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THE PROTEOME ANALYSIS OF RAT PLATELET WITH NANO-LIQUID CHROMATOGRAPHY-MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY TECHNIQUE

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Recently, the proteomic analysis has become an ideal tool to study the structure and function of platelets. We proposed a nano-liquid chromatography (nano-LC) technique coupled off-line with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) for rat platelet proteome analysis. In this study, we attempted to analyze the rat platelet proteome in two different subcellular fractions: cytosol and membrane. Platelet-rich plasma was collected from healthy rats. The platelet samples were extracted with Subcellular Proteome Extraction Kit to collect subcellular compartments. For further investigations, platelet lysate, cytosol and membrane fractions were used. Enzymatic digestion of proteins was performed using Filter Aided Sample Preparation method with trypsin as a proteolytic enzyme. Tryptic peptides were analyzed using nano-LC-MALDI-TOF/TOF-MS. Platelet proteins identification was performed using the Mascot engine. We identified 238 proteins in the platelet lysate, 210 in the cytosol, and 148 in the membrane fraction. Among them, 45 were unique for platelet lysate, 55 for cytosol, and 34 for the membrane fraction. The gene ontology analysis showed that there were differences in the proteome of cytosol and membrane fractions related to the molecular functions, *i.e.* coagulative activity. Our results may suggest that the membrane or cytosol location of the proteins with coagulative activity may be responsible for the acute or delayed platelet response to an agonist. The nano-LC-MALDI-TOF/TOF-MS method can be used for identifying proteins of subcellular fraction in rat platelets.

Key words: *platelet, proteome, mass spectrometry, nano-liquid chromatography, matrix-assisted laser desorption/ionization, tryptic peptides, coagulation factors*

INTRODUCTION

Blood platelets are indispensable in physiological hemostasis. However, under pathological conditions, their activation may induce thrombosis and vessel occlusion, which are the most common mechanisms leading to myocardial infarction and stroke (1, 2). Although the mechanism of platelet activation is still not fully understood, a considerable progress has been made in the last two decades. There are more and more platelet agonists known and, what is particularly important from a clinical point of view, literature data show new compounds with confirmed antiplatelet activity *e.g.* the phenolic fraction from the fruit of *Hippophae rhamnoides,* that could prevent the hyperactivation of blood platelets and cardiovascular disorders (3). It is well known that platelet hyperreactivity, reflected by enhanced platelet adhesion and aggregation, is a sum of precisely coordinated cell signaling events involving changes in platelet proteome and structural reorganization. The phenotypically altered platelets revealed differential expression of proteins, which are involved in platelet adhesion, aggregation, coagulation, acute phase response, transmembrane signaling, calcium homeostasis, and cytoskeletal reorganization (4-6). In addition to well-known standard laboratory test of coagulation, such as activated partial thromboplastin time, prothrombin time, and number of platelets, the functional test *e.g.* rotational thromboelastometry and platelet aggregometry are becoming gold standards in evaluation of platelet activation status and coagulation disorders (7). Moreover, new attempts are being made to use in clinical practice correlations of already known markers of platelet activation with other factors. It was shown in obese patients a positive correlation between P-selectin and adiponectin, what could be considered as a novel indicator for atherosclerotic plaque formation as well as platelet activity in obesity (8). Recently, the proteome analysis has become an ideal research tool for studying non-nucleated cells, platelets as well (9-11). The platelet proteomic analysis showed differential regulation of proteins in response to agonists as well as in diseases such as acute coronary syndrome, which makes this

method effective in evaluating platelet function. Although there are few experimental procedures for proteomic analysis of platelets, including liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization (ESI), in this paper, we propose an alternative method, a matrix-assisted laser desorption/ionization (MALDI) procedure, for qualitative proteomic analysis of platelets. We were one of the first who applied this technique for proteomic analysis of microvesicles and activated human platelets (12). Here, we used it for proteomic analysis of subcellular fractions of rat platelets. Therefore, the aim of the present study was to verify if the nano-LC-MALDI-TOF/TOF-MS technique is suitable for the proteome analysis of membrane and cytosol fractions of rat washed platelets.

MATERIALS AND METHODS

General protocol

A protocol was based on the bottom-up strategy of proteomic mass spectrometry analysis. Enzymatic digestion was performed using Filter Aided Sample Preparation (FASP) method with trypsin as a proteolytic enzyme (13). For peptide fractionation a two-dimensional chromatographic separation was used and the last dimension was carried out with nano-liquid chromatoghraphy (nano-LC) coupled off-line with MALDI-TOF/TOF mass spectrometer (12).

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research (14).

Sample collection and subcellular compartments extraction

Blood samples for platelet rich plasma (PRP) preparation were collected from healthy rats after overnight fasting. Male, Wistar rats (250 – 270 g, n = 3) were anesthetized with pentobarbital (40 mg/kg, i.p.). Blood samples were drawn from the right ventricle of the heart to the plastic tubes (3.6 mL) containing 3.2% sodium citrate as anticoagulant and mixed in a volume ratio 9:1. This anticoagulant was shown to be effective to prevent platelet activation during isolation (15). PRP was separated by centrifugation at 165 g (room temperature) for 10 min (Eppendorf 5810, Germany). Then, platelets were pelleted at 750 g for 10 min and twice washed with modified HEPES-Tyrode's (JNL) buffer without Ca^{2+} (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM D-glucose, and 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, and 10 mM Tris, pH 7.4). The lysate of platelet samples, suspended in washed buffer, was extracted with ProteoExtract Subcellular Proteome Extraction Kit according to the manufacturer's protocol (Merck Millipore, USA) to collect subcellular compartments. The stepwise extraction resulted in two distinct subcellular proteomes: cytosol and membrane. For further investigations the platelet lysate, cytosol and membrane fractions (n = 3) were used.

Sample preparation for mass spectrometry

The platelet lysate, cytosol and membrane fractions were pelleted by means of centrifugation (Thermo Fisher Scientific, Europe) at 14,000 × g for 10 min at 4°C. The pellet was resuspended in 50 µL of 0.1 M Tris-HCl pH 8.0, 0.1 M DTT and 4% SDS, and incubated for 5 min at 99°C for denaturation. The protein concentration was determined using the bicinchoninic acid BCA Assay (Pierce Biotechnology, Thermo Scientific, USA). Average protein concentration in the studied platelet fractions was between 4.0 and 5.5 mg/mL, the total protein amount used for MS was 40 µg. The purification procedure and fractionation of tryptic peptides were performed according to Wisniewski *et al*. (13). Briefly, proteins were subjected to trypsin digestion using filter aided sample preparation (FASP) approach with spin ultrafiltration units of nominal molecular weight cutoff 30,000. The sample was mixed with 8 M urea solution in 0.1 M Tris-HCl pH 8.5 (UA buffer) in YM-30 microcon filter units (Merck Millipore, Europe) and centrifuged at 20°C at 14,000 g for 15 min. This step was repeated once. Filter-bound proteins were treated with 0.05 M iodoacetamide and incubated in darkness for 10 min at room temperature. Microcon filter was washed three times with UA buffer followed by three washes with 0.05 M Tris-HCl pH 8.5. Proteins were digested at 37°C overnight with trypsin in ratio 1:100 (Promega, Europe). After digestion, the tryptic peptides were collected by centrifugation, diluted with Britton & Robinson Universal Buffer (BRUB) composed of 0.02 M acetic acid, 0.02 M phosphoric acid, and 0.02 M boric acid at pH 5.0 and loaded on SAX column. Peptides were eluted from SAX column to C18 column first BRUB pH 5.0 then with BRUB pH 2.0 and then eluted from C18 columns with 60% ACN. All peptide fractions were stored at –32°C prior to the nano-LC-MALDI TOF/TOF-MS analysis.

Nano-liquid chromatography-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis and database search

The nano-LC-MALDI-TOF/TOF-MS analysis was performed as previously described (9). Briefly, a nano-liquid chromatograph (EASY-nLC II™, Bruker Daltonics, Germany) was used. Tryptic peptides were separated on the reverse phase Acclaim PepMap 100 C18 column (Thermo Fisher Scientific, USA). In total 18 µl of each sample was injected. The solvent system consisted of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in ACN/H_2O (90/10) (solvent B). Elution was performed with a 2 – 45% gradient (solvent B) over 64 min followed by a 45 – 100% gradient (solvent B) over 0.1 min. The column was maintained at 7°C and the flow rate was set at 300 nL/min. Fractions were collected on MTP AnchorChipTM 800/384 TF plate (Bruker Daltonics, Germany) by automated system for fraction collection PROTEINEER fc II (Bruker Daltonics, Germany). The collected fractions were analyzed by MALDI-TOF/TOF-MS™ (Bruker Daltonics, Germany) using WARP-LC software (version 1.2, Bruker) for acquisition and data processing. Peptide identifications were performed using BioTools (version 3.2, Bruker) together with MASCOT server (version 2.4.0, Matrix Science). The search included oxidation (M) as a variable modification and carbidomethyl (C) as a fixed modification. The precision tolerance was 100 ppm for peptide masses, and 0.7 Da for fragment ion masses. Individual peptide matches with scores above 28 were considered statistically significant. This threshold was determined based on the Mascot result report for a peptide mass fingerprint search. Proteins identification was performed manually, based on two unique peptides with the probability less than 0.05. The protein classification was performed by means of a free algorithm applied in the PANTHER Classification System (Version 1.2) (16).

RESULTS

Tryptic peptides from membrane and cytosol fraction of rat platelets as well as platelet lysate were identified as assigned to specific proteins. Proteins were identified using BioTools (version

Table 1. The number of identified proteins in platelet lysate and subcellular compartments (cytosol and membrane fractions) collected from the rat blood.

Type of sample	Isoelectric point [pH]	Number of identified peptides	Number of identified proteins	Number of unique proteins
Platelet lysate	5 > pI > 2 pI > 5	806 441	148 90	45
Cytosol fraction	5 > pI > 2 pI > 5	977 159	178 32	55
Membrane fraction	5 > pI > 2 pI > 5	607 90	123 25	34

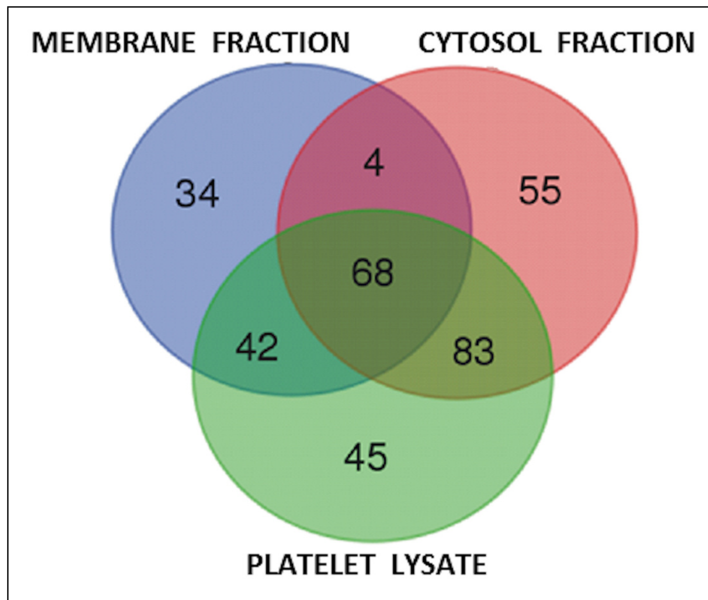

Fig. 1. The proportional distribution of mutual and non-mutual proteins in platelet lysate and subcellular fractions (Venn diagram) (17).

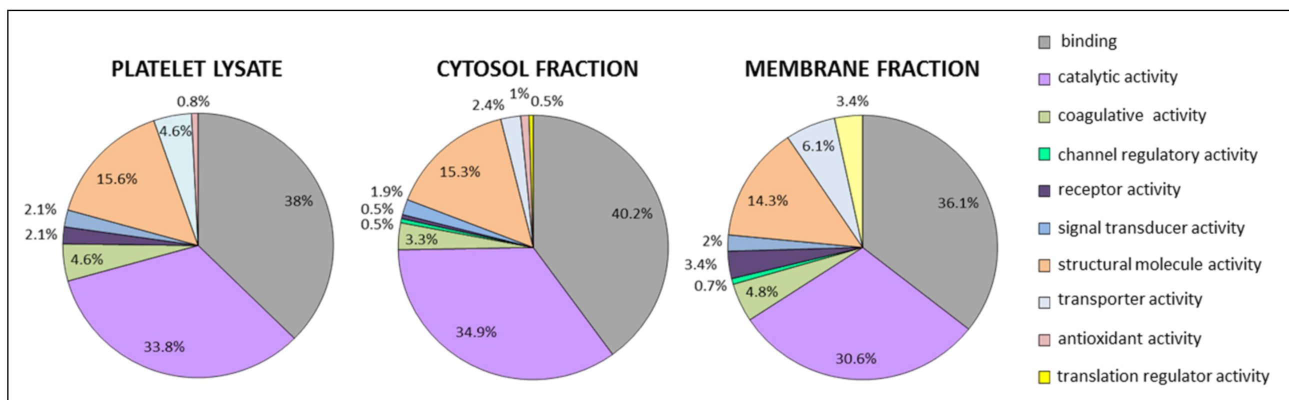

Fig. 2. The relative amount of proteins classified according to the molecular functions (PANTHER Classification System).

3.2, Bruker) together with MASCOT server (version 2.4.0, Matrix Science). Here, we identified 238 proteins in the platelet lysate, including 45 unique proteins, 210 in the cytosol fraction, including 55 unique proteins, and 148 in the membrane fraction of platelet, including 34 unique proteins (*Table 1*). Moreover, the comparison between subcellular platelet fractions revealed 68 mutual proteins as presented in Venn diagram (*Fig. 1*) (17).

The performed gene ontology analysis showed that there were differences in the proteome of cytosol and membrane fractions related to the molecular functions. Membrane fraction contained relatively more proteins with receptor activity (3.4% versus 0.5%), more proteins with translation regulator activity (3.4% versus 0.5%) and transporter activity (6.1% vs 2.4%), less binding proteins (36.1% versus 40.2%) and less proteins with catalytic activity (30.6% versus 34.9%), and no proteins with antioxidant activity compared to cytosol fraction (*Fig. 2*). We observed also more proteins with coagulative activity in the membrane fraction when compared to cytosol fraction (4.8% versus 3.3%). We found fibrinogen α, β and γ, P-selectin and platelet glycoprotein Ib beta chain in the platelet lysate as well as in both subcellular fractions (*Table 2*). Coagulation factor XIII (FXIII) was present in the platelet lysate and cytosol fraction, and not observed in the membrane compartment, while von Willebrand factor (vWF) and platelet factor 4 (PF4) were present

Table 2. Platelet proteins with coagulation activity differentially expressed in subcellular fractions ("+", means present; "–", means absent).

Protein	Platelet lysate	Cytosol fraction	Membrane fraction	General function
Fibrinogen α	+	+	+	Yield monomers that polymerize into fibrin and acts as a cofactor in platelet coagulation
Fibrinogen β	+	+	+	Yield monomers that polymerize into fibrin and acts as a cofactor in platelet coagulation
Fibrinogen γ	+	+	+	Yield monomers that polymerize into fibrin and acts as a cofactor in platelet coagulation
Platelet glycoprotein Ib beta chain	+	+	+	Participates in the formation of platelet plugs by binding to von Willebrand factor, which is already bound to the subendothelium
P-selectin	+	+	+	Mediates rolling of platelets on activated endothelial cells, determines size and stability of platelet aggregates
Coagulation factor XIII	+	+	-	Supports platelet adhesion under flow and potentiates the thrombogenic effects of established platelet ligands
Von Willebrand factor	+	–	+	Released from α-granules of activated platelets, binds to the glycoprotein IIb/IIIa complex, and forms a bridge between the subendothelial surface and the platelet, which initiates and supports platelet spreading, promotes platelet aggregation
Platelet factor 4	+	–	+	Released from α-granules of activated platelets, promotes blood coagulation by moderating the effects of heparin-like molecules
Platelet glycoprotein V	+	–	–	Subunit of the platelet receptor for von Willebrand factor and thrombin, cleaved from the platelet surface during activation by thrombin, binds to collagen
Apolipoprotein E	+	–	–	Secreted from platelets, enhances macrophage cholesterol accumulation

in the platelet lysate and membrane fraction, and not observed in the cytosol compartment. Interestingly, platelet glycoprotein V and apolipoprotein E (Apo E) were present in the platelet lysate but were not detected in subcellular fractions (*Table 2*).

DISCUSSION

In this study, we used the nano-LC-MALDI-TOF/TOF-MS method, based on the bottom-up proteomic analysis, for identification of proteins localized in the subcellular fractions of platelets. Here, we confirm that nano-LC is an alternative technique for identifying and differentiating proteins in subcellular fraction of rat platelet. Although, we used a well-described nano-LC-MALDI-TOF/TOF-MS method, we enlarged the protocol of sample preparation with subcellular fraction isolation. The subproteome analysis showed that membrane proteins due to their low abundance and low solubility could be masked, thus underrepresented in the whole platelet-proteome analysis (18). Thus, we performed proteomic analysis, identifying the rat platelet proteins in the platelet lysate, but also in the cytosol and membrane subcellular fractions. We showed previously that the nano-LC-MALDI-TOF/TOF-MS method is a sensitive and efficient technique for biomarker identifications in extracellular microvesicles and human platelets as well (12). However, the present study showed that the nano-LC-MALDI-TOF/TOF-MS technique was less sensitive than the two-dimensional liquid chromatography tandem mass spectrometry approach involving an LTQ Orbitrap mass spectrometer which was previously used by Yu *et al*. (19). Their analysis identified 837 proteins, making it the first comprehensive protein database so far for rat platelets. In fact, the review of platelet proteome literature shows different results of proteome analysis which depend on the way of samples isolation, proteomic techniques as well as platelet activation status (20, 21). It was demonstrated that the methods used to isolate and prepare protein samples may affect the results of proteomic studies. Delays in sample preparation and anticoagulant used may also influence the identification of proteins (22). The modern proteomic techniques are used not only for estimating protein abundance but also for comparing cell proteomes. A method combining two-dimensional electrophoresis-based proteomic assay with MALDI-TOF MS/MS protein identification was previously used for comparing platelet proteomes and evaluate the mechanism of platelet

activation in rat and rabbit (23, 24). Moreover, many of the proteins in the platelet proteome have not been reported at the mRNA level in platelets, and some, identified in the platelet secretome, have not previously been reported in platelets (25). This additionally makes it difficult to interpret and compare the results of proteomic analysis.

It is well known that the subcellular location of different proteins determines the signaling pathway and the final effect. Our subproteome analysis showed differences in the proteome of platelet cytosol and membrane fractions related to the biological process and signaling pathways. Different, by subcellular location, expression of proteins which are involved in the platelet activation and coagulation, was observed. Coagulation FXIII was present only in the platelet lysate and cytosol fraction. In fact, FXIII is highly abundant in platelets, and has been demonstrated as localized diffusely through the entire cytoplasmic matrix of platelet (26). The role of FXIII derived from platelets in platelet rich thrombus stabilization remains uncertain, however, it was reported that platelet-dependent clot retraction requires FXIII, which covalently associates fibrin polymers with protein located within the platelet plasma membrane (27). The lack of vWF and PF4 in the cytosol fraction may suggest that they were not released from α-granules, since the platelets were not activated. It seems that the platelet activation status may be responsible for differences in subcellular localization of proteins. Interestingly, we found some unique proteins in the platelet lysate, that were not present in the cytosol and membrane fractions. First, Apo E, which is a membrane protein belonging to the class of proteins involved in fat metabolism, was found only in the platelet lysate. Bearing in mind that Apo E is mostly produced in liver, macrophages and astrocytes, its presence in platelets should be considered as an incidental but important finding. The lack of Apo E in the membrane fraction could be related to the preparation procedures. We assume that Apo E could be washed out from the platelet surface, thus its presence in the membrane fraction was not detected. Next, the lack of platelet glycoprotein V, which is a part of the Ib-V-IX system of surface glycoproteins that constitute the receptor for vWF, in the membrane fraction is probably due to the lower sensitivity of this method than the previously published ESI-MS/MS study. In our next study, we are going to perform proteomic analysis of activated rat platelets to find out if the above discrepancies in subcellular localization are related to the platelet activation status, thus protein storage in subcellular compartments of resting platelets (*e.g.* α-granules or microvesicles).

Our study shows that the nano-LC-MALDI-TOF/TOF-MS technique could be used for identifying proteins in subcellular fractions of rat platelets. The nano-LC-MALDI-TOF/TOF-MS was less sensitive than previously published results of ESI or LC-MS analysis, which may be a limitation of this method. On the other hand, the sample handling in this method is minimized, since the preparation steps are carried out in solution instead of in gel. Moreover, the nano-LC-MALDI-TOF/TOF-MS technique allows removing all kinds of contamination from the samples and needs less reagents (green chemistry). In our next study, we are going to apply the nano-LC-MALDI-TOF/TOF-MS technique for proteins identification in subcellular fraction of activated rat platelets followed by quantitative analysis to find out if the observed differences in the protein subcellular localization depend on platelet activation status or method sensitivity.

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