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ORIGINAL ARTICLE

Platelet-released extracellular vesicles: the effects of thrombin activation

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Abstract

Platelets exert fundamental roles in thrombosis, inflammation, and angiogenesis, contributing to different pathologies from cardiovascular diseases to cancer. We previously reported that platelets release extracellular vesicles (pEVs) which contribute to thrombus formation. However, pEV composition remains poorly defined. Indeed, pEV quality and type, rather than quantity, may be relevant in intravascular cross-talk with either circulating or vascular cells. We aimed to define the phenotypic characteristics of pEVs released spontaneously and those induced by thrombin activation to better understand their role in disease dissemination. pEVs obtained from washed platelets from healthy donor blood were characterized by flow cytometry. pEVs from thrombin-activated platelets (T-pEVs) showed higher levels of P-selectin and active form of glycoprotein IIb/IIIa than baseline non-activated platelets (B-pEVs). Following mass spectrometry-based differential proteomic analysis, significant changes in the abundance of proteins secreted in T-pEVs compared to B-pEVs were found. These differential proteins were involved in coagulation, adhesion, cytoskeleton, signal transduction, metabolism, and vesicle-mediated transport. Interestingly, release of proteins relevant for cell adhesion, intrinsic pathway coagulation, and platelet activation signalling was significantly modified by thrombin stimulation. A novel pEV-associated protein (protocadherin-α4) was found to be significantly reduced in T-pEVs showing a shift towards increased expression in the membranes of activated platelets. In summary, platelet activation induced by thrombin triggers the shedding of pEVs with a complex proteomic pattern rich in procoagulant and proadhesive proteins. Crosstalk with other vascular and blood cells in a paracrine regulatory mode could extend the prothrombotic signalling as well as promote proteostasic changes in other cellular types.

Keywords Atherosclerosis · Extracellular vesicles · Microvesicles · Platelets · Thrombin · Thrombosis

Introduction

Extracellular vesicles (EVs) harbour a unique subset of lipids, nucleic acids, and proteins derived from the parental cell that can help to define solid organ diseases by liquid biopsy and contribute to cell-to-cell cross-talk and regulation of distal cell function [1–3]. Based on their size, cargo, and biogenesis, EVs (30 nm–5 µm range) are categorized either in exosomes, microvesicles, and apoptotic bodies, or in small, medium, and large extracellular vesicles, highlighting their heterogeneity. Platelet-derived extracellular vesicles (pEVs) constitute the majority of vesicles in the bloodstream [2–4]. Although pEVs are found in the blood of healthy individuals [5], higher numbers are found in the blood of patients with cardiovascular risk factors, with inflammation and with clinical syndromes as cardiovascular disease, cancer, and coronavirus disease-19 [6–8]. In recent years, it has become clear that pEVs have important biological functions, such as in inflammation-induced haematopoiesis [9], in blood coagulation [10] and as carriers of bioactive messages [11]. We previously showed that pEVs enhance thrombosis on atherosclerotic plaques [12], and that they are elevated in high-cardiovascular risk (HCVR) patients mapping asymptomatic lipid-rich atherosclerotic burden [6] and predicting adverse cardiovascular outcomes [13].

The proteome of pEVs released by washed platelets activated with adenosine diphosphate (ADP) [14, 15] and

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a comparative proteome analysis of plasma and pEVs were reported [16, 17]. Various studies have analysed the plasma EV proteome from healthy donors [18] and from patients with different pathologies [19]. The protein composition of EVs in plasma from healthy donors shows high variability [20]; however, a core set of plasma EV proteins found across populations rather than in a subset of individuals has been identified [21]. To increase its complexity, it seems that different size of pEVs contain different protein components [22] and that the different stimulation conditions induce very different types of pEVs [16]. In this regard, a biological link between the potency of the agonist and pEV composition in terms of proteins associated to platelet activation and degranulation has been reported [23]. *Releasates* (full secretome without removing EVs) from platelets activated by thrombin or thrombin receptor-activating peptide were characterized [24–26], although in these last studies, EVs were not specifically analysed.

Platelets, activated by a variety of biochemical agonists (thrombin, collagen, and ADP) and mechanical stimuli (shear stress), adhere and aggregate at sites of vascular injury [27] and are major components of thrombus growing on atherosclerotic plaques [28]. Thrombin potently activates platelets via cleavage of G protein-coupled protease-activated receptors (PARs) 1 and 4 contributing to signal transduction, platelet secretion and aggregation, and thrombosis on severely damaged atherosclerotic vessels [29, 30]. Based on our findings of an elevated level of pEVs in HCVR patients [6, 13] and the significant effect of pEVs in promoting thrombus formation on injured vessel wall [12], we have hypothesized that pEVs released from thrombin-activated platelets, as it happens in thrombosis induction by lipid- and TF-rich atherosclerotic plaques, may be unique cargoes capable of regulating blood–cell interactions and vascular permeability. Proteostasis, as the dynamic regulation of a balanced and functional proteome including networks regulating proteins present within and outside the cell, is important in the conformation of extracellular vesicles. Here, we approached the investigation of the extracellular vesicles released by human platelets and their proteome modification by activation with thrombin.

Methods

Platelet activation and platelet-derived extracellular vesicle subfractionation

Fresh human platelet concentrates from healthy donors were centrifuged (1200×*g*, 10 min, 20 °C), washed three times, and resuspended in Ca^{2+} free-HEPES-Tyrode's buffer (HTB; pH 7.3). Platelet function was determined by optical aggregometry. Washed platelets were counted and adjusted to a final concentration of 4.0×10^6 platelets/μL (Medonic–CA530-16). The platelet suspension was activated with human thrombin (T, 0.5 uNIH/mL) or its buffer (control or baseline, B) for 3 min at 37 °C with constant slow stirring, as previously described [31]. Immediately thereafter, platelets were pelleted by a centrifugation step (3220×*g*, 10 min, 20 °C) and stored at − 80 °C for further studies. The supernatant was centrifuged once more to assure platelet removal. One aliquot was taken for flow cytometry characterization and, thereafter, pEVs were isolated from the supernatants by ultracentrifugation (150,000×*g*, 90 min, 10 °C) [17]. The final pEV pellet was resuspended in lysis buffer containing protease inhibitors. pEV aliquots were snap-frozen in liquid nitrogen and stored at − 80 °C until proteomic studies were performed. Reporting of pEV isolation, characterization, and analysis follows the Minimal Information for Studies of Extracellular Vesicles standards and ISEV recommendations [1].

Flow cytometry characterization

pEVs were identified and characterized by flow cytometry according to the methodology previously described [12]. pEVs were identified and quantified based on their forward scatter (FSC)/side scatter (SSC) characteristics according to their size and their fluorescent binding to annexin V [32, 33] and cell-specific monoclonal antibodies (mAbs). Briefly, samples were incubated (20 min, room temperature [RT], dark) with phycoerythrin-conjugated Annexin V (Molecular Probes) and fluorescein isothiocyanate-conjugated anti-human mAbs or isotype-matched control antibodies (Supplementary Table 1). Samples were diluted in Tyrode's Buffer supplemented with 5 mM $CaCl_2$, and were immediately analysed in a FACSCantoII™ flow cytometer with FACSDiva™ analysis software (Becton Dickinson). Acquisition was performed for 1 min/sample at low flow rate. The instrument was calibrated with Flow-Check YG-Size-Range Calibration Kit (Polysciences). FSC/SSC light scatter and fluorescence channels were set at logarithmic gain. The lower detection limit was placed as a threshold above the electronic background noise, the upper threshold for FSC to 1 μm, and a threshold was set at the second logarithm for SSC parameter. Gating of pEVs in Supplementary Fig. 1 shows that cell and platelet contamination is minimal (less than 0.1%). pEV number was determined by comparison with calibrator FlowCount beads (Beckman Coulter) in a predetermined concentration. AV binding level was corrected for auto-fluorescence using fluorescence signals obtained with pEVs in a calcium-free buffer. To reduce background noise, buffers were prepared on the same day and 0.1 μm-filtered.

Proteomic analysis

Proteomic studies were performed as previously described [34] and are presented in accordance with the Minimum Information About a Proteomics Experiment principles [35].

Sample preparation pEV samples were subjected to IgG removal with protein G-sepharose and were cleaned by centrifugation using 3 kDa-centrifugal filters (Millipore) and sample buffer was exchanged to a urea-denaturing buffer. Prior protein separation, samples were desalted and decontaminated by ReadyPrep 2D-CleanUp Kit (Bio-Rad). Protein concentration was assessed in triplicate using QuantKit assay (GE Healthcare).

Two-dimensional gel electrophoresis (2-DE) Protein extracts (120 µg) were loaded by active rehydration on pH 3–10 linear range dry strips at 50 V during 16 h. Strips were isoelectric focused at 0.05 mA/strip for 70 kV/h at 20 °C using the Protean-IEF cell (Bio-Rad) and were equilibrated with a reducing and an alkylating solution (15 min/each). For the second dimension, strips were applied on top of 12% SDS–polyacrylamide separating gels and electrophoresis was performed using an EttanDalt-Six system (GE Healthcare). Gels were visualized by fluorescent staining (Flamingo Fluorescent Gel Stain, Bio-Rad) and were scanned for image analysis (Typhoon-9400, GE Healthcare). Analysis for differences in protein patterns was performed with PDQuest-8.0 software (Bio-Rad), using a single master including all gels. The relative volume of each single spot was compared to the volume of all spots in the gel, following background extraction and inter-gel normalization based on local regression method of the software, as previously reported [34].

Protein Identification by Maldi-ToF–MS Analysis Gel pieces containing protein spots of interest (based on differential analysis and being matched in > 90% of 2DE-gels) were excised from 2DE-gels using Spot-Picker. After distaining, proteins spots were washed and dried under vacuum before enzymatic digestion with sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion were applied to Prespotted-AnchorChip plates. Protein identification was performed by matrix-assisted laser desorption/ionization–time of flight mass spectrometry using an AutoFlex-III-SmartBeam MALDI-TOF/TOF (Bruker-Daltonics). All spectra were acquired with flexControl and each sample was processed with flexAnalysis (version 3.0, Bruker-Daltonics). After processing, spectra were sent to the interface BioTools-v.3.2 (Bruker-Daltonics) and MASCOT search on SwissProt-57.15 database was done.

Animal experimental design

Pigs received a single loading dose of 600 mg clopidogrel (Bristol Myers Squibb/Sanofi Pharmaceuticals). The study protocol was approved by the institutional ethics committee and all animal procedures were performed in accordance with the guidelines from Directive 2010/63/EU or the National Institutes of Health (NIH) guidelines (NIH No. 85–23, 1996). Blood samples were withdrawn into 3.8%-sodium citrate tubes at baseline and after 4 h of antiplatelet treatment. Blood cells were removed by low-speed centrifugation at 1258×*g* for 20 min at RT and platelet poor plasma was carefully aspirated. A second centrifugation step was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed within 1 h after extraction. A PFP aliquot was used for flow cytometric studies by isolating the EV fraction by a two-step high-speed centrifugation and lysed with sample buffer containing protease inhibitors.

Western blotting

Protein extracts were separated using Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electrotransferred to nitrocellulose membrane in semi-dry conditions (Semi-dry transfer system, Bio-Rad), and subsequently blocked with 5% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) for 1 h prior to primary antibody incubation (overnight at 4 °C). Membranes were exposed to rabbit anti-flotillin monoclonal antibody (Abcam) and anti-PCDHA4 polyclonal antibody (Creative-Diagnostics). Following incubation, membranes were washed with TBS-Tween (three times, 10 min each), incubated with horseradish peroxidase-labelled goat anti-rabbit secondary antibody (Millipore) for 1 h at RT and washed again as previously described. Blots were imaged by enhanced-chemiluminescence using Super-Signal (Pierce) and ChemiDoc™ XRS system (Bio-Rad) and quantified by densitometry (Image Lab software, Bio-Rad) [36]. Beta-actin was used as loading control for target protein quantification. Specifically, total protein signal was used for protein normalization in EVs.

Bioinformatics

Data mining Gene Ontology (GO) Slim/MGI-human database was used for GO assignments to identified proteins and determination of significantly under-and-over-represented functional categories. Signalling pathways were investigated using the KEGG and Phanter softwares. The identified protein datasets were compared to Vesiclepedia [37] and ExoCarta [38] databases, and subjected to FunRich enrichment analysis [39].

Protein–protein interactions Ingenuity pathways analysis (IPA) software (Ingenuity-Systems) and the search tool for retrieval of interacting genes (STRING) database (https://string-db.org) were used to explore possible interactions

and functional clusters among all the identified differentially expressed proteins.

Statistical analysis

Data are presented as scatter plots with bars and mean ± SEM. Unpaired and paired 2-tailed Student's T tests were used for comparison of continuous data between two groups. *n* indicates the number of experiments. Probability values < 0.05 were considered statistically significant. All analyses were performed with StatView (5.0.1, SAS Institute) software.

Results

Platelet-derived extracellular vesicle characterization

Flow cytometry determination of the EV-associated marker annexin V gave similar results among all pEV samples. The presence of this well-established marker confirms that the pEV-sample conform a properly isolated homogenous and pure fraction. The data showed that platelets shed high amounts of extracellular vesicles upon thrombin stimulation (T-pEVs) (Supplementary Fig. 1), carrying a significantly higher percentage of platelet activation markers than extracellular vesicles from non-activated platelets (B-pEVs). Figure 1 shows that T-pEVs had significantly increased PAC1$^+$ ($\alpha_{IIb}\beta_3$-integrin) and CD62P$^+$ (P-selectin) markers in their surface compared to B-pEVs (35.30 ± 1.10 vs 5.43 ± 1.67% and 10.13 ± 1.53 vs 2.54 ± 0.88%, respectively).

Comparison between pEVs and pEV-free platelet *releasate* fractions

SDS-PAGE–1D analysis showed a distinct band pattern (Supplementary Fig. 2) in pEV and in pEV-free soluble platelet *releasate* (SPR) extracts, indicating that EV shedding is a proteostatic actively regulated process different from mechanical release following cell rupture or lysis. Remarkably, EVs were rich in high-molecular-weight proteins (larger than 160 kDa) which may correspond to post-translationally modified proteins and integral membrane proteins. Interestingly, thrombin activation caused global changes in the proteomes of both pEVs and SPR.

The lipid-raft and caveolae marker Flotillin was analysed in EVs by Western blot. As shown in Supplementary Fig. 2, lipid-raft protein Flotillin is released in pEVs. Interestingly, Flotillin content tends to be lower in T-pEVs than B-pEVs, indicating that lipid-raft proteins, required for EV shedding, are kept within the activated platelet structure.

Platelet-derived extracellular vesicle proteome profiling

Using an untargeted proteomics approach, 175 proteins were identified from all samples of pEVs. When comparing our data to those in publicly available databases, 90% of pEVs proteins identified by our strategy were found in Vesiclepedia and 74% in ExoCarta. However, 18 proteins (10%) of our MS-identified proteins were not yet listed in Vesiclepedia and may be novel entries into the database. Moreover, 27 proteins found in Vesiclepedia were not present in Exocarta, whereas all proteins found in Exocarta were found in

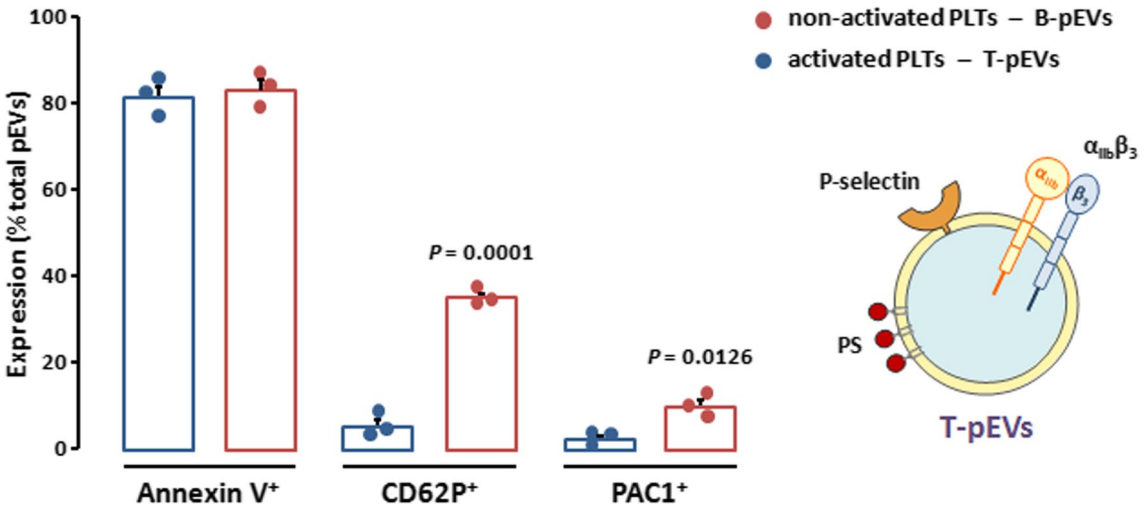

Fig. 1 Scatter plots with bars showing size-selected events with expression of phosphatidylserine (PS, annexin V$^+$) and platelet activation markers P-selectin (CD62P$^+$) and $\alpha_{IIb}\beta_3$-integrin (PAC1$^+$) in the fluorochrome-conjugated gate on pEV surface from baseline non-activated and thrombin-activated platelets ($n = 3$ independent experiments/group). Data are expressed as mean ± SEM of the labelling percentage of total population. *p* Value was calculated by 2-sided unpaired Student's *T* test

Vesiclepedia (Supplementary Fig. 3). Among the top-100 proteins more often identified in exosomes, 25 were also found in our pEV protein fraction.

Overall, platelets release pEVs with a highly complex and characteristic proteomic profile (Fig. 2A). Our identified proteins were then classified based on Gene Ontology Slim (GO-Slim) annotations for biological process (1165 entries), molecular function (239 entries), and cellular localization (214 entries), grouped into major GO annotations to identify the most represented ones (by number of proteins associated) in our pEVs dataset, as shown in Fig. 2B. Specifically, the five-top annotations for biological processes correspond to cell organization and biogenesis, protein metabolism, developmental processes, signal transduction, and transport. In accordance, signalling and cytoskeletal activity were the major molecular function categories. The proteins identified are associated with the major cellular organelles and with subcellular/extracellular localizations: 64 (19.8%) proteins were annotated as cell plasma membrane proteins and 45 (13.9%) as extracellular (secreted or plasma-related). Interestingly, a high percentage of proteins were cytosolic (41 proteins, 12.7%) and cytoskeletal (40 proteins, 12.4%).

As it could be expected from the high number of cytosolic and cytoskeletal-associated proteins, many proteins in pEVs were annotated to the metabolic and regulation of actin cytoskeleton pathways using the KEGG-pathway database (Supplementary Table 2). Furthermore, several proteins were annotated to the focal adhesion pathway. Interestingly, the complement and coagulation pathways were also found in resting pEVs. Finally, 5 proteins were annotated to the KEGG-pathway endocytosis. We determined likewise the over-represented pathway using Panther (Supplementary Table 3) with similarly comparable results to KEGG pathways and including pathways related to disease such as blood coagulation, metabolism like glycolysis, and cytoskeletal regulation as integrin signalling and Rho GTPase. Similarly, 10-top highly significant FunRich biological pathways were found in non-activated pEVs that were related to platelet function, coagulation system, thrombosis, and haemostasis

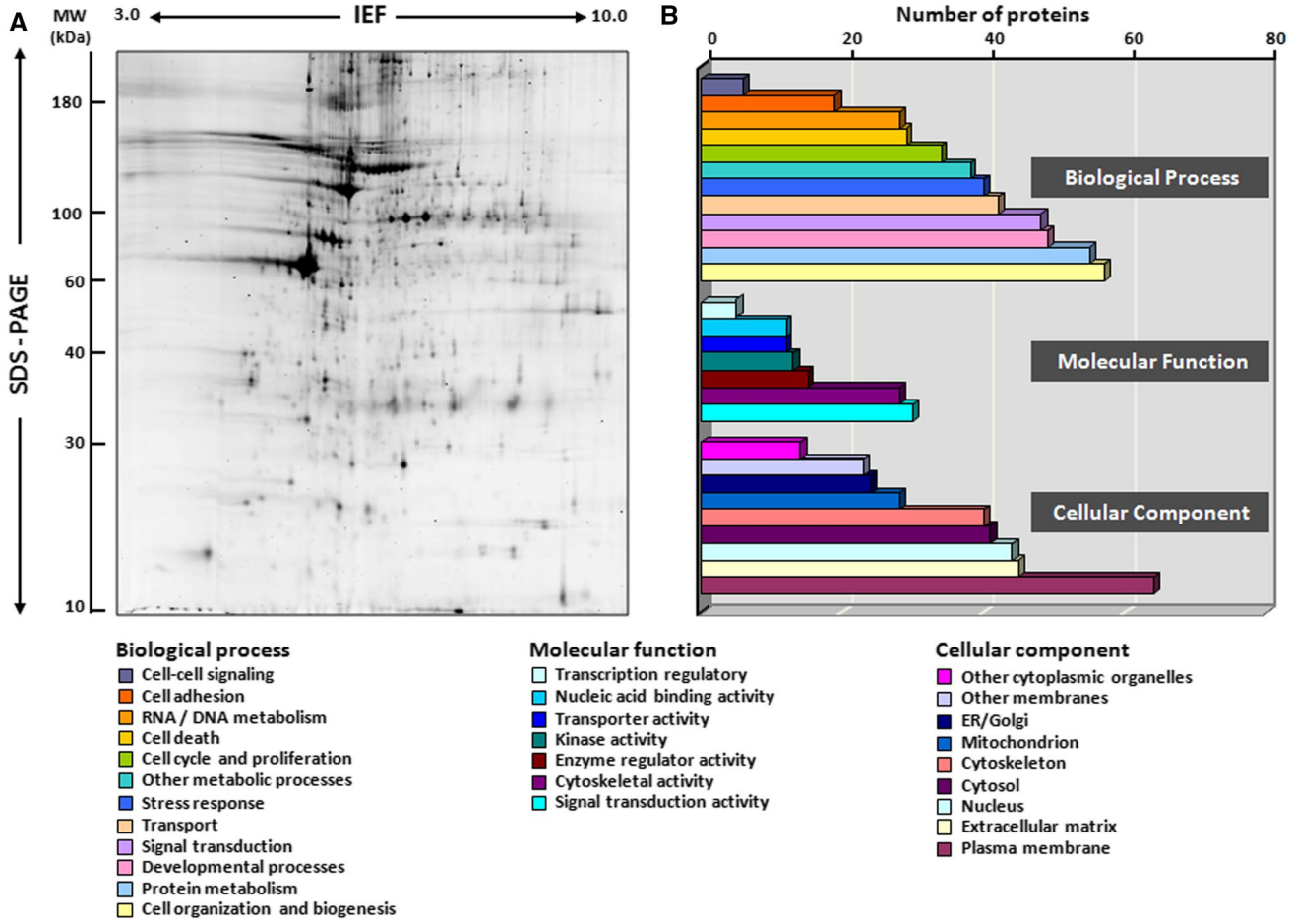

Fig. 2 Representative 2D proteome map of proteins in pEVs. **a** Proteins were separated in a pH range 3–10 on 12% SDS-PAGE. **b** Classification of identified proteins by gene ontology (GO) annotations—biological process, subcellular localization, and molecular function. Number of pEV proteins significantly enriched in all obtained GO-Slim categories

proving the validity of the bioinformatics analyses (Supplementary Table 4).

Thrombin-induced platelet activation and released pEV proteome

Proteomic analysis showed that pEVs released from platelets upon thrombin activation carry different proteins than pEVs from non-activated platelets (Fig. 3A). Remarkably, 63 protein features (spots) were identified as differentially processed between T-pEVs and B-pEVs (cut-offs: fold change of ≥ 1.5 and p value of < 0.05) (Fig. 3B). Of note, these were mainly involved in (1) cytoskeleton and cell organization, (2) signal transduction, (3) transport vesicle-mediated, and (4) metabolism. Functional pathways represented were coagulation and cell-adhesion proteins (Fig. 3C). The list of differentially represented proteins in thrombin-induced pEVs is shown in Fig. 4 and Table 1.

Extracellular vesicles carry proteins involved in signalling events in atherothrombosis

Among cytoskeleton, motility and cell organization proteins (Fig. 4), Coronin 1A (CORO1A), which forms the invaginations and protrusions of the plasma membrane, and catenin α-2, a linker between cadherin family of adhesion receptors and the cytoskeleton to control cell adhesion, are increased in pEVs upon platelet activation by thrombin stimulation, while myosin light chain 3 and myosin regulatory light chain (MRLC) polypeptide 9, the regulatory light chain of myosin, and the myosin regulatory subunit, respectively, are decreased in released T-pEVs. Regarding signalling proteins (Fig. 4), cAMP-dependent protein kinase type Iα regulatory subunit (PRKAR1A), a cAMP-dependent protein kinase regulator, and phosphatidylinositols-4-kinase α (PI4KA), involved in cadherin binding and cell adhesion, are released in T-pEVs. In contrast, transducin β-1 and kringle-containing transmembrane-1 (KREMEN1) are found decreased in T-pEVs. Similarly, vesicle transport protein USE1 and protein bicaudal-D homolog-1 (BICD1), both related to vesicle-mediated transport (Fig. 4) are also decreased in T-pEVs. In turn, the metabolic proteins ATP-synthase subunit α and glucose-regulated protein 78 (GRP78) are increasingly released in thrombin-activated platelet T-pEVs, while protein disulfide isomerase A1 (PDIA1) is decreased. Protein disulfide isomerase A3 (PDIA3) showed a trend to increase in T-pEVs (Fig. 4). Other proteins found differentially represented in T-pEVs include membrane glycoprotein gp140 (CDCP1), fermitin family homolog 3 (FERMT3 or Kindlin-3), long-chain-fatty-acid-CoA ligase-3, and annexin A4 and A5 (ANXA5), among others (Table 1), and relate to cell adhesion, organization, signalling, and vesicle trafficking,

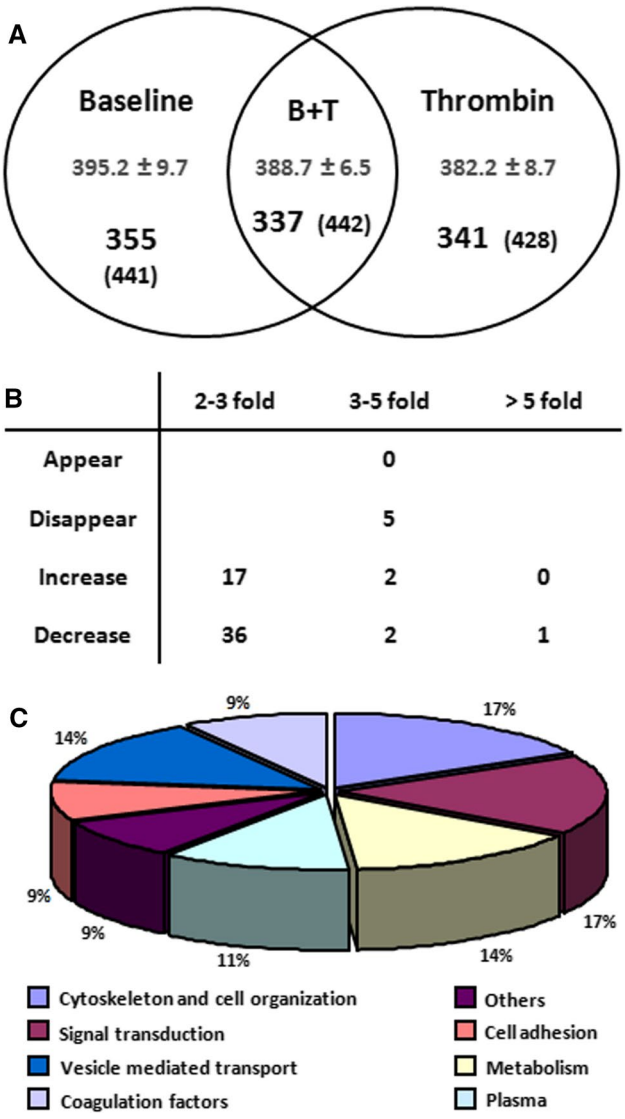

Fig. 3 Thrombin-induced effects on pEVs derived from baseline non-activated and thrombin-induced platelets. **a** Venn diagrams depicting overlap in vesicular protein spots in the different studied groups. **b** PDQuest differential analysis of pEV identified protein spots. **c** Distribution of thrombin-induced identified differential proteins by functional categories. Within each group (baseline non-activated and thrombin-stimulated), five individual experiments (biological replicates) were run. A total of ten gels per arm, two from each experiment, were analysed

playing fundamental roles in platelet activation and with coagulation system highly present.

Interestingly, there was lower loading of calcium-dependent cell-adhesion protein protocadherin α4 (PCDHA4) in T-pEVs, released by thrombin-activated platelets, than in B-pEVs from non-activated platelets (Table 1, Fig. 5A). PCDHA4 (UniProt number: Q9UN74) was identified as a single spot with an isoelectric point of 6.0 and a molecular weight of 52 kDa, and its levels were tenfold reduced

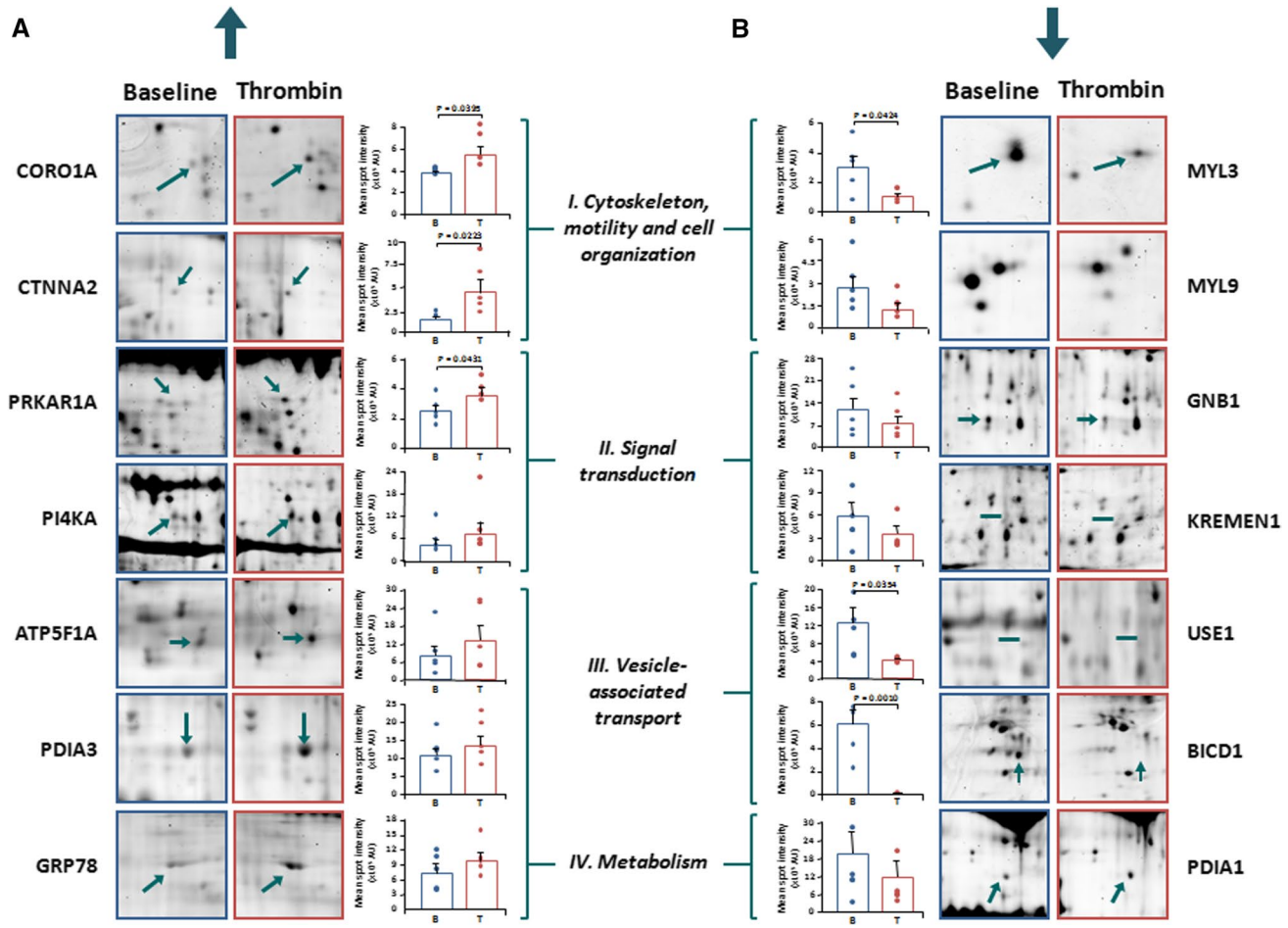

Fig. 4 Thrombin-induced effects on pEVs derived from baseline non-activated and thrombin-stimulated platelets. Selection of proteins differentially regulated between baseline non-activated and thrombin-induced platelets based on functional groups. Specifically, close-up views of representative 2-DE images corresponding to up-**a** and down-regulated **b** protein spots for cytoskeleton, motility and cell organization (**I**), signal transduction (**II**), vesicle-associated transport-related proteins (**III**), and metabolism (**IV**). Each panel shows scatter plots with bars for each spot showing variations in spot intensity in the different studied groups (*B* baseline non-activated and *T* thrombin-stimulated, $n = 5$ independent experiments/group). Data are expressed in arbitrary units (AU) as mean ± SEM. Differences were analysed by 2-sided unpaired Student's *T* test

in T-pEVs (Fig. 5A, Supplementary Fig. 4). Western blot validation in pEVs confirmed its reduction upon thrombin stimulation, though the change did not reach statistical significance (Fig. 5B). On the contrary, PCDHA4 was retained in the membrane of platelets activated by thrombin, as shown increased expression trend in the membranes of thrombin-activated platelets (Fig. 5B), while no differences were detected in the *releasate* fraction. To further study platelet proteostasis and deepen into the mechanism triggering PCDHA4 release to EVs, we tested the effect of a platelet inhibitor on PCDHA4 regulation. The effects on PCDHA4 processing in the platelets and circulating EVs were studied in vivo using clopidogrel (600 mg oral dose) in pigs. Annexin V-positive EV numbers in plasma were significantly reduced after treatment with the $P2Y_{12}$ antagonist. Pig platelets and their released EVs expressed PCDHA4 as human platelets. Treatment with clopidogrel did not significantly change the expression levels of PCDHA4 neither in the circulating EVs nor in the parental platelets (Fig. 5C), indicating that PCDHA4 regulation could be thrombin-specific. Platelet fraction showed a decreasing trend in PCDHA4 protein upon clopidogrel treatment, suggesting an agonist-dependent platelet aggregation impact on EV release and cargoes.

Functional groups identified

We run a systems biology analysis using IPA software to discover the most affected proteins in EVs released by thrombin-dependent platelet activation (Supplementary Table 4). The most significantly affected top canonical pathway was the intrinsic prothrombotic activation

Table 1 Thrombin-induced differential proteins in extracellular vesicles derived from thrombin-stimulated platelets

Function	Protein name	UniProt number	Mascot score	Fold[a] change	*p* Value[b]
Cytoskeleton, motility, and cell organization	Coronin 1A, CORO1A	P31146	59	+1.52	0.0881
	Filamin-A, FLNA	P21333	87	+1.67	0.0078
	Catenin alpha-2, CTNNA2	P26232	56	+3.03	0.1210
	Myosin light chain 3, MYL3	P08590	95	−2.94	0.0235
	MRLC polypeptide 9, MYL9	P24844	37	−2.63	0.0300
	Actin cytoplasmic 1, ACTB	P60709	144	−2.11	0.0094
Cell adhesion	CDCP1	Q9H5V8	56	+2.42	0.1011
	FERMT3	Q86UX7	57	+1.48	0.0479
	Protocadherin alpha 4, PCDHA4	Q9UN74	72	−7.69	<0.0001
Metabolism	ATP5F1A	P25705	101	+3.97	0.0235
	PDIA3	P30101	226	+1.46	0.1404
	PNP	P00491	80	−1.27	0.0092
	PDIA1	P07237	56	−1.96	0.0085
	ACSL3	O95573	60	−1.72	0.0025
	GRP78	P11021	59	+1.98	0.0037
Signal transduction	PRKAR1A	P10644	75	+1.49	0.0865
	PI4KA	P42356	74	+1.55	0.0135
	Annexin A5, ANXA5	P08758	30	+2.29	0.1942
	GNB1	P62873	56	−1.89	0.0043
	Ras suppressor protein 1, RSU1	Q15404	84	−1.61	0.0548
	KREMEN 1	Q96MU8	56	−1.89	0.0071
Vesicle-mediated transport	NAPA	P54920	58	+1.92	0.0488
	BICD1	Q96G01	60	D	<0.0001
	USE1	Q9NZ43	88	−3.70	0.0299
	RAB3IP	Q96QF0	80	−2.50	0.0020
	Annexin A4, ANXA4	P09525	52.6	−1.25	0.0255
Coagulation factors	Fibrin(ogen) gamma, FGG	P02679	92	D	<0.0001
	Anti-thrombin III, SERPINC1	P01008	65	−4.76	0.0074
	Fibrin(ogen) beta, FGB	P02675	129	−2.56	0.0026
Plasma	C4BPA	P04003	79	+1.62	0.0005
	Antitrypsin alpha 1, SERPINA1	P01009	110	+1.36	0.0198
	Kallikrein, KLK1	P06870	56	+1.62	0.0636
	OSCP1	Q8WVF1	61	−1.59	0.0164
Others	Ashwin, C2orf49	Q9BVC5	62	−1.25	0.0501
	HERV-K104	P61576	56	−2.13	0.0010
	FBXL20	Q96IG2	59	−1.82	0.0162

− decrease, + increase, *D* disappearance, *ACSL3* long-chain-fatty-acid-CoA ligase 3, *ATP5F1A* ATP-synthase subunit alpha, *BICD1* protein bicaudal-D homolog-1, *C4BPA* C4b-binding protein alpha chain, *CDCP1* CUB domain-containing protein 1 or membrane glycoprotein gp140, *FBXL20* F-box/LRR-repeat protein 20, *FERMT3* fermitin family homolog or kindlin-3 (KIND3), *GNB1* guanine nucleotide-binding protein G(1)/G(S)/G(T) subunit beta-1 or transducin beta chain 1, *GRP78* glucose-regulated protein 78 kDa, *HERV-K104* endogenous retrovirus group K member 104 Rec protein, *KREMEN1* kringle-containing transmembrane protein 1, *MRLC* myosin regulatory light chain, *NAPA* α-soluble NSF-attachment protein, *OSCP1* organic solute transport protein 1, *PRKAR1A* cAMP-dependent protein kinase type I-alpha regulatory subunit, *PDIA1* protein disulfide isomerase, *PDIA3* protein disulfide isomerase A3, *PI4KA* phosphatidylinositol-4-kinase alpha, *PNP* purine nucleoside phosphorylase, *RAB3IP* rab3a-interacting protein or SSX2-interacting protein, *USE1* vesicle transport protein

[a]Fold change over non-stimulated platelets

[b]*p* values after inter-gel normalization of intensity spots values by the local regression model method of the PDQuest software

pathway (Fig. 6A), in which the contents of coagulation factor fibrinogen γ and β and anti-thrombin-III are significantly reduced, whereas kallikrein-1 shows a trend to increase in T-pEVs (Fig. 6B). Significant biological pathways in thrombin-induced differential pEV proteins are coagulation and fibrin formation (FunRich, Supplementary

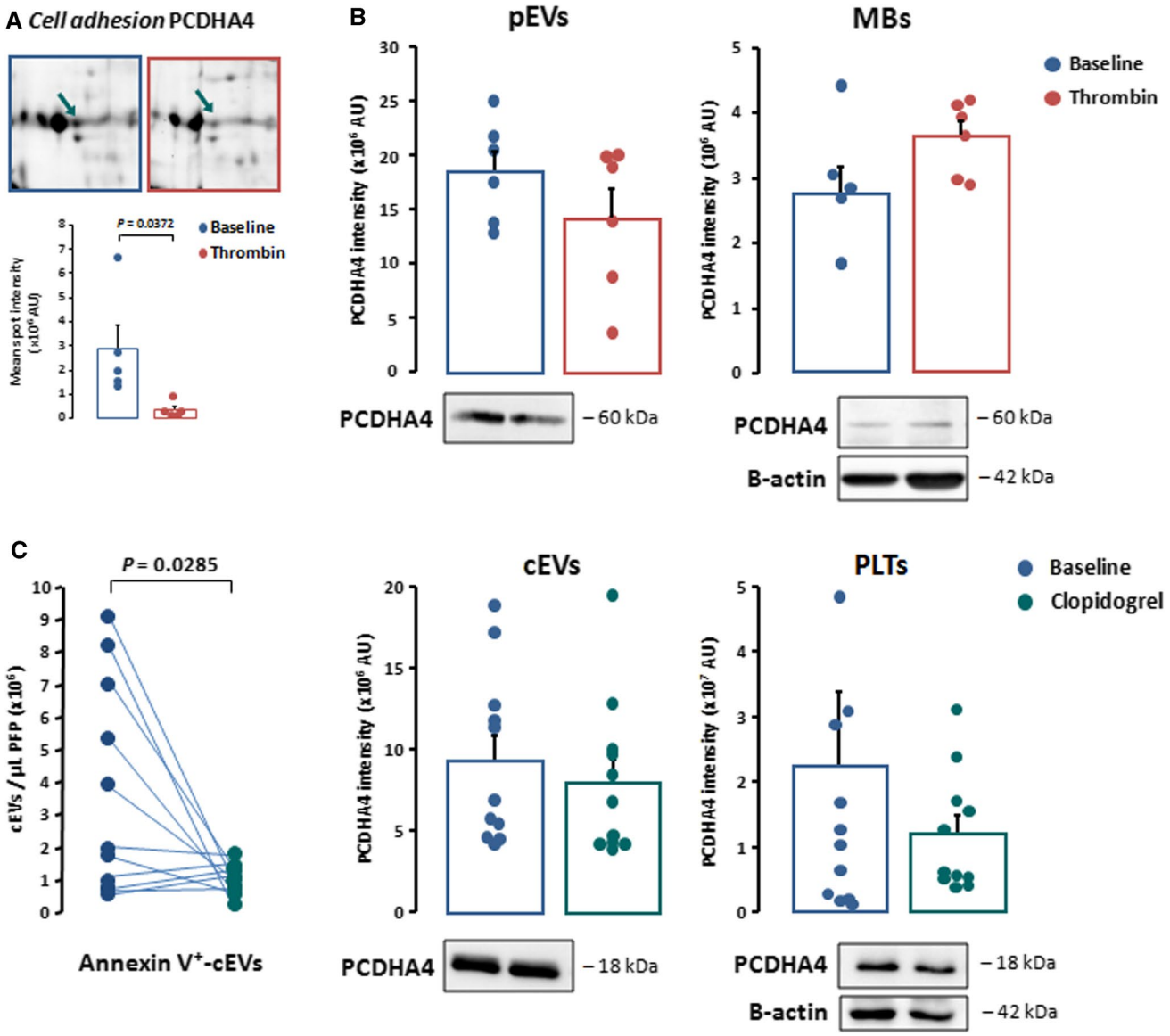

Fig. 5 Validation of differentially expressed protocadherin α4 protein. Analysis of the cell-adhesion protein protocadherin α4 (PCDHA4) by 2-DE (**a**) and western blot (**b** and **c**). **a** Enlargement of representative 2-DE images and scatter plots with bars showing variations in PCDHA4 spot intensity in the baseline non-activated and thrombin-stimulated pEVs ($n=5$ independent experiments/group). **b** Western blot analyses against PCDHA4 on pEV and platelet membrane (MB) samples. Scatter dots with bars showing the quantitative variations in band intensity in the baseline non-activated and thrombin-stimulated group ($n=6$ independent experiments/group). Data are expressed in arbitrary units (AU) as mean ± SEM. **c** Flow cytometric analysis of porcine AV^+-cEVs on plasma samples and western blot analyses against PCDHA4 on porcine circulating EV (cEV) and platelet (PLT) samples before and after clopidogrel administration ($n=11$ pigs). Scatter dots with lines showing the AV^+-cEV numbers (/µl platelet-free plasma [PFP]) before (at baseline) and after clopidogrel treatment. Scatter dots with bars showing the quantitative variations in band intensity (mean arbitrary units [AU] ± SEM) at baseline and after clopidogrel treatment ($n=11$ pigs). Total protein normalization of EVs was performed with *Ponceau S* and of MB and PLT fraction with β-actin. Differences were analysed by 2-sided unpaired (**a** and **b**) and paired (**c**) Student's *T* test

Table 6) and common pathway of fibrin and clot formation (Reactome, Supplementary Table 7). The top IPA connecting network for differentially regulated pEV proteome from thrombin-stimulated platelets shows functional association between coagulation, plasma membrane, cytoskeleton, and signalling proteins (Supplementary Fig. 5). When evaluating STRING network, beyond proteins involved in well-known signalling cascades, there are other thrombin-induced differential proteins neither clustered nor interconnected among them (Supplementary Fig. 6). As a compilation of our novel proteomic data and previous knowledge on the literature (*data harmonization*), the

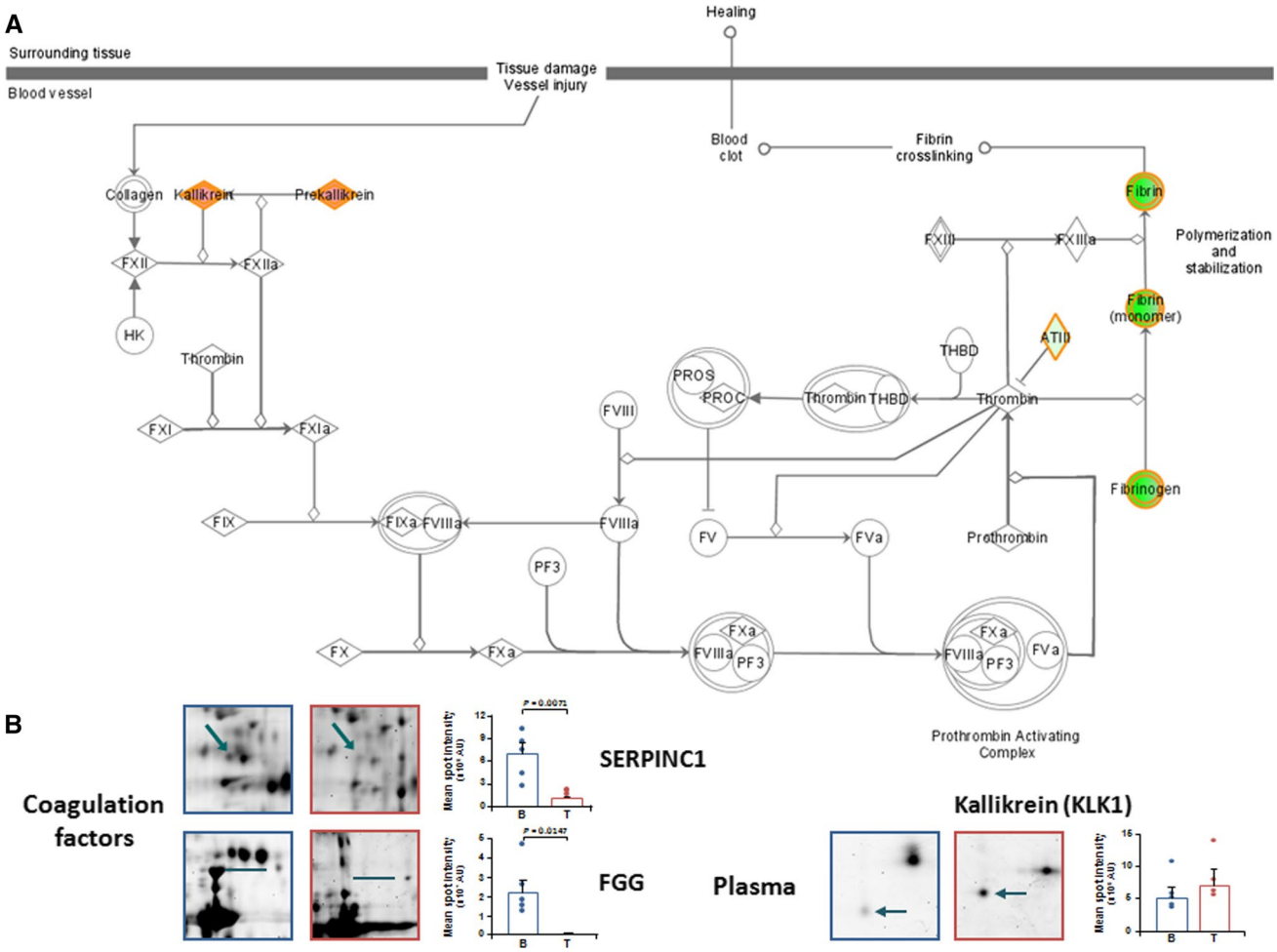

Fig. 6 Thrombin-induced effects on clotting factors and their inhibitor proteins of pEVs derived from baseline non-activated platelets and thrombin-stimulated platelets. **a** The top canonical pathway of proteins differentially regulated between baseline non-activated and thrombin-induced platelets by IPA software is the intrinsic prothrombin activation pathway. **b** Enlargement of representative 2-DE images corresponding to spots for coagulation factor fibrinogen gamma (FGG), anti-thrombin III (ATIII), and plasma protein Kallikrein (KLK1) with their corresponding scatter plots with bars indicating variations in spot intensity in the different studied groups (*B* baseline non-activated and *T* thrombin-stimulated, $n=5$ independent experiments/group). Data are expressed in arbitrary units (AU) as mean ± SEM. Differences were analysed by 2-sided unpaired Student's *T* test

potential functions triggered by these novel platelet entities are summarised in Fig. 7.

Discussion

Upon stimulation by biochemical or biomechanical agonists, platelets become activated and secrete proteins as soluble molecules or in extracellular vesicles generating a highly active secretome. pEVs are heterogeneous [40] and their protein load highly depends on the agonist triggering platelet activation [2, 3]. Because we found a significant effect of pEVs in promoting thrombosis on damaged vascular wall [12], where local thrombin formation has a major role, here we have investigated the proteostatic characteristics of EVs shed by thrombin-activated platelets to understand a possible systemic distal effect of local thrombin-induced platelet activation (Fig. 7).

Using a proteomic approach and bioinformatics, data mining, neural networks, and experimental validation, we have investigated proteins transported by extracellular vesicles when thrombin induces platelet activation. We have found that stress-related protein GRP78, a protein we previously described for the first time in platelets [41], is released in pEVs. Indeed, proteins found in the pEV belong to multiple subcellular localizations, from the endoplasmic reticulum (natural site for a stress-related protein) to the cytoplasm and cell surface; most of identified proteins were cytoskeleton and cytoskeleton-binding proteins (actin, cofilin, and MRLC), protein folding (isomerases, chaperons),

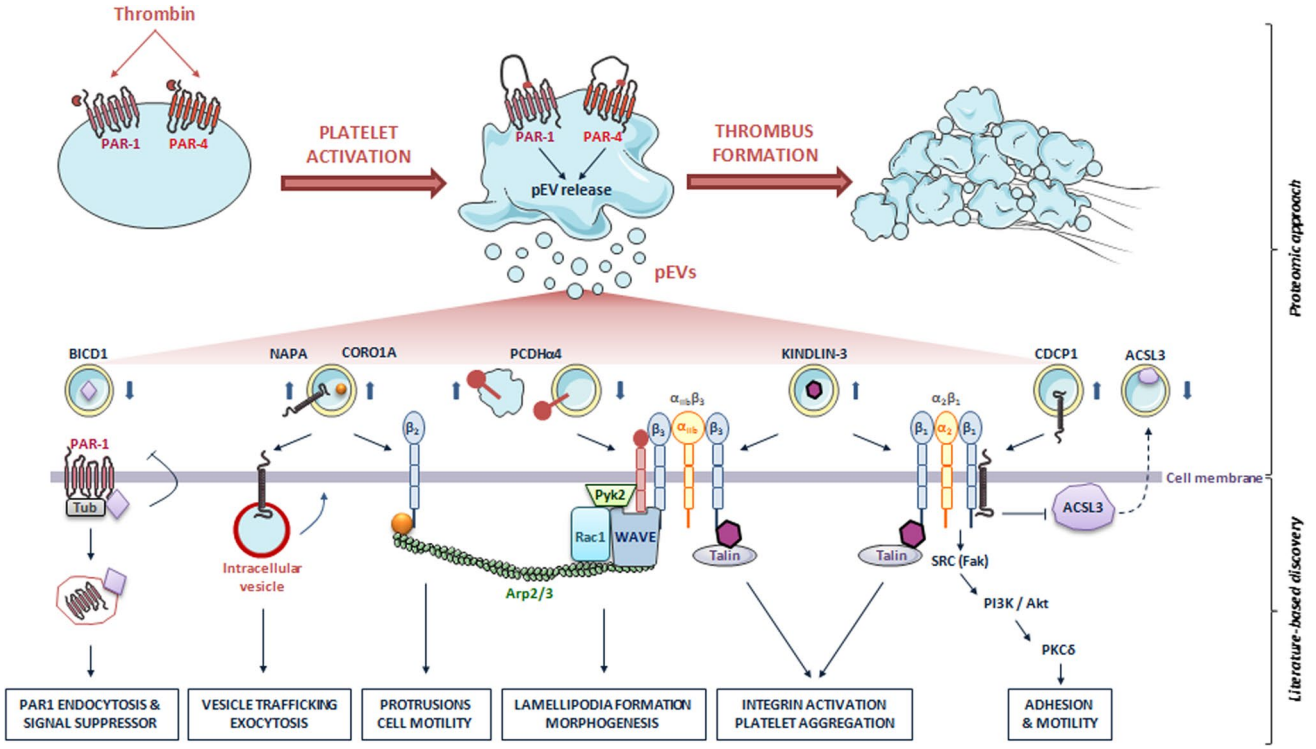

Fig. 7 Schematic representation of platelet-derived extracellular vesicle protein cargoes upon platelet activation with thrombin and potential associated cellular functions. Summary of the changes in platelet-derived extracellular vesicle proteins after thrombin-platelet activation compiled together with literature-based discovery of their cellular processes. $\alpha_2\beta_1$ integrin alpha-2/beta-1, $\alpha_{IIb}\beta_3$ integrin alpha-IIb/beta-3, *α-SNAP* alpha-soluble NSF-attachment protein, *ACSL* acyl-CoA synthetase long chain, *Akt* protein kinase B, *Arp2/3* actin-related proteins-2/3, *BICD1* protein bicaudal-D homolog-1, *CDCP1* CUB domain-containing protein 1, also known as membrane glycoprotein gp140, *CORO1A* coronin 1A, *Fak* focal adhesion kinase 1, *KINDLIN-3* fermitin family homolog 3 [FERMT3], *PAR* protease-activated receptor, *PCDHα4* protocadherin α4, *PI3K* phosphoinositide-3-kinase, *PKCδ* protein kinase C-delta, *pEV* platelet-derived microvesicle, *Pyk* pyruvate kinase, *Rac1* Ras-related C3 botulinum toxin substrate 1, *Tub* tubulin, *SRC* proto-oncogene tyrosine-protein kinase Src, *WAVE* Wiskott–Aldrich syndrome [WASP] family verprolin-homologous proteins

and membrane-associated proteins involved in intracellular transport, signalling and cell–cell interaction processes (annexins, CDCP1, fermitin, and protocadherin).

Low concentrations of thrombin activate platelets via the PAR-1 receptor. In contrast, high concentration of thrombin, as used herewith to activate platelets, results in stimulation of platelets via both PAR-1/-4 receptors. Interestingly, proteins from α-granules are found within EVs from platelets activated by either PAR-1 or PAR-4 [25], and the majority belong to metabolism, energy pathway, signal transduction, and communication systems [14]. Thrombin activation induces the release of EVs carrying cytoskeleton-related proteins involved in cell assembly and cell shape conformation. In fact, given the important role played by the cytoskeleton in cellular exocytosis, thrombin signalling induces EV generation via cytoskeletal remodelling. Some of the identified proteins are known to translocate from the soluble cytosol to the cytoskeleton associated to the actin scaffold in activated platelets; these are vinculin, α-actinin, filamin, the α, β and γ fibrinogen chains, the Arp2/3 complex, MRLC, and coronin. This association enable platelets to change their morphology, secrete granules and membrane blebs, as well as amplify the signals to adhere and aggregate. pEVs from thrombin-activated platelets showed increased surface expression of heterodimer αIIbβ3 integrin. Remarkably, GPIIb/IIIa exposed in activated platelets is involved in CRP monomerisation [42]. Thus, T-pEVs might enhance the prothrombotic effects of monomeric CRP, thereby linking inflammation and thrombosis.

We have previously shown that pEVs facilitate thrombus growth on thromboactive substrates [12]. Therefore, we have focused our attention on identifying proteins involved in thrombosis and signalling events underlying thrombus formation. Interestingly, we have identified proteins related to cell adhesion and outside-in signalling like CDCP1, FERMT3 and PCDHA4. CDCP1 is a glycoprotein CUB-domain-containing protein-1, also involved in cancer, which activates β1-integrin and induces motility signalling as well as regulates adhesion by forming complex with SRC-family kinases [43]. In the same line, FERMT3/Kindlin-3 plays a

central role in cell-adhesion in hematopoietic cells, by activating β1–β3-integrin and is required for platelet/leukocyte adhesion to endothelial cells [44] and platelet integrin activation and aggregation [45]. Of note, high expression of FERMT3 was detected in unstable atherosclerotic plaques [46].

For the first time, we have identified a novel cell-adhesion calcium ion-binding protein PCDHA4 in platelets and pEVs. PCDHA4 in nucleated cells exhibits both haemophilic and heterophilic interactions with membrane adhesion molecules and β1-integrin (through an RGD-motif), respectively [47]. The interaction of PCDHA4 intracellular domains with focal adhesion kinase (Fak) and proline-rich tyrosine kinase-2 (Pyk2), ensuing in kinase activity inhibition thereby affecting downstream Rho family small GTPases, has been described in neurons [48]. Specifically, clustered PCDHα family triggers a similar signalling cascade by interacting with WAVE complex and increasing Rac1, thereby modulating cytoplasmic dynamics and lamellipodial formation [49]. The Pyk2–Rac1–WAVE complex–Arp2/3 core signal has shown to mediate actin assembly in megakaryocytes [50]. Interestingly, we have detected significantly reduced levels of PCDHA4 in pEVs released from platelets upon thrombin activation, while thrombin-activated platelet membranes depicted an increase in PCDHA4 levels, suggesting activation of platelet cytoskeleton dynamics upon thrombin activation. Since all the described PCDHA4-interacting partners are important players in platelet activation, further studies aimed at unveiling the role of PCDHA4 in platelet biology and proteostasis are warranted.

Protein changes crucial for pEV cross-talk are highlighted by an increase in kallikrein and a reduction in fibrinogen γ and β as well as anti-thrombin-III. Kallikrein excises high-molecular-weight kininogen (HMWK) as an initial step in the intrinsic coagulation pathway [51]. Interestingly, our group has investigated the dynamics of coagulation proteins after acute myocardial infarction (MI), showing coordinately intrinsic coagulation pathway activation and an active HMWK cleavage early after post-MI [34]. Furthermore, other deregulated proteins are transported in pEVs, which include coagulation factor II (prothrombin), and the anticoagulant factors anti-thrombin-III, with also anti-inflammatory properties, and α2-macroglobulin. High α2-macroglobulin levels have been associated with the development of coronary artery disease [52]. In addition, we have identified in pEVs proteins of vesicle-mediated transport such as annexin A4 that promotes membrane fusion and is involved in exocytosis, Rab3a a key coordinator of secretion process and lysosome exocytosis, and α soluble NSF-attachment protein (NAPA) that also participates in intracellular membrane fusion and vesicular trafficking, actively contributing to paracrine signalling [53]. BICD1, primarily involved in Golgi-endoplasmic reticulum transport, was also found but only in pEVs from resting platelets. Remarkably, BICD1 adapter protein is a potent suppressor of PAR1 receptor signalling and transports PAR1 from the plasma membrane to the endosomal vesicles favouring its internalization [54]. Thus, its absence from T-pEVs prevents a down-regulation of the output signal of membrane PAR1 receptor.

Thrombin activation triggers also inside-out signalling and induces increase in protein kinase activity. Of interest is the detection and modified expression of PI4KA, PRKAR1A, ANXA5, transducin beta chain-1, and KREMEN1 in the T-pEVs. The latter one, KREMEN1 is involved in the Wnt-beta-catenin signalling [55, 56], which was identified in platelets and may enhance the amplification of platelet activation [57].

The endoplasmic reticulum resident and cell surface PDIA1 considered as a critical mediator of wound healing and as a chaperone that inhibits aggregation of misfolded proteins is reduced in T-pEVs. Another oxireductase that has been shown to participate in thrombus formation is PDIA3 [58]. Indeed, in a previous study of our group, Vilahur et al. showed that PDIA3 increases in the secretome of thrombin-activated platelets and an NO donor could modulate its release [59]. Now, in the present study, PDIA3 is found significantly increased in thrombin-induced pEVs, indicating that PDIA3 is secreted not only in a soluble form but also in pEV, in line with the previous findings [60]. The fact that PDIA1 levels are reduced and PDIA3 levels are increased upon platelet activation by thrombin suggests that platelet surface may undergo a redox remodelling state, which facilitates the different binding of thiol isomerases to mediate the disulphide rearrangements and activation of proteins such as $\alpha_{IIb}\beta_3$-integrin [61].

In silico analyses of proteostasis regulated proteins confirmed the involvement of membrane and cytoskeleton proteins in platelet function [62]. In fact, we have identified several proteins implicated in the regulation of actin-based motility by Rho as well as signalling proteins, conforming satellite hub proteins of the network. Small GTPases have been shown earlier to control actin reorganization necessary for pEV shedding [63]. The presence of members of this pathway reflects the mechanism of EV formation. Lipid rafts are also required for EV release from platelets [64]. Upon platelet activation by thrombin, flotillin-1 and -2, broadly found in resting platelet, are retained at the platelet plasma membrane instead of being shed into pEVs, compared to other lipid-raft proteins [65]. Similarly, we detected a lower presence of flotillin in T-pEVs that might reflect a specific lipid-raft sorting.

Proteostasis analysis and the understanding of the proteome changes by different stimulus are useful tools for the identification of proteins coordinately involved in platelet activation and vascular regulatory mechanisms [41]. EV-associated proteins are of high importance as EVs are

shed in biological fluids with potential local and distal effects [66]. Furthermore, focusing on a platelet subproteome found in pEVs enables to reduce complexity as well as to improve assessment of low-abundance proteins. We acknowledge as a limitation of our work that we have the proteomic analysis performed in mixed extracellular vesicles' samples. Uncovering the precise contribution of each vesicle subpopulation would have been valuable. Likewise, our isolation method could not distinguish whether any platelet-released protein was attached to pEV membranes, but, nonetheless, these proteins could also have a pathophysiological relevance as recently suggested [67, 68]. Since thrombin-activated pEV proteins may have a functional involvement in occlusive thrombus formation and atherosclerosis progression, targeting pEV proteins could be a novel therapeutic approach against the progression of atherothrombosis.

In summary, our results evidence that the proteomic approach used here may help to elucidate and anticipate some of the molecular mechanisms in which EV induced by thrombin-platelet activation can participate (Fig. 7). Further studies to validate the information generated in this study are warranted. Mechanistic studies to unveil the role(s) of the uncovered proteomic changes in EVs and their cross-talk with distant cells for paracrine regulation of function will help to unravel novel therapeutic targets.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-022-04222-4.

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Author contributions RS and LB were responsible for the study conception and design. Experiments, data collection, and analyses were performed by RS All authors (RS, TP, GV, and LB) were involved in the interpretation of data. RS and LB wrote the manuscript. All authors revised, edit, and approved the final submitted manuscript.

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Data availability Supplementary data to this article can be found available in the online version. The data underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Conflict of interest R.S. has no relevant financial or non-financial interests to disclose. L.B. received a research grant from AstraZeneca; hold advisory board work for Sanofi, Bayer, and AstraZeneca; received speaker fees from Lilly, MSD-Boehringer, and AstraZeneca; and is founder and shareholder of Glycardial Diagnostics SL and Ivestatin Therapeutics SL (all outside of this work). G.V. and T.P. are founders and shareholders of Glycardial Diagnostics SL and Ivestatin Therapeutics (all outside of this work).

Ethics approval and consent to participate Fresh human platelet concentrates from healthy blood donors were collected following the guidelines and standards for blood donation approved by Blood and Tissue Bank (BST, Spain) Ethical Committee. Authorised BST is accredited by ISO 9001, ISO 14001, and Transfusion Accreditation Committee (CAT) [from Spanish Haematology and Hemotherapy Foundation and Spanish Society of Blood Transfusion] certifications. The protocol for animal study was approved by the institutional ethics committee and all animal procedures were performed in accordance with the guidelines from Directive 2010/63/EU or the National Institutes of Health (NIH) guidelines (NIH No. 85–23, 1996).

Consent to publish The manuscript does not contain any individual person's data in any form (including any individual details, images, or videos).

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