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C1-inhibitor influence on platelet activation by thrombin receptors agonists

Ivan D. Tarandovskiy, PhD[1], Paul W. Buehler, PhD[2], and Elena Karnaukhova, PhD[3]

Abstract

Introduction: Protease activated receptors 1 (PAR1) and 4 (PAR4) agonists are used to study platelet activation. Data on platelet activation are extrapolated across experimental settings. C1-inhibitor (C1INH) is a protease inhibitor present in plasma but not in isolated platelet suspensions. Here we show that C1INH affects platelet activation through PAR1 and PAR4 agonists. **Methods:** Platelets were isolated from healthy donor whole blood and then labeled with anti-CD62P and PAC1 antibodies. The platelet suspensions were exposed to PAR1 agonists SFLLRN, TFLLR and TFLLRN; PAR4 agonists AYPGKF and GYPGQV; ADP and thrombin. Flow-cytometric measurements were performed in 5, 10 and 15 min after activation. **Results:** 0.25 mg/ml C1INH addition made platelets to faster expose CD62P and glycoprotein IIb/IIIa complex after activation with PAR1 agonists. Conversely, C1INH addition led to inhibition of platelet activation with PAR4 agonists and thrombin. Activation with ADP was not affected by C1INH. **Conclusions:** Our results suggest that C1INH can modify platelet activation in the presence of synthetic PAR agonists used in platelet research. These observations may be relevant to the development of new methods to assess platelet function.

Keywords

C1-inhibitor, platelet activation, thrombin receptors, PAR1, PAR4

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Introduction

Platelets are now widely studied in clinical laboratories and in experimental research settings. One of the methods to assess platelet function is to measure their activation. Different systems, from global hemostasis assays to aggregometry and flow cytometry, are employed to investigate this process.[1–4] Specific agonists of protease activated receptors 1 (PAR1) and 4 (PAR4) are often added to mimic the physiological activation by thrombin.[5–10] These compounds are utilized most often to assess platelet activity in whole blood, platelet-rich plasma or isolated platelet suspensions.[1] The results obtained under different experimental conditions are often extrapolated across models.[11] However, platelet reactivity is highly sensitive to experimental settings and activity can change upon the smallest of modifications. Understanding the possible inconsistency in the results obtained in different experimental settings and conditions is important for interpretation and comparison of study results and standardization of clinical methods. In this study, we investigated the effect of C1-inhibitor (C1INH) on platelet response within an isolated suspension.

C1INH is an anti-inflammatory plasma protein belonging to the serpin superfamily, it functions as a key regulator of the complement, contact (kallikrein-kinin), coagulation and fibrinolytic systems.[12,13] C1INH inhibits complement proteases C1r and C1s, as well as coagulation and the fibrinolytic agents thrombin,

[1] Hemostasis Branch, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, USA
[2] Department of Pathology and The Center for Blood Oxygen Transport and Hemostasis, Department of Pediatrics, School of Medicine, University of Maryland, Baltimore, Maryland, USA
[3] Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, USA

Corresponding Author:
Ivan D. Tarandovskiy, Hemostasis Branch, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, USA.
Email: ivan.tarandovskiy@fda.hhs.gov

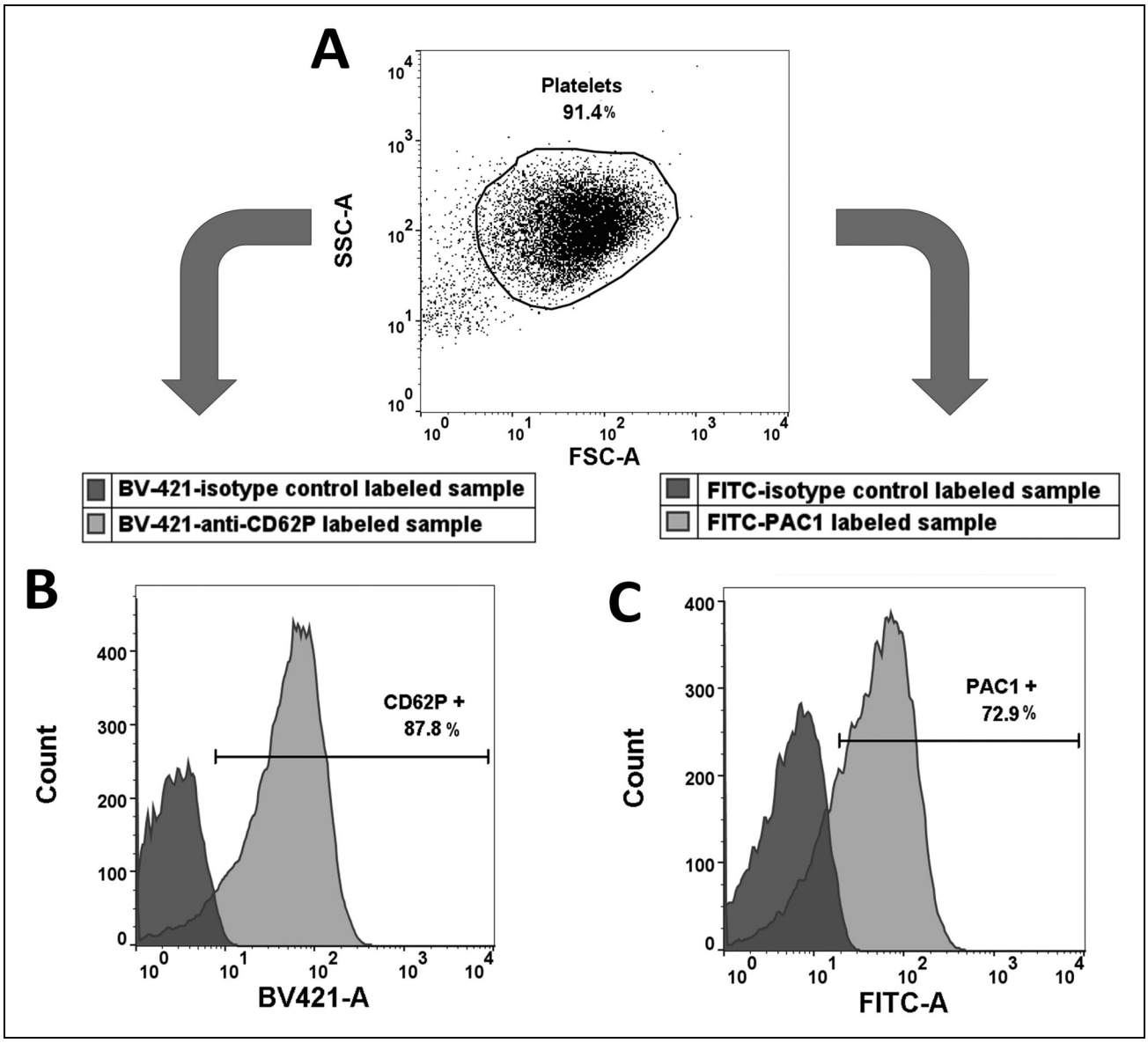

Figure 1. Processing of a representative flow-cytometric experiment. A. Shows the light scattering dot plot of a platelet sample with definition of the platelet population. B. Demonstrates the CD62P-positive platelet population comparing to isotype control. C. The PAC1-positive platelet population is shown compared to the isotype control.

plasmin, tissue plasminogen activator, kallikrein, activated factors XI and XII.[14,15] C1INH is currently approved for replacement therapy in patients with hereditary angioedema, moreover, due to its anti-inflammatory properties, C1INH is being investigated for other potential therapeutic uses.[14,16–22] C1INH is reported to inhibit the function of the protein C system enhancing the generation of thrombin but decreasing the generation of plasmin.[23,24] Moreover, glycosaminoglycans (e.g., heparin) and polyphosphate are reported to be a cofactor for C1INH.[25] C1INH has not been reported to modify platelet activation, however, it is known that C1INH is contained within platelet α-granules.[26] It is also reported that C1INH replacement therapy can increase platelet aggregation.[27] It is noteworthy that both the platelet activation and C1INH are related to the venous thromboembolic events: platelets are known to actively contribute to venous thrombosis,[28] whereas therapeutic use of C1INH is associated with thromboembolic events, under rare circumstances.[29] It remains unclear whether or to what extent the underlying mechanisms of the thromboembolic might include C1INH mediated platelet activation. In this study, we describe the effect of C1INH on isolated platelet suspensions with addition of either PAR1 or PAR4 agonists. To our knowledge, the effects observed are reported for the first time.

Methods

Platelets were isolated from whole blood collected in 3.8% sodium citrate vacutainer tubes from healthy donors in accordance with the FDA Research Involving Human Subjects

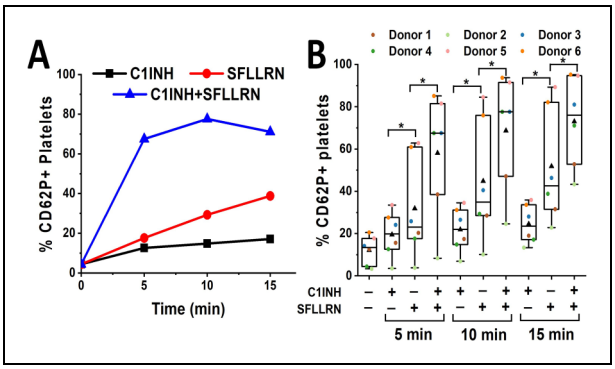

Figure 2. Influence of C1INH on the kinetics of CD62P positive platelet populations upon activation with 5 μM SFLLRN. A. Representative experiment on a platelet suspension from a single healthy donor with addition of C1INH alone (black line), SFLLRN (red line) and combined SFLLRN and C1INH (blue line). B. Box plots for 6 experiments on different healthy donors. Black triangle represents the mean value. Upper and lower horizontal lines mean 25th and 75th percentile. The horizontal line in the middle of the box represents the median value. The whisker caps mean the minimal and maximal values. One-Way Repeated Measures ANOVA was used to obtain statistics at the p-value set at 0.05.

Committee's (RIHSC) approval, RIHSC protocol #03-120B. All the samples were obtained under informed consent. After collection, prostaglandin E1 (100 nM final concentration, Sigma-Aldrich, St. Louis, MO, USA) was added to blood samples followed by centrifugation at 300 g for 25 min. The collected platelet rich plasma supernatant was centrifuged again under 1000 g for 20 min. After that, the supernatant was removed, and platelets were resuspended with Tyrode's buffer containing supplemented with 0.5% of bovine serum albumin (#T2397, Sigma-Aldrich, St. Louis, MO, USA, $CaCl_2$ concentration equal to 0.265 g/L) to reach concentration of 100000 platelets per μl which was measured on a Cell-Dyn, Hematology Analyzer (Abbott, Abbott Park, IL, USA). The activation was performed not later than 1 h after isolation. The platelets were labeled with anti-CD62P-PE only or anti-CD62P-BV421 with PAC1-FITC or isotype control antibodies conjugated with the fluorophores mentioned above. All the antibodies were obtained from BD Bioscience (Franklin Lakes, NJ, USA). Activation of platelets in suspension was achieved by gently mixing them with the equal volume of ADP (1 μM final concentration, Sigma-Aldrich, St. Louis, MO, USA); or one of the PAR1 agonists SFLLRN (5 μM final concentration), TFLLR (2.5 μM final concentration, Sigma-Aldrich, St. Louis, MO, USA) or TFLLRN (5 μM final concentration, Sigma-Aldrich, St. Louis, MO, USA); or PAR4 agonists AYPGKF (80 μM final concentration, Sigma-Aldrich, St. Louis, MO, USA) or GYPGQV (640 μM final concentration, Abgent, San Diego, CA, USA) or thrombin (Haematologic Technologies, Essex Junction, VT, USA, 0.5 nM final concentration). In some cases, the activation solution contained C1INH in the concentration of 1 IU/ml (0.25 mg/ml, within the normal plasma concentration, 0.15-0.30 mg/ml). C1INH (Berinert®, CSL Behring, LLC, Kankakee, IL, USA) was kindly provided by Dr. Dominik J. Schaer, Department of Internal Medicine, The University of Zurich Hospital; C1INH was also obtained from Athens Research & Technologies, Inc. (Athens, GA, USA) and Calbiochem (San Diego, CA, USA). Agonists" concentrations were chosen after preliminary titration on washed platelets to provide moderate activation from 30 to 70% of positive platelets to further enable both the activating and inhibiting effects of C1INH. Flow cytometric measurements were performed before and immediately after 5, 10 and 15 min from the moment of activation on a BD LSRII cytometer (BD Bioscience, Franklin Lakes, NJ, USA). For thrombin activation, the measurements were performed only after 5 min incubation. Before each experiment, flow cytometric measurements of CD62P and PAC1 exposure as well as visual dot FSC-SSC plots analysis were performed to verify that platelets were not preactivated. The results were processed using FlowJo V10-3 software (Figure 1, FlowJo, LLC, Ashland, OR, USA). After definition of the platelet population in the SSC-FSC dot plot (Figure 1A) the percentages of CD62P-positive (Figure 1B) and PAC1-positive (Figure 1B) cells were calculated by subtracting the isotype control. Each experiment was reproduced on the platelets from 3 healthy donors.

Results

C1INH facilitates platelet activation by SFLLRN

Figure 2 summarizes the results obtained after 5, 10 and 15 min of suspension labeled with anti-CD62P-PE incubation with 5 μM SFLLRN. The platelets from all 6 donors demonstrated increased CD62P positive platelet populations upon the addition of 1 IU/ml of C1INH (Athens). The control platelets from different donors demonstrated various activation rates, but the effect of C1INH was present in all the conducted experiments. C1INH alone did not demonstrate a strong ability to activate platelets.

C1INH facilitates platelet activation by PAR1 agonists and inhibits it by PAR4 agonists

Figure 3 shows the C1INH (Athens) influence on the kinetics of CD62P and PAC1 exposure obtained after 5, 10 and 15 min of the incubation of anti-CD62P-BV421- and PAC1-FITC-labeled suspensions activated with PAR1 agonists (Figure 3A to C), PAR4 agonists (Figure 3D and E), and ADP (Figure 3F). C1INH facilitated platelet activation with PAR1 agonists SFLLRN (Figure 3A), TFLLR (Figure 3B) and TFLLRN (Figure 3C). C1INH inhibited platelet activation with PAR4 agonists AYPGKF (Figure 3D) and GYPGQV (Figure 3E) conversely to PAR1 agonists. Activation with ADP was not influenced by C1INH in most of the cases (Figure 3F).

C1INH from different sources facilitates SFLLRN-induced platelet activation

Similar to C1INH (Athens), the C1INH preparations from other commercial sources, CSL Behring and Calbiochem (Figure 4),

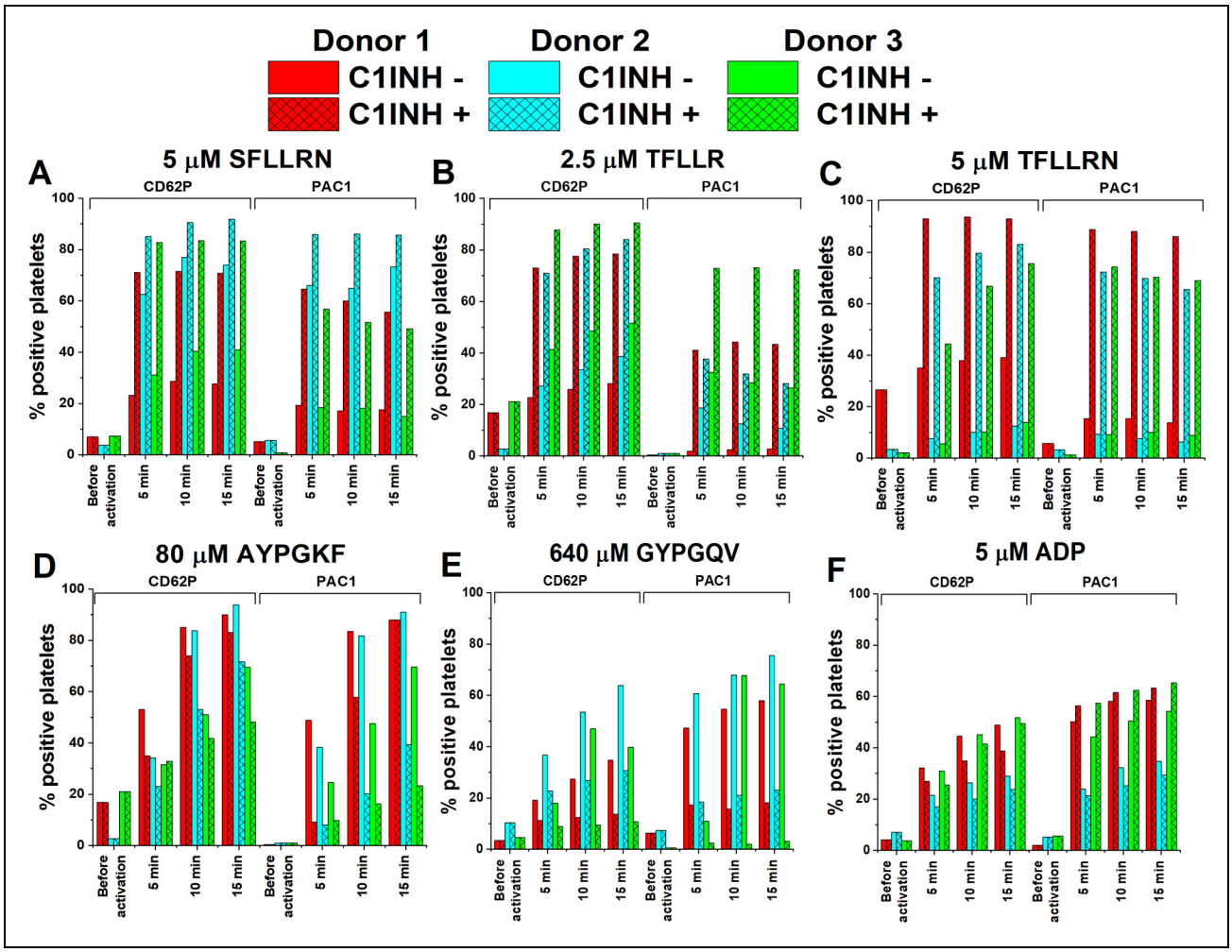

Figure 3. Influence of C1INH on the kinetics of CD62P and PAC1 positive platelet populations upon activation with PAR1 agonists SFLLRN (A), TFLLR (B) and TFLLRN (C); PAR4 agonists AYPGKF (D) and GYPGQV (E); and ADP (F) is presented for platelet suspensions from each of 3 healthy donors. Donors differ between the experiments with different platelet activators.

enhanced CD62P exposure on platelet surfaces after activation with SFLLRN in suspensions labeled with anti-CD62P-BV421.

C1INH inhibits thrombin-mediated platelet activation

C1INH (Athens) reduced the CD62P (Figure 5A) and PAC1 (Figure 5B) positive populations in platelet suspensions upon activation with thrombin.

Discussion

Our study points to a unique effect of C1INH that is relevant to the design of platelet activation studies. Platelet-rich plasma and whole blood contain C1INH while washed platelet suspensions do not, especially if gel filtration is performed. According to our results, changing the test material from isolated platelets to whole blood or blood plasma could introduce data inconsistency into the evaluation of platelet function. Further, specific peptide agonists of PAR1 and PAR4 are useful in various applications to mimic thrombin activation. The addition of thrombin to plasma is limited by fibrin clot formation thus PAR agonists are used to substitute thrombin. However, the use of PAR agonists can introduce variability depending on the test material evaluated (e.g., whole blood, platelet rich plasma or isolated platelets). Here we show that C1INH can modulate platelet function (activation or quiescence) in the presence of different PAR agonists. This is critical to ensure C1INH is present at consistent concentrations across biological specimen test materials.

We evaluated the effects of three PAR1 and two PAR4 agonists. Despite large variation in donor platelet response, the effect of C1INH was observed and qualitatively reproducible. In our experiments, we used two relatively independent platelet activation markers, including CD62P and glycoprotein IIb/IIIa complex exposure on platelet surfaces. In some donors, resting platelets demonstrated relatively high CD62P surface exposure (up to 25%), however, it corresponds to the results from published studies to date.[10,30] Both platelet activation markers used in the study demonstrated consistency in our flow cytometry assays after addition of C1INH. This illustrates the potential changes in intrinsic signaling pathways in platelets

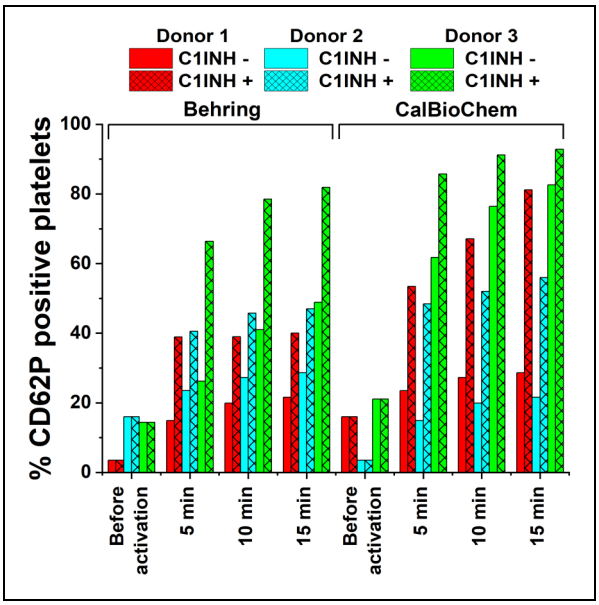

Figure 4. Influence of C1INH from Behring (Berinert®) and from Calbiochem on the kinetics of CD62P positive platelet population upon activation with 5 μM SFLLRN. Donors differ between the experiments with different C1INH sources.

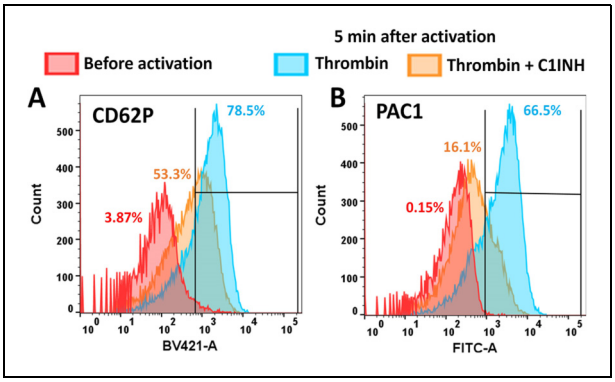

Figure 5. Representative flow-cytometric histograms for CD62P (A) and PAC1 (B) surface exposure in a platelet suspension from one of 3 healthy donors upon 0.5 nM thrombin activation.

if synthetic peptide PAR1 and PAR4 agonists are used together with C1INH. We also showed that platelet activation with thrombin is inhibited by C1INH. According to this experiment, C1INH suppresses platelet activation when both PAR1 and PAR4 receptors are activated. However, direct thrombin inhibition by C1INH[15] can also contribute to this effect.

The fact that C1INH alone did not affect platelet reactivity or ADP stimulation, might suggest that C1INH interacts with peptide agonists or with thrombin receptors. To our knowledge, there are no serpin-like inhibitors or other plasma proteins (which are not active proteases) that are reported to interact with synthetic peptides. Nonetheless, studies report on the potential role of the complement system through C4a activation of PAR1 and PAR4 pathways.[31,32] In other studies, complement was shown to interact with platelets through C3b and membrane attacking complex.[33–36] Our results suggest an additional C1INH specific mechanism of crosstalk between the complement system and platelets, specific to activation with synthetic peptide agonists of PAR1 and PAR4.

Our results also imply the potential for a cell surface receptor modifiable regulation of platelet activation by C1INH. Platelets are known to modulate complement and coagulation function by secretion of C1INH following activation. Here we demonstrate that C1INH has opposite unique effects on two different pathways of thrombin-mediated platelet activation. The PAR1 pathway is responsible for supporting platelet adhesion, aggregation, and granules secretion[37] and activation of the PAR1 pathway by C1INH induces its own release. The PAR4 pathway promotes plasma coagulation through phosphatidylserine (PS) expression and the release of coagulation factors by platelets.[37] Therefore, suppression of the PAR4 pathway by C1INH can lead to reduction of PS exposure and coagulation factors release suggesting an additional mechanism of C1INH modulation of blood coagulation. Thus, C1INH can potentially promote its own secretion by platelets, and at the same time indirectly facilitate its own anticoagulant activity through suppression of the PAR4 pathway. It should be also mentioned that C1INH promoted the inhibitory effect on thrombin mediated exposure of platelet fibrinogen receptors (PFR) measured by PAC1 binding, this serine protease inhibitor might play a role in pathophysiologic reductions of PFR exposure in the cases of myeloproliferative neoplasms.[38,39] Further studies in this area of research may afford new information about the mechanisms of hematologic cancers.

In the present study, we focused on the verification of the observed effect of C1INH on platelet activation in our experimental system, which approximates currently used methods. Further studies that include experiments with blood plasma and whole blood as well as direct evaluation of platelet aggregation and expression of PS over a range of C1INH (and other plasma serine protease inhibitors) concentrations will be of value. It should be also mentioned that due to the receptor-dependency of observed effects of C1INH on platelets, the investigation on molecular mechanisms should be carried on in the future. This should include studying the potential effects of α-granules containing C1INH.

In conclusion, this work introduces a novel effect of C1INH on platelet activation in the presence of synthetic PAR1 and PAR4 agonists. This effect is receptor-dependent and can either facilitate (PAR1) or suppress (PAR4). These findings are important for platelet activation research and should be considered when designing further research studies to include C1INH in platelet preparations. Further studies are needed to identify the molecular mechanisms of the observed C1INH effects described.

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Author Contributions

IT performed experiments and wrote the first draft of the manuscript. IT, EK and PWB discussed the data and edited the manuscript. All authors approved the final version.

Declaration of Conflicting Interests

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ORCID iD

Ivan D. Tarandovskiy https://orcid.org/0000-0003-0449-9673

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