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Mitochondrial respiration in human viable platelets—Methodology and influence of gender, age and storage

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ABSTRACT

Studying whole cell preparations with intact mitochondria and respiratory complexes has a clear benefit compared to isolated or disrupted mitochondria due to the dynamic interplay between mitochondria and other cellular compartments. Platelet mitochondria have a potential to serve as a source of human viable mitochondria when studying mitochondrial physiology and pathogenic mechanisms, as well as for the diagnostics of mitochondrial diseases. The objective of the present study was to perform a detailed evaluation of platelet mitochondrial respiration using high-resolution respirometry. Further, we aimed to explore the limits of sample size and the impact of storage as well as to establish a wide range of reference data from different pediatric and adult cohorts. Our results indicate that platelet mitochondria are well suited for ex-vivo analysis with the need for minute sample amounts and excellent reproducibility and stability.

1. Introduction

Mitochondrial dysfunction is recognized in primary respiratory chain diseases due to nuclear or mitochondrial DNA mutations and is also implicated in disorders such as Huntington's, Alzheimer's and Parkinson's disease as well as the result of excessive inflammation such as in sepsis (Brealey et al., 2002; Ferreira et al., 2010; Kones, 2010; Rosenstock et al., 2010).

The study of mitochondrial function is essential to both basic research of mitochondrial physiology and pathogenic mechanisms, as well as for the diagnostics of mitochondrial diseases. A common method to investigate mitochondrial function is determination of maximal enzymatic activity of the individual electron transport system (ETS) complexes in disrupted mitochondria by spectrophotometry. The benefit of this procedure is easy storage and transport to core laboratory facilities with high throughput analyses since the samples can be frozen (Haas et al., 2008). However, the mitochondrion and its components do not work as isolated units. Respiratory chain complexes are interconnected in the ETS that in turn gather to multienzyme- and supercomplexes (Lenaz and Genova, 2009). Mitochondria undergo fusion and fission, form networks and crosstalk with other subcellular compartments (Picard et al., 2011). This clearly highlights the need to analyse mitochondrial function without, or at least with minimal, cell disruption and with an as close as possible physiological environment. This can be achieved using polarographic measurements of mitochondrial respiration in cells (Kitchens and Newcomb, 1968). Respiration can be analysed in intact cells with natural surrounding media, such as plasma, utilizing endogenous substrates. Further, with permeabilization of the cell membrane direct

Abbreviations: ACD, acid citrate dextrose; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; ETS, electron transfer system; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; FFA, free fatty acids; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; PBS, phosphate buffered saline; PRP, platelet rich plasma; ROS, reactive oxygen species; SUIT, substrate, uncoupler, inhibitor titration.

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access to the mitochondria for exogenous substrates and inhibitors can be achieved, and individual complexes of the ETS investigated without the need for cell disruption and mitochondrial purification (Hutter et al., 2006).

When selecting which tissue to investigate for evidence of mitochondrial dysfunction the best choice is the tissue most profoundly affected by the disease process in a given patient. However, this is seldom possible due to the invasiveness and risks associated with biopsies from internal organs such as brain, liver and heart. Therefore other tissues are used, most commonly muscle cells and skin fibroblasts (Haas et al., 2008).

Platelets are an easily obtainable source of viable mitochondria and sampling is less invasive compared to muscle or skin biopsy. Platelet mitochondrial alterations have been demonstrated in a variety of diseases, primarily affecting other organ systems (Hauptmann et al., 2006; Krige et al., 1992) as well as in the process of ageing (Merlo Pich et al., 1996; Xu et al., 2007) and has therefore been proposed to serve as a potential marker of systemic mitochondrial dysfunction (Sjövall et al., 2010; Xu et al., 2007). Also, platelets are well suited for polarographic analysis (Kitchens and Newcomb, 1968; Sjövall et al., 2010).

Our aim of this study was to establish a methodology and make an in depth assessment of normal platelet respiratory function ex vivo in intact viable cells and individual complex function in permeabilized cells using high-resolution respirometry. Secondly, we studied the impact of storage in cold environment and room temperature, influence of gender and age and thirdly, the consistency of the method applied to different reference cohorts.

2. Materials and methods

2.1. Human sample acquisition

The study was approved by the regional ethical review board of Lund, Sweden (adults: 113/2008, and 644/2009, children: 59/2009) and the ethics committee of Tokyo Medical University, Japan (permit no. 1514). For Swedish adults, blood samples were collected from healthy blood donors at the blood donor central, Skåne University Hospital, Lund and healthy adults undergoing rehabilitation after knee injury. The Japanese cohort consisted of healthy adult volunteers. Samples were obtained after written informed consent was acquired. The experiments were carried out according to the same procedures and protocols and by the same researchers at both investigation sites. The pediatric control samples were obtained from patients undergoing minor elective surgery (inguinal hernia repair or phimosis surgery). Written informed consent was acquired from parents or guardian and blood was drawn before induction of anesthesia. Umbilical cord blood was sampled after delivery from healthy individuals undergoing a normal pregnancy. Samples were obtained after written informed consent was acquired.

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) if not stated otherwise.

2.2. Platelet preparation

For blood donors, samples were taken from the collection tubing at the same time as a planned blood donation and in the other adult cohorts and children via venous puncture. Umbilical cord blood was sampled directly after the child was delivered either vaginally or by caesarean section. A volume of 21 ml from adults, 6–12 ml from children, and 3–6 ml from umbilical cord was drawn in K_2EDTA tubes (Vacuette®, Greiner Bio-One GmbH, Kremmünster, Austria). In pilot studies, K_2EDTA were shown to result in the best yield and prohibit platelet activation compared to Heparin, Citrate and Acid Citrate Dextrose (ACD) as anticoagulants (data not shown). Blood samples were freshly prepared and analyzed within 3–5 h. The tubes were centrifuged 15 min at 300 ×*g* in room temperature, to yield a platelet-rich plasma (PRP). This PRP was pipetted off and centrifuged for 5 min at 4600 ×*g*, at room temperature, producing a close to cell free plasma and a platelet pellet. The pellet was dissolved in 1–3 ml of the control subject's own plasma by gentle pipetting to obtain a highly enriched PRP with a mean final concentration of 1864×10^6/ml (range 941–2498).

2.3. High-resolution respirometry

Respiration was measured at a constant temperature of 37 °C in a high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria (Gnaiger et al., 2000)) in 2 ml glass chambers with stirrer speed 750 rpm. Data was recorded with DatLab software 4.3. (Oroboros Instruments, Innsbruck, Austria) with sampling rate set to 2 s. All experiments were performed at an oxygen concentration in the range of 210–50 μM O_2. If necessary, reoxygenation was performed by partially raising the chamber stopper for a brief air equilibration. Instrumental background oxygen flux was measured in a separate set of experiments and automatically corrected for in the ensuing experiments according to the manufacturer's instructions. For respiration measurements in permeabilized cells, platelets were suspended in a mitochondrial respiration medium (MiR05) containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, $MgCl_2$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA 1 g/l, pH 7.1 (Gnaiger et al., 2000). For experiments in intact cells, platelets were suspended in either phosphate buffered saline (PBS) with addition of 5 mM glucose or in the control subject's own plasma. Calibration at air saturation was performed each day before starting experiments by letting Millipore water or respiration media stir with air in the oxygraph chamber until equilibration and a stable signal was obtained. Oxygen concentration was automatically calculated from barometric pressure and solubility factors that were set to 1.0 for water, 0.92 for MiR05 and PBS glucose and 0.89 for plasma (Baumgärtl and Lübbers, 1983).

2.3.1. Experimental protocol for intact platelets

Integrated respiration of intact cells with endogenous mitochondrial substrates was evaluated with two different titration protocols. Platelets were suspended in either PBS-glucose or the control subject's own plasma. Initially, samples were left to stabilise at a routine respiration state, revealing resting cellular energy demands on oxidative phosphorylation (OXPHOS). To evaluate the contribution of respiration independent of ADP phosphorylation, oligomycin (1 μg/ml, ATP synthase inhibitor) was sequentially added inducing LEAK respiration state (also known as oligomycin-induced state 4 respiration). Maximal capacity of the ETS was measured after careful titration of the protonophore, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) until no further increase in respiration was detected (in PBS mean concentration 6 μM, in plasma mean concentration 100 μM). Rotenone (2 μM, complex I [CI] inhibitor) and antimycin-A (1 μg/ml, complex III [CIII] inhibitor) were then sequentially added to inhibit the ETS providing the residual oxygen consumption which was subtracted from the different respiratory parameters in further analyses.

In order to evaluate the influence on maximal respiratory capacity by the inhibition of ATP-synthase, a second experimental protocol was performed, where ETS capacity was evaluated by direct titration of FCCP after stabilization of routine respiration, followed by the same inhibitors as above. Control ratios were derived from maximal, FCCP-stimulated, respiration divided by LEAK respiration (ETS/LEAK) and routine respiration (ETS/routine).

2.3.2. Experimental protocol for permeabilized platelets

To access the ETS with saturating exogenous substrates and inhibitors the plasma membrane was permeabilized with the detergent digitonin. A set of experiments were performed to establish the