Platelet Endothelial Cell Adhesion Molecule-1 Inhibits Platelet Response to Thrombin and von Willebrand Factor by Regulating the Internalization of Glycoprotein Ib via AKT/Glycogen Synthase Kinase-3/Dynamin and Integrin αIIbβ3

Chris I. Jones, Tanya Sage, Leonardo A. Moraes, Sakthivel Vaiyapuri, Umara Hussain, Katherine L. Tucker, Natasha E. Barrett, Jonathan M. Gibbins

Objective—Platelet endothelial cell adhesion molecule-1 (PECAM-1) regulates platelet response to multiple agonists. How this immunoreceptor tyrosine-based inhibitory motif–containing receptor inhibits GPCR-mediated thrombin-induced activation of platelets is unknown.

Approach and Results—Here, we show that the activation of PECAM-1 inhibits fibrinogen binding to integrin αIIbβ3 and P-selectin surface expression in response to thrombin (0.1–3 U/mL) but not thrombin receptor–activating peptides SFLLRN (3×10^{-7}–1×10^{-5} mol/L) and GYPGQV (3×10^{-6}–1×10^{-4} mol/L). We hypothesized a role for PECAM-1 in reducing the tethering of thrombin to glycoprotein Ibb (GPIbb) on the platelet surface. We show that PECAM-1 signaling regulates the binding of fluorescein isothiocyanate–labeled thrombin to the platelet surface and reduces the levels of cell surface GPIbb by promoting its internalization, while concomitantly reducing the binding of platelets to von Willebrand factor under flow in vitro. PECAM-1–mediated internalization of GPIbb was reduced in the presence of both EGTA and cytochalasin D and latrunculin, but not either individually, and was reduced in mice in which tyrosines 747 and 759 of the cytoplasmic tail of β3 integrin were mutated to phenylalanine. Furthermore, PECAM-1 cross-linking led to a significant reduction in the phosphorylation of glycoprotein synthesis kinase-3β Ser^{210}, but interestingly an increase in glycoprotein syntheses kinase-3α pSer^{77}. PECAM-1–mediated internalization of GPIbb was reduced by inhibitors of dynamin (Dynasore) and glycogen synthase kinase-3 (CHIR99021), an effect that was enhanced in the presence of EGTA.

Conclusions—PECAM-1 mediates internalization of GPIbb in platelets through dual AKT/glycogen synthase kinase-3/dynamin-dependent and αIIbβ3-dependent mechanisms. These findings expand our understanding of how PECAM-1 regulates nonimmunoreceptor signaling pathways and helps to explains how PECAM-1 regulates thrombosis. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: blood platelets • glycoproteins • signal transduction • thrombin • von Willebrand factor
association with the linker for activation of T cells and the scaffolding molecule Grb-2-associated binding protein-1.12 By disturbing the linker for activation of T cells–Grb-2–associated binding protein-1–phosphatidylinositol 3-kinase 2),12 thereby inhibiting immune-like signaling downstream γC.

collagen exposure,16 thrombi formed more rapidly, were more 
tide (CRP-XL) and convulxin.10, 13, 14

the GPVI-specific ligand cross-linked collagen-related pep-
ters the internalization of GPIb by dual AKT/glycogen syn-
inhibit activation stimulated by TRAP. Here, we investigate

inhibits the activation of platelets by thrombin, it does not
prevent homophilic ligation and the activation of PECAM-
1C). Similarly, the binding and cross-linking of antibodies against GPIbα, GPVI, PAR-1, and PAR-4 had no effect on the binding of thrombin to the platelet surface (Figure 1C).

PECAM-1 Cross-Linking Reduces the Levels of GPIbα at the Cell Surface

GPIbα as part of the GPIbα/V/IX complex plays an integral role in thrombin activation. It is a high-affinity receptor for thrombin19 and acts as a cofactor for PAR cleavage.20 Furthermore, PECAM-1 plays a role in GPIb signaling, becoming tyrosine phosphorylated on VWF binding to GPIbα, and PECAM-1–deficient mice show enhanced aggregation in response to VWF.9 We hypothesized that the reduction in thrombin binding to platelets may be attributable to reduced surface expression of GPIbα after PECAM-1 activation. PECAM-1 cross-linking resulted in a significant reduction in the binding of antibodies against GPIbα and a reduction in antibodies against GPIbβ (Figure 2A and Figure II in the online-only Data Supplement). Although the level of GPIbα expressed on the surface of resting platelets varied among subjects, the reduction in GPIbα after PECAM-1 cross-linking occurred in all subjects. The action of PECAM-1 seemed to be specific for GPIb and did not cause a reduction in the binding of antibodies against PAR-1 and PAR-4, or GPVI (Figure 2A).

To confirm that this reduction was attributable to PECAM-
1, PECAM-1 on the surface of wild-type mouse platelets was cross-linked using antibodies that recognize the sixth immunoglobulin domain of PECAM-1 (AB468), resulting in PECAM-1 phosphorylation,12–14 led to a small (5%–15%) but significant inhibition in platelet fibrinogen binding and P-selectin exposure in response to thrombin across a range of concentrations (P<0.0001 and P=0.001, respectively, 2-way ANOVA; Figure 1A). Again confirming our previous findings, PECAM-1 cross-linking did not inhibit platelet response to either PAR-1– or PAR-4–activating peptides (Figure 1A). The inhibition of thrombin activation of platelets without inhibiting PAR signaling suggests a role for PECAM-1 in modulating the binding of thrombin to platelets. Consistent with this cross-linking, PECAM-1 led to a significant reduction in the binding to fluorescein isothiocyanate–labeled thrombin and a concomitant reduction of platelet activation measured by the surface exposure of P-selectin (Figure 1B and Figure I in the online-only Data Supplement).

This reduction of thrombin binding was specific to the activation of PECAM-1. To control for the potential steric inhibition caused by the binding and cross-linking of the PECAM-1 antibody, we repeated these experiments using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation, thereby providing a control for any steric effects. No reduction in thrombin binding was seen when platelets were incubated with antibodies against the first or second immunoglobulin domain of PECAM-1 (WM59; which prevent homophilic ligation and the activation of PECAM-
14) and the cross-linking antibody (Figure 1C). Similarly, the binding and cross-linking of antibodies against GPIbα, GPVI, PAR-1, and PAR-4 had no effect on the binding of thrombin to the platelet surface (Figure 1C).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Activation of PECAM-1 Inhibits the Binding of Thrombin to Human Platelets and Their Subsequent Activation

Consistent with our previous work, activating PECAM-1 by cross-linking antibodies that recognize the sixth

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>GPVI</td>
<td>glycoprotein VI</td>
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<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
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<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>PECAM-1</td>
<td>platelet endothelial cell adhesion molecule-1</td>
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<tr>
<td>PMI</td>
<td>PECAM-1–mediated internalization</td>
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<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
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the effects of steric inhibitions caused by the binding and cross-linking of the PECAM-1 antibody, we repeated these experiments using a different PECAM-1 antibody (WM59) that binds PECAM-1 but prevents its activation. No reduction in GPIbα was seen when platelets were incubated with antibodies against the first or second immunoglobulin domain of PECAM-1 (WM59) and the cross-linking antibody (Figure 2C).

**PECAM-1 Activation Inhibits Platelet Binding to VWF**
Given these findings, we hypothesized that PECAM-1 cross-linking should also reduce platelet adhesion to VWF. The number of platelets in whole blood binding to VWF (100 μg/mL) under arterial flow conditions was indeed reduced after PECAM-1 cross-linking (Figure 2D–2G). This supports the role of PECAM-1 in regulating surface GPIbα and is in line with previous reports showing enhanced aggregation in response to VWF in PECAM-1–deficient mice.6

**PECAM-1–Mediated Reduction of Platelet Surface GPIbα Is Attributable to Internalization and Not Cleavage**
We sought to determine whether the PECAM-1–mediated reduction in the surface expression of GPIbα was attributable to internalization or cleavage of the receptor. Previous reports
have demonstrated the cleavage of GPIbα by ADAM17, generating a ≈95-kDa fragment and a ≈45-kDa N-terminal fragment.21 Using a purified cobra metalloproteinase, Nk (10 μg/mL)22 as a positive control, we identified both the ≈130-kDa intact GPIbα and the ≈95-kDa and ≈45-kDa fragments by Western blotting, using antibodies against the C-terminal and N-terminal of GPIbα respectively (Figure 3A). Neither of these cleaved forms of GPIbα was observed in lysates of resting, CRP-XL–stimulated, or PECAM-1–stimulated platelets, indicating that no detectable cleavage of GPIbα occurred (Figure 3A). Furthermore, there was no evidence of any GPIbα cleavage products in these samples. Although there is a smear in the lane of the PECAM-1–stimulated platelets, this appears at the same height when probing with both the N-terminal and C-terminal antibodies and is present in the antibody control lanes. We therefore conclude that this smear is not attributable to cleavage of GPIbα.

To further test whether GPIbα was cleaved or internalized, we measured the binding of GPIbα antibodies to nonpermeabilized and permeabilized platelets. As in all previous experiments, the binding of GPIbα antibodies to nonpermeabilized platelets (ie, binding solely to GPIbα expressed on the surface of platelets) decreased after PECAM-1 stimulation. There was, however, no reduction in binding of GPIbα antibodies to permeabilized platelets (ie, when the GPIb antibody could access both external and internal pools of GPIb) indicating that there

Figure 2. Activation of platelet endothelial cell adhesion molecule-1 (PECAM-1) reduces the surface expression of glycoprotein Ibα (GPIbα) and reduces platelet binding to von Willebrand factor (vWF). A, Human platelet surface expression of GPIbα, GPIbβ, GPVI, PAR-1, PAR-4 in the presence (black) or absence (gray) of PECAM-1 (AB468) cross-linking was measured by flow cytometry (n=12 for anti-GPIbα and n=3 for all other experiments). B, To confirm that this was PECAM-1 specific, the expression of GPIbα on the surface of mouse platelets from wild-type (WT; n=4) or PECAM-1 knockout (KO; n=6) mice after the cross-linking of isotype control (gray) or PECAM-1 stimulatory antibodies (black) was measured. C, To check that this reduction in the surface of GPIbα was specific to the activation of PECAM-1 and not the binding of antibodies to the platelet surface, human platelet surface expression of GP Ibα was measured in the presence of cross-linked isotype control (gray), PECAM-1 (WM59—inhibitory; black; n=4). The difference in median fluorescent intensity (MFI) between A and C is because they were performed on different flow cytometers, an FACSCalibur and an Accuri C6, respectively. D to G, Given the importance of GPIbα in platelet tethering to vWF, we assessed the impact of PECAM-1 cross-linking on the binding of platelets to vWF under flow. D, Representative images showing human platelets binding to vWF (100 μg/mL) under flow conditions after the cross-linking of isotype control or PECAM-1 stimulatory antibodies, or, as a positive control, antibodies that block the binding of GPIbα to vWF (Haematologic Technologies Inc). The number of human platelets binding to vWF (100 μg/mL; E), the area covered by (F), and the sum fluorescence intensity of (G) these platelets after the cross-linking of isotype control (gray) or PECAM-1 (black) stimulatory antibodies (n=3). *P<0.05, **P≤0.01, ***P<0.001.
was no loss of GPIbα from the platelets (Figure 3B). Taken together, these 2 pieces of evidence suggest that PECAM-1–mediated reduction of platelet surface GPIbα is attributable to internalization and not cleavage.

**PECAM-1 Internalization of GPIbα Is Dependent on Cytoskeletal Rearrangement and Exogenous Calcium Ions**

The internalization of GPIbα is well known after platelet activation,23 and PECAM-1 has, in other cells types, been shown to be involved in non–clathrin-mediated endocytosis.24 To elucidate the mechanism by which PECAM-1–mediated internalization (PMI) of GPIbα occurs in platelets, the reduction in surface expression of GPIbα was measured after PECAM-1 activation in the presence of EGTA to chelate extracellular calcium ions and cytochalasin D and latrunculin and to prevent actin polymerization. Individually none of the compounds significantly inhibited PMI of GPIbα. A combination of EGTA and either cytochalasin D or latrunculin did, however, completely abrogate the effect of PECAM-1 activation on the surface expression of GPIbα (Figure 4A). By contrast, GPIbα internalization after stimulation of GPVI by CRP-XL was not inhibited by EGTA alone but was completely inhibited by either cytochalasin D or latrunculin (Figure 4B). This indicates that although GPVI-mediated internalization of GPIbα is entirely dependent on cytoskeletal rearrangement, the activation of PECAM-1 initiates 2 separate mechanisms, one dependent on cytoskeleton and the other dependent on exogenous divalent cations, both of which are capable of modulating the surface exposure of GPIbα independently.

Chelating extracellular calcium inhibits the activation of integrin αIIbβ3, the activation of which is known to be enhanced by PECAM-1.15,25 To establish whether integrin αIIbβ3 is involved in PMI of GPIbα, we used knock-in mice (DiYF mice) in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, preventing tyrosine phosphorylation and selectively impairing outside-in αIIbβ3 signaling.26 Internalization of GPIbα after PECAM-1 cross-linking was reduced in heterozygous mice and abolished in knock-in mice (Figure 4C). In corroboration of our findings in humans, internalization of GPIbα after stimulation with CRP-XL was unaltered in DiYF mice (Figure 4D). There was no significant difference among wild-type, heterozygous, or homozygous knock-in mice in the surface expression of either αIIbβ3 or GPVI (Figure 4E and 4F).

**PMI of GPIbα Occurs in an AKT/GSK-3/Dynamin-Dependent Manner**

PECAM-1 clustering has been reported to induce dynamin-2–dependent endocytosis in endothelial cells,26,27 whereas in platelets, dynamin has been shown to be critical to the internalization of both P2Y1 and P2Y12.28 To investigate the role of dynamin in PMI of GPIbα, platelets were incubated with Dynasore (100 μmol/L) before antibody-mediated PECAM-1
Ser9 increased β with CRP-XL, the phosphorylation of GSK-3 turns regulate its kinase activity. After stimulation of platelets reduction in the phosphorylation of GSK-3, which would in from PECAM-1 activation in endothelial cells requires both clathrin-mediated endocytosis in neurons, non–clathrin-mediated bulk endocytosis (of the type previously shown to result in the presence of EGTA (2 mmol/L), cytochalasin D (50 μmol/L), or latrunculin (8.5 μmol/L) was measured by flow cytometry, (n=5). To test the involvement of cytoskeleton in PECAM-1–mediated internalization of GPIbα, platelets from DiYF mice, in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, underwent CRP-XL stimulation followed by measurement of surface GPIbα. The surface expression of β3 αβ cross-linking. PMI of GPIbα was reduced in the presence of Dynasore and almost abolished in the presence of Dynasore and EGTA (Figure 5B and 5C). To confirm the role of GSK-3 in PMI of GPIbα after the cross-linking of PECAM-1 (Figure 5D). Interestingly, the same pattern was not repeated for GSK-3α. Ser21 phosphorylation of GSK-3α did not increase significantly in response to CRP-XL but did increase significantly after PECAM-1 cross-linking (Figure 5B and 5C). To confirm the role of GSK-3 in PMI, we used the highly selective inhibitor of both GSK-3 isoforms, CHIR99021, which inhibited the internalization of GPIbα after the cross-linking of PECAM-1 (Figure 5D). Together, these data suggest that the internalization of GPIbα after PECAM-1 stimulation occurs in a non–clathrin-, AKT/GSK-3/dynamin-dependent manner which may be similar to bulk endocytosis reported in endothelial or neuronal cells.

**Discussion**

Two of the enduring questions surrounding the role of PECAM-1 in regulating platelet response are how this ITIM-containing molecule inhibits nonimmunoreceptor signaling and how a molecule with what seems to be a moderate impact on individual signaling pathways can have such a profound effect on thrombus formation in vitro and in vivo. By investigating the disparity between the action of PECAM-1 on platelet response to thrombin and TRAP, we have identified a PECAM-1–mediated receptor internalization pathway mediated via dual AKT/GSK-3/dynamin- and αIibβ3-dependent mechanisms that result in the internalization of GPIbα, a reduction in thrombin binding to, and activation of, platelets, and reduced platelet binding to VWF. The physiological impact of PMI of GPIbα on the binding of platelets to vessel-bond VWF is uncertain because it is unlikely that PECAM-1 is activated before platelets bind to VWF and adhere to the site of vessel damage. This novel role for PECAM-1 in platelets does, however, explain how PECAM-1 inhibits GPCR-mediated activation of platelets by thrombin and why it has such a potent impact on thrombosis. By simultaneously reducing thrombin stimulation through PMI of GPIbα, and GPVI signaling through its action as an ITIM-containing receptor, PECAM-1 directly regulates 2 of the major pathways by which platelets become activated and propagate thrombus formation.

PMI of GPIbα seems to occur via a distinct mechanism that is separate from the internalization of GPIbα that follows GPVI stimulation. This was seen in the internalization of GPIbα in response to CRP-XL in PECAM-1 knockout mice and showed no significant difference between the genotypes in resting or CRP-XL–stimulated conditions (wild type [WT]; n=5; heterozygous [Het]; n=5; knock in [KI]; n=5). Asterisks indicate significant difference from isotype control or resting platelets in all cases. *P<0.05, **P<0.01, ***P<0.001. MFI indicates median fluorescent intensity.

**Figure 4.** Actin polymerization and αIibβ3 are required for platelet endothelial cell adhesion molecule-1 (PECAM-1)–mediated internalization of glycoprotein Ibα (GPIbα). Surface expression of GPIbα on human platelets after (A) PECAM-1 cross-linking or (B) stimulation with CRP-XL in the presence of EGTA (2 mmol/L), cytochalasin D (50 μmol/L), or latrunculin (8.5 μmol/L) was measured by flow cytometry. (n=5). To test the involvement of cytoskeleton in PECAM-1–mediated internalization of GPIbα, platelets from DiYF mice, in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, underwent CRP-XL stimulation followed by measurement of surface GPIbα. The surface expression of (E) αIibβ3 and (F) GPVI was also measured in these mice and showed no significant difference between the genotypes in resting or CRP-XL–stimulated conditions (wild type [WT]; n=5; heterozygous [Het]; n=5; knock in [KI]; n=5). Asterisks indicate significant difference from isotype control or resting platelets in all cases. *P<0.05, **P<0.01, ***P<0.001. MFI indicates median fluorescent intensity.

**Figure 4.** Actin polymerization and αIibβ3 are required for platelet endothelial cell adhesion molecule-1 (PECAM-1)–mediated internalization of glycoprotein Ibα (GPIbα). Surface expression of GPIbα on human platelets after (A) PECAM-1 cross-linking or (B) stimulation with CRP-XL in the presence of EGTA (2 mmol/L), cytochalasin D (50 μmol/L), or latrunculin (8.5 μmol/L) was measured by flow cytometry. (n=5). To test the involvement of cytoskeleton in PECAM-1–mediated internalization of GPIbα, platelets from DiYF mice, in which tyrosines 747 and 759 of the cytoplasmic tail of the β3 integrin were mutated to phenylalanine, underwent CRP-XL stimulation followed by measurement of surface GPIbα. The surface expression of (E) αIibβ3 and (F) GPVI was also measured in these mice and showed no significant difference between the genotypes in resting or CRP-XL–stimulated conditions (wild type [WT]; n=5; heterozygous [Het]; n=5; knock in [KI]; n=5). Asterisks indicate significant difference from isotype control or resting platelets in all cases. *P<0.05, **P<0.01, ***P<0.001. MFI indicates median fluorescent intensity.
mice and the ability of cytochalasin D and latrunculin alone to inhibit CRP-XL–mediated internalization of GPIbβ, but not PMI. It is likely, however, that in vivo these 2 mechanisms work in concert, particularly because PECAM-1 becomes phosphorylated after GPVI stimulation and because PECAM-1 and GPVI have opposing effects on AKT and hence GSK-3β phosphorylation. Why such opposing signaling events result in such similar outcomes is not yet known. The importance of PMI during thrombus formation may in part come from the activation of PECAM-1 by homophilic ligation, which only occurs during close platelet–platelet contact. Under these circumstances, stimulation of receptor internalization or endocytosis by PECAM-1 at the site of contact may be a mechanism for recycling receptors that are not occupied or bound into focal adhesion complexes. Trafficking these receptors away from the site of contact to areas of the platelet that are not in contact with other cells provides an attractive mechanism by which to maximize the efficiency of platelet response to multiple simultaneous stimuli and orchestrate thrombus growth.

The mechanism by which integrin β3 regulates PMI is as yet unknown. Its effect, however, seems to be critical. One explanation for this perhaps lies with filamin. Filamin binds to GPIbα in resting platelets anchoring it to the cytoskeleton,14 helping to maintain the structure and integrity of the plasma membrane after platelet tethering to VWF under high shear.15 On stimulation, filamin becomes detached from the cytoplasmic tail GPIbα, allowing its translocation.16 By contrast, filamin binds to αIIbβ3 after platelet activation. One tempting hypothesis is that PECAM-1 plays a role, either directly or indirectly, in the decoupling of filamin from GPIbα and its subsequent binding to integrin β3. We have as yet been unable to confirm or refute this hypothesis, but it will be the focus of future work.

Of the 2 isoforms of GSK-3, it is the β-form that is thought to be predominant in platelets.17 Proteomic analysis has, however, identified that the copy number per platelet for each isoform is similar, 1300 for GSK-3β and 1000 for GSK-3α.18 Both isoforms are constitutively active in resting platelets but are inhibited by phosphorylation of Ser21 (GSK-3α) or Ser9 (GSK-3β) by protein kinase c or AKT on platelet stimulation.19–21 It is thought that GSK-3β acts as a negative regulator of platelets; GSK-3 inhibitors increase platelet responses, GSK-3β−/− mice display increased aggregation and thrombus formation, and platelets from mice in which GSK-3α Ser21 and GSK-3β Ser9 have been mutated to Ala show reduced response.17,39 The reduction on GSK-3β pSer9 after PECAM-1 cross-linking fits with this understanding of platelet GSK-3. PECAM-1, which has a well-established inhibitory effect on platelet signaling, reduces AKT phosphorylation and therefore reduces the phosphorylation and inhibition of GSK-3β, thereby enhancing its inhibitory effect, some of which may be mediated through dynamin and translocation of surface receptors, as we have shown to be the case with GPIbβ. The increase in GSK-3α phosphorylation after PECAM-1 cross-linking does not, however, fit with our current understanding. Part of the reason for this is that all previous work on GSK-3β mice has focused either on GSK-3β−/− alone, as in the case of the GSK-3β−/− mice, or has assumed that both isoforms work in a similar way and have therefore inhibited both isoforms. In the case of dual inhibition, the effect of GSK-3α may be masked by GSK-3β making any interpretation of the physiological relevance of GSK-3α impossible. Although there is 85% similarity in the amino acid sequence between the isoforms and 98% homology in the kinase domain,40 there is growing evidence
that the 2 isoforms of GSK-3 have distinct roles.41–43 Why the activation of PECAM-1 has such contrasting effects on the 2 GSK-3 isoforms and what significance GSK-3α/β phosphorylation has in PECAM-1 signaling are as yet unknown.

The inhibition of platelet activation by PECAM-1 is well known, but the mechanisms by which this ITIM-containing receptor inhibits platelet response to thrombin were not. Here, we have shown that the activation of PECAM-1 results in the internalization of GPⅠbα/dynamin- and c Tailβ3-dependent mechanisms, which result in the reduction of platelet binding of, and hence response to, thrombin and reduced platelet binding to VWF. Furthermore, we have for the first time shown the differential regulation of GSK-3α/β by PECAM-1 in platelets. This novel role for PECAM-1 expands our understanding of how this promiscuous molecule regulates multiple diverse signaling pathways and why it has such a potent impact on thrombosis. It may also suggest a role for PECAM-1 in regulating conditions where the generation of thrombin is central to pathology.

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Disclosures
None.

References
We show 2 new facets of platelet endothelial cell adhesion molecule-1 (PECAM-1) biology in platelets: (1) that PECAM-1 mediates the internalization of glycoprotein Ibα leading to a reduction in the activation of platelets by thrombin and a reduction in their binding to von Willebrand factor and (2) that PECAM-1 differentially regulates the phosphorylation of the 2 isoforms of glycogen synthase kinase-3. In so doing, this article answers several outstanding questions concerning the regulation of platelets by PECAM-1: how this immunoreceptor tyrosine-based inhibitory motif–containing molecule inhibits nonimmunoreceptor signaling and importantly, how PECAM-1 has such a profound effect on thrombus formation in vitro and in vivo.
Platelet Endothelial Cell Adhesion Molecule-1 Inhibits Platelet Response to Thrombin and von Willebrand Factor by Regulating the Internalization of Glycoprotein Ib via AKT/Glycogen Synthase Kinase-3/Dynamin and Integrin αIIbβ3

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Materials and methods

Reagents

CRP-XL (monomeric sequence GCI\[GPO\]_{10}GCiG) was prepared as described previously,\(^1\) thrombin was from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). FITC-labelled thrombin was labelled in house. Thrombin receptor activation peptides (TRAP, PAR-1 – SFLLRN, PAR-4 – GYPGQV) were from Bachem (St. Helens, UK). Gly-pro-arg-pro peptide (GPRP) (Sigma) was added where thrombin was present to prevent clotting, PECAM-1 monoclonal antibody (mAb) AB468 and appropriate isotype control antibodies (Millipore, Watford, UK) were dialysed to remove azide, as was PECAM-1 antibody (WM59) (Serotec, UK). PE/Cy5-anti-CD62P, FITC-anti-GPIbα and FITC-anti-GPIbβ were from BD Biosciences (Oxford, UK), and FITC-anti-fibrinogen was from Dako Ltd (Ely, UK). GPVI (HY101, M.L. Kahn, University of Pennsylvania), PAR1 (Life technologies, Paisley, UK) and PAR4 antibodies (Abcam, Cambridge, UK), respectively, were labelled using a Zenon Alexa Fluor 647 labelling kit (Life Technologies) prior to use. Antibodies against the C- or N-terminal of GPIbα came from Santa Cruz (Dallas, USA). Antibodies recognising mouse GPIbα, GPVI, and integrin αIIbβ3 came from Emfret analytics (Eibelstadt, Germany). VWF was from Haematologic Technologies Inc (Vermont, USA). The purified cobratoxin metalloproteinase, Nk, was a gift from Prof. Rob Andrews, Monash University, Australia.\(^2\) Cytochalasin D, Latrunculin and CHIR99021 all came from Millipore and Dynasore came from Sigma. GSK-3α/β and pSer\(^{21/9}\) GS-3a/β antibodies were from Cell Signalling Technologies (New England Biolabs, Hitchin, UK). Hepes buffered saline (HBS; 0.14M NaCl, 5mM KCl, 1mM MgSO4, 10mM HEPES (sodium salt), pH7.4) was used for all dilutions. PECAM-1 knockout mice were provided by Prof. T. Mak (University of Toronto, Canada) and the DiYF mice were generated in the laboratory of Dr. David R. Phillips (Portola Pharmaceuticals Inc. San Francisco, USA). All protocols involving the use of animals were approved by the University of Reading Local Ethical Review Panel and authorized by a Home Office license.

Phlebotomy

Fresh blood was taken via standardised phlebotomy into vacuette tubes (Greiner bio-one, Stonehouse, UK) containing 3.2% sodium citrate from drug free donors. Informed consent from human subjects was obtained and procedures approved by the University of Reading Research Ethics Committee.

Mouse platelet preparation

Blood from PECAM-1 Knockout or DiYF knockin or sibling matched wild-type or heterozygous mice was taken immediately after sacrifice by cardiac puncture into 4% citrate. All experiments were performed blind and genotyping was performed subsequently as previously described.\(^3,4\)

PECAM-1 cross-linking

Where applicable, PECAM-1 cross-linking was performed prior to platelet stimulation as follows. Human platelets, in whole blood or platelet rich plasma (PRP), were incubated with anti-PECAM-1 (AB468) or isotype control antibodies (10µg/ml) for 10 minutes followed by an excess of cross-linking antibody, goat-anti-mouse IgG antibody (20µg/ml, Sigma) for 10 minutes. The same procedure was performed for mouse platelets using anti-PECAM-1 antibody (M-185) or isotype-matched control IgG (both Santa Cruz, Dallas, USA) and goat-anti-rabbit cross-linking antibody (Sigma).

Flow cytometry

Citrated whole blood or PRP was diluted 1:10 (or 1:10,000 when using Dynasore, to reduce the inhibition of Dynasore by plasma proteins) in HBS. Where indicated platelets were incubated for 30 min and 37°C, following which PECAM-1 or control antibody cross-linking was performed as appropriate. Samples were then incubated for 20 minutes at room temperature either with CRP-XL, thrombin, or TRAP, together with FITC-anti-fibrinogen and
PE/Cy5-anti-CD62P antibodies, or surface receptor specific antibodies as indicated. Reactions were stopped by 100 fold dilution in 0.2% formyl saline.

To measure total GPIbα (internal stores as well as that expressed on the surface) citrated PRP was diluted 1:10 in HBS and PECAM-1 or isotype control antibody cross-linking was performed. Samples were fixed with 2% formyl saline, permeabilised using BD Phosflow perm buffer III (BD Bioscience, Oxford, UK) washed, resuspended in HBS, and incubated with FITC-anti-GPIbα antibodies for 20 minutes at room temperature.

Data were acquired on either a FACSCalibur or an Accuri C6 flow cytometer (BD) and were recorded as percentage of cells positive or median fluorescence intensity (MFI).

**Platelet adhesion to VWF under flow.**

DIOC₆ (Sigma Aldrich, UK) labelled human citrated blood, with or without PECAM-1 cross-linking, was perfused over VWF (100μg/ml) coated Vena8 BioChip (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm² for 10 minutes at 37°C. To prevent platelet-platelet binding, Integrelin (2μg/ml) was included in all samples. The channels were then washed with Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na₂HPO₄, 12mM NaHCO₃, 20mM HEPES, 5mM glucose and 1mM MgCl₂, pH 7.3) for 60 seconds and platelet adhesion was measured in six randomly selected fields of view per channel, using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The number of adherent platelets, the area covered and sum fluorescence intensity were calculated using Slidebook 5 software (Intelligent Imaging Innovations, USA).

**Platelet preparation and stimulation for Western blot analysis**

Washed platelets were prepared by differential centrifugation. Platelets were re-suspended to a density of 8x10⁸ per mL, in Tyrodes-HEPES buffer and rested for 30 minutes at 30°C before stimulation. Washed, rested platelets were either not activated or stimulated with CRP-XL following PECAM-1 cross-linking. The reaction was terminated by the addition of an equivalent volume of ice-cold lysis buffer (20mM Tris, 300mM NaCl, 10mM EDTA, 2% (v/v) Nonidet P40, 1mmol/L phenylmethylsulfonyl fluoride, 1μg/mL pepstatin A, 10μg/mL aprotinin, 10μg/mL leupeptin, and 2mmol/L sodium orthovanadate, pH 7.3). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting onto polyvinylidene difluoride (PVDF) membrane were performed using standard techniques. Membranes blocked using 5% (w/v) bovine serum albumin in Tris-buffered saline-Tween (BSA/TBS-T) (20mM Tris, 0.14M NaCl, 0.01% Tween, pH 7.6). Primary antibodies were diluted in 2% (w/v) BSA/TBS-T and incubated over night at 4°C. Species-specific fluorescently-labelled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were added for 1 hour at room temperature. Blots were visualised using a Typhoon Trio fluorescence scanner (GE healthcare, Buckinghamshire, UK) and analysed using Image Quant TL (GE).

**Statistics**

Data are presented as mean±standard deviation of the mean (SD). Statistical analyses were performed using PRISM 5 GRAPHPAD software (GraphPad Software Inc, La Jolla, CA, USA). Data were compared using a Student’s T-Test, One-way ANOVA or Two-way ANOVA and Bonferroni post-test analysis as appropriate.

**References**

1. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: Collagen
tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochemical Journal*. 1995;306:337-344


Supplement Material

Supplementary Figure I – Representative flow cytometry histograms showing the binding of FITC-labelled thrombin to platelets in the presence (black dotted line) or absence (grey solid line) of PECAM-1 (AB468) crosslinking.
Supplementary Figure II – Representative flow cytometry histograms showing the binding of FITC-labelled GPIbα antibodies to platelets in the presence (black dotted line) or absence (grey solid line) of PECAM-1 (AB468) crosslinking, on the two different flow cytometers used in this study.