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The neutrophil serine protease PR3 induces shape change of platelets via the Rho/Rho kinase and Ca²⁺ signaling pathways

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Abstract

Introduction: Proteinase 3 (PR3) is released from neutrophil azurophilic granules and exerts complex effects on the inflammatory process. PR3 catalyzes the degradation of a number of macromolecules, but the consequences on blood cells are less well defined. In the present study, the effect of PR3 on human platelets was thoroughly investigated.

Methods: The experiments were performed on washed platelets freshly isolated from blood donated by healthy human volunteers. Platelets shape change and aggregation was measured on a Chrono-Log aggregometer. The phosphorylated form of MYPT1 was visualized by immunostaining. Platelet activation was further evaluated by flow cytometry.

Results: PR3 induced platelet shape change but not aggregation. Flow cytometry analysis of fibrinogen binding to platelets confirmed that PR3 could not activate the RGD recognition integrin GPIIb/IIIa. Furthermore, Fura-2 measurement and immuno-blotting analysis, respectively, revealed that PR3 stimulated small intracellular Ca^{2+} mobilization and Thr696-specific phosphorylation of the myosin phosphatase target subunit 1 (MYPT1). Separate treatment of platelets with the Rho/Rho kinase inhibitor Y-27632 and the intracellular Ca^{2+} chelator BAPTA/AM reduced the shape change induced by PR3 whereas concurrent treatment completely inhibited it.

Conclusion: The data shows that the neutrophil protease PR3 is a direct modulator of human platelets and causes shape change through activation of the Rho/Rho kinase and Ca^{2+} signaling pathways. This finding highlights an additional mechanism in the complex interplay between neutrophils and platelets.

Keywords

ANCA-associated vasculitis, Proteinase PR3, Platelet shape change, Rho/Rho kinase signaling pathway, Ca²⁺ signaling pathway

Abbreviations

AAV, ANCA-associated vasculitis; ACD, acid-citrate dextrose; ANCA, Anti-Neutrophil Cytoplasmic antibodies; CG, cathepsin G; GPA, Granulomatosis with polyangiitis; HLE, Human Leukocyte Elastase; KRG, Krebs-Ringer glucose; MYPT1, myosin phosphatase target subunit 1; PAR1, protease-activated receptor 1; PR3, Proteinase 3; PRP, platelet-rich plasma; TXA₂, thromboxane A₂; VWF, von Willebrand factor.

Introduction

Beyond functions in haemostasis and thrombosis, platelets are considered to play an important role in inflammation by, for instance, modulating the functions of neutrophils [1,2]. The cross-talk between platelets and neutrophils may have profound consequences in several pathophysiological situations, such as sepsis and systemic autoimmune syndromes. In severe sepsis, platelets are able to trigger the formation of neutrophil extracellular traps (NETs), which bind and clear pathogens [3]. ANCA-associated vasculitis (AAV) encompasses a variety of autoimmune diseases characterized by the presence of Anti-Neutrophil Cytoplasmic Antibodies (ANCA) associated with small vessel damage [4,5]. Platelets and leukocytes co-localize and interact at sites of vessel injury, haemorrhage, thrombosis and inflammation. Neutrophils engaged by adhering platelets undergo activation, NET-osis and depletion of the content of their granules [6].

The serine protease PR3 was identified as a major target autoantigen for ANCA in Granulomatosis with polyangiitis (GPA, earlier called Wegener's granulomatosis). PR3 is a versatile protein produced by neutrophils and monocytes belonging to a large family of serine proteases including better known members such as trypsin, thrombin, Human Leukocyte Elastase (HLE) and cathepsin G (CG) [7]. After being synthesized as a prepro-enzyme, PR3 is processed into a mature form consisting of 222 amino acids in four consecutive steps. Together with HLE and CG, mature PR3 is stored in neutrophil azurophilic granules. They all are secreted extracellularly upon neutrophil activation [8], but only PR3 is constitutively expressed on the plasma membranes of a subpopulation of resting neutrophils [9,10]. The levels of plasma PR3, proPR3 and neutrophil

membrane PR3 are all elevated in patients with sepsis and AAV [10]. PR3 can enzymatically degrade the major macromolecules of extracellular matrix [8]. It also acts as an immunomodulator by cleaving cytokine precursors into their active form and by degrading active cytokines or cytokine receptors. Reported examples include IL-8, IL-1 β , TNF α and IL-6 [7]. Furthermore, proPR3 is a hematopoietic feed-back regulator of granulopoiesis [11] and PR3 has been shown to enter endothelial cells resulting in the induction of apoptosis [12].

A potential effect of PR3 on platelets has not been studied in detail. In a report from 1994, Renesto, et al. did not find any direct effect of PR3 on platelets, but a dose-dependent augmenting effect of PR3 on CG-induced platelet aggregation and serotonin release, especially at high concentrations (5-25 $\mu\text{g/ml}$ = 170-860 nM) [13]. In a later publication, the same group showed that incubation with PR3 (600 nM) reduced the number of thrombin receptors on the platelet surface and the rise in intracellular calcium in platelets induced by thrombin (5 nM), but not by a protease-activated receptor 1 (PAR1)-activating peptide (TRAP 42-55, 6.25 μM), and showed no effect on calcium levels by PR3 alone [14]. They also demonstrated a putative cleavage site in PAR1 for PR3, Val⁷²-Ser⁷³. The aim of the present investigation was to determine whether PR3 has a direct effect on human blood platelets. Specifically, the purpose was to elucidate functional response(s) induced by PR3 and to discern the underlying intracellular signaling pathway(s).

Materials and Methods

Reagents

Y-27632, α -thrombin from human plasma, apyrase (Grade III), Fura-2/AM (fura 2 acetoxymethyl

ester), acetylsalicylic acid, albumin from bovine serum (BSA), ADP, adrenaline and buffer chemicals were all obtained from Sigma-Aldrich (St Louis, MO, USA). Purified human Proteinase-3 (PR3) was kindly provided by Wieslab AB (Malmö, Sweden). PP2, PP3 and BAPTA/AM was from Calbiochem (La Jolla, CA, USA). The PAR1 thrombin-receptor-activating peptide (PAR1-AP, amino acid sequence SFLLRN) [15] and the PAR4 thrombin receptor activating peptide (PAR4-AP, amino acid sequence AYPGKF) [16] were synthesized by JPT (Berlin, Germany). The PAR1 antagonist SCH79797 dihydrochloride (N3-Cyclopropyl-7-[4-(1methylethyl) phenyl] methyl-7H-pyrrolo [3,2-f]quinazoline-1,3-diamine dihydrochloride) was obtained from Tocris Cookson Ltd. (Bristol, UK). Secretory leukocyte protease inhibitor (SLPI) was from R&D Systems (Minneapolis, USA). Specific synthetic PR3 inhibitor Ac-PYDA-Pcl (PYDA) and specific PR3 substrate Abz-VADnorVADYQ-EDDnp [17] were kindly provided by Dr. Brice Korkmaz (Centre d' Etude des Pathologies Respiratoires, INSERM U-1100/EA-6305, Tours, France). Anti-phospho-MYPT1 (Thr696) was from Millipore (Billerica, MA, USA). Secondary anti-rabbit-HRP antibody was from Santa Cruz Biotechnology Inc. (California, USA). ECL plus solution was from Perkin Elmer, Inc. (Waltham, MA, USA). Fluorescein (FITC)-conjugated chicken antibodies towards human fibrinogen were from Diapensia HB (Linköping, Sweden). APC-conjugated monoclonal mouse antibodies towards human P-selectin (CD62P, clone AK4) were from BD Biosciences (San Jose, USA) and phycoerythrin (PE)-conjugated monoclonal mouse antibodies towards human GPIIb (CD42b, clone AN51) were from Dako AS (Glostrup, Denmark).

Isolation of human platelets

Platelets were isolated from heparinised blood obtained from the blood bank at Linköping

University Hospital. (Delay between drawing of blood and start of platelet separation was always less than 1 hour). One part acid-citrate dextrose (ACD) solution (85 mM Na-citrate, 71 mM citric acid, and 111 mM glucose) and five parts blood were mixed and centrifuged at 150 g for 20 min to obtain platelet-rich plasma (PRP). The PRP was treated with 100 μ M acetylsalicylic acid and 0.5 U/ml apyrase to prevent activation of platelets by thromboxane A₂ (TXA₂) and ADP/ATP during the isolation procedure. The platelets were isolated by centrifugation at 520 g for 25 min and then resuspended in Krebs-Ringer glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, and 10 mM glucose; pH 7.3) supplemented with 1 U/ml apyrase.

Measurement of platelet shape change and aggregation

A Chrono-Log aggregometer (Chrono-Log Corporation, Pennsylvania, USA) was used to measure aggregation responses in suspended platelets (2.5×10^8 /ml) under constant stirring (800 rpm). The extracellular calcium concentration was adjusted to 1 mM in the samples, which were subsequently allowed to equilibrate at 37°C before adding PR3 (0.1–3.0 μ g/ml = 3.5 – 100 nM). The Rho-kinase inhibitors Y-27632 and the intracellular Ca²⁺ chelator BAPTA/AM were added 5 min before PR3. Serum and PYDA were incubated for 30 min at 37°C with PR3 prior to the addition to aliquots of platelets suspensions. Adrenaline and PAR1 antagonist SCH79797 were added 2 min before PR3. PR3 was added 1 min before ADP but for these experiments, isolated platelets were prepared as described before but resuspended in KRG buffer without apyrase. Changes in light transmission in response to PR3 were followed and recorded over time.

Immunoblotting and measurement of protein phosphorylation

PR3 (0.6 µg/ml) was added to aliquots of platelet suspensions (2.5×10^8 platelets/ml) incubated in aggregometer cuvettes (37°C; 800 rpm), and the reaction was stopped after 10 seconds by adding an equal volume of SDS-PAGE sample buffer (Bio-Rad, Hercules, CA, USA). The samples were immediately transferred to Eppendorf tubes and heated to 95°C for 5 min. Proteins were separated on a 10% Tris-HCl gel from Bio-Rad (Hercules, CA, USA) and then transferred by electroblotting to an Immobilon-P membrane from Millipore (Billerica, MA, USA), which was subsequently blocked with 5% (wt/vol) BSA solution (albumin from bovine serum diluted in 1× TBST buffer). The phosphorylated form of MYPT1 was visualized by immunostaining. Anti-phospho-MYPT1 (Thr696) and the secondary anti-rabbit-HRP antibody were used at a concentration of 1:2,000. The membranes were developed with ECL plus solution, and the chemiluminescence was detected using a Fuji LAS-1000 system from Fuji Photo Film (Tokyo, Japan). Densitometric analysis of the protein bands was done with Image Gauge 4.0 software from Fuji Photo Film (Tokyo, Japan).

Measurement of changes in cytosolic Ca^{2+}

Platelets were loaded with Fura-2/AM by incubating platelet-rich plasma with 3 µM Fura-2/AM for 45 min at room temperature and were subsequently isolated as described above. Platelets were pre-incubated and stimulated as indicated at 37°C and fluorescence was recorded using a Hitachi F-7000 spectrofluorometer at 510 nm with simultaneous excitation at 340 nm and 380 nm. The cytosolic calcium concentration $[\text{Ca}^{2+}]_i$ was expressed as a fluorescence ratio (340/380 nm).

Flow cytometry measurement of platelet activation

Platelet activation was evaluated by flow cytometry using platelet-bound fibrinogen as a marker for fibrinogen receptor GPIIb/IIIa transformation into its active conformation [18] and P-selectin expression as a marker for platelet α -granule release. Isolated platelets prepared as described before and resuspended in KRG buffer with 1 mM CaCl_2 but without apyrase were pre-incubated with PR3 for 10 min at room temperature in the dark (final concentrations 0.6 or 3 $\mu\text{g/ml}$). Control samples were also incubated with buffers with different concentrations of Triton X-100, corresponding to the concentrations in the different PR3 preparations tested. Five μl of platelet suspension ($2.5 \times 10^8/\text{ml}$) was transferred to plastic tubes containing anti-fibrinogen-FITC (final concentration 2 $\mu\text{g/ml}$), anti-P-selectin-APC (final concentration 0.02 $\mu\text{g/ml}$), anti-GPIb-PE (final concentration 1.3 $\mu\text{g/ml}$), platelet agonists (final concentrations; PAR1-AP: 2.5, 10 or 30 μM , PAR4-AP: 10, 50 or 200 μM , thrombin: 0.05, 0.1, 0.2, 0.5 or 2 U/ml) and HEPES buffer to a final volume of 70 μl . For the negative control sample, an isotype control antibody (IgG₁-APC) with corresponding fluorescence replaced the anti-P-selectin antibody and the buffer was supplemented with 10 mM EDTA to prevent binding of the anti-fibrinogen antibody. After exactly 10 min incubation in the dark, the reaction was stopped by addition of 2 ml HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 5.6 mM glucose, 1 g/l bovine serum albumin and 20 mM HEPES, pH 7.40). All steps were performed at room temperature and with only gentle mixing of the samples to avoid contact between platelets. For flow cytometry analysis, platelets were identified by their forward scatter and anti-GPIb-PE fluorescence. Platelet particles were analysed in histograms for FITC and APC fluorescence. An analytical marker was set in the histograms to divide the platelets in the negative control sample into two fractions, one that contained 98-99%

of the platelets and the other containing the brightest 1-2% of the platelets. Platelets with fluorescence intensity higher than the marker were identified as fibrinogen binding (FITC-positive) or P-selectin expressing (APC-positive). All samples were run in duplicate.

Statistical methods

Statistical analysis was done using SPSS 16.0 for Windows software (IBM Corporation, New York, USA). Significant differences were calculated either by applying paired Student's t-test or analysis of variance (ANOVA) and Dunnett's multiple comparison as post-hoc test when applicable (★ $p < 0.05$, ★★ $p < 0.01$). Results are presented as means $\pm$ standard error of the mean (SEM).

Results

PR3 induces a rapid shape change but no subsequent aggregation in platelets

Addition of PR3 (0–3.0 $\mu\text{g/ml}$ = 0–100 nM) to isolated human platelets induced an immediate and dose-dependent decrease in light transmission, usually interpreted as platelet shape change (Fig. 1 A and B). As compared to the response to 0.2 U/ml thrombin shown as the last trace, the initial phase of platelet activation induced by PR3 was not followed by the steep and rapid increase in light transmission indicating platelet aggregation. In control experiments, platelets were exposed to PR3 for up to 20 minutes or aliquots of platelet suspensions were supplemented with fibrinogen (100 $\mu\text{g/ml}$) without causing aggregation (data not shown). Pre-incubation with adrenaline (10 μM) enhanced the magnitude of the decrease in light transmission induced by PR3, but the adrenaline/PR3 combination did not cause aggregation (Figure 1C). Pre-incubation with PAR1 antagonist SCH79797 (10 μM) showed no influence on the shape change induced by PR3

(data not shown, n=3). Also, PR3 showed no enhancing effect on ADP-induced platelet aggregation in platelets resuspended in KRG buffer without apyrase (data not shown, n=3).

PR3 does not affect P-selectin expression or fibrinogen binding in platelets

Platelet activation by PR3 induced a rapid decrease in light transmission but no aggregation as detected by light transmission aggregometry. To explore possible mechanisms by which PR3 could affect platelets, we used flow cytometry to study whether PR3 could affect platelet activation induced by thrombin or specific PAR1- or PAR4-activating peptides (SFLLRN and AYPGKF, respectively). Release of alpha granule contents and activation of the fibrinogen receptor was studied using flow cytometry by measuring expression of P-selectin and binding of fibrinogen to the platelets, respectively. In this system the P-selectin expression and fibrinogen binding was not altered by platelet incubation with Triton X-100 in the concentrations present in the PR3 suspensions, showing that Triton X-100 did not have any measurable effect on its own (data not shown). Isolated platelets were then incubated with PR3 in two different doses (0.6 and 3 µg/ml) and their activation in response to different platelet agonists was determined. Pre-incubation with PR3 showed no significant effects on platelet P-selectin expression or binding of fibrinogen. It did not change the spontaneous activation response in samples where only buffer was added for activation, nor did it change the activation in response to PAR1-activating peptide (SFLLRN: 2.5, 10 or 30 µM), PAR4-activating peptide (AYPGKF: 10, 50 or 200 µM) or thrombin (0.05, 0.1, 0.2, 0.5 or 2 U/ml) at either low or high concentrations. Figures 2 A and B show the results with the intermediate doses of platelet agonists, but no effects by PR3 were observed at any of the

concentrations (data not shown).

PR3-induced shape change in platelets is dependent on PR3 enzymatic activity

Most effects of PR3 are dependent on its enzymatic activity. To discern whether this was the case with platelet shape changes, purified PR3 was incubated with serum (1:1) or PYDA (a synthetic low molecular weight PR3-specific inhibitor, 1 μ M) for 30 minutes at 37°C prior to its addition to the platelets (Fig. 3A). Pre-incubation with serum diminished the shape change and PYDA pre-incubation showed an almost complete prevention. The purity of the PR3 preparations are routinely tested during purification by ELISA using antibodies against bactericidal/permeability-increasing protein, azurocidin, CG, lactoferrin, elastase and myeloperoxidase. All gave negative results for the batches used in our experiments. Furthermore, control experiments were conducted using a colorimetric assay to measure the enzymatic activity of PR3. The results showed that PYDA (1 μ M) but not SLPI (10 μ g/ml) could inhibit the enzymatic activity of our PR3 preparation (data not shown). SLPI is known to inhibit CG[19] and HLE but not PR3 [20]. Hence, our PR3 preparation had no enzymatic impurity that could account for the activity attributed to PR3. However, the stock solution of PR3 contains 0.1% Triton X-100, a detergent used to block adhesion of PR3 to surfaces during storage. We found that Triton X-100 in higher concentrations also could have some effect on light transmission in platelet suspensions. However, when PR3 was diluted to low concentrations (0.6-1 μ g/ml), we still observed changes in light transmission, but the corresponding concentrations of Triton X-100 ($6.7 \times 10^{-5}\%$) alone left the platelets unaffected. We also tested a Triton X-100-free PR3 batch and could confirm the same biological effect (data not shown).

PR3-induced platelet shape change relies on activation of Rho-kinase and calcium signaling cascades

Fura-2-measurements revealed that PR3 induced a small but distinct elevation of the intracellular calcium concentration in platelets (a typical curve is shown as an insert in Fig. 3C). Furthermore, pre-incubation with the intracellular calcium chelator BAPTA/AM (20 μ M) partly reduced the change in light transmission induced by PR3 (Fig. 3B). Pre-incubation with the Rho-kinase inhibitor Y-27632 (20 μ M) also reduced the PR3-provoked responses (Fig. 3B and C). When platelets were pre-exposed to both BAPTA/AM and Y-27632, the PR3-induced platelet response was completely abolished (Fig. 3B). Inhibition of Src family kinases by PP2 showed no effect on the PR3-induced shape change (n=5, data not shown).

Shape change mediated by the Rho/Rho-kinase pathway involves the inhibition of myosin phosphates through Thr696-specific phosphorylation of MYPT1. We analysed phosphorylation of MYPT1 by western blotting using an antibody specific for MYPT1 phosphorylated at the Thr696 residue. The result revealed that PR3 induced a rapid, Y-27632-sensitive increase in Thr696 phosphorylation, which according to densitometric analysis, was comparable to the rise in Thr696 phosphorylation provoked by an intermediate dose of thrombin (0.2 U/ml). Furthermore, PR3-induced Thr696 phosphorylation was PYDA-sensitive, confirming that this effect results from the enzymatic activity of PR3 (Fig. 3D).

Discussion

This study provides evidence for a role of the neutrophil protease PR3 in modulating a specific function of human platelets. Specifically, we found that PR3 induced a rapid decrease in light transmission in suspensions of isolated human platelets, which is usually considered as an indication of early activation and shape change.

Activation of platelets is mainly correlated to aggregation and the formation of the primary hemostatic plug. However, adhesion, complex shape change, extensive secretion as well as post-aggregatory events (e.g. induction of clot retraction) are normal components of platelet physiology. In our study we present evidence that PR3 dose-dependently induced platelet shape change without other manifestations of platelet activation. The platelets were pre-treated with aspirin and apyrase, thus the observed shape change was a direct consequence of PR3 and not caused by secondary autocrine feedback activation from TXA₂ or ADP. Light transmission as well as flow cytometry measurements revealed that PR3 did not induce activation of the fibrinogen (RGD)-binding integrin GPIIb/IIIa. This implies that PR3 alone cannot induce platelet aggregation.

Similar to our findings, many previous studies have shown that certain activators, independent of dose, only induce increased adhesion/shape change [21-24]. This diverse group of endogenous molecules includes adrenaline, ATP, serotonin, lysophosphatidic acid, alpha1-acid glycoprotein, and many others and they are often referred to as “weak activators”, “potentiating/priming stimuli” and “positive modulators” [25]. Based on the present findings, we propose that PR3 should be considered a direct, positive modulator of human blood platelets.

Elucidating the physiological role(s) of PR3-induced platelet activation is beyond the scope of the present investigation. There is a large body of evidence that positive modulators, such as adrenaline, facilitate for secretion and aggregation when platelets are subsequently exposed to

strong activators like thrombin, ADP and collagen [26,27]. However, PR3 was not shown to alter activation of the fibrinogen receptor in response to thrombin, PAR1- or PAR4-activating peptides at any concentrations, nor did it change the magnitude of ADP-induced aggregation (in the absence of apyrase). Based on these findings, we conclude that PR3 is not a priming stimulus of platelet secretion and aggregation. PR3 as well as other proteases are secreted upon neutrophil activation [28], but only PR3 is constitutively expressed on the plasma membranes of a subpopulation of resting neutrophils [9]. Consequently, it is possible that PR3 plays a role in the reciprocal cross-talk between neutrophils and platelets. If membrane-expressed PR3 induces adhesion and discrete activation of platelets to the surface of neutrophils remains to be determined.

The intracellular signal transduction pathways leading to platelet shape change include Ca^{2+} mobilization and Rho/Rho-kinase activation [29,30]. The latter signaling pathway causes specific Thr696 phosphorylation of MYPT1. We found that PR3 induced a minor Ca^{2+} response and a marked increase in Thr696 phosphorylation of MYPT1. Furthermore, inhibition of Rho-kinase and Ca^{2+} mobilization abolished the shape change induced by PR3. Therefore, we conclude that PR3 stimulates shape change of human platelets through activation of the Rho/Rho kinase and Ca^{2+} signaling pathways. It is well established that adrenaline enhances platelet activation by other, more efficient activators [27]. We found that adrenaline reinforced platelet shape change by PR3 but this was not followed by aggregation. Consequently, the signaling power attributable to PR3 (i.e. Rho activation and minimal Ca^{2+} mobilization) is not linked to formation of platelet aggregates. Interestingly, we recently showed that the acute-phase reactant alpha1-acid glycoprotein caused platelet shape change, but not secretion or aggregation, via

activation of the same intracellular signaling pathways [31]. This may imply that acute inflammation is associated with priming of platelets via molecules such as PR3 and alpha1-acid glycoprotein. Such priming will not cause aggregation but may potentially lead to increased platelet/neutrophil adhesion interactions with other cells and/or improved responsiveness of platelets towards other activators. This needs to be further tested in future studies.

Only a few previous studies have examined the effect of PR3 on human platelets. Renesto et al. reported that high doses of PR3 (up to 25 µg/ml = 860 nM) were unable to induce platelet activation measured as aggregation and serotonin release [13]. This finding is in line with our findings. However, in a later publication the same group failed to see any change in intracellular calcium in platelets with 600 nM PR3 (= 17 µg/ml) and they saw diminished capacity of thrombin to elicit a calcium response after pretreatment with PR3 [14]. A possible reason for the different results may be a difference in the preparation. PR3 is highly hydrophobic and detergents are necessary during preparation and storage, and our results highlight the possible influence of detergents in proteinase preparations. It should also be pointed out that the calcium signal we detected was modest and that we tested much lower concentrations of PR3, at which the presence of detergents was confirmed not to influence the results.

We found that both the low-molecular weight PR3 inhibitor PYDA and serum (presence of alpha1-antitrypsin) inhibited platelet shape change induced by PR3. Hence, we conclude that the proteolytic activity of PR3 is essential for its ability to modulate platelets. As mentioned above it has been shown that PR3 inhibits subsequent Ca²⁺ mobilization in platelets provoked by thrombin [14], and the underlying molecular mechanism was suggested to be PR3-induced proteolysis of PARs which removes the cleavage site for thrombin. Furthermore, PR3 can activate dendritic cells

through cleavage of PAR2 but also disable PAR1 by proteolytic removal of the tethered ligand domain from the receptor [32]. Recently, it was demonstrated that PR3 can activate endothelial cells through non-canonical cleavage of PAR1 [33]. It is thus possible that PR3 modulates platelets via PAR cleavage and subsequent intracellular signaling. However, even when using flow cytometry to enable detection of subtle changes in platelet fibrinogen receptor activation, we found no evidence that PR3 could modulate platelet activation through the thrombin PAR1 or PAR4 receptors. Also, inhibition of PAR1 by SCH79797 showed no effect on PR3-induced shape change. Activation of platelet PAR1/4 by thrombin leads to powerful platelet activation and aggregation, while our findings point to a restrictive, modulating effect of PR3. Collectively, the present results point to a PAR 1/4 independent mechanism underlying platelet shape change by PR3. In a recent publication, trypsin was shown to activate platelets enzymatically through a non-PAR-mediated mechanism, most probably through cleavage of another receptor. This activation was calcium-independent and involving the Src family kinases, as shown by its complete inhibition in the presence of 10 μ M PP2 [34]. In contrast to this, we found no evidence for a role of Src in platelet shape change induced by PR3. Furthermore, and opposite to our findings concerning PR3-mediated effects, trypsin did not provoke any calcium response in PAR4-deficient mice platelets. Taken together, it may be possible that PR3 can modulate platelets via PAR cleavage, but our flow cytometry experiments provide no support for a direct effect on PAR1 or PAR4, thus further studies are needed in order to establish the receptor responsible for the PR3-induced shape change. In conclusion, we suggest that the neutrophil protease PR3 is a direct modulator of human platelets and causes shape change through activation of the Rho/Rho kinase and Ca^{2+} signaling pathways. This finding highlights an additional mechanism in the

complex interplay between neutrophils and platelets, which in turn might have an impact on haemostasis and inflammation. The physiological relevance and potential importance of PR3 in this interplay should be further investigated in future studies.

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Conflict of interest

The authors have no conflict of interest.

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Figure legends

Figure 1: PR3 induces shape change in isolated human platelets. (A) Traces showing changes in light transmission in platelet suspensions treated with different doses of PR3 (0–3.0 $\mu\text{g/ml}$ = 0–100 nM) and thrombin (0.2 U/ml). Arrows indicate addition of PR3 or thrombin. (B) Histogram quantifying the changes in transmission provoked by increased doses of PR3. The bars represent means $\pm$ SEM (n=4). (C) Summarized effects of adrenaline on the PR3-induced decrease in light transmission. The data represent means $\pm$ SEM, and statistical significance was tested using paired Student's t-test (★★, $p < 0.01$; n=4).

Figure 2: P-selectin expression and fibrinogen binding is unaffected by platelet pre-incubation with PR3. Isolated platelets pre-incubated for 10 min with or without PR3 (0.6 or 3 $\mu\text{g/ml}$ = 20–100 nM) were activated by PAR1 or PAR4 activating peptides, or thrombin and the activation response, measured as P-selectin expression (A) and fibrinogen binding (B), was determined by flow cytometry. Data shown are means $\pm$ SEM, n=3 (except for fibrinogen binding for thrombin at PR3 0 and 3 $\mu\text{g/ml}$, where n=2).

Figure 3: PR3 induced light transmission change is dependent on PR3 enzymatic activity. Rho-kinase activation and intracellular Ca^{2+} mobilization. PR3 induces pronounced Rho-kinase-dependent phosphorylation of MYPT1.

(A) Histogram quantifying the change in light transmission provoked by PR3 (0.6 $\mu\text{g/ml}$ = 20 nM) pre-incubated with control buffer, PYDA (1 μM) or serum (1:1 volume ratio) for 30 minutes at 37°C. Serum contains several protease inhibitors (e.g. alpha-1-antitrypsin) and PYDA is a low molecular weight protease inhibitor specific for PR3. The bars represent means $\pm$ SEM, and statistical significance was tested using paired Student's t-test (★, $p < 0.05$; $n = 3$).

(B) Summarized effects of BAPTA/AM and Y-27632 on the PR3-induced decrease in light transmission. Short (5 minutes) pre-incubation with the calcium chelator BAPTA/AM (20 μM), or Rho-kinase inhibitor Y-27632 (20 μM), antagonized the PR3- (0.6 $\mu\text{g/ml}$ = 20 nM) induced changes in platelets. Pre-incubation with a combination of Y-27632 (20 μM) and BAPTA/AM (20 μM) abolished the response. Bars represent means $\pm$ SEM ($n = 4$), and ★ represent a statistical significant difference compared to control when using paired Student's t-test ($p < 0.05$).

(C) PR3 (0.6 $\mu\text{g/ml}$) provoked a PYDA (1 μM)- and Y-27632 (20 μM)-sensitive Thr696 phosphorylation on MYPT1 measured 10 seconds after stimulation in platelets. The bars represent means $\pm$ SEM of densitometric values from western blots repeated thrice. The inset trace shows intracellular calcium mobilization in Fura-2-loaded platelet suspensions. PR3 (0.3 $\mu\text{g/ml}$ = 10 nM) provoked a minor calcium rise in platelets. ANOVA with Dunnett's multiple comparison test was used to test the statistical significance of results from inhibitor-pre-incubated samples compared to the control (0.6 $\mu\text{g/ml}$ PR3 without inhibitors) (★, $p < 0.05$; $n = 3$).

(D) Original data from one western blot is shown under the bar graph. Y-27: Y-27632 (20 μM); Thr: thrombin (0.2 U/ml).

1.

A

B

C

Figure 1: PR3 induces shape change in isolated human platelets. (A) Traces showing changes in light transmission in platelet suspensions treated with different doses of PR3 (0–3.0 $\mu\text{g}/\text{ml}$ = 0–100 nM) and thrombin (0.2 U/ml). Arrows indicate addition of PR3 or thrombin. (B) Histogram quantifying the changes in transmission provoked by increased doses of PR3. The bars represent means $\pm$ SEM (n=4). (C) Summarized effects of adrenaline on the PR3-induced decrease in light transmission. The data represent means $\pm$ SEM, and statistical significance was tested using paired Student's t-test (★, $p < 0.01$; n=4).

2.

2

Figure 2: P-selectin expression and fibrinogen binding is unaffected by platelet pre-incubation with PR3. Isolated platelets pre-incubated for 10 min with or without PR3 (0.6 or 3 μ g/ml = 20-100 nM) were activated by PAR1 or PAR4 activating peptides, or thrombin and the activation response, measured as P-selectin expression (A) and fibrinogen binding (B), was determined by flow cytometry. Data shown are means $\pm$ SEM, n=3 (except for fibrinogen binding for thrombin at PR3 0 and 3 μ g/ml, where n=2).

3. **Figure 3: PR3 induced light transmission change is dependent on PR3 enzymatic activity, Rho-kinase activation and intracellular Ca^{2+} mobilization. PR3 induces pronounced Rho-kinase-dependent phosphorylation of MYPT1.**

A

(A) Histogram quantifying the changes in light transmission provoked by PR3 (0.6 $\mu\text{g/ml}$ = 20 nM) pre-incubated with control buffer, PYDA (1 μM) or serum (1:1 volume ratio) for 30 minutes at 37°C. Serum contains several protease inhibitors (e.g. alpha-1-antitrypsin) and PYDA is a low molecular weight protease inhibitor specific for PR3. The bars represent means $\pm$ SEM, and statistical significance was tested using paired Student's t-test (★, $p < 0.05$; $n = 3$).

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(C) PR3 (0.6 μ g/ml) provoked a PYDA (1 μ M)- and Y-27632 (20 μ M)-sensitive Thr696 phosphorylation on MYPT1 measured 10 seconds after stimulation in platelets. The bars represent means $\pm$ SEM of densitometric values from western blots repeated thrice. The inset trace shows intracellular calcium mobilization in Fura-2-loaded platelet suspensions. PR3 (0.3 μ g/ml = 10 nM) provoked a minor

calcium rise in platelets. ANOVA with Dunnett's multiple comparison test was used to test the statistical significance of results from inhibitor-pre-incubated samples compared to the control (0.6 $\mu\text{g/ml}$ PR3 without inhibitors) ($\star$, $p < 0.05$; $n = 3$).

(D) Original data from one blot is shown under the bar graph. Y-27: Y-27632 (20 μM); Thr: thrombin (0.2 U/ml)