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Quantitative proteomics analysis reveals similar release profiles following specific PAR-1 or PAR-4 stimulation of platelets

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Aims Platelets are a natural source of growth factors, cytokines and chemokines, that regulate angiogenesis and inflammation. It has been suggested that differential release of pro- and anti-angiogenic growth factors from platelet α-granules by protease-activated receptors (PAR) 1 and 4 may be important for the regulation of angiogenesis. We aimed to compare the releasates of unstimulated platelets with PAR-1- and PAR-4-stimulated platelets.

Methods and results The release of β-thromboglobulin, platelet factor (PF)-4, thrombospondin, platelet-derived growth factor (PDGF)-A/B, regulated and normal T-cell expressed and secreted (RANTES/CCL5), endostatin, CXCL12, and vascular endothelial growth factor (VEGF) was measured with enzyme-linked immunosorbent assay (ELISA). Mass spectrometry (MS)-based quantitative proteomics identified 93 proteins from platelets stimulated with PAR-1 and PAR-4. A strong correlation between the factors released after either stimulus was observed (Spearman's r 0.94, $P < 0.001$). Analysis with ELISA showed that stimulation with PAR-1 or PAR-4 lead to non-differential release of β-thromboglobulin, PF-4, thrombospondin, PDGF-A/B, RANTES/CCL5, endostatin, CXCL12, and VEGF. Release of thrombospondin was slightly lower after PAR-1 stimulation (7.2 μg/mL), compared with PAR-4 induced release (9.8 μg/mL; $P < 0.05$).

Conclusions Both ELISA on established α-granule proteins and MS-based quantitative proteomics showed that the most abundant α-granule proteins are released in similar quantities from platelets after stimulation with either PAR-1 or PAR-4. Our findings provide evidence against the hypothesis that PAR-1 and PAR-4 stimulation of platelets trigger differential release of alpha-granule, but further studies are needed to draw conclusions for physiological conditions.

Keywords Quantitative proteomics • Platelets • Thrombosis • Protease-activated receptor • Releasate

1. Introduction

Platelets are essential for the prevention of lethal blood loss upon injury. In addition, they are a natural source of growth factors, cytokines and chemokines, which are important for the regulation of angiogenesis and inflammation.[1,2] Thrombin is one of the most potent activators of platelets. Platelet activation by thrombin depends on protease-activated receptors 1 (PAR-1) and 4 (PAR-4). Low concentrations of thrombin cleave the SFLLRN peptide from PAR-1. The released SFLLRN then activates platelets via the same PAR-1 receptor. Higher concentrations of thrombin do not only release SFLLRN from PAR-1, but also AYPGKF from PAR-4, resulting in combined activation of platelets via the receptors PAR-1 and PAR-4, respectively.

It has been argued that there are distinct subsets of α-granules that either contain pro-angiogenic or anti-angiogenic content, and which are preferentially released after either stimulation of protease activating

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receptor (PAR)-1 or PAR-4.[3–5] This hypothesis suggests functional organization of pro- and anti-angiogenic proteins, depending on the concentration of thrombin. In contrast to this hypothesis, pairwise comparison of angiogenic relevant proteins that reside in α-granules suggested that there are no α-granule subsets with distinct functional cargo.[6,7]

The evidence for differential release by PAR-1 and PAR-4 stimulation is currently based on a limited number of studies that measured only a few low abundant target proteins, despite the fact that α-granules are abundantly filled with different proteins.[3–5,8] Therefore, a more unbiased, global analysis of the platelet releasate upon activation of these two thrombin receptors could shed light on this conundrum. We compared the released proteome by mass spectrometry (MS)-based quantitative proteomics as to identify thematic groups of factors after either PAR-1 or PAR-4 stimulation. Our results do not support the hypothesis of differential granule release upon platelet activation by these two protease-activated receptors.

2. Methods

2.1 Materials

Materials used in the various experiments are described in the Supplementary material online, Methods.

2.2 Blood sample preparation

Venous blood was collected from healthy volunteers, who did not use any medication in the past 10 days. The collection of blood from the volunteers was performed conform the declaration of Helsinki and this part of the study was approved by the ethics review board of the UMC Utrecht. Blood for MS-based proteomics analysis was collected with an open system, anticoagulated with 3.2% trisodium citrate. Blood for other experiments was collected using vacuum tubes with 3.2% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 *g* for 15 min at room temperature, no brake. For MS-based proteomics analysis, platelets were prepared from PRP after addition of ACD (8.5 mM trisodium citrate, 7.1 mM citric acid, 5.5 mM D-glucose; final concentration) and centrifugation for 15 min with 340 *g* at room temperature, no brake. The platelet pellet was resuspended in Tris buffer (145 mM NaCl, 5 mM KCl, 260 nM NaH_2PO_4, 1 mM $MgSO_4$, 100 mM Tris, 5.5 mM D-glucose, pH 6.5) with 10 ng/mL of prostacyclin. Platelets were centrifuged for 15 min at 340 *g* at room temperature and resuspended up to 200 × 10^9/L in Tris buffer (pH 7.3), with 100 μM acetylsalicylic acid and 1 μM AR-C69931MX. Platelets were not used until 30 min after isolation.

2.3 Platelet responsiveness

Platelet responsiveness was determined with agonist concentration series for PAR-1 or PAR-4 agonist. Serial dilutions of PAR-1 agonist (SFLLRN) ranging from 625 μM to 38 nM were prepared in 50 μL of HEPES-buffered solution (HBS: 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4) with 2 μL of PE-labelled anti-P-selectin antibodies. Similarly, serial dilutions of PAR-4 agonist (AYPGKF) ranging from 4 mM to 0.2 μM were prepared in 50 μL of HBS with 2 μL of PE-labelled anti-P-selectin antibodies. The platelet responsiveness assay was initiated by adding 5 μL of fresh, citrate anticoagulated whole blood to each sample of serial dilutions. After 20 min of incubation, the samples were fixed with 500 μL of 0.2% formyl saline (0.2% formaldehyde and 0.9% NaCl) and kept at room temperature until flow cytometry analysis. All samples were analysed on the same day of processing. Single platelets were gated based on forward and side scatter properties. The median fluorescence intensity in the platelet gate was measured with flow cytometry analysis.

2.4 Platelet stimulation for proteomics

The platelet protein releasate after PAR-1 or PAR-4 stimulation was determined in three separate experiments, in platelets isolated from different three donors to do all experiments in triplicate. Platelet suspensions were stimulated with 625 μM of PAR-1 peptide and 4 mM of PAR-4 peptide for 5 min. Optimal PAR-1 agonist concentration was determined from *Figure 1A*, and optimal PAR-4 agonist concentration was determined from *Figure 1B*. Unstimulated platelet suspensions were taken as control for the same time point. After stimulation, platelet suspensions were centrifuged at 4000 *g* for 2 min. For MS-based proteomics analysis, the supernatant was aspirated and the pellet was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Platelet pellets were reconstituted in a buffer containing 100 mM Tris, 10 mM dithiothreitol, 2% SDS at pH 8.0 with complete mini-protease inhibitors and PhosSTOP phosphatase inhibitor cocktail. Cell suspensions were sonicated, heated at 95°C for 5 min, and cleared by centrifugation. Protein concentrations were determined using a 2D Quant Kit (GE Healthcare, Diegem, Belgium).

Lysates were subsequently alkylated and digested using the filter-aided sample preparation approach.[1] Digestion was performed for 4 h with Lys-C, followed by overnight trypsin digestion. Peptides were desalted using Sep-Pak C18 cartridges and subsequently labelled on column with stable isotope dimethyl labelling as described previously.[2] Resting platelets were labelled 'light', whereas PAR-1- and PAR-4-stimulated platelets received the 'intermediate' and 'heavy' labels, respectively. Labelling efficiency was checked by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) before mixing the pools in a 1 : 1 : 1 ratio. The samples were dried *in vacuo* and reconstituted in 10% formic acid prior to strong cation exchange (SCX) chromatography.

2.5 SCX chromatography and MS

For assessment of the platelet releasate, 250 μg of pooled, labelled digests were subjected to SCX as described previously.[3] A total number of 50 SCX fractions were collected and dried in a vacuum centrifuge. Twenty fractions were reconstituted in 10% formic acid and analysed by LC–MS/MS on an LTQ-Orbitrap Velos instrument, using a data-dependent decision-tree analysis with the orthogonal fragmentation methods high energy collisional dissociation (HCD) and electron transfer dissociation, as described previously and in Supplementary material online, Methods.[4,5]

2.6 Data analysis

Peak lists were generated from the raw data files using the Proteome Discoverer software package version 1.3.339. Peptide identification was performed by searching the individual peak lists (HCD, ion trap electron transfer dissociation, and FT electron transfer dissociation) against a concatenated target–decoy database containing the human sequences in the Uniprot database (release 2012_06) supplemented with a common contaminant database using the Mascot search engine version 2.3 via the Proteome Discoverer interface. The search parameters included the use of trypsin as a proteolytic enzyme allowing up to a maximum of two missed cleavages. Carbamidomethylation of cysteines was set as a fixed modification, whereas oxidation of methionines, phosphorylation, as well as the dimethyl 'light', 'intermediate', and 'heavy' labels on N-termini and lysine residues were set as variable modifications.[9] Further details are described in Supplementary material online, Methods. The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000228 and doi:10.6019/PXD000228.

2.7 Releasate determination

For each biological replicate, a 95% confidence interval was used to determine the 'outliers'.[7] The ratios were binned according to the number of quantification counts in such a fashion that each bin consists of at least 200 proteins and proteins with an identical quantification count can only

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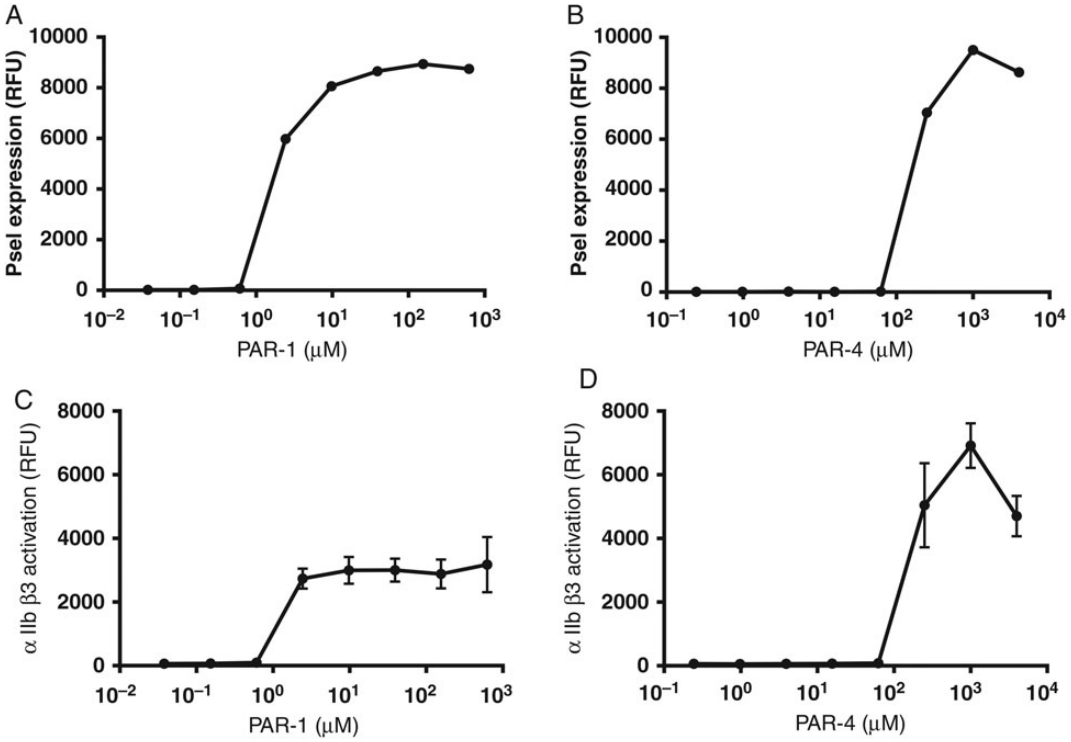

Figure 1 Dose–response assays to determine the optimal concentrations of PAR-1 and PAR-4 agonist for platelet activation. Granule release assessed by P-selectin measurement and αIIbβ3 activation of platelets in response to increasing concentrations of both agonists. Platelet activation reached a maximum at 625 μM of PAR-1 agonist, whereas the maximum PAR-4 response was reached at 4 mM of agonist, both showing comparable P-selectin expression (*A* and *B*) and αIIbβ3 activation (*C* and *D*).

be contributed to 1 bin. Proteins that were determined as outlier with a down-regulated (activated/resting) ratio in at least two of three biological replicates of the PAR-1- or PAR-4-stimulated platelets were attributed to the releasate (see Supplementary material online, *Table S2*).[7] The percentage release was subsequently calculated by (1 − the average ratio of the three replicates) × 100%. Only proteins with a positive % were considered.

2.8 Enzyme-linked immunosorbent assay

Washed platelets were stimulated with 625 μM of PAR-1 peptide and 4 mM of PAR-4 peptide for 5 min. Platelet suspensions were centrifuged at 4000 *g* for 2 min and the supernatant was collected. Maxisorb plates were coated with mouse anti-human beta-thromboglobulin (β-TG), mouse anti-human PF4, mouse anti-human thrombospondin-1, mouse anti-human platelet-derived growth factor (PDGF)-BB, mouse anti-human regulated and normal T-cell expressed and secreted (RANTES/CCL5), mouse anti-human endostatin, mouse anti-human CXCL12 [stromal cell-derived (SDF)-1], mouse anti-human vascular endothelial growth factor (VEGF), or rabbit-anti-human von Willebrand Factor (vWF). Plates were blocked with 1% bovine serum albumin, and incubated with supernatants. Plates were washed with phosphate-buffered saline (PBS) pH 7.4 with 0.05% Tween 20. Bound factors were detected with biotin-coupled goat anti-human β-TG, goat anti-human PF4, biotin-coupled goat anti-human thrombospondin-1, biotin-coupled goat anti-human PDGF-AA, goat anti-human RANTES/CCL5, biotin-coupled goat anti-human endostatin, biotin-coupled goat anti-human CXCL12, biotin-coupled goat anti-human VEGF, or peroxidase-conjugated rabbit-anti-human vWF. Plates were washed with PBS pH 7.4 with 0.05% Tween 20. Biotin-coupled antibodies were bound with streptavidin–Horse reddish peroxidase (HRP), or goat–anti-human antibodies with rabbit-anti-goat HRP (DAKO, P0449). Detection was performed with SuperSignal West Pico Chemiluminescent substrate, and read with a luminometer.

3. Results

3.1 Platelet response to PAR-1 and PAR-4

The agonist concentration for optimal PAR-1 or PAR-4 granule release was determined by P-selectin expression and αIIbβ3 activation of platelets in response to increasing concentrations of both agonists (*Figure 1A–D*). Platelet activation reached a maximum at 625 μM of PAR-1 agonist, whereas the maximum PAR-4 response was reached at 4 mM of agonist, both showing comparable P-selectin expression and αIIbβ3 activation and thus activation status. These concentrations were used to further study the releasates of platelets. We also did a time-dependent platelet activation response with 625 μM of PAR-1 and 4 mM of PAR-1. We measured time-dependent P-selectin expression (*Figure 2A*), fibrinogen (FGB) binding (*Figure 2B*), platelet factor (PF)-4 release (*Figure 2C*), β-thromboglobulin release (*Figure 2D*), VEGF release (*Figure 2E*), and CXCL12 release (*Figure 2F*). Based on these outcomes, we set the sub-optimal incubation time at 5 min.

3.2 Analysis of platelet releasates using MS-based quantitative proteomics and enzyme-linked immunosorbent assay

We used MS-based quantitative proteomics to identify subsets of factors released by either PAR-1 or PAR-4 stimulation. Employing the 'reversed protein profiling' strategy recently published by Wijten *et al.*,[9] we determined significantly decreased levels of proteins in platelets after PAR-1 or PAR-4 stimulation compared with unstimulated platelets in three

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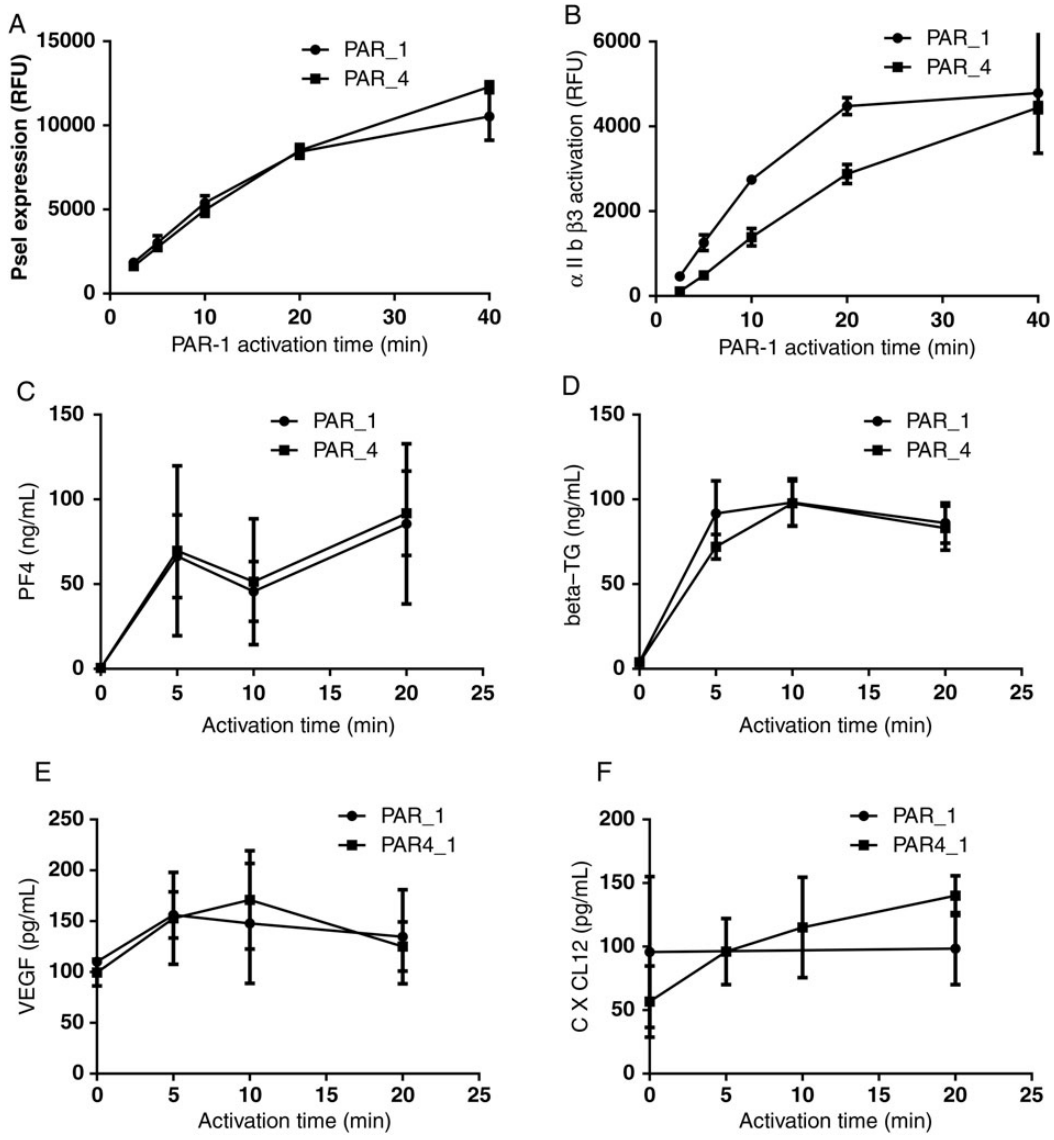

Figure 2 A time-dependent platelet activation response to 625 μM of PAR-1 and 4 mM of PAR-4 on P-selectin expression (*A*) and αIIbβ3 activation (*B*) was plotted to determine the optimal incubation time to compare differences in release between PAR-1- and PAR-4-dependent activation of platelets. Similarly, we have plotted time-dependent PF4 (*C*), beta-TG (*D*), VEGF (*E*), and CXCL12 (*F*) release upon platelet activation by 625 μM of PAR-1 and 4 mM of PAR-4. In none of the time curves, there was a significant difference in P-selectin expression and protein release between PAR-1- and PAR-4-stimulated platelets. All experiments were done in blood of three independent donors.

individuals. In total, 3142 unique proteins were quantified, of which 2296 (73%) could be quantified in at least 2 of 3 individuals. For each protein, the intensity ratios between the light (unstimulated), intermediate (PAR-1 stimulated), and heavy (PAR-4 stimulated) stable isotopic labelled peptides were compared. The vast majority of proteins (>95%) had a (activated/resting) ratio around 1.0 (see Supplementary material online, *Table S1*) for both PAR-1 and PAR-4 stimuli, meaning these did not get substantially released upon either stimulus. Using the statistical criteria described in the Methods section, 93 proteins were determined as being significantly released from platelets activated with PAR-1 and/or PAR-4 (see Supplementary material online, *Table S2*), with release percentages ranging from ca. 20–90% as determined from an average over all ratios obtained from peptide isotopomers of each released protein.

We identified most established α-granule proteins, such as vWF, RANTES/CCL5, PDGF, FGB, and platelet basic protein (PPBP). The release of a few hallmark α-granule proteins from platelets activated with either agonist is displayed in *Figure 3A*. The majority of the quantified proteins were released from platelets to a similar extent upon stimulation with either PAR-1 or PAR-4 agonist, with the release of PPBP, PF-4, VEGFC, and thrombospondin being slightly more efficient after PAR-4 activation. A correlation analysis of the release of all identified releasate proteins (see Supplementary material online, *Table S2*) upon PAR-1 and PAR-4 stimulation gave a coefficient of 0.93 (r^2: 0.86, Spearman $P < 0.0001$) (*Figure 3B*). The release of granule components after full PAR-4 stimulation is more efficient than after PAR-1 stimulation, causing the correlation to shift to the right indicating more of the granule content remained within the platelet after PAR-1 stimulation.

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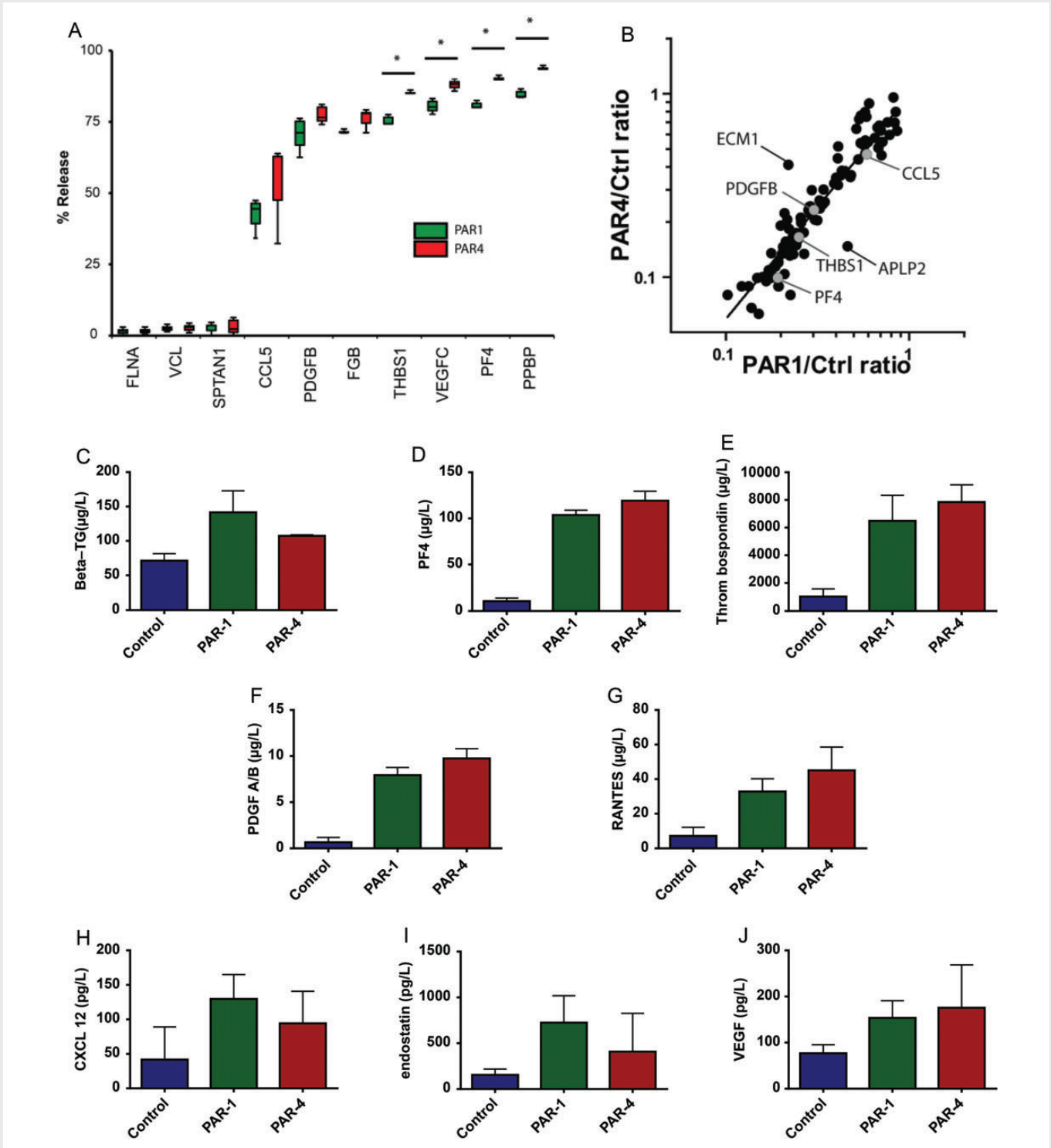

Figure 3 Releasates of PAR-1- and PAR-4-activated platelets are qualitatively and quantitatively similar. (*A*) The extent to which several hallmark α-granule proteins are released from PAR-1- (green boxplots) and PAR-4 (red boxplots)-activated platelets, as assessed from the quantitative proteomics data, is displayed and compared with a few non-released matrix proteins. Stars indicate a statistically significant difference in %-release (Student's *t*-test, $P < 0.05$; see Supplementary material online, *Table S2*). FLNA, filamin-A; VCL, vinculin; SPTAN1, spectrin alpha chain, non-erythrocytic 1; CCL5, C–C motif chemokine 5; PDGFB, platelet-derived growth factor beta subunit beta; FGB, fibrinogen beta chain; THBS1, thrombospondin-1; VEGFC, vascular endothelial growth factor C; PF-4, platelet factor 4; PPBP, platelet basic protein (*: Student's *t*-test $P < 0.05$). (*B*) Correlation between protein ratios obtained for significantly released proteins from PAR-1- and PAR-4-stimulated platelets. The overall correlation *r* is 0.93. Proteins that were subsequently probed by ELISA are shown in grey (*C*) Determination of protein release from platelets by ELISA of five individuals for the proteins β-TG (*C*), PF-4 (*D*), thrombospondin (*E*), PDGF-AB (*F*), RANTES/CCL5 (*G*), endostatin (*H*), CXCL12 (*I*), and VEGF (*J*) after stimulation with PAR-1 and PAR-4. In none of the releasates, there was a significant difference between PAR-1- and PAR-4-stimulated platelets. All experiments were done in three independent experiments.

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Although release levels differed slightly, we did not find evidence of any uniquely released proteins with one of the two stimuli, and thus for release of distinct α-granule populations. Next, we analysed the releasate of either PAR-1- or PAR-4-stimulated platelets with enzyme-linked immunosorbent assay (ELISA; *Figures 2H* and *3C*). The release of β-TG, PF-4, thrombospondin-1, PDGF-A/B, RANTES/CCL5, CXCL12, endostatin, and VEGF after PAR-1 and PAR-4 stimulation was also similar.

4. Discussion

Platelet α-granules are the natural source of large amount of growth factors, cytokines and chemokines, with opposing effects on angiogenesis and atherosclerosis, such as VEGF, SDF-1α, PF-4, and endostatin. It has been suggested that release of these angiogenic factors with opposing functions is controlled by differential release via PAR-1 and PAR-4 stimulation.[3–5] The goal of the present research was to comprehensively identify functional clusters of proteins that are preferentially released after PAR-1 or PAR-4 stimulation. For this we have utilized a 'reversed protein profiling' strategy to identify platelet releasate components.[9] Using this approach we could not confirm differential release after PAR-1 or PAR-4 stimulation. The lack of differential release was confirmed with ELISA on β-TG, PF-4, thrombospondin, PDGF-A/B, RANTES/CCL5, endostatin, CXCL12, and VEGF. Under these assay conditions, maximum PAR-4 stimulation, which was obtained from dose–response measurements, seemed to be a slightly stronger activator of platelet granule release than maximum PAR-1. This was reflected in the absolute release amounts observed by ELISA and the slightly higher % release found in the proteomics data (*Figure 3*).

The topic of differential release by PAR-1 and PAR-4 has been studied with a few factors with a focus on SDF-1α, VEGF, PF-4, and endostatin.[3–5] With the exception of PF-4, those factors are very low abundant in platelet granules.[9] Recently, Jonnalagadda *et al.*[8] have researched differential release induced by PAR-1 and PAR-4 agonist with micro-ELISA's, where they were able to determine release of 17 proteins after PAR-4, and 23 after PAR-1, activation. We took a comprehensive approach using MS-based quantitative proteomics, which enabled us to identify and quantify 93 proteins in the releasate. We hypothesized that with the large palette of proteins being present in α-granules more proteins would be subjected to differential release. These would have to show as clusters containing either pro- or anti-angiogenic factors in a graph where the relative release after PAR-1 or PAR-4 stimulation is plotted (*Figure 3B*). We were not able to identify such clusters of proteins.

The existence of subsets of α-granules that are packed with antagonistic factors has been debated. Van Nispen tot Pannerden *et al.*[7] identified two types of morphologically different α-granules, but one type was identified in only 16% of the platelets. In addition, Kamykowski *et al.*[6] argued that there is no functional co-clustering of factors in subsets of α-granules, rather antagonistic factors are stochastically packed into the same large granules. Recently, the release kinetics of different α-granule proteins was investigated, which showed that although PAR-1 and PAR-4 induce release of proteins with different kinetics, there are no functional patterns in release kinetics between PAR-1 and PAR-4 stimulation.[8] Our data support the earlier reported random sorting of α-granule content, and the lack of thematic patterns of release kinetics for PAR-1- or PAR-4-stimulated platelet releasates.[8] The differences in release kinetics also observed here (*Figure 3A*) and the release in response to different agonist concentrations as reported by Jonnalagadda *et al.* provide context as into how certain activation conditions could result into what may appear thematically differential release of α-granule.[6,8]

To date, it is not known why platelets have two separate thrombin receptors. Our findings that platelet activation via PAR-1 and PAR-4 show comparable release of α-granule protein patterns argue against the hypothesis that different α-granules contain either pro- or anti-angiogenic content, which are preferentially released after either stimulation of protease activating receptor (PAR)-1 or PAR-4.[3–5] It is known that PAR-1 on the platelet membrane is activated at low thrombin concentrations, while PAR-4 can mediate platelet activation at high thrombin concentrations, indicating that the receptors act at different stages in the haemostasis. Blocking the activation of PAR-4 receptor has no effect on platelet function,[10] which raises the question whether we need PAR-4 on platelets. It is possible that PAR-4 is a biological back-up mechanism or that PAR-4 provides a further amplification step of platelet alpha-granule release at high concentrations of thrombin. In addition, PAR-4 activation is involved in non-thrombin-dependent platelet activation via cathepsin G or other proteases.[11] Furthermore, it has been shown that, at high thrombin concentrations, PAR-4, and not PAR-1, is the thrombin receptor that signals human platelet aggregation via calcium mobilization and may be regulated through purinergic feedback, and therefore affected by P2Y12 blockade.[12]

In summary, we show with an ELISA-based quantification of established α-granule proteins and with a MS-based quantitative proteomics approach that the most abundant α-granule proteins are released in similar quantities from platelets after stimulation with PAR-1 or PAR-4. Our findings provide evidence against the hypothesis that PAR-1 and PAR-4 stimulation of platelets trigger differential release of α-granule proteins, but further studies are needed to draw conclusions for physiological conditions.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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