PHARMACOBIOCHEMICAL STUDY

Platelet mitochondrial bioenergetic analysis in patients with nephropathies and non-communicable diseases: a new method

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ABSTRACT
OBJECTIVES: To test the hypothesis if mitochondrial bioenergetic function analyzed in circulating platelets may represent peripheral signature of mitochondrial dysfunction in nephropathy associated to non-communicable human diseases such as cardiovascular diseases, diabetes and with statins treatment.

METHODS: High-resolution respirometry was used for analysis of mitochondrial bioenergetics in human platelets isolated from peripheral blood. This method is less-invasively compared to skeletal muscle biopsy. Patients with nephropathies and in combination with non-communicable diseases were included in the study.

RESULTS: This pilot study showed platelet mitochondrial bioenergy dysfunction in patients with nephropathies and non-communicable diseases. Positive effect of treatment with 10 mg atorvastatin on platelet mitochondrial respiratory chain Complex I-linked respiration and ATP production in patients with nephropathies, diabetes and 80 mg atorvastatin in patient with nephropathy and dialysis was found. Positive effect of 80 mg fluvastatin treatment, and negative effect of thrombocytopenia and renal transplantation on platelet mitochondrial bioenergy was determined.

CONCLUSION: High-resolution respirometry allowed detection of small changes in platelet mitochondrial function. This method could be used as a sensitive bioenergetic test of mitochondrial function for diagnosis and monitoring the therapy in patients with nephropathy (Tab. 1, Fig. 3, Ref. 39). Text in PDF www.ells.sk.

KEY WORDS: platelets, mitochondria, high-resolution respirometry, nephropathies, non-communicable diseases.

Introduction

Chronic non-communicable diseases (NCDs) are responsible for 71 % of all deaths in the world. NCDs are non-infectious and non-transmissible among people. These diseases are result of combination of genetic, physiological, environmental and behaviours factors. According to the World Health Organization (WHO), the four main types of NCDs are included. Cardiovascular diseases are reason of deaths of 17.9 million people annually, followed by 9.0 million of deaths for cancers, 3.9 million deaths for respiratory tract diseases and 1.6 million deaths for diabetes (1).

Metabolic factors (raised blood pressure, hyperglycemia, hyperlipidemia and overweight or obesity) and behaviours factors increase the risk of NCDs. Over 7.2 million deaths every year are related to tobacco smoking, including the effect of second-hand smoke, 4.1 million deaths annually to excessive salt intake, unhealthy diet (increased fat and sodium, with low fruit and vegetable intake), harmful use of alcohol, 1.6 million deaths to insufficient physical activity (2). NCDs effect on cellular metabolism includes mitochondrial dysfunction of various organs and increased concentration of reactive oxygen species (ROS) (3). Mitochondrial dysfunction and ROS production has a critical role in the pathobiological and sociomedical mechanisms of development and progression of diseases.

The number of patients suffering from chronic kidney diseases (CKD) raises every year. CKD is characterized by a progressive loss of renal function, chronic inflammation, oxidative stress and vascular remodeling (4, 5). The main clinical manifestation of CKD is progressive decrease of glomerular filtration rate (GFR) which allows estimation of CKD progression (6). Among the most frequent causes of CKD are diabetes, hypertension, atherosclerosis, glomerulonephritis and polycystic kidneys (7, 8).

High-resolution respirometry offers sensitive diagnostic tests of mitochondrial respiratory chain function and oxidative phosphorylation in circulating human blood cells such as peripheral blood mononuclear cells and platelets (9–12).

Platelets are attractive source of viable mitochondria, which can be obtained much less invasively compared to skeletal muscle biopsy (13, 14). Platelets as circulating anucleate fragments, contain a small amount of mitochondria, important for energy
Fig. 1. The analysis of bioenergetics in platelets mitochondria by high-resolution respirometry. Legend: Trace from the measurement of PLT respiration at 37 °C in respiration medium MiR05+20 mM Cr. Blue line represents oxygen concentration (μM), the red trace represents oxygen consumption as flow per cells (pmol O₂∙s⁻¹∙10⁻⁶ cells). The modified SUIT protocol RP1 includes following steps: 1) Oxygen consumption rate of intact PLT- ROUTINE respiration, 2) Respiration rate of mitochondria in permeabilized PLTs with digitonin, 3) LEAK at Complex I (State 4) reflects rate of mitochondrial respiration with exogenous substrates (pyruvate + malate), 4) OXPHOS at Complex I (State 3) after ADP addition reflects ATP production at Complex I, 5) Cytochrome c addition – a test for the integrity of outer mitochondrial membrane, 6) The rate after CCCP addition (uncoupled of OXPHOS) represents maximal oxidative capacity at Complex I with substrates pyruvate + malate, 7) Noncoupled oxygen consumption at Complex I after addition of substrate glutamate, 8) Noncoupled oxygen consumption at Complex I and Complex II after addition of CH substrate succinate, 9) Noncoupled oxygen consumption at Complex II after addition of rotenone – inhibitor of Complex I, 10) Residual oxygen consumption (ROX) determined after addition of Antimycin A - an inhibitor of Complex III.

Fig. 2. Platelet mitochondrial bioenergetic function in patients with nephropathies and non-communicable diseases. Legends: Parameters of platelet respiration determined following modified SUIT protocol RP1. Oxygen flux was normalized on activity of mitochondrial marker citrate synthase. Steps in the protocol are in legend for Figure 1.

In this study we tested the hypothesis if analysis of mitochondrial bioenergetic function in circulating platelets may represent peripheral signature of mitochondrial dysfunction in patients with nephropathies and non-communicable human diseases.

production (15). Platelets are metabolically active cells with high energy consumption. During resting state of platelets, their energy is derived both from glycolysis and from mitochondrial oxidative phosphorylation (16).
Material and methods

1) Control group: Elderly volunteers, number of 9 (3 men, 6 women), age from 56 to 82 years (68.37±1.32, mean±SEM) were included in the study. All evaluated parameters are shown as mean±SEM.

Patients with nephropathies and combinations of non-communicable diseases with nephropathies were treated by standard nephropathy therapy. In this cases we focused only to statins therapy,

2) Case: Nephropathy + CHD + 10 mg Atorvastatin (CHD – chronic heart disease)
3) Case: Nephropathy
4) Case: Nephropathy + Diabetes + 10 mg Atorvastatin
5) Case: Nephropathy + Dialysis + 80 mg Atorvastatin
6) Case: Fluvastatin 80 mg
7) Case: Thrombocytopenia
8) Case: Renal transplantation

Methods

Observed parameters

Following metabolic blood parameters were measured in clinical biochemical laboratory, using standard methods: hemoglobin, leukocytes and platelets count, serum c-reactive protein (CRP), triacylglycerol (TAG), LDL-Cholesterol, HDL-Cholesterol, total Cholesterol, glucose concentration and activities of liver enzymes: AST, ALT, GMT. Determined kidney parameters included: creatinine, uric acid and estimated glomerular filtration rate – eGFR.

Platelets preparation

For platelets (PLT) isolation 18 mL of venous blood was collected to K2-EDTA (triptotassium ethylenediaminetetraacetic acid) tubes in the morning after overnight fasting. Fresh blood was centrifuged at 200 g, 25 °C for 10 min using swing-out rotor without breaking. Platelets rich plasma (PRP) was transferred into a new plastic tube and mixed with 100 mM EGTA (ethylene glycol-bis(2-aminoethyl ether) N,N,N′,N′-tetraacetic acid) to final concentration 10 mmol/L. After centrifugation in swing-out rotor with soft breaking (brake 2 of scale 9) at 1200g for 10 min, the sediment containing PLT was resuspended in 4 mL of Dulbecco’s Phosphate Buffered Saline (DPBS) + 10 mmol/L EGTA and centrifugated again. The final sediment was resuspended in 0.4 mL of DPBS + 10 mmol/L EGTA (modified after 17) and counted on hematological analyzer Mindray BC-2800 (Mindray, China).

Platelets mitochondrial respiration and oxidative phosphorylation

High-resolution respirometry represents a sensitive technique to determine mitochondrial dysfunction (9, 10, 18). For mitochondrial respirometric analysis 200x10^6 platelets was used in 2 mL chamber of O2k-Respirometer (Oroboros Instruments, Austria). The respiration was measured at 37°C in mitochondrial respiration medium MiR05 + 20 mM creatine using modified SUIT (Substrate-Uncoupler-Inhibitor-Titration) protocol RP1 (11) (Fig. 1).

Citrate synthase (CS)

All parameters of oxygen consumption are evaluated as JO2/CS (nmol.s⁻¹.IU⁻¹). The activity of mitochondrial marker citrate synthase was evaluated spectrophotometrically according method of (19), described in detail by (20).

The study was carried out according to the principles expressed in the Declaration of Helsinki and the study protocol was approved by the Ethical Committee of the Academic Ladislav Dérer’s Hospital, Bratislava, Slovakia. Written informed consent from each subject was obtained prior to inclusion.

Results

Table 1 shows metabolic characteristics of selected patients with nephropathies and non-communicable diseases. Concentrations of hemoglobin, leukocytes and platelets were in reference values, only in patient with thrombocytopenia the count of platelets was very low (48·10⁹ cells/L). The patient with diabetes complication on statin therapy had very high CRP (101.14 mg/L). CRP was over reference values also in patient with CHD on statin therapy (7.55 mg/L) and in dialyzed patient (5.17 mg/L). Dyslipidemia (increased TAG and cholesterol levels) was recorded in patients on fluvastatin therapy, after renal transplantation and in patient with CHD complication. Activities of liver enzymes were not affected by diseases. In patients with nephropathies creatinine and uric acid concentrations were increased. eGFR was decreased in all cases (table 1).

Platelet mitochondrial bioenergetic analysis in patients with nephropathies and non-communicable diseases shows Figure 2.

1. Intact PLT

Oxygen consumption in intact platelets was the highest in patient treated with fluvastatin (+50.1 % in comparison with control group); Oxygen consumption increased in patient with nephropathy, diabetes and atorvastatin therapy +12.8 %; in dialyzed patient with nephropathy and atorvastatin therapy +10.9 %. In other cases of patients the respiration in intact platelets was almost at control levels, in patient after renal transplantation it was decreased by 15.2 % (Fig. 2).

3. LEAK CI (P+M)

Mitochondrial oxygen consumption in permeabilized platelets with Complex-I substrates (LEAK CI-PM, State 4), was increased in patient with nephropathy after fluvastatin therapy by 38.5 % vs control group. Low LEAK respiration was in patient after renal transplantation - it accounted only 38.5 % of control group value; and the lowest LEAK respiration was in patient with thrombocytopenia (5.8 % of control group value). In other cases, LEAK respiration was close to control values.

4. OXPHOS CI

Platelet mitochondrial respiration associated with ATP production (OXPHOS CI) was increased in patients with nephropathy, dialyzed and with atorvastatin therapy by 44.6 %; in patient with fluvastatin therapy by 34.8 %; in patient with diabetes complication and atorvastatin therapy by 29.8 % and in patient with cardiovascular diseases complication by 15.2 %. In other cases of patients OXPHOS CI was decreased in comparison with control group:
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in patient with nephropathy OXPHOS at CI accounted 67.3 % of control values, in patient with thrombocytopenia 38.1%. In patient after renal transplantation CI-linked OXPHOS was not detectable.

5. Cyt c
The integrity of outer mitochondrial membrane was measured by cytochrome c addition. The increase in respiration after addition of cytochrome c did not exceed 10 % in any patient.

6. CCCP CI – (P+M)
Maximal oxidative capacity (noncoupled mitochondrial respiration) after uncoupler titration was increased in dialyzed patient on atorvastatin treatment by 79.9 %; in patient with diabetes complication and on atorvastatin therapy by 42.1 %; in patient with fluvastatin therapy by 38.7 % and in patient with cardiovascular complication by 34.7 % vs control group; in patient with thrombocytopenia this parameter accounted 71% of value in control group; after renal transplantation only 21.8 % of control values.

7. Noncoupled CI – (P+M+G)
After addition of glutamate the relative increase of CI-linked respiration was similar in control and patients (+13 – 22.4 %) except for the patient with renal transplantation. In this patient after glutamate addition the respiration decreased by 23.5 %.

8. Noncoupled CI+II- (P+M+G+S)
Addition of CII substrate succinate enabled determination of convergent electron flow from both CI and CII to coenzyme Q. Noncoupled CI+II respiration was stimulated vs control in patient with cardiovascular complication by 6.1 %; in patient with diabetes by 7.7 %; in patient with fluvastatin therapy by 16.4 %. This parameter was decreased in patient with nephropathy by 11.3 %; in patient with dialysis by 32.6 %; in patient with thrombocytopenia by 31.8 %, and in patient after renal transplantation by 56.7 %.

9. Noncoupled CI - S(Rot)
The CII-linked noncoupled respiration determined after inhibition of Complex I with rotenone was increased vs control group in patient with cardiovascular complication by 24 %; in patient with fluvastatin therapy by 6.7 %. This parameter was decreased in patient with thrombocytopenia by 32.4 %, in patient with ne-
Discussion

Mitochondria are important not only for energy production, but also responsible for regulation of intracellular signaling through reactive oxygen species (ROS), which initiate apoptosis through the release of cytochrome c (21, 22). The diabetic nephropathy is the leading cause of CKD and end-stage renal disease. People with CKD are at high risk of death from stroke or heart attack (4). In CKD muscle weakness and abnormality of skeletal muscle mitochondria were documented (23). The progression of the disease contributes to changes in cellular energy metabolism, oxidative stress, which develops through dysfunctional kidney mitochondria (24, 25), reduced antioxidants protection of the organism and damage to mitochondrial function in the kidneys (Fig. 3) – mitochondrial nephropathy (8, 23, 26, 27 28).

In the past years impairment of mitochondrial function is used as a disease biomarker for the evaluation of disease prognosis and therapeutic response. High-resolution respirometry offers the possibility to use peripheral blood mononuclear cells and platelets for studying mitochondrial bioenergetic metabolism in humans (29). Platelets are attractive source of mitochondria which can be obtained less-invasively compared to muscle biopsy. Limited studies are about mitochondrial function in platelets in pathological states (21).

In this study we compared platelet mitochondrial function in patients with CKD and with in patient with CKD associated to non-communicable diseases (diabetes, CHD, thrombocytopenia, renal transplantation) and treatment with statins. Increased respiration in intact platelets, which reflects increased platelets membrane permeability, was found in patient after fluvastatin treatment, followed by patients with diabetes and atorvastatin treatment, next by dialyzed patient and atorvastatin treatment. In another study opposite results, decreased respiration in intact platelets was reported in depressive patients (30).

In permeabilized platelets, mitochondrial CI-linked oxygen consumption (State 4 at Complex I) was increased after fluvastatin treatment (Fig. 2). Opposite results, decreased Complex I-linked respiration in permeabilized platelets in humans after statins treatment were published, suggesting respirometric analysis of mitochondrial function in platelets for studying changes in cellular energy metabolism in patients treated with statins (31). We found damaged CI-linked respiration in patient after renal transplantation and strongly impaired CI-linked respiration in patient with thrombocytopenia (Fig. 2).

In patients with nephropathy and non-communicable diseases we found different effects of statins therapy. In dialyzed patient, in nephropathy and diabetes and in CHD, atorvastatin and fluvastatin had positive effect on platelet mitochondrial CI-linked OXPHOS (respiration associated with ATP production). However, in patient with nephropathy without statins therapy, in patient with thrombocytopenia CI-linked OXPHOS was low, and after renal transplantation CI-linked OXPHOS was not detectable. These results show damaged function of mitochondrial OXPHOS at Complex I (Fig. 2). We suppose that damaged mitochondrial function in patients with nephropathies may be associated with increased oxidative stress and damaged coenzyme Q<sub>10</sub> biosynthesis, essential factor for the normal mitochondrial function.

Altered mitochondrial respiratory chain function in platelets of patients with type 2 diabetes, Alzheimer’s, Parkinson’s (32, 33), Huntington’s (34), schizophrenia showed an increase in Complex I activity (37). In platelets of patients with septic shock and cardiogenic shock activities of mitochondrial complexes were reduced (38). The activities of respiratory chain Complexes I and II in isolated platelets were significantly higher in females with anorexia nervosa in comparison with control group. No differences were found in the activities of Complexes IV, I+III, and citrate synthase (39).

In our study maximal oxidative capacity (noncoupled mitochondrial respiration) was increased in patients with CKD and statins therapy and after statins therapy. CI-linked as well as CII-linked noncoupled respiration was decreased in patient with thrombocytopenia and after renal transplantation showing strongly impaired mitochondrial respiratory chain function at Complexes I and II in these patients (Fig. 2).

The study of physiological and pathological mitochondrial function is essential for the diagnostics and targeting therapy of mitochondrial diseases. It was shown that circulating platelets can act as sensors of the inflammatory and metabolic stresses associated with various chronic diseases, as cardiovascular disease, neurodegenerative diseases and diabetes (39). The respirometry of human platelets allows dynamic monitoring of mitochondrial function. Platelets are used for the assessment of organ-specific mitochondrial dysfunction relevant to clinical outcome (29). Results of this study contribute to the knowledge about the use of respirometric analysis of platelet mitochondrial function as bioenergetic marker of mitochondrial nephropathy.

Conclusion

This pilot study shows platelet mitochondrial bioenergy dysfunction in patients with nephropathies associated to non-communicable diseases. Positive effect of atorvastatin on platelet mitochondrial respiration at Complex I associated with ATP production in CKD patients with CHD, diabetes, and dialysis was found. Positive effect of high dose of fluvastatin treatment on PLT respiration was documented. In patients with thrombocytopenia and after renal transplantation, platelet mitochondrial respiratory chain function at Complex I and Complex II was strongly impaired.

In conclusion, respirometric analysis of bioenergetics of platelets mitochondria by High-resolution respirometry allowed detection of signs of platelet mitochondrial dysfunction, which could be used as bioenergetic markers for diagnostics and monitoring the therapy in patients with nephropathy and non-communicable diseases.
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