Assessment of Mitochondrial Respiration in Human Platelets

ALEXANDRA PETRUS[1,2#], CORINA RATIU[1#], LAVINIA NOVEANU[1,2], RODICA LIGHEZAN[2,3], MARIANA ROSCA[4], DANINA MUNTEAN[1,2*], OANA DUICU[1,2]

[1] Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Medicine, Department of Pathophysiology, 14 Splaiul Tudor Vladimirescu, 300173, Timisoara, Romania

[2] Victor Babes University of Medicine and Pharmacy Timisoara, Center for Translational Research and Systems Medicine, 14 Splaiul Tudor Vladimirescu, 300173, Timisoara, Roamnai

[3] Victor Babes University of Medicine and Pharmacy Timisoara, Faculty of Medicine, Department of Parasitology, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

[4] Department of Foundational Sciences, Central Michigan University College of Medicine CMED Research Building, Room 105, Mount Pleasant, MI 48859, USA

It has been long recognized that the impairment of platelet mitochondrial function occurs in a broad spectrum of diseases. Accordingly, the assessment of platelet respiratory dys/function has emerged as a putative approach allowing the characterization of the early impairment of human bioenergetic profile in several chronic pathologies. The aim of this study was to standardize the methodology for platelet isolation from peripheral blood and the measurement of mitochondrial oxygen consumption by means of high-resolution respirometry, respectively. The platelet isolation protocol consisted of two consecutive centrifugations of the whole blood collected from adult healthy females (n = 10) yielding a platelet-rich plasma sample. Respiration was measured at 37^{0}C using the Oxygraph-2k (Oroboros Instruments, Austria) according to a classic substrate-uncoupler-inhibitor-titration protocol. Platelets permeabilized with digitonin were allowed to respire in the presence of complex I (glutamate and malate) and complex II (succinate) substrates. We obtained a respiratory control ratio of 2.77 ± 3.65 that indicates an accurate coupling efficiency of oxidative phosphorylation. The in vitro measurement of platelet respiration is a reliable method to evaluate the bioenergetic profile in humans. The standardized technique will be further used to assess the occurrence of mitochondrial dysfunction in peripheral blood in the setting of various chronic non-communicable diseases.

Key words: platelets, mitochondria, high-resolution respirometry

Extensive experimental research over the last decades has overturned the traditional view of mitochondria just as the *power-house* of the cell. Nowadays, the participation of mitochondria to an increasing number of pathologies is widely recognized. Accordingly, mitochondrial dysfunction is a central event in the pathogenesis of a large number of diseases, such as diabetes mellitus, cardiovascular diseases, neurodegenerative, as well as malignant disorders. Thereby, the study of mitochondrial respiration by polarographic measurements of oxygen consumption, has become a current approach in the assessment of mitochondrial dysfunction. However, the use of mitochondria isolated from tissues (heart, skeletal muscle) has been criticized, because of the invasive procedure for tissue harvesting and the methodology for assessing the function of organelles devoid of their physiologic cellular environment. Subsequently, researchers currently attempt to study mitochondrial function in homogenized tissues and cells, with minimal cell disruption, mimicking the physiological conditions [1]. Circulating blood cells are thought to be a reliable system to study the energetic metabolism. Nevertheless, despite the vast knowledge about the role mitochondrial (dys)function in metabolically active tissues such as the heart, brain, and liver, the exact role of mitochondria in circulating blood cells, such as platelets, still remains elusive. Among blood cells, platelets contain a small number of functional mitochondria that are fairly metabolically active by possessing a higher rate of ATP turnover [2]. One advantage in assessing the platelet mitochondrial function is represented by the sampling accessibility as compared to muscle or skin biopsies, the most commonly used samples to assess mitochondrial dysfunction [3].

Platelet mitochondrial function may be a reliable biomarker of disease and a potential useful diagnostic tool to assess the overall bioenergetic health of an individual [4]. Several studies have characterized the platelet mitochondrial function in pathological conditions, such as diabetes, septic shock, fibromyalgia, Parkinson and Alzheimer diseases [5-9]. Thus, platelets are considered an early predictive marker of mitochondrial dysfunction induced by metabolic stress [10-13].

In platelets isolated from patients with type 2 diabetes mellitus there is a decrease in both basal and maximal respiratory rates *vs.* healthy subjects [15]. Also, the activity of respiratory chain was found to be impaired in platelets isolated from patients with Parkinson disease [16, 17], Alzheimer disease [18, 19], schizophrenia [20], Huntington disease [21] and migraine headaches [22]. Interestingly, while platelets presented a decreased CI activity in patients with Parkinson disease [16, 17], a decreased CIV activity in patients with Alzheimer's disease [23], and a decreased CIII activity in patients with migraine headaches and aura, respectively, in patients with schizophrenia CI activity was shown to be increased [20]. At variance, an increased efficiency of some respiratory chain complexes in platelets of female patients with anorexia nervosa was reported [24]. A decrease of the electron transport system (ETS) maximal capacity in platelets harvested from patients with

* email daninamuntean@umft.ro ; Phone: (+40)256493085 # Authors with equal contribution

Alzheimer was associated with disease progression, thus suggesting that quantifying ETS capacity may enable the characterization of mitochondrial dysfunction in the initial stage of Alzheimer disease [25]. The study by Sjovall et al. demonstrated that mitochondrial uncoupling parallel with a gradual and pronounced increase in mitochondrial respiratory capacity was negatively correlated with the clinical outcome in patients with sepsis, suggesting that platelet mitochondrial function can potentially be used as a biomarker for the severity of sepsis [26]. Interestingly, recent data showed that sepsis-induced mitochondrial changes in platelets (i.e., a decrease in CI, II, III and IV activity) [27] were not accompanied by mitochondrial changes in other organs, suggesting that platelets' energetic ability may be a marker of sepsis progression [28]. Thus, the assessment of the platelet energetic profile have emerged as potential biomarker for both presence and prognostic of the disease.

The objective of this pilot study was to standardize the methodology for the *in vitro* assessment of platelet respiratory function that can be further used to examine the imapirment of respiration that occur in relation with several chronic pathologies.

Experimental part

Platelet harvesting and preparation

All the experimental procedures were conducted in agreement with the Helsinki Declaration and were approved by the Committee for Research Ethics of Victor Babes University for Medicine and Pharmacy, Timisoara, Romania.

Blood samples were collected from healthy adult female donors (n = 10, age range: 33-51) after obtaining the written informed consent. A volume of 12 mL of blood was harvested in K_2EDTA tubes (Vacutest Kima, Italy) and analysed within 3-5 h. The platelet isolation protocol comprised of two consecutive centrifugations at room temperature. The tubes were centrifuged for 10 min at 500 *g* yielding a platelet-rich plasma that was collected into new Falcon tubes and centrifuged again for 5 min at 4600 *g*. The resulted supernatant (almost cell free plasma) was discarded, and the platelet pellet obtained suspended in 1 mL plasma, thus obtaining a highly enriched platelet plasma (mean final concentration of approximately 1000 x 10^6/mL counted by turbidimetry).

High-Resolution Respirometry Studies

Respiration was measured at 37^{0}C using the high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Austria). A standard oxygen calibration for experimental conditions was performed prior to each experiment. Platelets (200 x 10^6/mL) were incubated in a mitochondrial respiration medium (D-Sucrose 110 mM, taurine 20 mM, EGTA 0.5 mM, HEPES 20 mM, $MgCl_2$ 3 mM, Lactobionic acid 60 mM, KH_2PO_4 10 mM, BSA 1 g/L, *p*H 7.1 adjusted with 5 M KOH). The oxygen concentration was kept in the range 210-50 μM and the oxygen solubility factor in the experimental media was set to 0.92 for all experiments. The data was recorded using the software support DatLab 4 (Oroboros Instruments, Austria).

Experimental protocol for permeabilized platelets

In order to allow the exchange of soluble molecules between platelet cytosol and external media and perform a comprehensive assessment of OXPHOS capacity, platelets plasma membrane was permeabilized with a mild detergent: digitonin (1μg/L x 10^6 platelets). The concentration used, induced a maximal permeabilization without producing any damage to the outer/inner mitochondrial membrane, as previously established by the group [1].

To study the respiratory control, we used a substrate-uncoupler-inhibitor-titration (SUIT) protocol that allowed the measurement of the respiratory rates when electrons were transferred from both complex I (CI) and complex II (CII), separately and conjunctively (CI+II). The platelets were first suspended in media without substrates, for 10-15 min, to allow the measurement of routine respiratory rate. Titration started with the addition of digitonin followed by the concomitant addition of CI substrates: glutamate (G, 5 mM) and malate (M, 5mM), thus yielding the State 2 respiratory rate that reflects the respiration in basal conditions. ADP (1 mM) was added to measure the active respiration/State 3/OXPHOS capacity, driven by CI substrates ($OXPHOS_{CI}$). The subsequent addition of succinate (S, 10 mM), a CII substrate, induced the maximal OXPHOS capacity, as a consequence of the convergent electron flow of CI and CII at the Q-junction ($OXPHOS_{CI+CII}$). The return to a basal respiration (State 4_{CI+CII}) was obtained by the addition of an ATP synthase inhibitor, oligomycin (1 μg/mL). With successive titrations of the uncoupler FCCP (carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone, 1 μM/titration step) we were able to measure the maximal convergent respiratory capacity of the electron transport system (ETS_{CI+CII}). The following addition of CI inhibitor rotenone (2μM) allowed the measurement of ETS capacity supported only by CII (ETS_{CII}). Finally, the respiration was inhibited by the addition of a complex III inhibitor, antimycin A (1μg/mL), resulting in the measurement of residual oxygen concentration (ROX). All the values obtained, were corrected for ROX and used for further analysis.

Data Analysis

The results are expressed as mean ± SD. The statistical analysis was performed using the GraphPad Prism 5 software (La Jolla, USA).

Reagents

All the chemicals used in this study were purchased from Sigma-Aldrich (St Louis, USA).

Results and discussions

Since the number of mitochondria per platelet is rather small, we used a platelet concentration of 200 million/mlL In order to gather as much information as possible about the capacity of respiratory complexes, the SUIT protocol was adapted after the one used by the group of Sjövall et al. [1], as depicted in figure 1. Platelets were suspended in mitochondrial respiration media and left for 10-15 min to respire on endogenous and exogenous (from media) substrates, thus establishing the routine respiration rate (fig. 1). The levels of respiration and phosphorylation in the physiological routine state is controlled by the physiological energy demand, energy turnover, and the degree of coupling (intrinsic uncoupling and pathological dyscoupling) [29]. Although plasma membrane permeabilization with digitonin can affect the outer and inner mitochondrial membrane, Sjövall el at showed that digitonin presents a large safety margin and the concentration used in this had no effect on mitochondrial membranes [1].

After the stabilization of routine respiration, CI substrates, glutamate and malate, were added and the cells were permeabilized using digitonin. A slight decline in respiration followed after these additions, due to the release of cytosolic substrates and adenine nucleotides in the media,

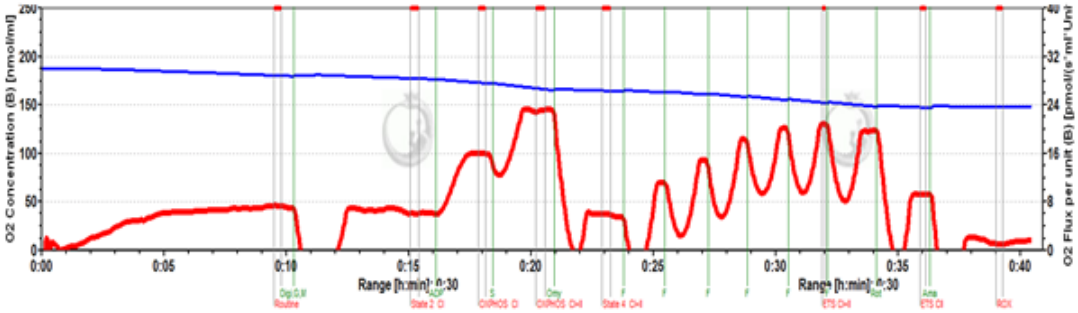

Fig. 1. Representative diagram of the SUIT protocol in permeabilized platelets. Oxygen concentration (nmol.mL^{-1} – blue trace) and O_2 flux per Volume (pmol.s^{-1}.Units^{-1} – red trace) are represented as a function of time. Additions are as follows: Digi – digitonin (1\µg/L x 10^6 platelets), G – glutamate (5 mM), M – malate (5 mM), ADP (1 mM), Omy – oligomycin (1 µg/mL), F – FCCP (1 µM/titration step), Rot – rotenone (2 µM), Ama – antimycin A (1 µg/mL)

as previously demonstrated [1]. Next, ADP addition stimulated respiration by ~100% compared to the routine respiration ($OXPHOS_{CI}$, fig. 1). The subsequent addition of CII substrate, succinate, resulted in a supplementary increase of active respiration by 50%, compared to the respiration with only CI substrates, due to the convergent electron flow from both CI and CII, respectively. State 4_{CI+CII}, obtained after oligomycin was added in the oxygraph, was 26% of maximal coupled respiration ($OXPHOS_{CI+CII}$), as presented in table 1. The ETS_{CI+CII} was produced by successive titrations of the uncoupler FCCP. The maximal respiratory capacity obtained for both complexes (ETS_{CI+CII}) was ~12% lower compared to $OXPHOS_{CI+CII}$ (Table 1), possibly due to platelet transport at room temperature conditions [30]. ETS_{CII} was measured after the addition of CI inhibitor rotenone. The maximal capacity of CII was about 45% of ETS_{CI+CII}. Using the assessed respiratory capacities we calculated the flux control ratios (FCRs), that express the respiratory control independent of mitochondrial content and cell size [29]. The routine control ratio is a measure of mitochondrial dysfunction, showing how close routine respiration operates to ETS capacity; the value of 0.37 obtained in this study falls in the normal range, as reported by Pesta et al [29]. The respiratory control ratio (RCR) reflects OXPHOS coupling efficiency and ranges from 1.0 to infinity. The obtained value of 2.77 indicates a good coupling efficiency. The $OXPHOS_{CI+CII}$/ETS_{CI+CII} of ~1 indicates that at saturating levels of exogenous substrates, the phosphorylation system exerts no flux limitation.

The advantage of using permeabilized cells, compared to intact cells, is that proton circuit and ETS can be measured without a rate-limitation of substrates, since the citric acid cycle is saturated with exogenous substrates and in parallel, both glycolysis and β-oxidation are by-passed. Compared to intact cells, both the OXPHOS and ETS could be increased with 50% in permeabilized cells. Moreover, it was showed that ETS respiration values of intact cells are almost equal to the values of $OXPHOS_{CI}$ in permeabilized cells [1].

Our results are in line with those reported by other studies [1, 30], and clearly demonstrate that measurement of platelet respiration is suited to assess the human mitochondrial function *in vitro* in physiological conditions. To date, there is no available biomarker of energetic function, that can be used as a diagnostic, prognostic and/or treatment response quantification tool in clinical practice.

Table 1

MITOCHONDRIAL RESPIRATORY RATES OF PERMEABILIZED PLATELETS. DATA ARE EXPRESSED AS pmol O_2/s/1*10^8 PLATELETS

Respiratory parameters	Mean value ± SD
Routine (R)	5.75 ± 0.96
State 2_{CI}	6.30 ± 1.58
OXPHOS CI	11.51 ± 3.81
$OXPHOS_{CI+CII}$	17.48 ± 5.76
State 4_{CI+CII}	4.48 ± 0.97
ETS_{CI+CII}	15.51 ± 6.09
ETS_{CII}	6.89 ± 1.24
Routine control ratio (R/ETS_{CI+CII})	0.37 ± 0.16
RCR ($OXPHOS_{CI+CII}$/STATE 2_{CI})	2.77 ± 3.65
$OXPHOS_{CI+CII}$/ETS_{CI+CII}	1.13 ± 0.95

Conclusions

The *in vitro* measurement of platelet respiration is a reliable method to evaluate the bioenergetic profile in humans. The standardized technique will be further used to assess the occurrence of mitochondrial dysfunction in peripheral blood in the setting of non-communicable diseases.

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