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ORIGINAL ARTICLE

Heparin and heparin proteoglycan-mimetics activate platelets via PEAR1 and PI3Kβ

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Abstract

Background: Platelet endothelial aggregation receptor 1 (PEAR1) is a single-transmembrane orphan receptor primarily expressed on platelets and endothelial cells. Genetic variants of *PEAR1* have repeatedly and independently been identified to be associated with cardiovascular diseases, including coronary artery disease.

Objectives: We have identified sulfated fucoidans and their mimetics as ligands for PEAR1 and proposed that its endogenous ligand is a sulfated proteoglycan. The aim of this study was to test this hypothesis.

Methods: A heparin proteoglycan-mimetic (HPGM) was created by linking unfractionated heparin (UFH) to albumin. The ability of the HPGM, UFH and selectively desulfated heparins to stimulate platelet aggregation and protein phosphorylation was investigated. Nanobodies against the 12th to 13th epidermal growth factor-like repeat of PEAR1 and phosphoinositide 3-kinase (PI3K) isoform-selective inhibitors were tested for the inhibition of platelet activation.

Results: We show that HPGM, heparin conjugated to an albumin protein core, stimulates aggregation and phosphorylation of PEAR1 in washed platelets. Platelet aggregation was abolished by an anti-PEAR1 nanobody, Nb138. UFH stimulated platelet aggregation in washed platelets, but desulfated UFH did not. Furthermore, HPGM, but not UFH, stimulated maximal aggregation in platelet-rich plasma. However, both HPGM and UFH increased integrin αIIbβ3 activation in whole blood. By using PI3K isoform-selective inhibitors, we show that PEAR1 activates PI3Kβ, leading to Akt phosphorylation.

Conclusion: Our findings reveal that PEAR1 is a receptor for heparin and HPGM and that PI3Kβ is a key signaling molecule downstream of PEAR1 in platelets. These findings may have important implications for our understanding of the role of PEAR1 in cardiovascular disease.

KEYWORDS
Fucoidan, glycosaminoglycan, polysaccharides, signal transduction, sulfates

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1 | INTRODUCTION

Platelet endothelial aggregation receptor 1 (PEAR1) is a 15 epidermal growth factor (EGF)-like domain repeat protein that mediates powerful platelet activation [1]. PEAR1 has been described as a platelet aggregate-stabilizing receptor that is tyrosine phosphorylated on platelet contact [1,2], suggesting that the endogenous ligand for PEAR1 is present in platelets. In 2015, the high-affinity immunoglobulin E receptor subunit α (FcεRIα) was identified as an endogenous PEAR1 ligand using an AVidity-based EXtracellular protein Interaction Screen [3]. However, FcεRIα does not induce platelet aggregation on its own but enhances platelet aggregation in response to other agonists [3]. In contrast, sulfated polysaccharides of seaweed and bacterial origin, fucoidans and dextran sulfate, respectively, as well as synthetic sulfated glycopolymers, stimulate the powerful activation of platelets through PEAR1 [4–6]. In a previous study, we showed that fucoidan-mimetic sulfated glycopolymers activate PEAR1 by binding to the 13th EGF-like repeat [4], which contains a BXXBB sequence (where B is a positively charged amino acid) that is similar to previously known heparin-binding sequences [7,8].

We have previously proposed that the endogenous ligand for PEAR1 is a sulfated polysaccharide with activation requiring an average of >2 sulfate groups per monomeric residue and a branched structure [6]. One of the highest negatively charged molecules in the human body is the glycosaminoglycan (GAG) heparin [9,10], and we have thus hypothesized that heparin might be a physiological ligand for PEAR1. However, the reported effects of heparin on platelets have been mixed, ranging from none [11,12] to increased responsiveness and activation [13,14]. To reconcile these contradicting findings, we have reasoned that the ability of heparin to activate platelets may be increased if several heparin chains are covalently conjugated to a protein. This would resemble the physiological form of heparin in which heparin chains are attached to the serglycin core protein [6], hence resulting in a highly branched proteoglycan structure with increased potential to crosslink several PEAR1 molecules. To mimic a proteoglycan form of heparin, we have conjugated heparin with albumin and studied its ability to activate PEAR1 in blood platelets.

We have previously studied the signaling pathways downstream of PEAR1 engagement using fucoidans and dextran sulfate as ligands. These 2 sulfated polysaccharides induce Src-family kinase-dependent phosphorylation of Tyr943 in the PEAR1 tail. Tyr943 forms part of a YXXM motif, a consensus binding sequence for the SH2 domain of the regulatory p110 subunit of phosphoinositide 3-kinase (PI3K) [1,2,4]. In line with this, previous studies have shown that PEAR1 signaling is abolished by the pan-PI3K-inhibitors LY294002 and wortmannin [2,4,5,15]. PI3Kβ and PI3Kγ are the major isoforms of PI3K in platelets, and they mediate platelet activation via tyrosine kinase-linked and G protein-coupled receptors, respectively [16].

In the present study, we show that a heparin proteoglycan-mimetic (HPGM) stimulates rapid activation of platelets through a PI3Kβ-based pathway in association with phosphorylation of PEAR1, hence introducing the notion that a heparin proteoglycan is an endogenous ligand for PEAR1.

Essentials

- Variants of *PEAR1* are associated with cardiovascular disease and increased platelet aggregability.
- Unfractionated heparin and heparin proteoglycan-mimetics cause platelet activation via PEAR1.
- Both agonists cause aggregation in washed platelets and αIIbβ3 activation in whole blood.
- PEAR1 aggregation is mediated via PI3Kβ, a PI3K isoform known to induce platelet priming.

2 | MATERIALS AND METHODS

2.1 | Ethical approvals

Ethical approval for collecting blood samples from healthy volunteers was granted by the University of Birmingham Internal Ethical Review (ERN_11-0175). All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and with United Kingdom Home Office approval (PP9677279).

2.2 | Agonists and inhibitors

All agonists and inhibitors used in this study are specified in the Supplementary Data.

2.3 | Generation of heparin proteoglycan-mimetic

In brief, HPGM was generated using heparin with an average size of 18 kDa (#H3393, Merck), albumin (#A9731, Merck), SPDP (succinimidyl 3-(2-pyridyldithio)propionate) (#21857, Thermo Scientific), and ZebaTM Spin Desalting Columns (#89882, Thermo Scientific) according to the manufacturer's protocol for conjugating 2 reagents, neither of which contains sulfhydryls. The HPGM was fractionated by size using a Hiload 26/600 Superdex 75 pg gel filtration column.

The fraction used for platelet treatment had a heparin content of ∼235 μg/mL and an albumin content of ∼610 μg/mL and was used at 10 to 20 μg/mL final dose based on the heparin content of the HPGM product. The heparin doses used in this study are equivalent to ∼1.8 to 3.6 U/mL. A more detailed description is available in the Supplementary Data.

2.4 | Generation of nanobodies against PEAR1

Nanobodies were raised against EGF-like repeat 12 to 13 of PEAR1, which was expressed as previously described [3,4]. The nanobodies were generated by VIB Nanobody Core. Nanobodies were expressed, and binding to full-length PEAR1 was confirmed using ELISA and AVidity-based EXtracellular protein Interaction Screen. A more detailed description is available in the Supplementary Data.

2.5 | Human platelet-rich plasma and platelet preparation

Platelet-rich plasma (PRP) and isolated platelets were prepared from citrated blood by centrifugation. The platelet pellet was resuspended in a modified Tyrode's buffer (129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, and 1 mM $MgCl_2$; pH 7.3) and was used at a platelet count of 2.5 x 10^8 platelets/mL. A more detailed description is available in the Supplementary Data.

2.6 | Mouse platelet preparation

Blood was drawn from the vena cava of CO_2 terminally anesthetized wild-type mice (C57BL/6J and C57BL/6N, 8-12 weeks old, both female and male) using acid citrate dextrose, volume ratio 1:10. Platelets were isolated as described previously [4].

2.7 | Light transmission aggregometry

Aggregometry was performed by using a lumi-aggregometer (Model 700; Chronolog) at 37 °C with stirring at 1200 rpm [4]. Platelets were pretreated for 3 minutes using inhibitors before agonist addition.

2.8 | Flow cytometry

Whole blood flow cytometry was performed on citrated whole blood in static conditions using a Beckman Coulter CytoFLEX flow cytometer. Blood was diluted in phosphate buffered saline (PBS) and stimulated for 30 minutes using agonists and was stained for activated integrin αIIbβ3 using PAC-1 FITC (#340507, BD Biosciences), p-selectin upregulation for alpha granule secretion using an anti-CD62p APC antibody (Clone AK4, # 550888, BD Biosciences). Reactions were stopped by using 2% form aldehyde PBS. Samples were fixed for 10 minutes before the analysis. Increases in p-selectin levels were determined by using a matching APC isotype control (#555751, BD Biosciences); PAC-1 levels were determined by comparing with an untreated sample. An anti-CD41a PE antibody was used to gate for platelets (#A07781, Beckman Coulter). For measuring PEAR1 levels, we used an anti-PEAR1 Alexia Fluor 488-conjugated antibody (clone 492621, #FAB4527G, R&D Systems) with matching isotype control (#IC0041G, R&D Systems). Median fluorescence intensity (MFI) and percentage-positive cells positive for both CD41a and activation markers were analyzed using CytExpert (version 2.5).

2.9 | Protein phosphorylation

Washed human and mouse platelets were treated with agonists at 37 °C under stirring conditions. Where indicated, platelet aliquots were pretreated for 3 minutes with inhibitors before stimulation using agonists. Reactions were stopped using lysis buffer. Samples were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot. All reagents are specified in the Supplementary Data.

2.10 | Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8 (version 8.3.0) and Excel. Data are presented as mean ± SD. Statistical significance was calculated using a 2-tailed *t*-test and Benjamini-Hochberg correction for multiple comparisons. $q < 0.05$ was considered notable.

3 | RESULTS

3.1 | A heparin proteoglycan-mimetic and heparin activate human platelets

We have previously proposed that PEAR1 is a proteoglycan receptor [6]. To test this hypothesis, we generated an HPGM by linking unfractionated heparin (UFH) to an albumin core. The product was size-fractionated, and the highest molecular weight product was used (heparin content ~235 μg/mL; albumin content ~610 μg/mL). Fractionation trace and batch comparison results are shown in Supplementary Figure S1.

HPGM was tested in a light transmission platelet aggregation assay. HPGM (10 μg/mL) stimulated a slow increase in light transmission of washed platelets over 10 to 25 minutes, which was followed by a second rapid phase resulting in full platelet aggregation in 7 of 8 donors (Figure 1Ai, Bi). In the remaining donors, the slow phase of aggregation continued for 50 minutes, reaching the near maximal aggregation.

Albumin, which was used in the synthesis of the HPGM, had no effect on light transmission at a concentration of 25 μg/mL (Figure 1Bi, Ci), suggesting that the protein moiety of the HPGM did not contribute to platelet aggregation. By contrast, the nonconjugated heparin (UFH 1, 10 μg/mL, from Merck) used for generating the proteoglycan-mimetic caused a slow primary phase of platelet aggregation, which was followed by a rapid secondary phase that reached full aggregation in 3 of the 7 donors (Figure 1Ai, Bi). Notably, the secondary phase occurred after a longer delay than for HPGM (Figure 1Ai). In the 4 remaining donors, the slow primary phase continued for up to 50 minutes (Figure 1Ai). Similar results were observed with a second source of UFH, termed UFH 2 (from Wockhardt; Figure 1Bi). In comparison, low molecular weight heparin (10 μg/mL) induced aggregation in only 1 of the 5 donors after a delay of 40 minutes (Figure 1Ai, Bi). The latter suggests that the length of the glycosaminoglycan (GAG) is important for platelet activation, which is consistent with previous observations [4].

The observation that heparins induce platelet aggregation is unexpected, given their wide use as anticoagulants. To explore this further, studies were undertaken to investigate whether UFH or HPGM induce aggregation in PRP. As shown in Figure 1Aii, Bii, HPGM (10 μg/mL) stimulated maximal aggregation in PRP after a delay of 10

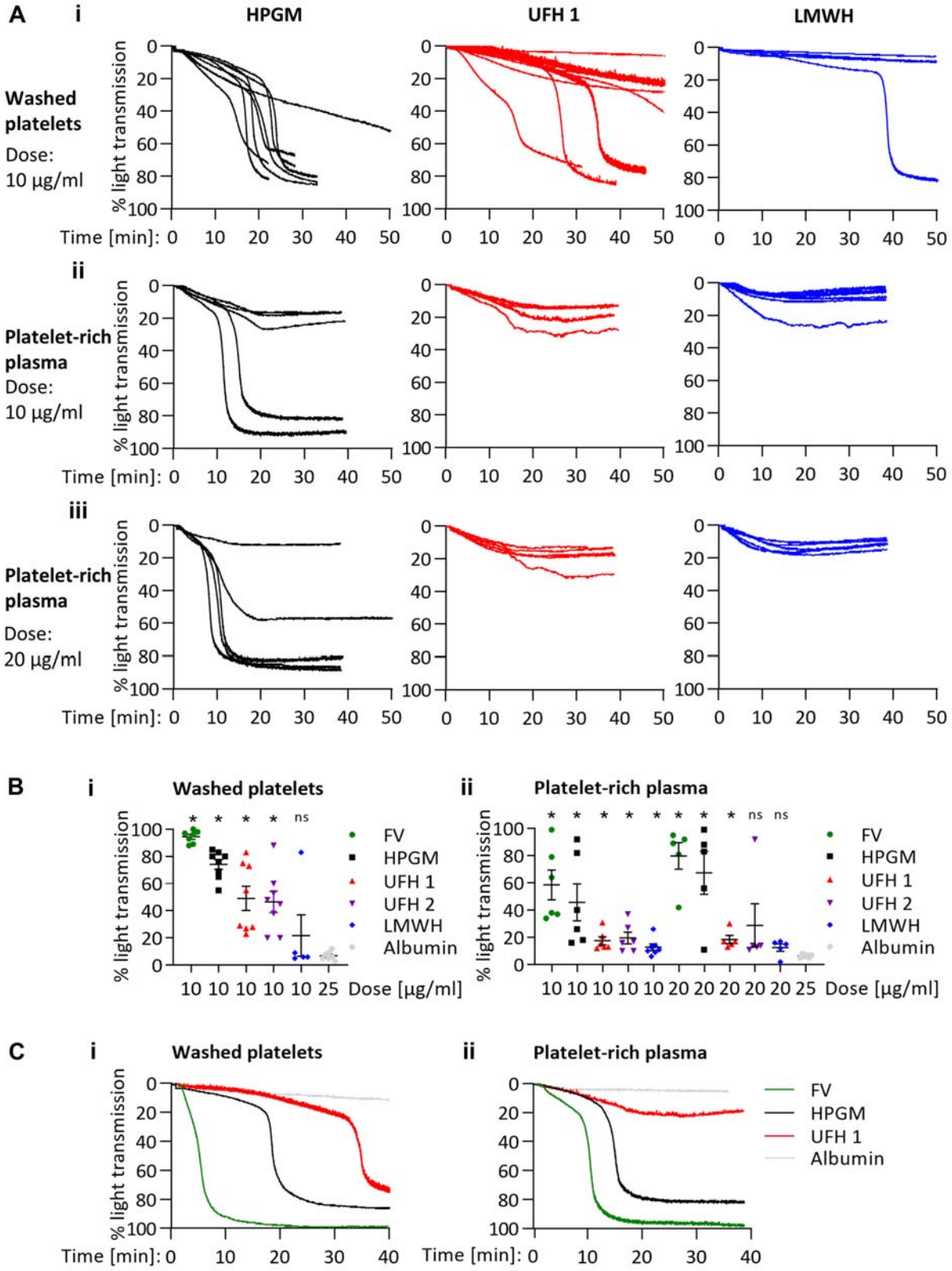

FIGURE 1 A heparin proteoglycan-mimetic causes platelet aggregation in both washed platelets and platelet-rich plasma. Aliquots of platelet-rich plasma or platelet suspension from human volunteers were stimulated using fucoidan from Fucus vesiculosus, heparin proteoglycan-mimetic (HPGM), unfractionated heparin (UFH) of 2 brands, low molecular weight heparin (LMWH), or albumin. (Ai) Original traces after stimulation in washed platelets using 10 µg/ml of the different heparins, each trace of the same color represents a different individual (n =5-8). (Aii) Original traces after stimulation of platelet-rich plasma using 10 µg/ml of the different heparins, each trace of the same color represents a different individual (n = 6-7). (Aiii) Original traces after stimulation in platelet-rich plasma using 20 µg/ml of the different

to 15 minutes in 2 donors but had no effect on 4 other donors. A 2-fold higher dose (20 μg/mL) stimulated maximal aggregation within 15 minutes in 3 donors, and partial aggregation in 1 donor, but had no effect in one other (Figure 1Aiii, Bii). This demonstrates that HPGM causes platelet aggregation in PRP, but with low efficacy. By contrast, the nonconjugated heparin (UFH) caused only a minimal increase in light transmission in 7 donors at 10 or 20 μg/mL >40 minutes (Figure 1Aii–iii, Bii). The difference in response between washed platelets and PRP is illustrated in platelets from a single donor (Figure 1Ci–ii).

On investigation of the effect of HPGM and UFH in citrated whole blood using flow cytometry, a notable increase in integrin αIIbβ3 activation, as measured by the increase in PAC-1 binding, was observed after 30 minutes by both UFH and HPGM. The percentage of positive platelets in comparison to vehicle control was determined. For HPGM, both doses tested (10 and 20 μg/mL) caused PAC-1 binding to ∼30% of platelets, whereas UFH caused the binding to ∼45% of platelets when used at 10 μg/mL (Supplementary Fig. S2Ai). The MFI showed a small but notable increase at the lower doses (1–3 μg/mL). The higher doses were not statistically notable after statistical correction for multiple comparisons, which is likely to be due to the heterogenous donor responses (Supplementary Fig. S2Aii). The binding of CD62p, a marker for p-selectin exposure on alpha granule secretion, was not notably increased after stimulation in comparison to matching isotype control. However, UFH 3 caused substantial secretion in 1 donor (Supplementary Fig. S2Bi, Bii). A combination of collagen-related peptide (10 μg/mL) and 2-MeS-adenosine diphosphate (10 μM) was used as a positive control. The experiments were carried out at room temperature in static conditions.

To explore the potential difference in PEAR1 receptor levels between high responders (short primary aggregation phase) and low responders (long primary aggregation phase), we measured PEAR1 MFI levels on platelets in whole blood. However, we were not able to detect a clear difference between the low and high responder groups (Supplementary Fig. S3).

The ability of HPGM to activate mouse platelets was also tested. However, HPGM did not cause mouse platelet aggregation within 60 minutes when used up to 100 μg/mL and did not induce notable Akt phosphorylation within 10 minutes at 20 μg/mL (n = 3 mice, data not shown).

Taken together, these findings suggest that HPGM, representing multiple heparin chains conjugated to a protein core, stimulates human platelet aggregation both in washed platelets and in PRP, whereas nonconjugated heparin (UFH) has a lower ability to induce aggregation of washed platelets and causes a small increase in light transmission in PRP. Furthermore, our findings demonstrate that HPGM does not stimulate aggregation in mouse platelets.

3.2 | Heparin proteoglycan-mimetic and unfractionated heparin cause platelet PEAR1 activation

Experiments were performed to investigate whether the activation of platelets by the HPGM is associated with PEAR1 activation. For this, we raised nanobodies against the 12th to 13th EGF-like repeats of PEAR1, which is the site of the binding site of fucoidans [4]. In total, 24 nanobodies were raised and characterized, as described in the Supplementary Data (Supplementary Figs. S4A, B). Two of the nanobodies, denoted Nb20 and Nb138, were found to reduce fucoidan-induced aggregation when used at 3 μM (Supplementary Fig. S4B). Because Nb138 was more potent at reducing FCεR1α-PEAR1 binding, and therefore most likely interferes with the 13th EGF-like repeat of PEAR1, it was used in subsequent experiments (Supplementary Fig. S4).

As shown in Figure 2A, platelet aggregation in response to HPGM was abolished in the presence of 3 μM of Nb138, demonstrating that platelet activation via HPGM is associated with PEAR1 activation. By contrast, platelet activation by collagen was not affected by Nb138, showing that the inhibition was not due to an off-target effect (Figure 2A).

To measure PEAR1 phosphorylation, immunoprecipitation of PEAR1 was performed, and tyrosine phosphorylation was measured by using the pan-phosphotyrosine antibody 4G10. HPGM stimulated a notable increase in phosphorylation of PEAR1, which was detected after 30 seconds and maintained for 500 seconds (Figure 2B). The degree of phosphorylation in response to the HPGM was lower than that in response to fucoidan (Figure 2B), consistent with the slower onset rate of platelet aggregation (Figure 1B). Stimulation by UFH 1 caused a weak phosphorylation of PEAR1 (Figure 2B). In addition, we investigated the effect of Nb138 (3 μM), Cangrelor (10 μM), indomethacin (10 μM), and eptifibatide (9 μM) on HPGM-induced PEAR1 phosphorylation. Supplementary Figure S5 shows that Nb138 caused a notable decrease in PEAR1 phosphorylation, whereas Cangrelor or indomethacin had no notable effect. Eptifibatide gave rise to an increase in PEAR1 phosphorylation, most likely because of the loss of dephosphorylation that is observed in aggregate formation [17].

Together, these findings demonstrate that HPGM activates human platelets via PEAR1.

3.3 | Heparin-induced platelet aggregation is abolished by the removal of sulfate groups

We have previously shown that sulfation is necessary for glycopolymer-PEAR1 binding and platelet aggregation [4]. Experiments were therefore designed to investigate the importance of

heparins, each trace of the same color represents a different individual (n = 5). (Bi-ii) Quantification of maximal aggregation within 60 minutes presented as percentage increase of light transmission ± SD. All traces from panel Ai-iii were quantified; the panel also includes maximal aggregation to a second UFH from another supplier (UFH 2) (n = 5-8). Statistical significance was calculated comparing treatments to albumin using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, q <.05 was considered statistically significant. (Ci, ii) Examples of traces from the platelet-rich plasma and washed platelets from the same donor using 10 μg/ml of the different heparins and fucoidan from *Fucus vesiculosus* (FV). Albumin was used at 25 μg/ml. 172 × 219 mm (300 × 300 DPI).

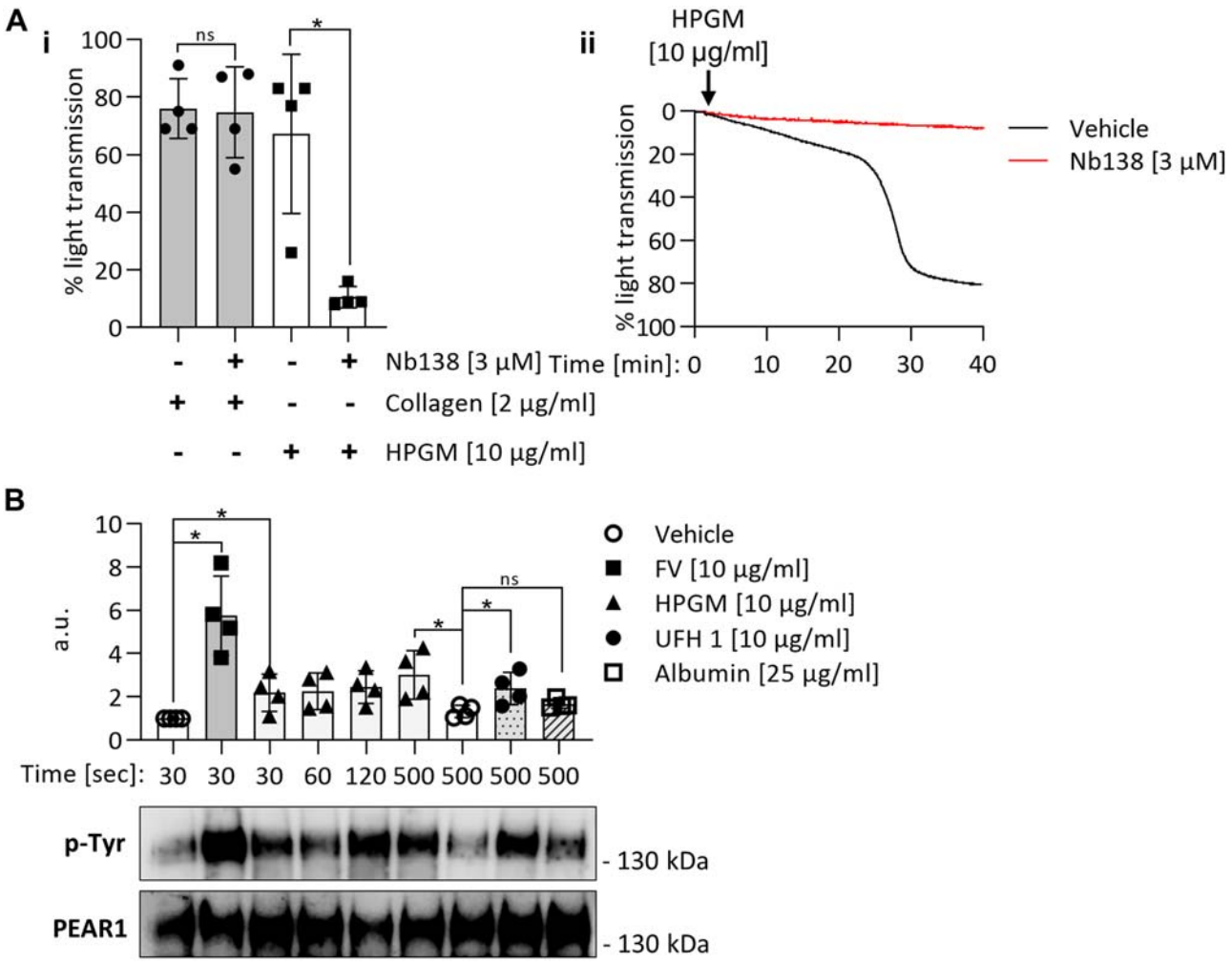

FIGURE 2 A heparin proteoglycan-mimetic cause PEAR1-mediated platelet aggregation and receptor phosphorylation. (Ai) Aliquots of platelet suspensions were incubated with nanobody 138 (Nb138, 3 μM) or a vehicle control (PBS) for 3 minute before stimulation using a heparin proteoglycan-mimetic (HPGM, 10 μg/ml) or collagen (2 μg/ml). Platelet aggregation was measured as increase in percentage light transmission presented as mean percentage increase in light transmission ± SD (n = 4). (Aii) Example trace of HPGM-stimulated washed platelets in the presence and absence of Nb138. (B) Aliquots of human platelet suspensions were stimulated using a HPGM (10 μg/ml), fucoidan from *Fucus vesiculosus* (10 μg/ml), unfractionated heparin from Merck (UFH 1, 10 μg/ml), or albumin (25 μg/ml). Reactions were stopped at specific time points followed by immunoprecipitation for PEAR1. Samples were analyzed by SDS-page followed by Western blot (n = 4). Quantification of the optical density of the Western blot bands are presented as mean ± SD. Statistical significance was calculated using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, $q < 0.05$ was considered statistically significant. 172 × 129 mm (300 × 300 DPI).

different sulfation sites of heparin by using 3 different desulfated heparins. All the heparins used in this series of experiments are derived from the same type of high-grade UFH (UFH 3 from Iduron) with an average size of 15.7 kDa. The modified heparins had been desulfated on C2 of uronic acids (2-O desulfated), C6 of glucosamine (6-O desulfated), and at the aminosulfate (N-desulfated). Following N-desulfation, the heparin was reacetylated.

Platelet aggregation in response to UFH 3 was dose dependent, reaching maximal aggregation at 10 μg/mL (Figure 3Ai). The time course of the response was similar to that of UFH 1 and UFH 2, which were used above. By contrast, neither of the 3 modified heparins stimulated platelet aggregation (Figure 3ii–iii). Furthermore, high-grade heparin stimulated a time-dependent increase in Akt phosphorylation, which peaked at 10 minutes and remained elevated at the 30 minutes time point (Figure 3Bi). In comparison, neither of the modified heparins induced Akt phosphorylation (Figure 3Bii). Fucoidan from *Fucus vesuclosus*, a known PEAR1 agonist, was used as a positive control for Akt phosphorylation. On pretreatment of the platelets using the PEAR1-specific Nb138 (3 μM) followed by stimulation using UFH 3 (10 μg/mL), Akt phosphorylation was diminished (Figure 3Ci). As a control experiment, we tested the inhibitory effect of 2 unrelated nanobodies, Nb28 against GPVI, and Nb32 against CLEC-2. No inhibition of HPGM- or UFH-induced Akt phosphorylation was observed (Supplementary Figure S6). Collectively, these data show that the

ability of heparin to induce platelet aggregation, and concomitant Akt signaling is dependent on its sulfate groups.

3.4 | Heparin proteoglycan-mimetic-induced aggregation is dependent on secondary mediators and Src-family kinases

Next, experiments were designed to test the role of secondary mediators and tyrosine kinases in HPGM-induced platelet aggregation. As depicted in Figure 3A, the slow platelet aggregation caused by HPGM was blocked by the integrin αIIbβ3 antagonist eptifibatide (Figure 4A). In addition, HPGM-induced platelet aggregation was inhibited by the purinergic receptor $P2Y_{12}$ antagonist Cangrelor, suggesting a role for platelet-released ADP in HPGM-induced platelet aggregation. Furthermore, platelet aggregation was blocked by the cyclooxygenase inhibitor indomethacin (Figure 4A). As expected, indomethacin and Cangrelor also reduced platelet aggregation in response to collagen (Figure 4A).

Owing to the observed strong inhibition of HPGM-induced platelet aggregation by Cangrelor, we tested the effect of Cangrelor on downstream Akt signaling to verify that the observed Akt phosphorylation in response to HPGM is PEAR1-driven rather than ADP-mediated. On pretreatment with Cangrelor followed by stimulation using HPGM or UFH 3, no notable reduction in Akt phosphorylation could be observed (Figure 4Ci–ii). In addition, the inhibitors indomethacin or eptifibatide had no notable effect on HPGM- or UFH-induced Akt phosphorylation (Supplementary Figure S6). The activation of PEAR1 by fucoidan has previously been shown to be mediated through Src-dependent and Syk-independent pathways [4]. In line with this, the Syk-inhibitor PRT-060318 had no effect on HPGM-induced aggregation, whereas PP2, a Src-family kinase inhibitor, abolished the response (Figure 4B). By contrast, PRT-060318 completely inhibited platelet aggregation in response to collagen but not fucoidan (Figure 4B). Altogether, these results indicate that HPGM induces a Src-dependent signaling pathway, which is reinforced by the release of the secondary mediators ADP and thromboxane A_2. However, the downstream Akt phosphorylation is mainly driven by PEAR1-activation rather than by secondary ADP stimulation of purinergic receptors.

3.5 | PEAR1 selectively signals via PI3Kβ

PEAR1 has a conserved YXXM motif in its intracellular tail which, on phosphorylation, can bind directly to PI3K leading to Akt phosphorylation followed by platelet aggregation [1]. We set out to identify which PI3K isoform is activated by PEAR1 in response to fucoidan and HPGM. To this end, human platelets were stimulated by fucoidan or HPGM in the presence of PI3K isoform-specific inhibitors: A66 (p110α), TGX 221 (p110β), and AS 252424 (p110γ). All inhibitors were used at optimized concentrations for the inhibition of PI3K isoforms [18–23].

As shown in Figure 5A, B, only TGX 221 caused an inhibition of fucoidan-induced platelet aggregation in a concentration-dependent manner. Similarly, HPGM-induced platelet aggregation was abolished by the PI3Kβ inhibitor TGX 221, although unaffected by A66 and AS 252424 (Figure 5C). TGX 221 also inhibited fucoidan- and HPGM-induced Akt phosphorylation but A66 and AS 252424 did not (Figure 6Ai–ii). Phosphorylation of Akt by HPGM was also abolished by TGX 221, whereas no reduction was observed by A66 or AS 252424 (Figure 6B). In addition, HPGM-induced Akt phosphorylation was inhibited by the Src-inhibitor PP2 (Figure 6B). Together, these results show that fucoidan and HPGM signal through Src kinases and PI3Kβ. The proposed signaling cascade has been illustrated in Supplementary Figure S7.

3.6 | PEAR1 phosphorylation to classical agonists is minimal

Previous studies have shown that PEAR1 is phosphorylated during platelet aggregation, suggesting that platelets express an endogenous ligand for the glycoprotein receptor [1,2]. However, there was no discernible increase in the phosphorylation of a band corresponding to the molecular weight of PEAR1 (130 kDa) during aggregation in response to thrombin or collagen-related peptide when compared with fucoidan, which was used as a positive control (Figure 7Ai). To confirm these results, we immunoprecipitated PEAR1 after stimulation by thrombin under platelet-aggregating and nonaggregating conditions and blotted for tyrosine phosphorylation. Fucoidan was included as a positive control [4]. These experiments revealed that fucoidan induced strong tyrosine phosphorylation of PEAR1, in both aggregating and nonaggregating conditions. By contrast, there was no notable increase in tyrosine phosphorylation of PEAR1 in response to thrombin at 180 or 300 seconds (Figure 7Aii).

We extended these studies to mouse platelets to investigate a possible species difference. Figure 7B shows a prominent increase in the phosphorylation of PEAR1 in response to dextran sulfate, which was used as a positive control [4,5]. On the contrary, thrombin did not induce a notable increase in PEAR1 phosphorylation in mouse platelets at 30 or 180 seconds under aggregating conditions (Figure 7B).

Together, these results indicate that an endogenous ligand for PEAR1 is not expressed or released from activated human or mouse platelets.

4 | DISCUSSION

This study shows that: 1) HPGM and UFH cause platelet aggregation and PEAR1 phosphorylation in washed platelets and notable integrin αIIbβ3 activation in whole blood; 2) PEAR1 stimulates platelet aggregation through a Src-PI3Kβ-Akt signaling cascade; and 3) classical agonists, such as thrombin, do not induce a notable increase in PEAR1 phosphorylation in platelets, this being in contrast to previous reports. Therefore, the present study provides evidence in support of the

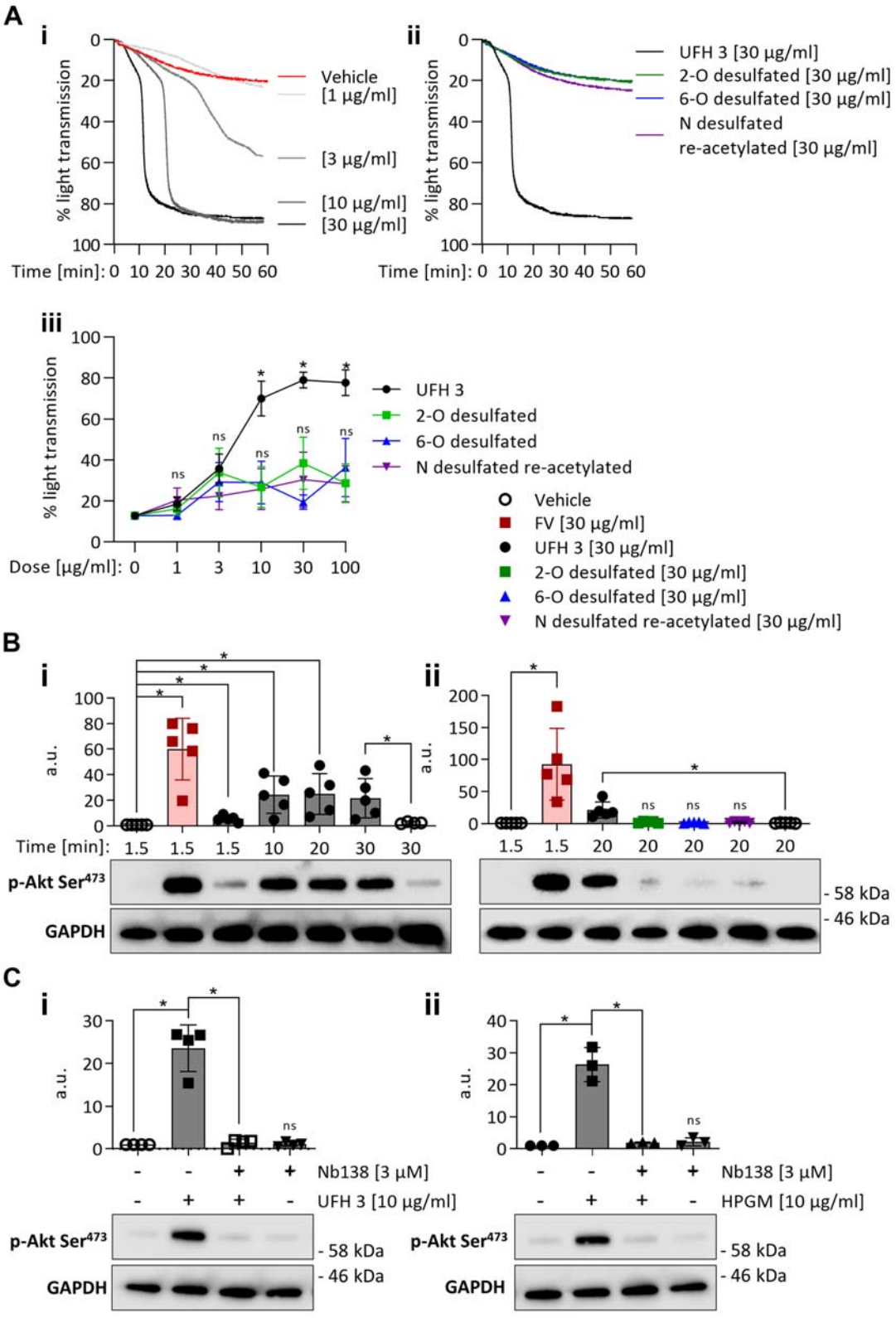

FIGURE 3 Reduction in effect of heparin-induced platelet aggregation and downstream Akt phosphorylation upon removal of specific sulfate groups or nanobody inhibition of PEAR1. (Ai) Aliquots of washed platelet suspensions were stimulated with increasing doses of unmodified unfractionated heparin (UFH 3, average molecular weight 15.7 kDa). Representative traces of platelet aggregation (n = 5). (Aii) Aliquots of washed platelet suspensions were stimulated using UFH 3 and modified unfractionated heparins. The heparins were modified by 2-O, 6-O, or N-desulfation. Comparison of representative traces of platelet aggregation at the same dose (30 µg/ml, n = 5). (Aiii) Quantification of maximal aggregation to UFH 3 and modified heparins within 60 minutes presented as percentage increase of light transmission ± SD (n = 5). (Bi) Aliquots of human platelet suspensions were treated for 1.5 minutes to 30 minutes using UFH 3. Reactions were stopped using lysis buffer

proposal that the endogenous ligand for PEAR1 is a heparin-based proteoglycan that causes PI3Kβ-driven Akt phosphorylation.

Both UFH and HPGM caused activation of PEAR1, as shown by phosphorylation and inhibition of HPGM-induced aggregation using nanobodies. The length of the primary aggregation phase was variable between donors but more rapid for the HPGM in comparison to UFH. Donors that responded rapidly to HPGM in PRP also responded rapidly in washed platelets and the highest responder also had the strongest PEAR1 phosphorylation. In addition, stimulation of washed platelets in the same donor on different days yielded very similar results (data not shown).

The difference between HPGM and nonconjugated GAGs, represented by UFH, is that the heparinized protein core has several GAG chains attached to the same core protein, thereby achieving "branching," enabling a higher extent of multivalency for interaction with platelets. In line with previous hypotheses, this indicates that such branching is of importance for the platelet aggregation potency [6], most likely because of an increased ability to cause receptor clustering.

In concordance with previously published studies from our laboratory, the length and the negative charge of the carbohydrate seem important [4], that is, shorter heparin fragments (low molecular weight heparin) were less potent in causing platelet aggregation in comparison to full-length heparin (UFH) when used at equal weight dose (Figure 1). Notably, both HPGM and heparins cause a much slower aggregation response than fucoidan from *Fucus vesiculosus*. We believe that this may be because of the greater size and branching of fucoidan (average size ∼82.5 kDa) [24]. In comparison to HPGM (UFH 1 average size ∼18 kDa), it has previously been shown that the potency of glycopolymer-induced PEAR1-mediated platelet aggregation correlates with glycopolymer size [4] and that fucoidan size is correlated to biological effects [25]. In addition, fucoidan also interacts with GPIbα, which may contribute to a faster aggregation rate [4]. In the present study, we show that the removal of charge from the heparin through desulfation diminishes the ability of heparin to cause aggregation and downstream phosphorylation. Other studies using similar desulfated heparins have shown that the desulfation changes the anti-inflammatory and anticoagulant properties [26]. Their study showed that the removal of 2-O sulfation reduced anticoagulant ability of heparin, whereas N-desulfation and re-acetylation abolished the anticoagulant effect [26]. In the present study, all 3 positions were important for PEAR1-induced platelet aggregation, highlighting the importance of the GAG origin. Only mast cells can produce this level of endogenous sulfation in the human body [9,27].

We show here that UFH from 3 commercial sources stimulated platelet aggregation in washed platelets. However, in contrast to the HPGM, the aggregatory effect of UFH was largely lost in PRP. Although we cannot with certainty explain the reason for this, a plausible scenario could be that the effect of UFH may be neutralized by binding to other components present in plasma and that such components might be washed away during platelet preparation. A recent study has shown that PEAR1 itself is present in plasma [28], which could be one of the components binding both UFH and HPGM reducing their effectiveness in PRP. However, the effect of the HPGM was retained at higher doses. The present study also shows that a PEAR1-specific nanobody inhibits HPGM-induced platelet activation. This nanobody represents a framework for the development of PEAR1 antagonists and the potential treatment of PEAR1-driven thrombosis.

The notion that PEAR1 acts as a receptor for heparin could explain the observation that the proteoglycan-mimetic, dual Anti-Platelet, and AntiCoagulant (APAC) cause powerful activation of mouse platelets deficient in the inhibitory ITIM-containing receptor G6b-B. Notably, APAC-induced platelet activation was associated with the phosphorylation of a protein of 130 kDa, which is equivalent to the size of PEAR1 [12], raising the possibility that APAC in fact causes PEAR1 activation. Moreover, the finding that APAC stimulated the inhibiting receptor G6b-B in mouse platelets [12] could potentially explain why we did not observe platelet aggregation and phosphorylation in HPGM-stimulated mouse platelets, which is in sharp contrast to the effects of HPGM on human platelets. Although we are at present not able to explain such species differences, one contributing factor could be that the G6b-B protein expression is more than twice as high in mice (∼30 000 copies) [29] than that in humans (∼13 700 copies) [30].

In seeming contrast to the present study, a study by Gao et al. [4] showed that heparin promotes platelet responsiveness through integrin αIIbβ3. However, although our study suggest that PEAR1 triggers platelet activation by heparin on platelets, we cannot exclude that the signal may be amplified by an interaction of heparin with integrin αIIbβ3 [14].

The present study shows for the first time that PEAR1 stimulates PI3Kβ-induced signaling. There are 3 major isoforms of PI3K in platelets; PI3Kα, PI3Kβ, and PI3Kγ [16], and our findings reveal that the selective PI3Kβ-inhibitor TGX 221 blocks HPGM-induced platelet activation. The PI3Kβ isoform is ubiquitously expressed and has been shown to be involved in inflammation, cancer, and immune cell reactive oxygen species production [31]. Multiple studies have shown that the use of PI3Kβ-selective inhibitors or deletion of PI3Kβ *in vivo*

and the samples were analyzed using SDS-PAGE followed by Western blot (n = 5). (Bii) Aliquots of human platelet suspensions were treated for 20 minutes using UFH 3 and modified heparins. Reactions were stopped using lysis buffer and the samples were analyzed using SDS-PAGE followed by Western blot (n = 5). Fucoidan from *Fucus vesiculosus* (FV) was used as a positive control for maximal Akt phosphorylation. (Ci, ii) Aliquots of human platelet suspensions were pretreated with nanobody 138 (Nb138) for 3 minutes followed by stimulation with heparin proteoglycan-mimetic (HPGM) or UFH 3 for 20 minutes. Reactions were stopped using lysis buffer and the samples were subjected to SDS-PAGE followed by Western blot (n = 3-4). Vehicle controls were included for both the longest and shortest time points. Protein densitometry was measured and is presented as arbitrary units (a.u.) ± SD. Vehicle sample = 1 a.u. Statistical significance was calculated comparing treatments with albumin using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, $q < 0.05$ was considered statistically significant. 172 × 250 mm (300 × 300 DPI).

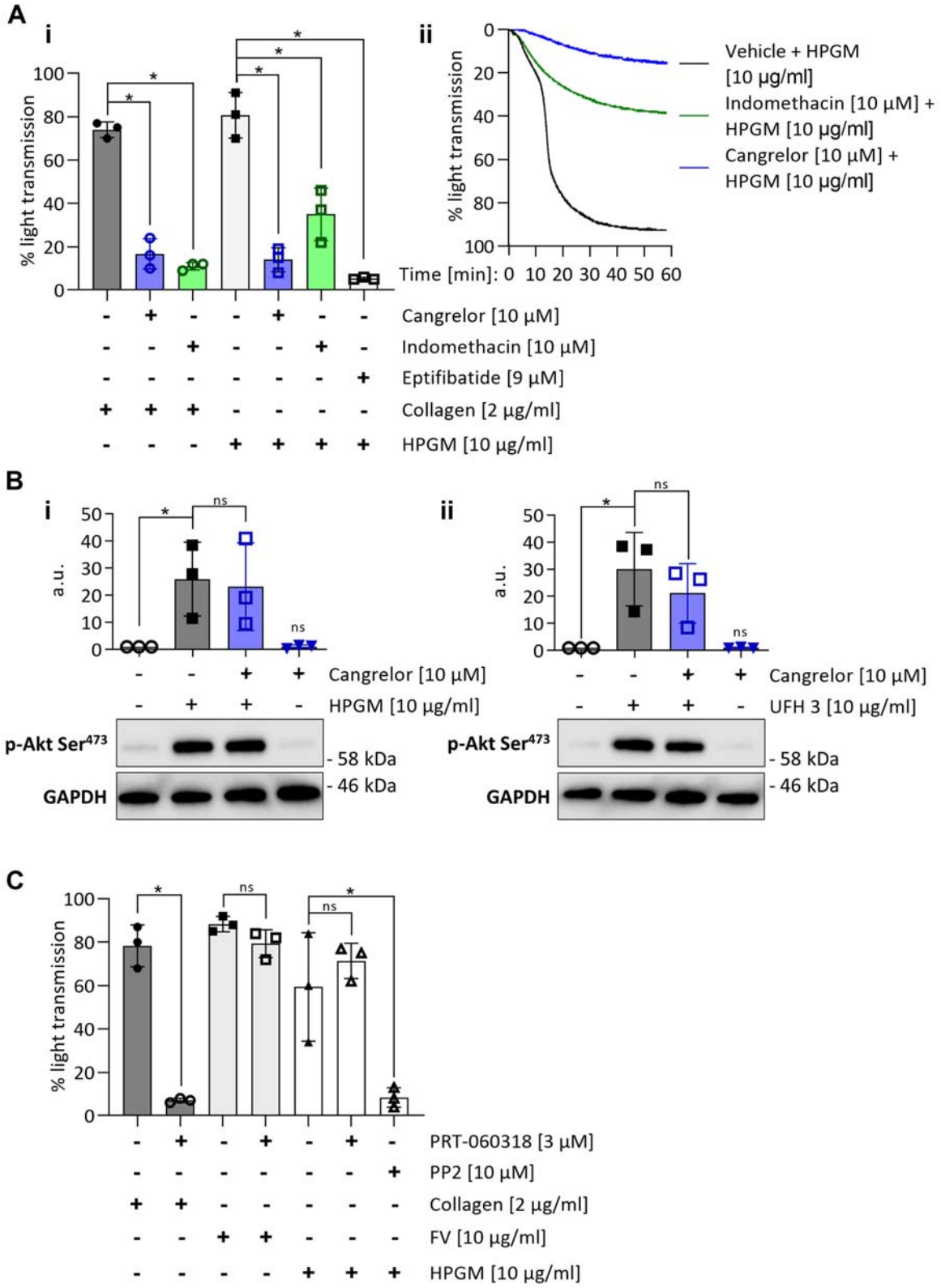

FIGURE 4 Heparin proteoglycan-mimetic-induced platelet aggregation is integrin-depended, reliant on secondary mediators, Src-mediated, and Syk-independent. (Ai) Aliquots of human platelet suspensions were treated for 3 minutes with either indomethacin, Cangrelor, or eptifibatide, followed by stimulation with heparin proteoglycan-mimetic (HPGM). Aggregation is presented as percentage increase of light transmission ± SD. (Aii) Original aggregation traces showing inhibition HPGM-induced aggregation by Cangrelor and indomethacin. (Bi-ii) Aliquots of human platelet suspensions were pretreated with Cangrelor for 3 minutes followed by stimulation using HPGM or unfractionated heparin (UFH 3) for 20 minutes. Reactions were stopped using lysis buffer and the samples were analyzed using SDS-PAGE followed by

affects thrombus stability [18,32,33]. The strong PI3Kβ-component of PEAR1 signaling may further support the hypothesis that PEAR1 contributes to thrombus stability. In addition, recent studies by Moore et al. have shown that thrombopoietin stimulation of platelets causes PI3Kβ-dependent platelet priming [34]. Possibly, induction of PEAR1-mediated activation of PI3Kβ by HPGM and UFH could have a similar priming effect on platelets, and this may explain the small but notable increase of light transmission observed on UFH-stimulation in PRP and the integrin αIIbβ3 activation we observed by flow cytometry.

Previous studies have shown that PEAR1 is phosphorylated on platelet stimulation using classical agonists, such as thrombin [1,2]. Contrary to those studies, when using gels with a higher resolution of the 130-kDa region and when performing quantification of PEAR1 phosphorylation after immunoprecipitation by a PEAR1 antibody, we were unable to detect any statistically notable phosphorylation of PEAR1 by thrombin. We cannot fully explain this apparent discrepancy between our results and previously published data. However, results suggest that activated platelets do not express endogenous ligands to PEAR1.

There are several limitations in this study. First, we have not used human endogenous heparin proteoglycan for our studies because of the challenge of isolating this compound from heparin-producing human cells (mast cells). In addition, the present study observed donor variations in response to heparins, which could be because of unknown *PEAR1* polymorphisms in the participants. Owing to the species difference between human and mouse and the strong association between *PEAR1* and cardiovascular disease, an important future step is to test PEAR1-induced platelet aggregation in blood donors or patients who have *PEAR1* polymorphisms.

The interest in PEAR1 has been fueled by the close association between *PEAR1* and cardiovascular disease seen in genome-wide association studies [35–40]. However, one of the conundrums that remain to be answered is the functional connection between *PEAR1* polymorphisms and the observed associations. PEAR1 is not only mainly expressed on platelets and endothelial cells but also in megakaryocytes and neurons [1,2,41,42]. More recently, Goudswaard et al. [28] showed that PEAR1 levels in plasma are negatively associated with an increase in body mass index—a major risk factor in cardiovascular disease. In addition, increased body mass index has also been shown to be associated with autoimmune diseases and increased incidence of heparin-induced thrombocytopenia [43]. The present study shows for the first time that heparins activate PEAR1, and we believe that this can be of major importance in understanding both the physiological role of PEAR1 and the physiological role of endogenous heparin. Our findings may also be of importance for an understanding of the background to why certain patients exhibit adverse events to heparin, whereas other patients do not. After heparin treatment, up to 10% of patients experience heparin-associated nonimmune thrombocytopenia, where heparin directly interacts with platelets through an unknown mechanism [44]. The role PEAR1 plasma levels and the effects of PEAR1 variants on cardiovascular disease, and their potential involvement in heparin-associated nonimmune thrombocytopenia, would be important future fields of study.

One way to approach the functional aspects of *PEAR1* polymorphisms would be to examine the expression of the proposed endogenous ligands for PEAR1. Heparin and heparin proteoglycans, together with FcεRIα, represent 3 separate potential ligands for PEAR1, and it is notable that all these types of PEAR1 ligands are expressed by one cell type—the mast cell. Hence, this introduces the notion that mast cells may have the capacity to cause platelet activation, by engaging PEAR1. Although serglycin is ubiquitously expressed by many cell types in the human body, it only acquires the highly sulfated heparin chains when being expressed by mast cells [27,45]. For example, although serglycin is present in platelets, it contains chondroitin sulfate rather than heparin when expressed in this cellular niche. This may explain why we did not observe any substantial phosphorylation of PEAR1 on the stimulation of platelets by classical agonists.

A crucial question is as follows: Where would mast cells interact with PEAR1, and when would this be relevant in cardiovascular disease? Studies so far have shown a potential role for PEAR1 in neoangiogenesis [15,46]. Specifically, effects related to PEAR1 expression levels have been implicated after shear alterations and hypoxia [15] and in models of sterile inflammation and reactive oxygen species production [46], which are all important components in cardiovascular disease. In addition, the process of neoangiogenesis during wound healing was severely altered in PEAR1-deficient mice, and PEAR1-deficiency also led to differential immune cell recruitment [15]. Interestingly, all of these processes are known to be affected by mast cells and mast cell degranulation [47,48]. Mast cells have been shown to increase in numbers in processes of pathologic vessel remodeling during aortic aneurysm formation [49,50] and to be key drivers in atherosclerotic plaque formation [51]. Together, these findings lead us to propose that PEAR1 could represent a functional link between mast cell-mediated immune-orchestration and the roles of platelets and endothelium in the inflammatory processes of cardiovascular diseases.

In conclusion, this study shows that PEAR1 is activated by the mast cell GAG heparin and that PI3Kβ is a key signaling molecule downstream of PEAR1 in platelets. However, the absence of notable PEAR1 phosphorylation after the addition of the classical agonist thrombin indicates the absence of a potential endogenous PEAR1 ligand in activated platelets. Instead, we show that PEAR1 is activated

Western blot (n = 3). Vehicle control was performed for the longest time point. Protein densitometry was measured and is presented as arbitrary units (a.u.) ± SD. Vehicle sample = 1 a.u. (C) Aliquots of human platelet suspensions were treated for 3 minutes using PRT-080318 or PP2 followed by stimulation using a HPGM, fucoidan from *Fucus vesiculosus* (FV), or collagen. Control samples contain an equal percentage of dimethyl sulfoxide. Aggregation is presented as percentage increase of light transmission ± SD. Statistical significance was calculated using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, $q < .05$ was considered statistically significant (n = 3). 172 × 230 mm (300 × 300 DPI).

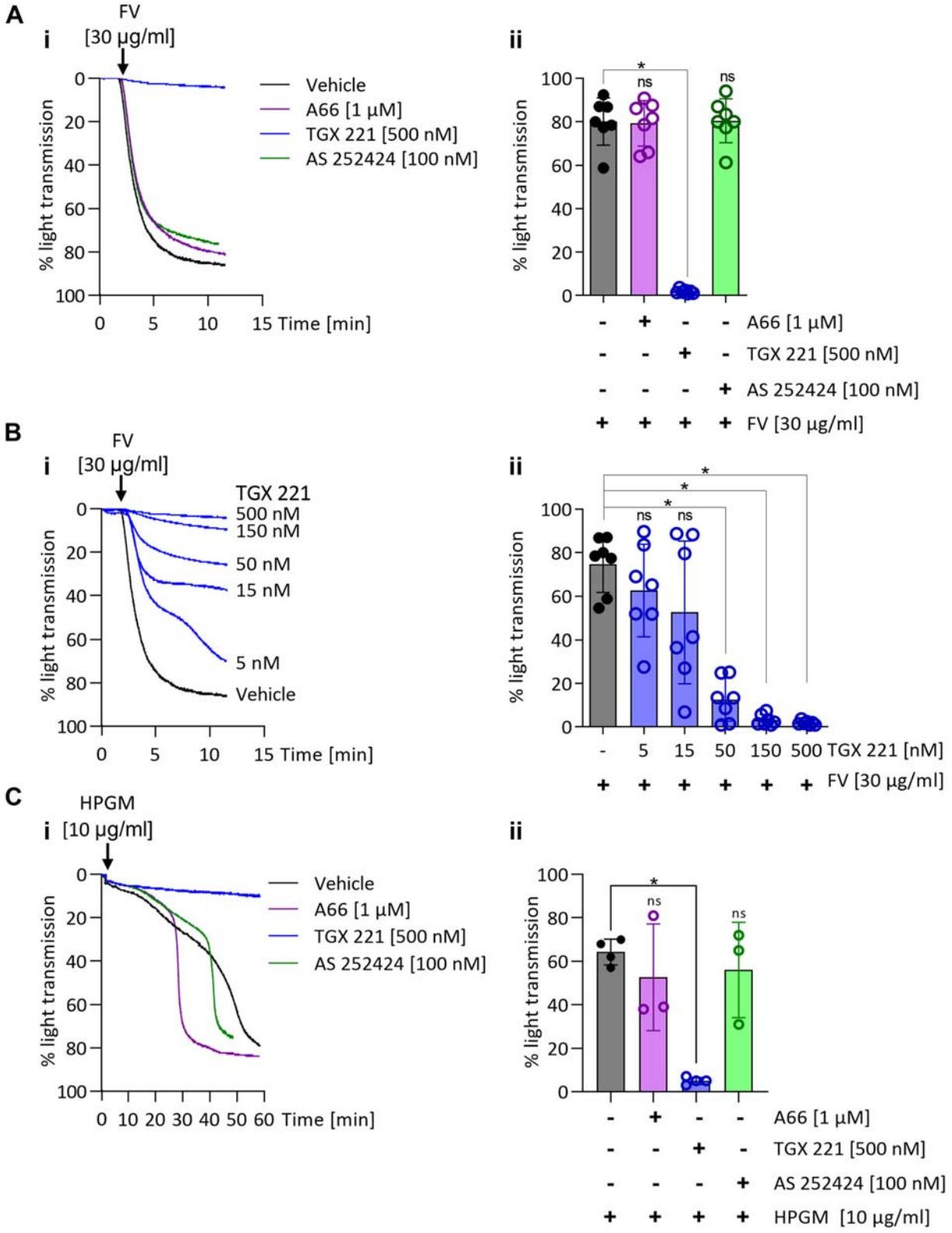

FIGURE 5 PEAR1-mediated platelet aggregation is dependent on PI3Kβ. (A, B) Aliquots of human platelet suspensions were treated for 3 min with either p110α inhibitor A66, p110β inhibitor TGX 221, or p110γ inhibitor AS 252424 followed by stimulation with fucoidan from *Fucus vesiculosus* (FV) (n = 7). (C) Aliquots of human platelet suspensions were treated for 3 minutes using A66, TGX 221, or AS 252424 followed by stimulation with a heparin proteoglycan-mimetic (HPGM) (n = 3). Control samples contain an equal percentage of dimethyl sulfoxide. Aggregation is presented as percentage increase of light transmission ± SD. Statistical significance was calculated using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, q <.05 was considered statistically significant. 172 × 215 mm (300 × 300 DPI).

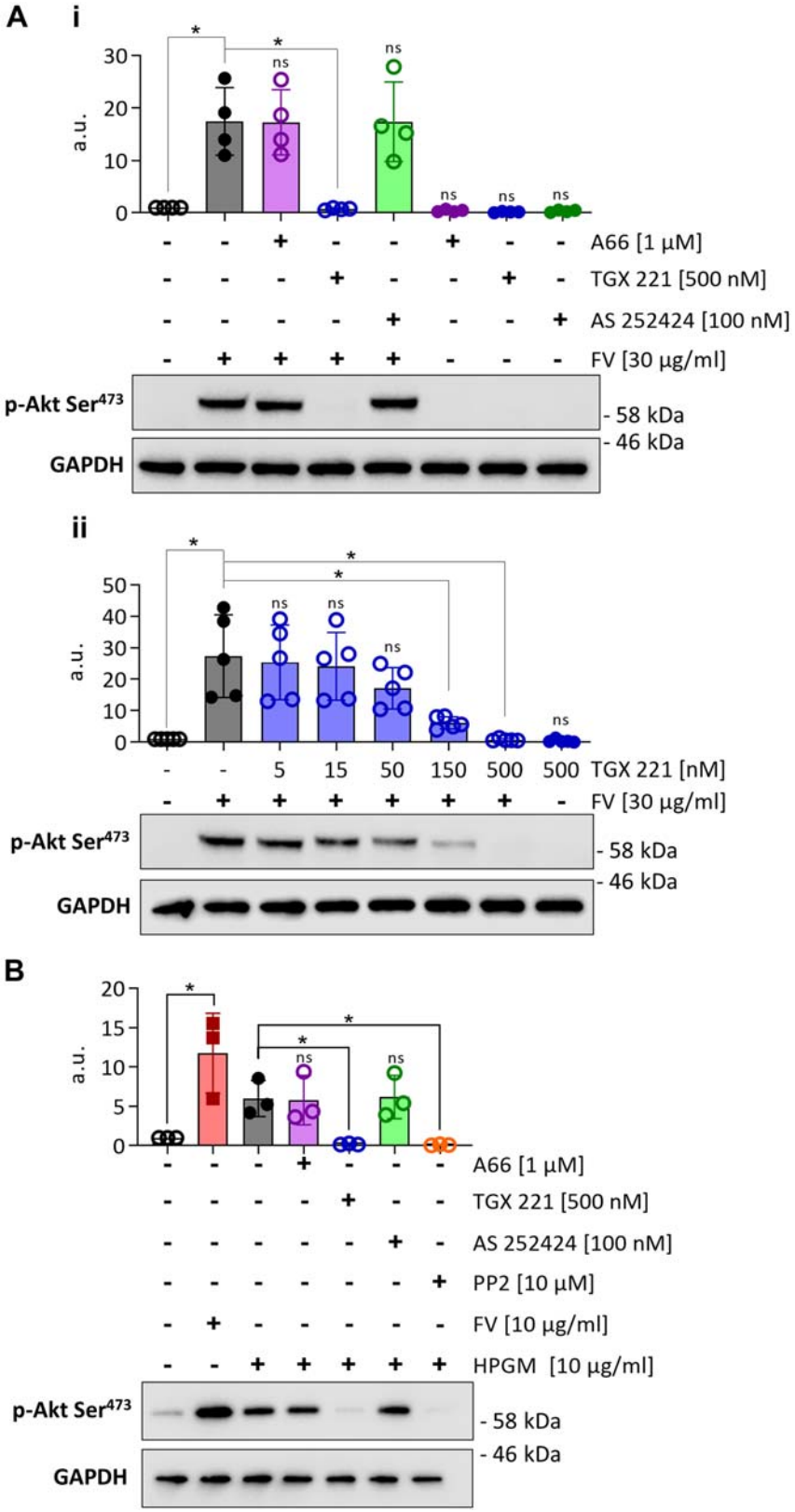

FIGURE 6 Inhibitor to PI3Kβ blocks downstream Akt signaling induced via PEAR1. (A) Aliquots of human platelet suspensions were treated for 3 minutes with either p110α inhibitor A66, p110β inhibitor TGX 221, or p110γ inhibitor AS 252424, followed by stimulation with fucoidan from *Fucus vesiculosus* (FV) for 90 seconds (n = 5). (B) Aliquots of human platelet suspensions were treated for 3 minutes with A66, TGX 221, AS252424, or the Src inhibitor PP2, followed by stimulation with HPGM. FV was used as a positive control (n = 3). Control samples contain an equal percentage of dimethyl sulfoxide. Reactions were stopped using lysis buffer and the samples were analyzed using SDS-PAGE followed by Western blot. Protein densitometry was measured and is presented as arbitrary units (a.u.) ± SD. Vehicle sample = 1 a.u. Statistical significance was calculated using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, $q < 0.05$ was considered statistically significant. 172 × 230 mm (300 × 300 DPI)

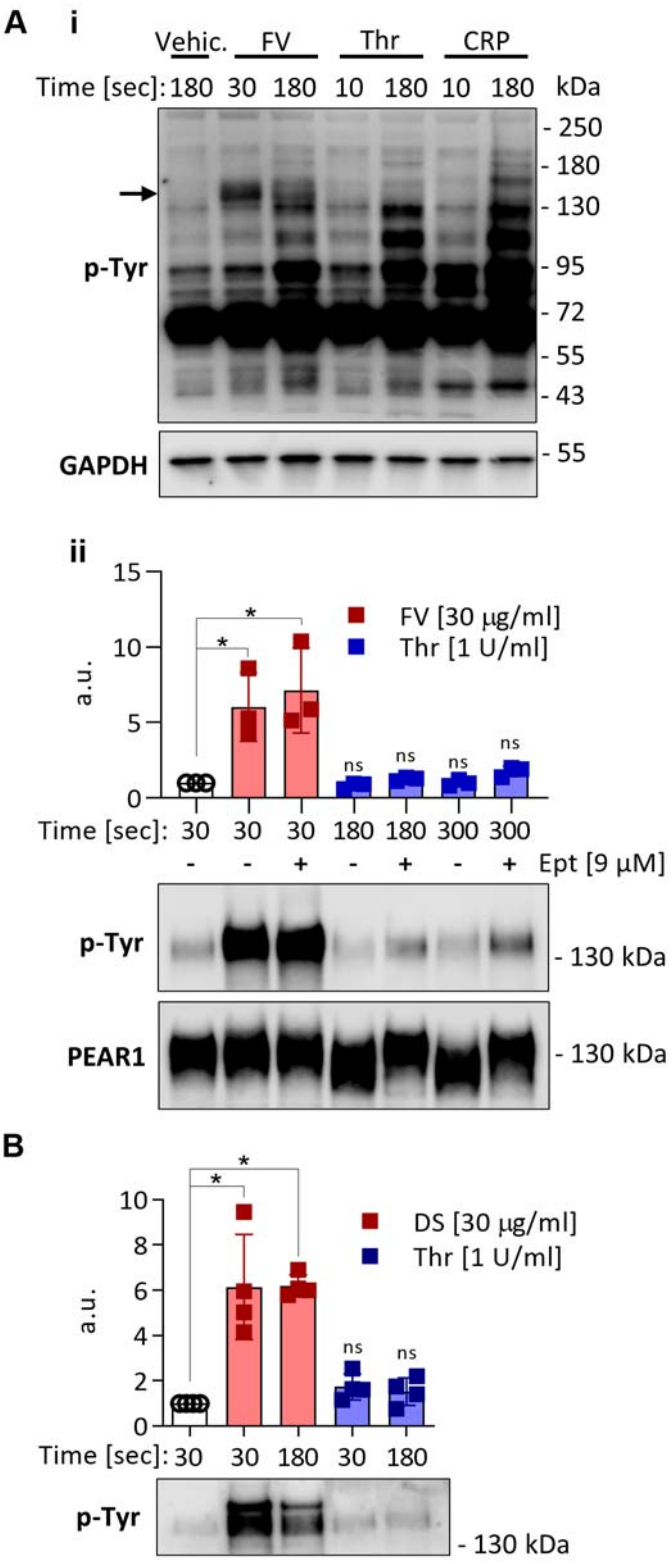

FIGURE 7 PEAR1 is phosphorylated by sulfated polysaccharides but not by classical platelet agonists. (Ai) Aliquots of human platelet suspensions were stimulated with fucoidan from *Fucus vesiculosus* (FV, 30 μg/ml), thrombin (Thr. 1 U/ml), or collagen-related peptide (CRP, 10 μg/ml). The reactions were stopped in the early phase of aggregation after shape change (30 seconds for FV and 10 seconds for thrombin and CRP), and 180 seconds using SDS-PAGE lysis buffer and samples were analyzed by SDS-PAGE followed by Western blot. A black arrow indicates the molecular weight of PEAR1 (representative image of n = 2). (Aii) Aliquots of human platelet suspensions were stimulated with FV (30 μg/ml), Thr (1 U/ml), with or without pretreatment with eptifibatide (Ept, 9 μM) for 180 seconds. Reactions were stopped at specific time points followed by immunoprecipitation for PEAR1. Samples were analyzed by SDS-PAGE followed by Western blot (n = 3). (B) Aliquots of mouse platelet suspensions were stimulated with dextran sulfate (DS, 30 μg/ml) or Thr (1 U/ml). Reactions were stopped at specific time points followed by immunoprecipitation for PEAR1. Samples were subsequently analyzed by SDS-PAGE followed by Western blot (n = 4). Vehicle sample = 1 a.u. Quantification of the optical density of the PEAR1 band presented as mean ± SD. Statistical significance was calculated using 2-tailed *t*-tests followed by Benjamini-Hochberg correction for multiplicity, $q < 0.05$ was considered statistically significant. Membranes were probed using the pan-phosphotyrosine antibody 4G10. 113 × 209 mm (300 × 300 DPI)

by an HPGM, suggesting that the endogenous ligand for PEAR1 may be a proteoglycan—possibly heparin-containing serglycin proteoglycans expressed by mast cells.

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AUTHOR CONTRIBUTIONS

C.K., A.E., J.C., A.M.A-W., C.W.S., A.S., E.M.M., and Y.S. performed experiments. C.K., A.E., and A.S. analyzed the results and made the figures. C.K., S.S., A.B., G.P., and S.P.W. designed the research. C.K., and S.P.W, wrote the paper, and A.E., J.C., C.W.S., A.S., E.M.M., S.S., A.B., G.P., and Y.S. provided feedback and intellectual input on the text. All authors read and approved the final version of the paper.

DECLARATION OF COMPETING INTEREST

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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