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Complementary roles of platelet $\alpha_{IIb}\beta_3$ integrin, phosphatidylserine exposure and cytoskeletal rearrangement in the release of extracellular vesicles

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HIGHLIGHTS

- Inhibition or genetic deficiency of integrin $\alpha_{IIb}\beta_3$ results in a strong decrease of vesicle formation by activated platelets.
- Cytoskeletal rearrangement is required for vesicle release after platelet activation.
- Vesicle release requires integrin activation and closure, negative phospholipid exposure and cytoskeletal rearrangement.

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ABSTRACT

Background and aims: Platelets can release extracellular vesicles (EVs) upon stimulation with various agonists. Interestingly, platelets from patients with Glanzmann thrombasthenia have reduced EV release. These platelets lack functional $\alpha_{IIb}\beta_3$ integrins, indicating that $\alpha_{IIb}\beta_3$ integrin is critical in vesicle release. Integrin activation is central in platelet function and is associated with e.g. adhesion, aggregation and cytoskeletal rearrangement. However, while platelet activation pathways are widely known, the mechanisms underlying EV release remain uncharacterized. We investigated the role of integrin $\alpha_{IIb}\beta_3$, phosphatidyl serine (PS) exposure, cytoskeletal rearrangement and their associated signalling pathways in EV release.
Methods: EVs were isolated from activated platelets. Platelet activation status was measured by multicolour flow cytometry. A panel of pharmacologic inhibitors was used to interfere in specific signalling pathways. EV release was quantified enzymatically based on membrane PS content and nanoparticle tracking analysis. In addition, real-time visualization of EV shedding with confocal microscopy and EVs with Cryo-TEM imaging was performed.
Results: Platelet activation with convulxin resulted in higher EV release than with activation by thrombin. Kinetic measurements indicated that EV release followed the pattern of $\alpha_{IIb}\beta_3$ integrin activation and subsequent closure paralleled by PS exposure. Prevention of $\alpha_{IIb}\beta_3$ activation with the inhibitor tirofiban dramatically suppressed EV release. Similar results were obtained using $\alpha_{IIb}\beta_3$-deficient platelets from patients with Glanzmann thrombasthenia. Inhibition of actin cytoskeleton rearrangement decreased EV release, whereas inhibition of individual signalling targets upstream of cytoskeletal rearrangement showed no such effects.
Conclusion: Platelet EV release requires three main events: integrin activation and closure, PS exposure, and cytoskeletal rearrangement.

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1. Introduction

Besides platelets role in haemostasis and thrombosis, there is a growing body of literature recognizing that they contribute to several other processes like vascular integrity, wound healing, inflammation and immune processes [1–7]. Platelets release extracellular vesicles (EVs) after activation by different agonist like collagen or thrombin, or spontaneously during prolonged storage as in platelet concentrates used for transfusion [8–10]. Although many cells can shed EVs, platelets are considered to be a major source of EVs in circulation [11,12]. Platelet-derived EVs can contain many proteins, such as chemokines, and are believed to be involved in cell-to-cell communication. EVs play a role in normal haemostasis and in disease processes such as thrombosis, cancer, rheumatoid arthritis, hypertension or diabetes mellitus [13–20].

EVs are small, phospholipid bi-layered membrane vesicles ranging between 50 and 1000 nm in size and can be released from different cells into circulation. The EV outer membrane is similar in composition as its cell of origin, e.g. EVs derived from platelets can possess procoagulant properties (phosphatidylserine) and display of various integrins (e.g. $\alpha_{IIb}\beta_3$) on their outer surface. Similarly to platelets, EVs can bind to endothelial surface and deposit chemokines CCL5 and CXCL4 onto the endothelium [21,22]. Furthermore, platelet EVs can provoke a phenotypic switch of smooth muscle cells towards a pro-inflammatory phenotype [23].

In recent years, studies on EVs were primarily focused on their functions during health and disease. However, the mechanisms of EV release from platelets are still unclear. Platelet activation involves activation of integrin receptors, such as integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa), release of eicosanoids, secretion of dense and α-granules, and finally exposure of negatively charged phosphatidylserine. Integrin $\alpha_{IIb}\beta_3$ is among the most abundantly expressed integrins (50,000–100,000 copies) on the platelet surface, and is known to transmit signals in a bidirectional manner [24]. Furthermore, $\alpha_{IIb}\beta_3$ integrin is entwined with many other signal transduction pathways, hence, is an important player in platelet functions [25]. In circulating platelets, $\alpha_{IIb}\beta_3$ integrin is in a low-affinity state which, after platelet activation, changes into a high affinity conformation via inside-out signalling [26]. Ligand binding and integrin clustering initiate an outside-in signalling cascade triggering different platelet functions (e.g. platelet aggregation and cytoskeletal rearrangement) [25,27]. In a previous study, $\alpha_{IIb}\beta_3$ integrin has been implicated in the release of EVs from platelets. Platelets from patients with Glanzmann thrombasthenia, a genetic platelet disorder caused by dysfunctional or poorly expressed $\alpha_{IIb}\beta_3$, were shown to display a notable impairment of EV release [27]. Although the bidirectional signalling during $\alpha_{IIb}\beta_3$ activation has been well studied, its contribution to EV release has remained unclear.

In the present study, the role of $\alpha_{IIb}\beta_3$ integrin, its underlying outside-in signalling, and platelet-procoagulant state was investigated to characterize the pathways involved in EV release by activated platelets. This study shows that an active conformation of integrin $\alpha_{IIb}\beta_3$ is important. Inhibition or lack of this integrin impairs EV release. However, integrin activation alone was not sufficient for full EV release as platelets that showed PS exposure produced more EVs after activation compared to platelets that did not show PS exposure. Furthermore, this study implies an involvement of cytoskeletal rearrangement in EV release. Inhibition of platelet cytoskeletal rearrangement abolished EV release. However, only minor effects were observed when selected upstream factors governing platelet spreading and shape change were inhibited.

2. Materials and methods

2.1. Platelet isolation

Blood was collected from healthy volunteers and patients with Glanzmann thrombasthenia with a 21 Gauge needle (vacutainer precision glide, BD) into citrate tubes (9 ml coagulation sodium citrate 3.2% vacuette, Greiner Bio-one). Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 350*g* for 15 min. Washed platelets were obtained by centrifugation of PRP at 1240*g* for 15 min, and a wash step with platelet buffer pH 6.6 (10 mM HEPES buffer, 2 mM $CaCl_2$, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$ supplemented with 0.5% BSA and 0.2% glucose). All centrifugation steps were performed in the presence of anticoagulant acid citrate buffer (80 mM trisodium citrate, 52 mM citric acid and 183 mM glucose) to prevent platelet activation during isolation procedure. After pelleting, platelets were resuspended in platelet buffer pH 7.45 (10 mM HEPES buffer, 2 mM $CaCl_2$, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$ supplemented with 0.5% BSA and 0.2% glucose) at a concentration of 2×10^8 platelets/ml. The involvement of human subjects was approved after full informed consent by the local Maastricht ethics committee (METC), and studies were performed in accordance with the declaration of Helsinki.

2.2. Generation of platelet-derived extracellular vesicles

Washed platelets (2×10^8/ml) were activated with different agonists (100 ng/ml convulxin, Enzo Life Sciences), 50 μM TRAP-6 (AnaSpec Inc.), or 5 nM thrombin (Haematologic Technologies) for 30 min at 37 °C. Integrin $\alpha_{IIb}\beta_3$ ligand binding was blocked with 10 nM tirofiban (CAS 144494-65-5, Correvio Int.). Integrin dependent outside-in signalling pathways were blocked with either 10 μM cytochalasin D (CAS 22144-77-0, Sigma), 10 μM PTPN1 inhibitor (CAS 765317-72-4, Merck), 10 μM SYK inhibitor (CAS 622387-85-3, Cayman Chemicals), 10 μM calpain inhibitor I (CAS 110044-82-1, Cayman chemicals), 1 μM ROCK (GSK429286A, selleckchem) or 10 μM $G\alpha_{13}$ inhibitor (made in-house according to Shen B. et al. [28], see below). Platelets were pre-incubated for 5 min at 37 °C with indicated inhibitors prior to activation, as described. Activated platelets were spun down by centrifugation at 300*g* for 5 min, after which the EV-containing supernatant was filtered with PK50 MiniSart sterile 0.8 μm filters (Sartorius). The filtrate was centrifuged at 20,000*g* for 1 h at 4 °C to pellet the EVs [22,23,29]. Pellets were resuspended in platelet buffer pH 7.45, snap frozen into liquid nitrogen and stored at −80 °C.

2.3. Gα13 inhibitor peptide

A novel $G\alpha_{13}$ inhibitor peptide described by Shen et al. was synthesized by Solid-Phase Peptide Synthesis as previously described with minor changes [28,30]. The modified peptide Myr-MP6 (Myr-KFEEERA-NH_2) was synthesized by manual solid-phase peptide synthesis on a 0.20 mmol scale, using an *in-situ* neutralization/activation procedure for Boc-Bzl peptide synthesis. However, as a coupling reagent for amino acids and myristic acid, HCTU was used instead of HBTU. An MBHA resin (0.63 meq/g) was used as solid support. After synthesis, the resin-bound peptide was washed with DMF, DCM and 1:1 *v/v* DCM/MeOH, and subsequently dried.

The synthetized peptide was deprotected and cleaved from the resin by treatment with anhydrous HF for 1 h at 0 °C, using 4 *v*-% *p*-cresol as a scavenger. Following cleavage, the peptide was precipitated with ice-cold diethylether, dissolved into 0.1 M sodium acetate buffer (pH 4) containing 6 M guanidine-HCl. Purification was performed by preparative HPLC; fractions containing the desired product were identified by UPLC-MS, pooled and lyophilized.

2.4. Quantitation of platelet-derived extracellular vesicles

2.4.1. Standard phospholipid vesicle preparation

Extracted lipids from isolated porcine platelets were used to prepare lipid calibration curves. Phospholipid concentrations were determined by phosphate analysis, according to Rouser et al. [31]. Preparation of phospholipid vesicles was as follows: a known volume of the

phospholipid mixture in chloroform/methanol was dried by nitrogen flow, and the resulting phospholipid film was dissolved in 25 mM HEPES and 175 mM NaCl buffer pH 7.7 to obtain a phospholipid stock suspension of 0.1 mM. This suspension was sonicated (7.5 μm peak to peak amplitude for 10 min) on ice to generate unilamellar phospholipid vesicles.

2.4.2. Prothrombinase based assay

In prothrombinase-based assay (PTase), formation of thrombin on a lipid surface was measured, wherein platelet EVs served as a lipid surface, as described [32,33]. Prothrombinase complex consisted of purified bovine coagulation factors Xa (0.05 nM) and Va (1.0 nM), in 25 mM HEPES pH 7.7 and 150 mM NaCl, 5 mg/ml BSA and 5 mM $CaCl_2$. Incubations with (known) amounts of platelet phospholipids vesicles or EVs for 10 min at 37 °C. Human prothrombin (500 nM) (Haematologic Technologies) was added, and subsamples were taken into cold 50 mM Tris, 175 mM NaCl, 20 mM EDTA buffer pH 7.9 and 0.5 mg/ml ovalbumin. Thrombin substrate P2238 (Pepscan BV) was added, and subsamples were analysed for chromogenic activity in a plate reader every 30 s for 15 min, at absorbance wavelengths of 405 nm and 490 nm at 37 °C. Amounts of lipids in the samples were calculated from the changes in the absorbance, compared to the lipid calibration curve.

2.4.3. Nanoparticle tracking analysis

Size distribution and quantification of EVs was conducted by nanoparticle tracking analysis (NTA) with NanoSight NS300 analyser (Malvern Technologies, Malvern, UK) with a high sensitivity sCMOS camera and 488 nm laser. For size distribution determination samples of EVs, isolated from 5 donors, were pooled and centrifuged for 1 h at 20 000 g at 4 °C. EV pellets were resuspended into particle free 25 mM HEPES pH 7.7 buffer. Sample dilution was 1:20. For quantification of EVs, samples were diluted 1:20 in particle free 25 mM HEPES pH 7.7 buffer. Tracking of Brownian movements was performed by capturing 60-s videos in triplicate per EV sample. Measurements were done at 25 °C with camera level at 11 for all samples. Data analysis was performed with NTA 2.3 software with a detection threshold between 2 and 5.

2.5. Fluorescent imaging of vesiculation from activated platelets

Microscopic images of EV vesiculation during activated platelets were taken with confocal microscopy (LSM7 Live line-scanning confocal system, Carl Zeiss, Oberkochen, Germany) with Plan-Apochromat 100x/1.4 oil immersion objective. Isolated platelets (1×10^8 platelets/ml) were spread on a glass coverslip (25 min for healthy platelets and 60 min for Glanzmann platelets) and labelled with CD41-Alexa Fluor 488 (Thermo Scientific, 1:200). Platelets were washed with HEPES buffer pH 7.45 described before and supplemented with 2 mM $CaCl_2$. Platelets were activated with convulxin (100 ng/ml) or thrombin (5 nM) in the presence of annexin-A5 Alexa Fluor 647 (Life Technologies, 1:100) in HEPES buffer as described and supplemented with 2 mM $CaCl_2$. During stimulation fluorescent images were recorded at a frequency of 2.0 Hz for 4 min. Additional fluorescent images were captured after 20 and 30 min. Images were captured using ZEN software 2010 B SP1 and analysed with ImageJ 2.0.0.

2.6. Cryo-transmission electron microscopy (Cryo-TEM)

EVs from convulxin (100 ng/ml nM) activated platelets were visualized by Cryo-TEM. On a glow-discharged R2/2 200u Mesh grid (Quantifiol) a thin film of 2.5 μl sample was applied using a Vitrobot (FEI company, Eindhoven, Netherlands). Sample grids were blotted with a blot force of 5 for 4 s and vitrified with liquid ethane (−182 °C). Sample application was performed under humid (100%) conditions. The vitreous sample grids were imaged with Arctica 200 KV (FEI company) with a Falcon III camera (FEI company) and temperature between −180 and −190 C. Pictures were taken with a resolution of 4096x4096 pixels.

2.7. Assessment of platelet activation state

Isolated platelets from healthy volunteers or Glanzmann thrombasthenia (1×10^8 platelets/ml) were stimulated with convulxin (100 ng/ml) or thrombin (5 nM) in the presence of 2 mM $CaCl_2$. In time (5, 15, 30 and 60 min) samples were taken for detection of PS surface expression with annexin-A5 Alexa Fluor 647 (Life Technologies) and for integrin $\alpha_{IIb}\beta_3$ activation with PAC-1 FITC mAB (BD biosciences). Samples were analysed with flow cytometry (BD Accuri Cytometers).

2.8. Statistical analysis

Experiments were performed using platelets from at least 3 different blood donors. Experimental data were represented as mean ± SD. Statistical analysis of the data were as stated in figures. A p-value < 0.05 was considered significant. Statistical analysis was performed with Graphpad Prism 8 software.

3. Results

3.1. Integrin $\alpha_{IIb}\beta_3$ activation and procoagulant state contribute to EV release by stimulated platelets

Platelet EV suspensions obtained from activated platelets were quantified by PTase and NTA. These data revealed that CVX activated platelets release more EVs compared to thrombin and Trap-6– activated platelets (Fig. 1A and B). Furthermore, NTA revealed a heterogeneous mix of different sizes of EVs ranging between 50 nm and 500 nm, which does not differ between different agonists used (Fig. 1C). Cryo-TEM imaging showed the heterogeneity of the different sizes of the CVX activated platelet EVs in size, and both larger and smaller are membrane-enclosed (Fig. 1D).

During thrombus formation, two distinct platelet populations have been described, namely co-aggregated platelets with activated integrins and PS-exposing platelets with low adhesion properties [34]. The importance of integrin $\alpha_{IIb}\beta_3$ in EV release was seen in Glanzmann thrombasthenia [27]. To investigate how integrin activation and inactivation followed by PS exposure are related to EV release, washed platelets were stimulated with CVX or thrombin and integrin activation and PS exposure were followed in time by flow cytometry. CVX stimulated platelets showed high integrin activation (63%) within 5 min, which gradually decreased over time (to 36%) (Fig. 2A). Simultaneously, PS exposure gradually increased during the first 15 min of platelet activation from 42% to a maximum of 77% (Fig. 2B). Interestingly, at the time point of 15 min, $\alpha_{IIb}\beta_3$ integrin is partially inactivated along with high PS exposure, and EV are released (Fig. 2C).

Flow cytometry also indicated that thrombin stimulation led to an instantaneous and persistent activation of $\alpha_{IIb}\beta_3$ integrin, over 90% after 5 min and remained high during the 60 min experiment (Fig. 3A). This indicates that integrin inactivation does not occur after thrombin activation. In addition, PS exposure and EV release remained minimal for up to 60 min after activation (Fig. 3B and C).

To further visualise the relation between integrin $\alpha_{IIb}\beta_3$ activation, PS exposure, and EV release, washed platelets (1×10^8 platelets/ml) were spread on a glass cover slip prior to stimulation with CVX or thrombin. Confocal microscopic images were taken up to 30 min after platelet activation. Integrin $\alpha_{IIb}\beta_3$ on activated platelets was labelled with anti-CD41 (green) and PS exposure was visualized with annexin A5 (red). Only platelets that were PS-positive were observed to shed EVs (Fig. 2D–F). In addition, although microvesicle formation appeared to occur through membrane protrusions (supplemental movie), platelet balloons formed after prolonged activation [35] were also associated

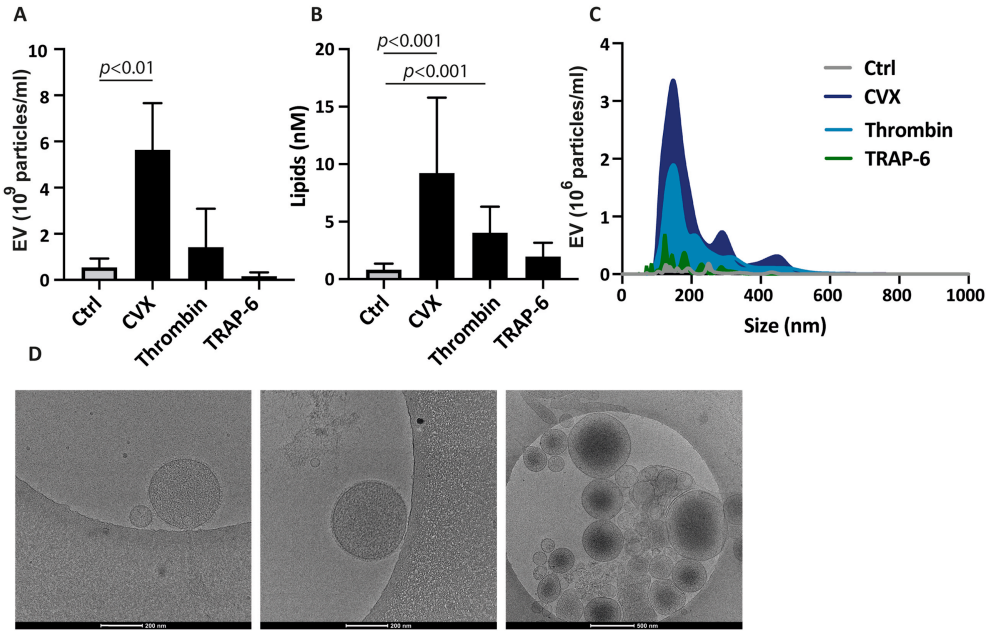

Fig. 1. Determination of EVs released from differently activated platelets, comparing two methods. Washed platelets (2 × 10^8/ml) were activated with CVX (100 ng/ml), thrombin (5 nM), or TRAP-6 (50 μM) for 30 min at 37 °C. As control condition, non-activated platelets were incubated 30 min at 37 °C. EVs were isolated via centrifugation and determined with (A) NTA, (B) based on lipid content. (C) EV size distribution was determined with NTA. (D) Platelets were activated with CVX as described and EVs were isolated via centrifugation, samples were frozen on a grid at 100% humidity and captured with Cryo-TEM. Bars are 200 nm, 200 nm and 500 nm, respectively. Data is show as means ± SD (N ≥ 5), *p*-values were calculated by Kruskal Wallis with Dunn's correction.

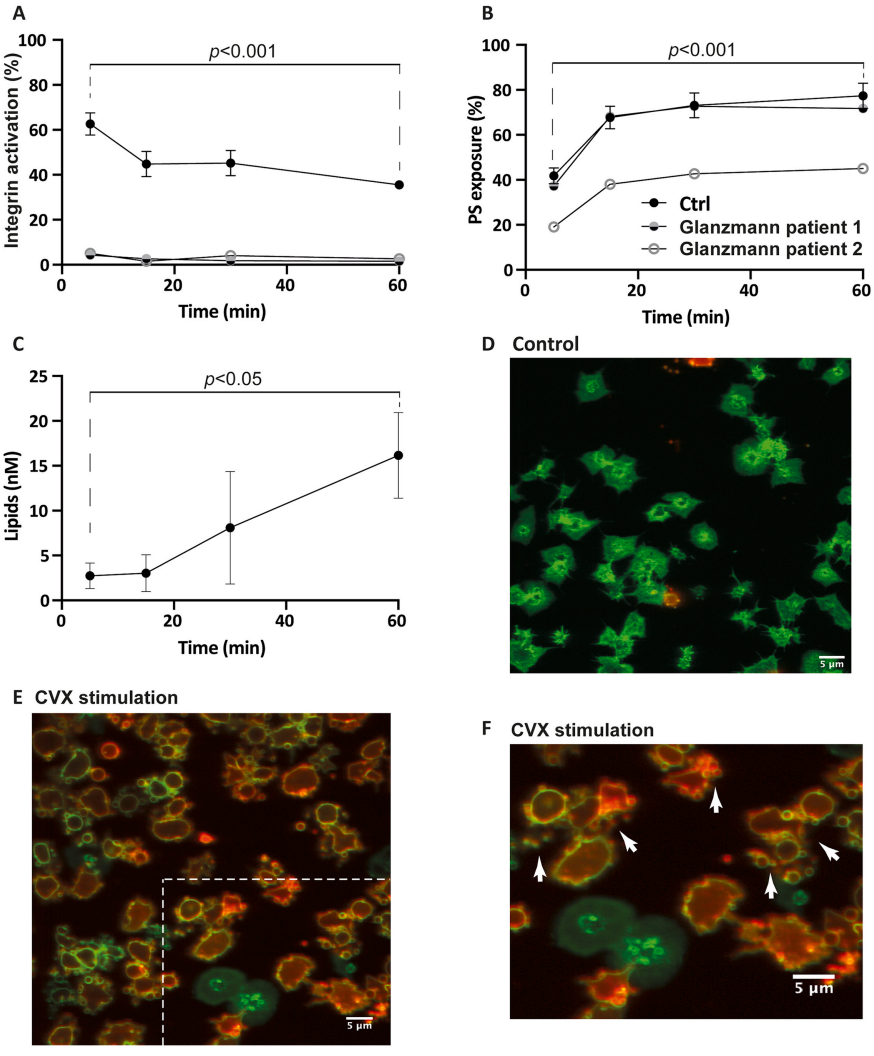

Fig. 2. Closure of activated $\alpha_{IIb}\beta_3$ integrin and PS exposure related to EV release from convulxin-activated platelets. Washed platelets (1 × 10^8/ml) were activated with CVX (100 ng/ml) in the presence of 2 mM $CaCl_2$. Two-color flow cytometry after stimulation at different time points as indicated. Activated platelets were labelled with PAC-1 (FITC) and Annexin A5 (AF647). Representative graphs of PAC-1 positive platelets in time (A). PS positive platelets in time (B). Closed circles represent control platelets, half closed circles represent Glanzmann patient 1, and open circles represent Glanzmann patient 2. For EV release in time, washed platelets (2 × 10^8/ml) were activated as described. Representative graph of EV release in time (C). Fluorescent images of washed platelets (1 × 10^8/ml) were spread on a glass coverslip and activated with CVX (100 ng/ml) as described. Platelets are labelled with CD41 (AF 488, green) and Annexin A5 (AF 647, red). After 25 min, images were taken with confocal microscopy. Representative images of non-activated platelets (control) (D) and convulxin activated platelets (E), and zoomed image (F). White arrows indicate vesiculation. Mean ± SD (N = 3), *p*-values were calculated by Kruskal Wallis with Dunn's correction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

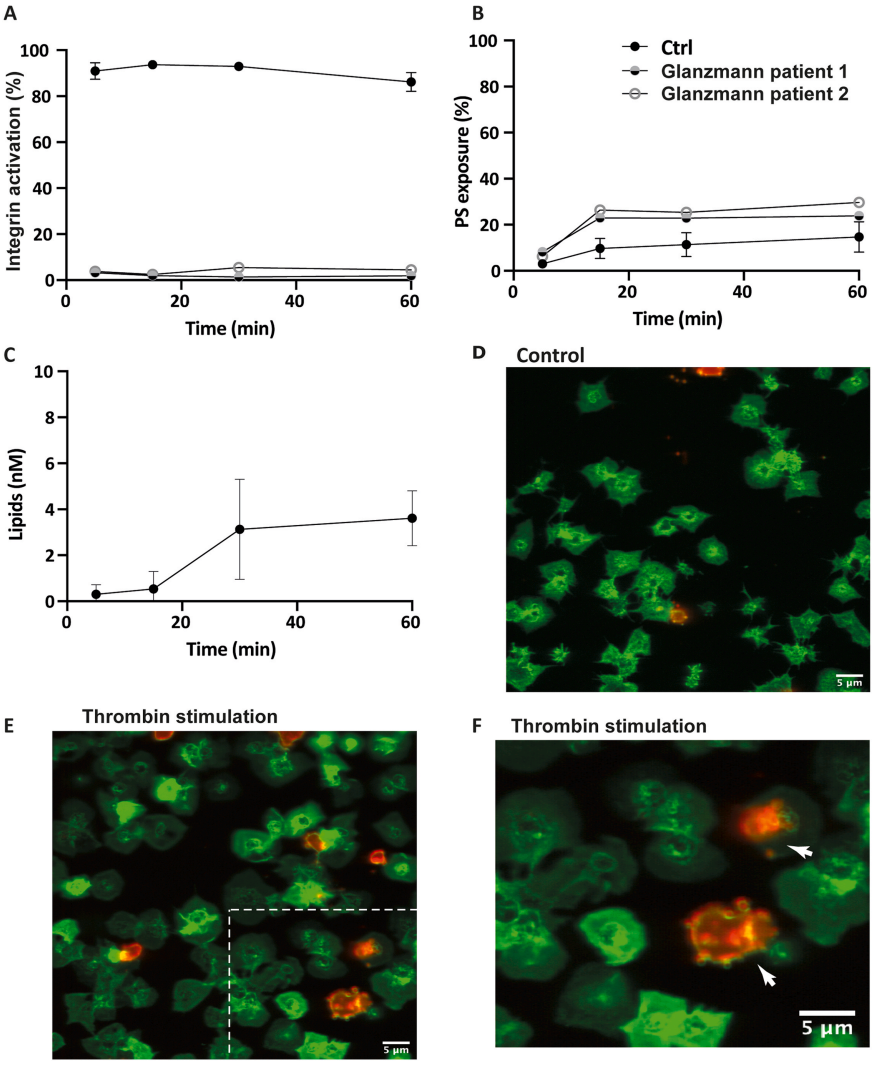

Fig. 3. Closure of activated $\alpha_{IIb}\beta_3$ integrin and PS exposure related to EV release from thrombin-activated platelets. Washed platelets (1×10^8/ml) were activated with thrombin (5 nM) in the presence of 2 mM $CaCl_2$. Two-color flow cytometry after stimulation at different time points as indicated. Activated platelets were labelled with PAC-1 (FITC) and Annexin A5 (AF647). Representative graphs of PAC-1 positive platelets in time (A). PS positive platelets in time (B). Closed circles represent control platelets, half closed circles represent Glanzmann patient 1, and open circles represent Glanzmann patient 2. For EV release in time, washed platelets (2×10^8/ml) were activated as described. Representative graph of EV release in time (C). Fluorescent images of washed platelets (1×10^8/ml) were spread on a glass coverslip and activated with thrombin (5 nM) as described. Platelets are labelled with CD41 (AF 488, green) and Annexin A5 (AF 647, red). After 25 min, images were taken with confocal microscopy. Representative images of non-activated platelets (control) (D) and thrombin activated platelets (E) and zoomed image (F). White arrows indicate vesiculation. Mean ± SD (N = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with EV (Supplemental Fig. 2A). Microscopy also confirmed that thrombin-activation mainly resulted in PS-negative platelets, which did not shed EVs (Fig. 3D–F).

3.2. Inhibition or absence of $\alpha_{IIb}\beta_3$ integrin function reduces agonist induced EV release

Platelet integrin $\alpha_{IIb}\beta_3$ activity was investigated by stimulating washed platelets from healthy donors with CVX and thrombin in the presence of integrin inhibitor tirofiban. EV release triggered by CVX was significantly reduced by tirofiban (Fig. 4A and B). To support this observation, platelets isolated from two patients with Glanzmann thrombasthenia were activated with CVX, and EVs were isolated. The absence of (active) $\alpha_{IIb}\beta_3$ integrin was confirmed with flow cytometry (less than 3% after 60 min) (Figs. 2A and 3A). However, PS exposure was similar to that of platelets from control donors (Figs. 2B and 3B) and ballooning was also observed (Supplemental Fig. 2B), yet EV release from Glanzmann platelets was strongly decreased (Fig. 4C). This could be confirmed from microscopic images from Glanzmann platelets stimulated with CVX (Fig. 4D and E). In summary, these results indicate that activation of $\alpha_{IIb}\beta_3$ integrin plays a pivotal role in EV release, despite normal to moderately impaired PS exposure.

3.3. Involvement of cytoskeletal rearrangement and upstream signalling in EV release by activated platelets

Cytoskeletal rearrangement with contribution of integrin $\alpha_{IIb}\beta_3$, enables platelets to release their contents. To invest the involvement of cytoskeletal rearrangement in EV release, washed platelets were pre-incubated with the established actin polymerization inhibitor cytochalasin D prior to activation with CVX or thrombin. In line with previous findings, activated platelets showed a strongly reduced EV release in the presence of cytochalasin D (Fig. 5A). Inhibition of the Rho-associated protein kinase (ROCK), which acts downstream of the Rho-like small GTPases that regulate shape change, affected EV release from CVX stimulated platelets, albeit not significantly. However, ROCK inhibition did not affect EV release in thrombin-activated platelets (Fig. 5B).

Platelet spreading and clot retraction are responses mediated by $\alpha_{IIb}\beta_3$ outside-in signalling through interconnected signalling pathways [36]. To see whether these pathways are linked to EV release, the interaction of $G\alpha_{13}$ with $\alpha_{IIb}\beta_3$ was inhibited using a synthetic $G\alpha_{13}$ peptide (MP6) during platelet activation [37]. Activity of MP6 was confirmed by its ability to suppress platelet spreading (Supplemental Fig. 1). Surprisingly, EV release was increased after pre-treatment with MP6, after activation with CVX, however, unchanged with thrombin activation (Fig. 5B).

Tyrosine kinases, in particular Syk, are involved in $\alpha_{IIb}\beta_3$ integrin's ability to regulate platelet adhesion and spreading. Upon activation,

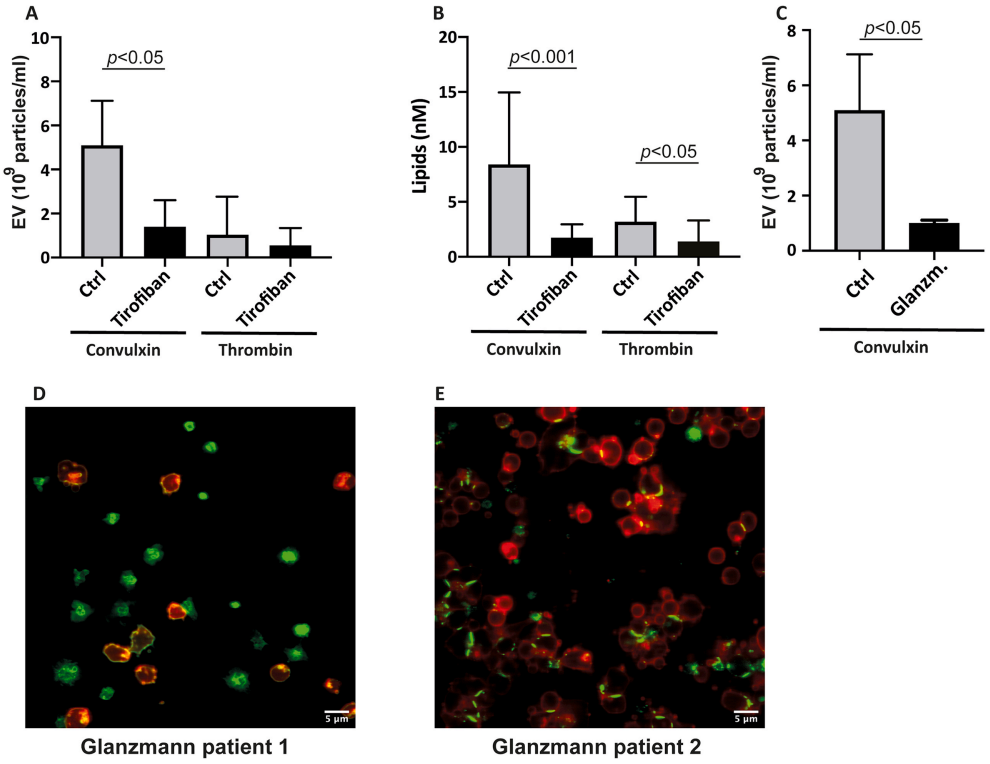

Fig. 4. Loss of integrin function results in reduced EV release.
Washed platelets (2 × 10^8/ml) were activated with CVX (100 ng/ml) or thrombin (5 nM) as described. EVs were isolated via centrifugation and determined with NTA and PTase. Integrin inhibition on healthy platelets was done by the use of integrin inhibitor tirofiban (10 nM) during platelet activation with CVX (100 ng/ml) or thrombin (5 nM) as described. EVs were determined with NTA (A) and PTase (B). Glanzmann platelets were activated only with CVX (100 ng/ml) and EVs were determined with NTA (C). Platelet visualization with confocal microscopy, platelets from Glanzmann patients were washed (1 × 10^8/ml), spread for 60 min on a glass coverslip and activated with CVX (100 ng/ml). Platelets are labelled with CD41 (AF 488, green) and Annexin A5 (AF 647, red). After 25 min of activation, microscopic images were taken from patient 1 (D) and patient 2 (E). Data of healthy platelets EV release is shown as means ± SD (N ≥ 5), *p*-values were calculated by Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

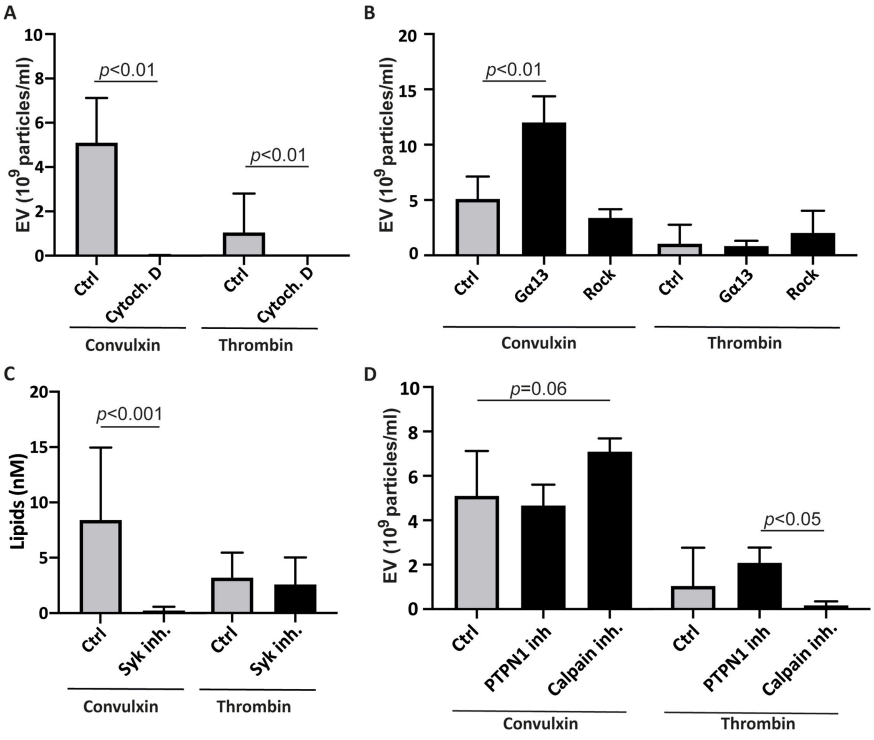

Fig. 5. Influence of cytoskeletal rearrangement and upstream signalling inhibition on EV release. Washed platelets (2 × 10^8/ml) were activated with CVX (100 ng/ml) or thrombin (5 nM) for 30 min at 37 °C. Platelets were pre-treated for 5 min with cytochalasin D (A), or inhibitors of Gα13 (MP6), ROCK (B) Syk (C) PTPN1, or calpain (D). EV were isolated via centrifugation and quantified with NTA. Data is shown as means ± SD (N ≥ 3). *p*-values were calculated by Mann-Whitney test or ANOVA with Bonferroni post-test.

Syk associates with $\alpha_{IIb}\beta_3$ integrin and triggers a signalling cascade to induce cytoskeletal remodeling. Since inhibition of cytoskeletal rearrangement reduced EV release, Syk might be an upstream component of this signalling pathway. Syk inhibition resulted in strongly decreased EV release after CVX activation, but not after thrombin stimulation (Fig. 5C).

Proteins PTPN1 and calpain are seen as regulators of platelet spreading and clot retraction pathways by controlling the activation of c-Src [38–40]. To investigate whether these c-Src regulators influence EV release, platelets were incubated with PTPN1 or calpain inhibitors prior to activation with CVX or thrombin. Inhibition of neither PTPN1 nor calpain significantly influenced EV release from activated platelets (Fig. 5D), likely excluding a role of the c-Src pathway.

In summary, these data indicate that inhibition of platelet cytoskeletal rearrangement results in a reduction of EV release triggered by either CVX or thrombin. However, manipulation of downstream targets yielded different results depending on the platelet stimulus. For example, $G\alpha_{13}$ might be involved in EV release via the GPVI activation

pathway but not via PAR1/PAR4. Inhibition of Syk kinase only influenced GPVI-induced EV-release, which is plausible since Syk is an integral part of GPVI signalling leading to active $\alpha_{IIb}\beta_3$ integrin. Furthermore, inhibition of PTPN1 or calpain did not significantly influence EV release in our experimental setting.

4. Discussion

Over the past years, EVs have become recognized as important components in cell-to-cell communication, and research interest in EVs has vastly increased. In circulation, EVs are predominantly derived from platelets [11,12]. However, still little is known about the mechanism underlying EV release from activated platelets. The main focus of this study was to investigate involvement of $\alpha_{IIb}\beta_3$ integrin and downstream outside-in signalling pathways in the release of EVs from activated platelets. The most interesting finding of this study is the complementary roles of $\alpha_{IIb}\beta_3$ integrin activation/closure and PS exposure in EV release from activated platelets. In procoagulant platelets, integrin $\alpha_{IIb}\beta_3$ becomes inactive, which is paralleled by PS exposure [34]. Platelets that were activated with CVX showed this inactivation mechanism of $\alpha_{IIb}\beta_3$ integrin after PS exposure and robust EV release was observed both from membrane protrusions and from balloons. After activation of platelets with thrombin, $\alpha_{IIb}\beta_3$ integrin closure does not occur and stays in an active conformation. Furthermore, activation by thrombin leads to low PS exposure on the platelet surface and low EV release compared to CVX. This finding is consistent with previously reported observations for patients with Scott syndrome. Platelets from these patients are not able to expose PS and to form balloons and furthermore, show an impaired $\alpha_{IIb}\beta_3$ integrin closure and a reduced EV release [34,41–43].

The importance of $\alpha_{IIb}\beta_3$ integrin was further confirmed using the $\alpha_{IIb}\beta_3$ inhibitor tirofiban and with platelets from Glanzmann patients, in both cases resulting in a significant reduction of EV release. Despite having no or low active $\alpha_{IIb}\beta_3$ integrin, platelets from Glanzmann patients show PS exposure and ballooning, but virtually no EV release. These results are consistent with those obtained by Gemmell et al. [27] who showed impaired EV release in Glanzmann platelets. Together, these data indicate that $\alpha_{IIb}\beta_3$ integrin activation followed by integrin closure and PS exposure are fundamental for strong EV release from activated platelets. Whether integrin closure and platelet ballooning occur around the same time and are mechanistically related remains a subject for future studies.

A further aim of this study was to investigate the role of the outside-in signalling in EV release. Ligand binding on $\alpha_{IIb}\beta_3$ integrin and clustering trigger an outside-in signalling cascade, thereby inducing platelet spreading and clot retraction, granule release, and cytoskeletal rearrangement [44,45]. Activation of platelets results in transformation from a biconcave disk to an irregularly shaped platelet. This drastic change in appearance enables a strong increase of their surface area and enables platelets to support aggregation and recruitment of other platelets [46]. Furthermore, cytoskeletal rearrangement also enables platelets to release their platelet content e.g. granules, coagulation factors, or EVs. Integrin $\alpha_{IIb}\beta_3$, which has an established role in platelet aggregation, also contributes to this cytoskeletal rearrangement [47]. After pre-incubation of platelets with cytochalasin D, which prevents polymerization of actin, this shape change was found to be suppressed in a study by Yano and colleagues [48]. In line with that study, activated platelets were not found to release EVs after inhibition of actin polymerization.

Earlier studies have shown that the cytoskeletal rearrangements in platelet spreading indeed rely on $\alpha_{IIb}\beta_3$ integrin outside-in signalling pathways [24,49,50]. Furthermore, recent studies have indicated that $G\alpha_{13}$ interacts with $\alpha_{IIb}\beta_3$ integrin and regulates platelet spreading and clot retraction. Inhibition of $G\alpha_{13}$, or interference of binding to $\alpha_{IIb}\beta_3$ integrin resulted in inhibition of platelet spreading and accelerating platelet retraction [37,51]. In this study, platelet EV release is almost abolished when actin cytoskeletal rearrangements are prevented in activated platelets. In contrast, inhibition of $G\alpha_{13}$ (with a peptide abrogating platelet spreading) enhanced rather than suppressed release after activation via GPIV, while it did not affect EV release induced by thrombin. The mechanism underlying this observation remains unclear. It can be speculated that the interaction of $G\alpha_{13}$ with $\alpha_{IIb}\beta_3$ integrin might be involved in regulating integrin closure-related mechanisms, which might lead to exaggerated EV release after stimulation with CVX, yet minor effects after stimulation with thrombin.

Syk kinase is involved in platelet spreading. Our data showed that Syk inhibition abolished EV release after CVX, but not after thrombin stimulation. CVX activates platelets through the GPVI receptor and Syk is required for efficient downstream signal transduction [52,53]. Since Syk inhibition did not influence EV release induced by thrombin, our findings might indicate that Syk is involved in GPVI receptor signal transduction, rather than in outside-in signals through $\alpha_{IIb}\beta_3$ integrin. Alternatively, Syk kinase might be involved in $\alpha_{IIb}\beta_3$ integrin outside-in signalling only when platelets are activated through GPVI.

Finally, previous studies revealed that protein dephosphorylation and degradation of cytoskeletal proteins were involved in EV release by platelets [54,55]. In particular, PTPN1 and calpain have been implied to regulate platelet spreading and clot retraction via c-Src. However, inhibition of these enzymes did not significantly influence EV release from activated platelets in our study. Studies by Fox et al. in the early 1990s showed that inhibition of calpain by calpeptin reduced the amount of procoagulant activity released from platelets (i.e. microvesicles) after stimulation by ionophore or collagen [56,57]. These observations were largely confirmed by Yano and colleagues, wo besides demonstrating a role for cytoskeletal remodeling in EV formation, also suggested that calpain mainly plays a role in early events leading to EV release [58]. Calpain has many different targets, including numerous substrates involved in cytoskeletal rearrangement (e.g. filamin, RhoA) [59] and it is challenging to pinpoint the effect on these individual targets after calpain inhibition. Actin remodeling is regulated by (de-)phosphorylation events, which are in consequence also involved in the formation of EV. Several (tyrosine) phosphatases are targets of calpain, yet the use of different phosphatase inhibitors led to contrasting effects [48,55]. In our study, inhibition of PTPN1 did not impact EV release. Given our observations that the inhibition of calpain did not inhibit EV release induced by convulxin, yet did appear to reduce thrombin-induced EV release, we believe that the effects of calpain inhibition are, at least in part, receptor-specific. Another explanation for the apparent discrepancy with former studies might be that these studies mainly used calpeptin as a calpain inhibitor, which also blocks particular tyrosine phosphatases [60]. Taken together, there might be redundancy in the actin-remodeling pathways and multiple phosphatases and enzymes need to be blocked for full inhibition of EV release. Despite the observation by us and others that cytoskeletal rearrangement plays a role in EV release [50], pharmacologic manipulation of several upstream signalling events of cytoskeletal rearrangement did not result in reduced release of EVs.

To conclude, this study shows evidence for complementary pathways in EV release from activated platelets; integrin activation and closure, PS exposure on the outer membrane, and platelets must be capable of cytoskeletal rearrangement.

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CRediT authorship contribution statement

Alexandra C.A. Heinzmann: performed experiments, analysed data and drafted the manuscript. **Mieke F.A. Karel:** performed experiments. **Daniëlle M. Coenen:** performed experiments. **Tanja Vajen:** performed experiments. **Nicole M.M. Meulendijks:** performed NTA analysis. **Magdolna Nagy:** tested the MP6 peptide. **Dennis P.L. Suylen:** synthesized Gα13 inhibitor. **Judith M.E.M. Cosemans:** provided critical reagents and intellectual input. **Johan W.M. Heemskerk:** provided critical reagents and intellectual input. **Tilman M. Hackeng:** provided intellectual input. **Rory R. Koenen:** supervised this study, obtained funding, and finalized the manuscript.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2020.07.015.

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