± 8.8	12	$^{\$}$84.7 ± 20.7	12	61.9 ± 17.8	12	78.9 ± 21.3	12	67.1 ± 20.5	10
	Day 4	**11.3 ± 4.8	8	40.0 ± 13.8	8	89.5 ± 31.4	8	69.4 ± 31.3	8	80.1 ± 27.4	8	67.9 ± 29.6	7
PMP	Day 1	29.8 ± 16.2	7	51.7 ± 15.3	7	70.3 ± 16.3	7	49.1 ± 8.9	7	$^{\#}$67.0 ± 18.7	7	52.4 ± 18.4	7
	Day 2	$^{\#}$17.8 ± 10.9	8	44.1 ± 9.9	8	63.9 ± 12.3	8	48.7 ± 14.0	8	$^{\#}$61.1 ± 15.8	8	48.8 ± 16.6	8
	Day 3	12.4 ± 11.4	4	42.6 ± 11.5	6	56.3 ± 13.3	6	44.5 ± 20.0	6	52.2 ± 17.3	6	41.2 ± 20.5	6
	Day 4	$^{\#}$*9.2 ± 10.3	5	46.0 ± 5.0	5	59.5 ± 5.8	5	54.6 ± 9.7	5	56.8 ± 7.4	5	52.0 ± 9.6	5

Respiration FCRs in CMF, PMS, and PMP were normalized relative to CS activity of isolated pig brain mitochondria at different storage times. Mean of N measurements ± standard deviation is shown. The differences between means were evaluated using ANOVA and Dunnett or Scheffé post-hoc test; *P < 0.05, **P < 0.01 when compared with Day 1 of the same mitochondrial sample; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 when compared with CMF at the same time point; $^{\$}$P < 0.05 for comparison between PMS and PMP at the same time point.

Table 3. Mean values of respiration flux control ratios at various time points after mitochondria isolation

Sample	Time	*CI respiration/ ETS capacity*	N	*OXPHOS capacity low/ETS capacity*	N	*OXPHOS capacity/ETS capacity*	N	*LEAK/ETS capacity*	N	*CI inhibition/ ETS capacity*	N	*OXPHOS capacity/ OXPHOS capacity low*	N
CMF	Day 1	0.393 ± 0.157	15	0.647 ± 0.044	15	0.852 ± 0.054	15	0.490 ± 0.229	15	0.587 ± 0.149	10	1.323 ± 0.130	15
	Day 2	0.402 ± 0.156	12	0.616 ± 0.076	12	0.872 ± 0.057	12	0.466 ± 0.187	12	0.603 ± 0.141	9	1.438 ± 0.221	12
	Day 3	0.362 ± 0.175	11	0.596 ± 0.084	11	0.904 ± 0.090	11	0.536 ± 0.182	11	0.659 ± 0.129	9	*1.544 ± 0.269	11
	Day 4	0.305 ± 0.163	5	0.613 ± 0.143	5	**0.966 ± 0.100	5	0.632 ± 0.091	5	*0.794 ± 0.122	4	*1.619 ± 0.255	5
PMS	Day 1	$^{\$\$\#\#}$0.228 ± 0.072	15	$^{\$\$\$\#\#\#}$0.484 ± 0.064	15	$^{\#\#\#}$1.037 ± 0.033	15	$^{\#}$0.701 ± 0.179	15	$^{\#\#}$0.778 ± 0.082	10	$^{\$\$\$\#\#\#}$2.175 ± 0.260	15
	Day 2	$^{\#\#\#}$0.188 ± 0.091	15	$^{\$\$\$\#\#\#}$0.439 ± 0.096	15	$^{\#\#\#}$1.040 ± 0.089	15	$^{\#\#}$0.738 ± 0.164	15	$^{\#\#\#}$0.829 ± 0.079	12	$^{\$\$\$\#\#\#}$2.474 ± 0.547	15
	Day 3	$^{\#\#}$*0.154 ± 0.052	12	$^{\$\$\$\#\#}$0.457 ± 0.081	12	$^{\#\#\#}$1.079 ± 0.063	12	$^{\#\#}$0.791 ± 0.153	12	$^{\#}$0.842 ± 0.054	10	$^{\$\$\$\#\#\#}$2.420 ± 0.359	12
	Day 4	*0.151 ± 0.070	8	$^{\$\$\$}$0.501 ± 0.042	8	$^{\#}$*1.118 ± 0.082	8	$^{\#\#}$0.848 ± 0.113	8	0.802 ± 0.142	7	$^{\$\$\$\#\#}$2.249 ± 0.306	8
PMP	Day 1	0.434 ± 0.159	7	$^{\#\#}$0.778 ± 0.126	7	$^{\#\#\#}$1.066 ± 0.097	7	$^{\#}$0.775 ± 0.204	7	$^{\#}$0.771 ± 0.098	7	1.387 ± 0.163	7
	Day 2	0.295 ± 0.151	8	$^{\#}$0.729 ± 0.059	8	$^{\#\#\#}$1.066 ± 0.118	8	$^{\#\#\#}$0.814 ± 0.164	8	$^{\#\#}$0.791 ± 0.115	8	1.465 ± 0.144	8
	Day 3	0.211 ± 0.200	4	$^{\#\#\#}$0.831 ± 0.074	6	$^{\#\#\#}$1.110 ± 0.120	6	$^{\#\#}$0.830 ± 0.171	6	0.754 ± 0.171	6	1.337 ± 0.104	6
	Day 4	*0.175 ± 0.217	5	$^{\#}$0.815 ± 0.081	5	1.052 ± 0.044	5	$^{\#\#\#}$0.957 ± 0.080	5	0.912 ± 0.085	5	1.298 ± 0.106	5

Respiration FCRs in CMF, PMS and PMP were normalized relative to *ETS capacity* of isolated pig brain mitochondria at different storage times. The ratio *OXPHOS capacity/OXPHOS capacity low* reflects the damage to the outer mitochondrial membrane. Mean of N measurements ± standard deviation is shown. The differences between means were evaluated using ANOVA and Dunnett or Scheffé post-hoc test; *P < 0.05, **P < 0.01 when compared with Day 1 of the same mitochondrial sample; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 when compared with CMF at the same time point; $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001 for comparison between PMS and PMP at the same time point.

Flux control ratio normalized to ETS capacity

The respiratory rate expressed relative to *ETS capacity* (FCR) was found more sensitive to changes caused by both sample storage and technique of mitochondrial isolation. Compared to Day 1, we observed significantly lower *CI respiration/ETS capacity* in PMS on Days 3 and 4; significantly higher values were found for *OXPHOS capacity/ETS capacity* in CMF and PMS on Day 4 and for *CI inhibition/ETS capacity* in CMF on Day 4. Significant positive correlation with time was observed for the ratio *OXPHOS capacity/OXPHOS capacity low* in CMF (r = 0.43, P = 0.004, N = 43), but not in PMS and PMP. The ratio *OXPHOS capacity/OXPHOS capacity low* in CMF was significantly higher on Days 3 and 4 compared to Day 1 (Table 3).

The ratio *CI respiration/ETS capacity* was significantly lower in PMS compared to CMF (Table 3). Ratios *OXPHOS capacity/ETS capacity*, *LEAK/ETS capacity*, and *CI inhibition/ETS capacity* were significantly lower in CMF compared to both PMS and PMP. The ratio *OXPHOS capacity low/ETS capacity* as well as *OXPHOS capacity/OXPHOS capacity low* was significantly higher

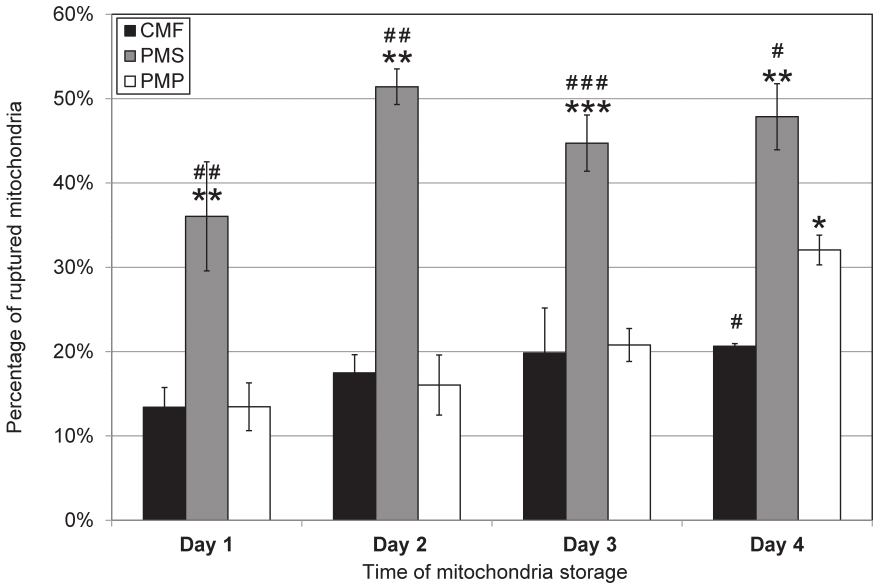

Fig. 3. Damage to the inner mitochondrial membrane (percentage of ruptured mitochondria) determined as a ratio between citrate synthase activities before and after treatment with 0.05% Triton X-100 at various time points after isolation of mitochondria. Crude mitochondrial fraction (CMF), mitochondria purified in sucrose gradient (PMS), and mitochondria purified in Percoll gradient (PMP) were compared using ANOVA and post-hoc Scheffé test. Mean ± standard deviation is shown (N = 3–4). Significantly different mean values are marked by * when compared with CMF or # when compared with PMP; *P < 0.05, **P < 0.01, ***P < 0.001, #P < 0.05, ##P < 0.01, ###P < 0.001.

in PMS compared to both CMF and PMP. PMS showed less Complex I-linked respiration compared PMP, especially shortly after isolation (on Day 1).

Damage to the inner mitochondrial membrane

Damage to the inner mitochondrial membrane was characterized as a ratio of CS activity in the mitochondrial sample stored at 2 °C for various time periods to CS activity in the same sample after membrane permeabilization by 0.05% Triton X-100 (Fig. 3). Significantly higher damage to the inner mitochondrial membrane was found in PMS compared with both CMF and PMP. CMF and PMP did not show significant differences, except for higher damage to PMP on Day 4 (P = 0.042). We did not observe a significant change in the damage to the inner mitochondrial membrane with time, except for PMP with increased relative CS activity on Day 4 compared to Day 1 (P = 0.00056), Day 2 (P = 0.0031) and Day 3 (P = 0.0091).

Discussion

Mitochondrial experiments usually require minimum storage time of the samples and accurate isolation procedures to preserve the structure and function of mitochondria. It has been reported that preservation of isolated mitochondria could be significantly prolonged in respirometric assays by cold and proper mitochondrial preservation solution (Gnaiger et al., 2000). Our study was aimed to select a suitable biological model for measuring the influence of drugs on mitochondrial function, particularly the ETS activity.

We compared oxygen flux normalized to CS activity and respiration flux control ratios in the crude mitochondrial fraction (CMF) and mitochondria purified in sucrose gradient (PMS) or Percoll gradient (PMP) during long-term storage of samples on ice. Our results indicate that impurities do not significantly interfere with the mitochondrial respirometry analyses. However, contaminants can contribute to the overall protein content. In addition, CMF contains synaptosomes, which comprise mitochondria. In previous experiments, we found that permeabilization of plasma membranes by digitonin increased the respiration rate above 40 % for CMF, contrary to very low digitonin-induced increase (about 7 %) of respiration in PMS. Therefore, mass-specific oxygen flux, calculated as the oxygen consumption rate per 1 mg of total protein in the sample, is underestimated in CMF samples. Normalization of oxygen flux to the activity of CS (Table 2) was used in our study to avoid alterations caused by different contents of non-mitochondrial proteins. Internal normalization to the *ETS capacity* (Table 3) was used as an alternative for correction of both mitochondrial content and contamination within an experimental run to minimize several experimental errors (Gnaiger, 2009; Pesta and Gnaiger, 2012). We found that the respiration flux control ratio was more sensitive to changes caused by sample storage or technique of mitochondria isolation.

Mitochondrial coupling and functionality were evaluated by the respiratory control ratio, depending on the isolation technique and time of storage (Fig. 2). No significant dependence of RCR on the time of storage was found in any of the three mitochondrial samples. A slight

reduction in RCR with time confirmed that the samples can be used to respirometry measurement even three days after isolation. We suppose that the significantly higher RCR in CMF compared to purified mitochondria (PMS, PMP) is related to higher damage to mitochondria during their purification.

Complex I-linked respiration was significantly lower in purified mitochondria, especially in PMS, compared to CMF, and decreased after long-term storage of all mitochondrial samples, most markedly in PMP (Tables 2 and 3). Our results indicate that Complex I-linked respiration is more susceptible to damage by both the isolation procedure and time of sample storage. This is consistent with the known high sensitivity of Complex I activity to drugs and other interactions. It can be speculated that the decrease of Complex I-linked respiration due to the isolation procedure or due to the time of storage of mitochondria may be caused by disruption of supercomplexes stabilizing Complex I.

The damage to mitochondrial membranes was determined in all three mitochondrial samples (CMF, PMS, and PMP) as a function of the time of storage. Changes in permeability of the outer mitochondrial membrane were measured using the cyt *c* test and evaluated by the ratio of OXPHOS capacity after and before cyt *c* addition (*OXPHOS capacity/OXPHOS capacity low*, Table 3). The extent of damage to the inner mitochondrial membrane was evaluated as a ratio of CS activity in the absence or presence of Triton X-100 (Fig. 3).

For PMS, we found the largest both the cyt *c*-induced increase in the respiration rate and the percentage of ruptured mitochondria. The increased damage to mitochondrial membranes in PMS can be explained by the exposure of mitochondria to hyperosmotic environment (1.2 M sucrose) during their purification. This is supported by the finding that there were no significant differences in the mitochondrial membrane damage between CFM and PMP for more than 50 hours after isolation of mitochondria.

Except for Complex I-linked respiration in PMS, respiration oxygen fluxes (Table 2), respiration flux control ratios (Table 3), and the percentage of ruptured mitochondria (Fig. 3) showed insignificant changes during more than 50 h of mitochondria storage. This confirms that the cyt *c* release from mitochondria and increased CS activity are events caused especially by the initial damage to the mitochondria during isolation and only to a small extent by processes associated with the storage time.

Significant positive correlation with time was observed for the ratio *OXPHOS capacity/OXPHOS capacity low* (characterizing the damage to the outer mitochondrial membrane) in CMF, but not in PMS and PMP. It indicates that the damage to the outer mitochondrial membrane was increased with time in CMF, but not in PMS and PMP, which may be due to interference with cytosolic factors in CMF.

Another parameter characterizing mitochondrial damage is *LEAK* respiration. The *LEAK* respiration as well as the flux control ratio *LEAK/ETS capacity* did not significantly increase with the time of storage of mitochondria, regardless of their isolation method (Tables 2 and 3). We found significantly higher *LEAK* respiration in PMS compared with CMF. We suppose that the lower inhibitory effect of oligomycin in PMS reflected dissipation of the inner membrane potential, which cannot be increased to a value sufficient to inhibit further transport of protons across the inner membrane. This may be due to increased permeability of the inner membrane for protons and/or to a decrease of the proton-motive force by removing protons from the intermembrane space. The ratio *OXPHOS capacity/ETS capacity* was significantly lower in CMF compared to both PMS (due to increased *LEAK* respiration) and PMP (due to decreased *ETS capacity*). *LEAK/ETS capacity* was found to be about 0.49 for CMF, 0.70 for PMS and 0.77 for PMP early after mitochondria isolation. Thus, we confirmed that the coupling is partially disturbed in isolated mitochondria, probably as a result of mechanical damage during the isolation. Note that using a similar experimental protocol we found much lower *LEAK/ETS capacity* in blood platelets, 0.02 in intact platelets and 0.16 in permeabilized platelets (Fišar et al., 2016). Thus, our results indicate that there is an uncoupling effect linked to mitochondria isolation/purification.

The integrity of the inner and outer mitochondrial membrane should be ensured in isolated mitochondria. However, functional mitochondrial competence rather than viability is important for the study of drug effects on mitochondrial functions and cellular bioenergetics. It is essential that ETS of isolated mitochondria (CMF, PMS, PMP) is functional enough, which was evidenced by the effects of substrates and inhibitors of the respiratory chain complexes on the oxygen consumption rate. Some advantages and disadvantages (in terms of ETS activity measurements) of mitochondria prepared by different techniques are summarized in Table 4.

When lower *LEAK* respiration and higher Complex I-linked respiration is preferred, then the CFM may be a better choice than PMS or PMP. Purified mitochondria should be used in order to minimize contamination of mitochondria by synaptosomes, other non-mitochondrial membranes, and free cytosolic molecules, which can increase the risk of distortion of specific respirometric measurements. We speculate that gradient purification of mitochondria may be recommended for measuring the effect of lipophilic or amphiphilic drugs on mitochondrial respiration to avoid binding of the drug to non-mitochondrial structures. The high stability of mitochondrial respiratory control ratios over time means that isolated pig brain mitochondria (CMF, PMS or PMP) can be used for more than 48 h after isolation, when stored in isolation medium on ice, and especially when Complex II-linked respiration is the subject of research. We suppose that large amounts of dense mitochondria contributed to the long-term stability of samples stored on ice.

Table 4. Advantages and disadvantages of pig brain mitochondria isolated by different techniques in term of respirometry experiments

Sample	Advantage	Disadvantage
CMF	• Higher yield of mitochondria • Shorter preparation time • Higher and more stable Complex I-linked respiration • Better integrity of outer mitochondrial membrane • Lower *LEAK* respiration	• Lower purity • Lower mass-specific oxygen flux
PMS	• Higher purity • Higher mass-specific oxygen flux • More stable Complex I-linked respiration (compared to PMP)	• Lower yield of mitochondria • Longer preparation time • Lower Complex I-linked respiration (compared to CMF) • Worse integrity of both outer and inner mitochondrial membrane
PMP	• Higher purity • Higher mass-specific oxygen flux • Better integrity of outer mitochondrial membrane (compared to PMS)	• Lower yield of mitochondria • Longer preparation time • Lower and less stable Complex I-linked respiration (compared to CMF) • Lower *ETS capacity*

It can be concluded that the isolated pig brain mitochondria are suitable for studying the influence of drugs on the function of respiratory complexes and/or ETS activity, but they should be used with caution during measurements of mitochondrial parameters strongly influenced by coupling/uncoupling, such as ATP production or mitochondrial membrane potential. The advantage of using the pig brain as a source of mitochondria is clearly represented by the possibility of isolation of sufficient quantities of mitochondria, which ensure high stability of the respiratory parameters for a long time. Gradient purification of mitochondria is not necessary for a number of respirometric measurements.

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