GPVI Potentiation of Platelet Activation by Thrombin and Adhesion Molecules Independent of Src Kinases and Syk

Sascha C. Hughan, Craig E. Hughes, Owen J.T. McCarty, Edina Schweighoffer, Izoumroud Soultanova, Jerry Ware, Victor L.J. Tybulewicz, Steve P. Watson

Objective—The present study investigates the role of Src and Syk tyrosine kinases in signaling by G-protein coupled and platelet adhesion receptors.

Methods and Results—Using Syk−/− platelets or the Src kinase inhibitor PP2, we demonstrate a critical role for Src and Syk kinases in mediating lamellipodia formation on VWF, collagen, CRP, fibrinogen, and fibronectin. In all cases, the spreading defect was overcome by addition of thrombin. Conversely, platelet aggregation and αIIbβ3 activation induced by thrombin was similar to controls, arguing against a functional role for Src and Syk in αIIbβ3 activation. Unexpectedly, CRP potentiates integrin αIIbβ3 activation and platelet aggregation induced by subthreshold concentrations of thrombin in Syk−/− platelets or in the presence of the Src kinase inhibitor PP2. Potentiation in the presence of PP2 was lost in the absence of FcRγ chain or GPVI confirming that it was mediated through the immunoglobulin receptor. Further delineation of this PP2-resistant synergy revealed that PAR4 could trigger the enhanced response in combination with CRP.

Conclusions—We show that Syk is critical for lamellipodia formation on a range of immobilized proteins but that this can be overcome by addition of thrombin. Further, we reveal a novel role for GPVI in supporting thrombin-induced activation, independent of Syk and Src kinases. (Arterioscler Thromb Vasc Biol. 2007;27:422-429.)

Key Words: platelets ■ GPVI signaling ■ Src kinases ■ Syk ■ thrombin

The tyrosine kinase Syk contains two SH2 domains and a C-terminal kinase domain and is expressed in cells of the hematopoietic lineage. The absence of Syk results in the abrogated development of B cells, functional defects in immune receptors, and perinatal lethality.1,2 Interestingly, mice deficient in Syk, or the Syk substrate SLP-76, also show a failure to separate emerging blood vessels from those of the emerging lymphatic system, thereby triggering embryonic hemorrhaging.3

In platelets, Syk is recognized for its role in mediating signaling of the collagen receptor complex, GPVI-FcRγ chain, by binding to the immunoreceptor tyrosine-based activation motif (ITAM) within the intracellular portion of the FcRγ chain. Signaling by this receptor complex parallels that of other SYK kinases: A common tyrosine receptor complex that by immune receptors, such as the T- and B-cell antigen receptor and Fc receptors. Reviewed by Watson et al4,5 the two Src kinases Fyn and Lyn, are believed to initially phosphorylate the ITAM of the FcRγ chain. Syk then binds to the dually phosphorylated tyrosine residues via its SH2 domains, resulting in autophosphorylation of Syk and sequential activation and phosphorylation of several adapter proteins, including LAT, SLP-76, and a number of signaling proteins, including Tec kinases and PI-3 kinase. This ultimately leads to phosphorylation of phospholipase C (PLC) γ isoforms and a rise in intracellular calcium.

Syk is also implicated in signaling by other classes of adhesion and G protein–coupled receptors in platelets. For example, Syk plays a critical role in signaling by the major platelet integrin, αIIbβ3. This has been shown by a series of elegant studies in Chinese hamster ovary (CHO) cell models for integrin αIIbβ3 and biochemical analyses of human and murine platelets.6–8 Syk undergoes tyrosine phosphorylation and activation during adhesion of αIIbβ3-expressing CHO cells to the matrix protein fibrinogen, implicating Syk in the proximal signaling events of αIIbβ3.6 Phosphotyrosine-independent binding of Syk to the cytoplasmic tail of the β3 subunit of αIIbβ3 initiates a downstream cascade that leads to tyrosine phosphorylation of Vav1, Vav3, SLP-76, ADAP, and PLCγ2.7,8

More recently, the interaction of GPIb/V/IX with VWF in the presence of the snake venom toxin botrocetin10 and the interaction of collagen with integrin α2β11 have been shown to trigger Syk autophosphorylation and kinase activity. In both cases, the interactions of Syk with GPIb/V/IX or α2β1...
are implicated in mediating spreading, but definitive evidence for this is not available. Recent studies show that Syk plays a significant role in mediating signaling downstream of the novel platelet receptor CLEC-2, after platelet stimulation by the snake venom toxin, rhodocytin. 12 Lastly, Syk becomes activated and phosphorylated in platelets by G protein–coupled receptors agonists, including thrombin, ADP, and TxA2.13,14 However, despite all that is recognized on the phosphorylation and activation of Syk, the functional role for Syk in signaling by many of these receptors is unclear.

The present study was designed to determine more clearly the role of Syk in signaling by G protein–coupled receptors and to extend our understanding of the contribution of Syk to signaling by platelet adhesion receptors, including integrin \( \alpha_{IIb}\beta_{3} \), GPIb/V/IX, and GPVI. Using platelets obtained from radiation chimeric mice transplanted with wild-type or Syk \(-/-\) embryonic liver cells, we demonstrate a critical role for Syk in mediating spreading, but not adhesion, on a wide variety of extracellular matrix proteins, although it does not appear to play a role in supporting platelet activation by thrombin. Unexpectedly, we reveal a novel synergy between GPVI and thrombin that exists in the absence of Src kinases and Syk. Given the central role of Syk in numerous signaling cascades, it is increasingly viewed as a target for the development of novel antithrombotic therapeutics. This study furthers this notion and may suggest that Syk presents a worthwhile antithrombotic target when used concurrently with antithrombin therapies.

Materials and Methods

For detailed descriptions of the materials and methods, please see supplemental material (available online at http://atvb.ahajournals.org).

Mouse Strains

Syk \(-/-\) and wild-type radiation chimeras were generated as described.15 Hemopoietic progenitor cells were obtained from fetal liver from wild-type B10.D2 or Syk-deficient 15 to 16 day embryos, and injected intravenously into lethally-irradiated wild-type mice. FcR\( y \) chain \(-/-\) mice and GPVI \(-/-\) mice have been described previously.16,17

Preparation of Washed Platelets

Murine platelets were obtained and prepared as described,18 in accordance with Animals (Scientific Procedures) Act 1986, United Kingdom. Human platelets were prepared as described,19 and all donors provided informed written consent, according to the Declaration of Helsinki.

Platelet Aggregation and Stimulation Studies

Murine platelets were stimulated with thrombin or collagen related peptide (CRP), in the presence/absence of an integrin \( \alpha_{IIb}\beta_{3} \) inhibitor or PP2, and aggregation determined. Platelets were lysed and tyrosine phosphorylation determined after protein separation by SDS-PAGE and Western blotting, followed by immunoblotting with an antiphosphotyrosine antibody. Human platelets were aggregated with thrombin, protease activation receptors (PAR) activation peptides (TRAP and PAR4-AP), and/or CRP, in the presence/absence of vehicle, PP2 and/or ADP receptor antagonists plus the TxA2 inhibitor, indomethacin.

Platelet Spreading and Imaging

Static adhesion assays were performed as described11,20 with the additional incubation of some samples with thrombin, ADP, or PP2.

Adherent platelets were fixed and imaged using differential interference contrast (DIC) microscopy.21

Measurement of Integrin \( \alpha_{IIb}\beta_{3} \) Activation

Murine platelets were stimulated with thrombin, CRP, ADP, thrombin/CRP, or ADP/CRP. The PE-conjugated JON/A antibody or Oregon-green fibrinogen (OG-FGN) was added and allowed to incubate. The reaction was terminated and samples were read in FL2/FL1. Control samples containing resting platelets labeled with JON/A or PE-labeled Rat IgG were tested. Human platelets were stimulated with thrombin, CRP, or thrombin/CRP, plus/minus PP2, before addition of OG-FGN. Samples were read using a FACs machine.

Statistical Analysis

Statistical significance of results was determined using one-way ANOVA, using the Prism Software program.

Results

The Requirement for Syk in Mouse Platelet Spreading Is Bypassed by Thrombin

We examined the role for Syk in platelet spreading on a number of matrix proteins under static conditions both in the absence and presence of G protein receptor activation. Platelets obtained from radiation chimeric mice reconstituted with wild-type or Syk \(-/-\) fetal liver cells were allowed to adhere and spread on immobilized Von Willebrand Factor (VWF), collagen, CRP, fibronectin, or fibrinogen. As demonstrated in Figure 1A, wild-type platelets extend filopodia and lamellipodia when plated on all adhesive surfaces. In contrast,
Syk−/− platelets were unable to generate lamellipodia on any of the matrices tested, although there was evidence of filopodia formation (Figure 1A, arrow heads). The inclusion of the G protein agonist thrombin restored maximal platelet spreading in Syk−/− murine platelets on all surfaces, as measured by the percentage of platelets with lamellipodia (Figure 1B and supplemental Table I). Spreading was also restored by ADP but to a limited extent (supplemental Table I). These data demonstrate that the defect in platelet spreading observed in the absence of Syk is overcome through activation of G protein coupled receptors.

It is widely accepted that Syk phosphorylation occurs distally to Src kinase activation in platelet activation pathways. Therefore, to examine whether the compensatory mechanism triggered by G protein coupled receptor agonists occurred proximally or distally to Src kinases, we examined whether human platelet spreading on fibrinogen, VWF, and collagen in the presence of a Src kinase inhibitor (PP2) could be restored by thrombin. PP2-treatment blocked lamellipodia formation (see supplemental data) and reduced adhesion on all matrices tested. However, thrombin was able to fully compensate for blockade of Src kinases with respect to all matrices tested. However, thrombin was able to fully compensate for blockade of Src kinases with respect to lamellipodia formation, with 96.0 ± 1.6%, 97.2 ± 0.6%, and 92.5 ± 2.3% of PP2-treated platelets undergoing spreading on VWF, fibrinogen, and collagen, respectively (supplemental Table II). As observed for the Syk−/− platelets, spreading of PP2-treated platelets was also restored by ADP, but to a limited extent (supplemental Table II).

Thrombin Stimulates Tyrosine Phosphorylation Downstream of Src Kinases

Having shown that thrombin and ADP could compensate for the absence of Syk and Src kinases in platelet spreading, we examined the underlying signaling processes. First we determined the contribution of Syk to general signaling triggered by thrombin-induced activation of platelets. There was no marked alteration in aggregation to a range of concentrations of thrombin in Syk−/− murine platelets (Figure 2A). There was, however, a partial reduction in thrombin-mediated whole cell tyrosine phosphorylation, measured under nonaggregating conditions, in the absence of Syk, in contrast to the complete abolition in the presence of the Src kinase inhibitor, PP2 (Figure 2B). These data demonstrate that although Syk contributes to thrombin-induced tyrosine phosphorylation in platelets, it is neither required for aggregation nor for thrombin-induced platelet spreading. These findings were also true for inhibition of Src kinases. We observed that PP2 had no deleterious effect on thrombin-induced aggregation of human platelets, despite inhibition of tyrosine phosphorylation (Figure 2B through 2C). This suggested that Src kinases, like Syk, contribute to thrombin-induced tyrosine phosphorylation in platelets but are not required for aggregation.

Potentiation of Integrin αIIBβ3 Inside-Out Signaling in Syk−/− Murine Platelets

Having demonstrated that Syk is not required for thrombin-induced platelet aggregation, we chose to use a more sensitive measure of integrin αIIBβ3 activation in platelets, namely the αIIBβ3 activation-specific antibody, JON/A. Under the conditions used, JON/A binds specifically to the activated conformation of αIIBβ3. As shown in Figure 3, platelets stimulated with increasing thrombin concentrations showed a concomitant increase in JON/A binding, which was not altered in the absence of Syk. In contrast, the response to CRP was completely blocked in Syk−/− platelets. Strikingly, however, a
concentration of thrombin (0.01 U/mL) that was unable to induce significant activation of $\alpha_{IIb}\beta_3$, was able to restore JON/A binding observed following CRP stimulation in Syk$^{-/-}$ platelets (Figure 3), thereby revealing a novel and unexpected synergy in integrin activation.

**CRP Potentiates Thrombin Aggregation of Human Platelets Through Src Kinase-Dependent and Independent Pathways**

To determine the dependency of the observed synergy between GPVI and thrombin on Src kinases, we first examined the ability of control human platelets to undergo aggregation to subthreshold concentrations of CRP and thrombin, either alone or in combination. As demonstrated in Figure 4A, we observed a significant level of synergy between CRP and thrombin under these assay conditions. When we treated platelets with PP2, the observed synergy remained, although to a lesser extent (Figure 4B), demonstrating a novel Src-kinase independent synergy between CRP and thrombin.

The contribution of secondary mediators, ADP and thromboxane A2 (TxA2), to the PP2-independent synergy was investigated. PP2-treated platelets were incubated with two ADP receptor antagonists, ARC69931MX and MRS2179, in combination with indomethacin to inhibit Thromboxane A2 (TxA2), before aggregations with subthreshold concentrations of thrombin and CRP, alone or in combination. A combination of the inhibitors caused a marked reduction in aggregation to thrombin and CRP from 61.8±13.7% (+PP2) to 43.4±5.5% (+PP2/ARC/MRS/Indo; Figure 4B). The partial nature of the inhibition indicates the existence of an ADP/TxA2- and Src kinase-independent signaling process occurring in response to thrombin/CRP.

To extend these observations, we examined Oregon green fibrinogen (OG-FGN) binding by flow cytometry in human platelets. OG-FGN was used in these studies instead of JON/A as a marker for integrin inside-out activation, as the latter does not recognize human $\alpha_{IIb}\beta_3$. We confirmed that OG-FGN binding to activated $\alpha_{IIb}\beta_3$ was specific, as it was blocked by the integrin inhibitor, 7E3 (data not shown). The results demonstrated that in control platelets, thrombin and CRP in combination produced no additional effect on integrin inside-out activation leading to OG-FGN binding (Figure 4C). Conversely, in the presence of PP2, the com-

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**Figure 3.** CRP potentiates thrombin-induced integrin $\alpha_{IIb}\beta_3$ activation in Syk$^{-/-}$ platelets. Platelet suspensions from wild-type (black) or Syk$^{-/-}$ (white) mice were allowed to rest in the presence of vehicle (Resting) or the PE-labeled Rat IgG negative control (2Ab alone). Alternatively, platelets were stimulated with thrombin (0.01 to 0.1U/mL), CRP (10 μg/mL), or CRP/thrombin (10 μg/mL and 0.01U/mL, respectively). Suspensions were incubated with JON/A antibody and samples read in FL2. Results show integrin $\alpha_{IIb}\beta_3$ activation, measured in arbitrary fluorescence units (mean±SEM); 3 mice per genotype.

**Figure 4.** Novel synergy between CRP and thrombin involves Src kinases and secondary mediators. A and B, Human platelets were aggregated with thrombin (0.015 to 0.02U/mL), CRP (0.5 to 1.5 μg/mL), thrombin/CRP in combination, in the absence or presence of PP2 (10 μmol/L) and/or ARC69931MX (10 μmol/L), MRS2179 (100 μmol/L), and indomethacin (10 μmol/L). Platelet aggregation was plotted as the maximal extent of aggregation. C, Washed human platelets were treated with DMSO (black bars), PP2 (gray bars), or ARC69931MX, MRS2176, Indomethacin, and PP2 (AMIP) (white bars) before stimulation with thrombin (0.04U/mL), CRP (2.0 μg/mL), or CRP/thrombin (2.0 μg/mL and 0.04U/mL, respectively). Platelets bound OG-FGN for 5 minutes before samples were read using FL1. Results are arbitrary units (mean±SEM), n=3; *P<0.05; **P<0.001.
bined effect of thrombin and CRP on OG-FGN binding was synergistic, when compared with the levels triggered by either thrombin/PP2 \((P<0.05)\) or CRP/PP2 \((P<0.001)\) alone. The synergism observed in the presence of PP2 was completely lost in the presence of ADP receptor antagonists and indomethacin \((P<0.001)\), thereby revealing a critical role for secondary mediators in this response. It should be noted that we observed a reduction in thrombin-induced OG-FGN binding in the presence of PP2 (Figure 4C), which was not apparent when aggregation was measured turbidometrically (Figure 4B). This inhibition may reflect the use of a very low concentration of thrombin and the absence of integrin \(\alpha_{IIb}\beta_3\) outside-in signaling.

**CRP and Thrombin Potentiate Integrin \(\alpha_{IIb}\beta_3\) Activation Through GPVI**

To confirm that the PP2-independent synergy was mediated through CRP acting on GPVI, we investigated responses to CRP and thrombin in mice deficient in GPVI\(^{16}\) or the FcR\(\gamma\) chain.\(^{16}\) Fluorescence-activated-cell sorter analysis of OG-FGN (Figure 5) binding of age and/or litter-matched murine platelets treated with PP2 demonstrated synergistic activation of integrin \(\alpha_{IIb}\beta_3\) by CRP and thrombin (Figure 5A and 5B). Importantly, this Src-kinase independent synergy was completely lost in the GPVI\(^{-/-}\) (Figure 5A) and FcR\(\gamma\) chain\(^{-/-}\) (Figure 5B) platelets, thereby confirming that the action of CRP is mediated through GPVI. We also confirmed that OG-FGN binding to activated integrin \(\alpha_{IIb}\beta_3\) was specific, as it was blocked by the presence of EGTA/Mg\(^{2+}\) (Figure 5B).

**PAR4 Synergizes With CRP to Promote Integrin \(\alpha_{IIb}\beta_3\) Activation in Human Platelets Independent of Src Kinases**

To examine the potential mechanism behind the thrombin-induced synergy with CRP, we determined the involvement of two main thrombin receptors, protease activated receptor (PAR1) and PAR4, in this response in human platelets. Using specific PAR1 and PAR4 agonist peptides, SLLRN (TRAP) and AYPGKF (PAR4AP), respectively, we first determined that PAR1 and PAR4 could reproducibly synergize with CRP to promote maximal platelet aggregation (data not shown). Interestingly, when platelets were treated with PP2, the synergistic response evoked by subthreshold PAR4AP/CRP addition could be maintained (Figure 6B and 6C); however, the aggregation response to subthreshold TRAP/CRP was significantly reduced (Figure 6A and 6C). These data therefore suggested that PAR4, and not PAR1, is important for mediating the Src kinase-independent signaling by GPVI in combination with thrombin.

**Discussion**

The studies presented here provide further insight into the role of Syk and Src tyrosine kinases in platelet signaling cascades emanating from the adhesion receptors GPVI, GPIb/\(\alpha\)/\(\gamma\), and integrin \(\alpha_{IIb}\beta_3\). Specifically, the data confirm and extend previous observations\(^{8}\) for an important role for Syk in mediating lamellipodia formation on fibrinogen and collagen. In addition, we have now characterized a role for Syk in lamellipodia formation on VWF, CRP, and fibronectin, and have shown that the spreading defect can be overcome through the addition of G protein receptor agonists, thrombin, and, to a lesser extent, ADP. Significantly, these studies also reveal that the GPVI-agonist CRP potentiates signaling by G protein–coupled receptors, notably thrombin through PAR4, independent of Syk and Src kinases. This novel finding was evident in studies measuring inside-out activation of \(\alpha_{IIb}\beta_3\) and in platelet aggregation studies. Moreover, there appears to be a critical contribution of ADP and TxA\(_2\) to this synergy.

A major finding from the present study is the observation that subthreshold concentrations of thrombin synergize with CRP in promoting integrin \(\alpha_{IIb}\beta_3\) activation in the absence of...
Syk and Src kinases. The observed synergy between thrombin and collagen is not unique, as a number of other studies have made similar findings; however, our study is the first to show a synergistic effect in the absence of Syk or Src kinases. Further studies on GPVI and FcR γ-chain–deficient platelets demonstrate that the Src kinase- and Syk-independent signals are mediated downstream of the collagen receptor GPVI. Moreover, it would appear that secondary mediators, ADP and TxA₂, play a significant role in this event.

Further delineation of the synergy suggested that PAR4, one of two main thrombin receptors in human and murine platelets, mediates the effect independent of Src kinases. Although it is recognized that PAR1 and PAR4 both contribute to thrombin signaling in human platelets, they have been shown to elicit their effects through different temporal profiles linked to calcium release and receptor internalization.

It would therefore appear that the characteristics defining PAR4 signaling, notably slower internalization and a prolonged calcium release, synergize with GPVI signaling to promote integrin activation. This finding is consistent with Graff et al., who established that PAR4 acts synergistically with collagen to promote CD62 and Pac-1 expression, in a manner that depended on integrin α₄β₁, the P2Y₁₂ ADP receptor and TxA₂. In addition, Keuren et al. claimed that PAR1 and collagen could synergize to promote procoagulant platelet activity. Taken together, these data suggest that both PARs can participate in synergistic signaling with collagen. Our data has extended this by first demonstrating that GPVI alone can synergize with thrombin, as compared with studies using collagen which activates platelet receptors additional to GPVI. Second, we suggest that PAR4 and GPVI synergize in vitro, independently of Src kinases, which may affect thrombosis growth in vivo.

One possible mechanism for the Src kinase- and Syk-independent signaling downstream of GPVI and thrombin relates to the recognized interactions GPVI has with other signaling and adapter molecules, most notably calmodulin. Calmodulin is a calcium-binding protein that is involved in a wide variety of cellular processes, including cellular growth, movement, and proliferation. Recent studies in platelets have shown that calmodulin interacts with the GPIb/V/IX complex and a basic region within the cytoplasmic tail of GPVI, and dissociates from the latter following collagen-, CRP-, but not ionophore-mediated platelet activation. In addition, calmodulin dissociation from GPVI follows GPVI-mediated tyrosine phosphorylation and is blocked by the Src kinase inhibitor, PP1, suggesting that there is an activation-dependent mechanism of dissociation.

Although the role for calmodulin in platelets remain unclear at this stage, mostly because of the contradictory findings within the literature concerning the use of “selective” calmodulin inhibitors, there is some evidence suggesting a role for the protein in platelet aggregation, T cell aggregation, and protection of GPVI from proteolytic cleavage. It therefore remains an interesting possibility, that in the studies presented herein, CRP binding to GPVI may trigger calmodulin activation, which when combined with thrombin signaling, may trigger platelet aggregation. This therefore establishes a possible means for GPVI to potentiate signaling by thrombin, in the absence of critical components of the GPVI-FcRγ chain pathway.

The present findings have important implications for the clinical management of thrombosis. Specifically, these studies support recent findings by Mangin et al., who have observed that thrombin plays an important role in overcoming the hemostatic and thrombotic defect linked to a deficiency in the GPVI-FcRγ signaling cascade. Importantly, these studies raise the interesting scenario that complete prevention of thrombosis will require administration of both anti-GPVI agents and antithrombin agents (anticoagulants). This notion is supported by in vivo studies that have highlighted that the relative importance of signaling proteins in vivo...
thrombosis models varies with a function of lesion severity. Nonne et al. show that the major PLC isofrom which signals downstream of GPVI receptor engagement, PLCγ2, plays an important role in platelet thrombosis under conditions of superficial injury, whereas inhibitors of thrombin significantly compromise thrombosis after severe injury. As such, we could predict that: first, concurrent anti-GPVI/antithrombin therapy would prevent thrombosis in vivo after both superficial and severe injury; second, both the compensation elicited by thrombin in the absence of Syk and Src kinases, and the synergistic signaling cascade triggered by the combination of sub-threshold GPVI agonists with thrombin (PAR4), both described in this article, would be blocked under these therapeutic conditions.

In summary, this study further dissects the critical role of Syk in platelet activation downstream of adhesion and G protein–coupled platelet receptors. Specifically we show that platelet spreading on a variety of adhesion molecules requires Src and Syk kinases, yet their deficit can be overcome by G protein–coupled receptor agonists. In addition, we demonstrated that thrombin stimulates tyrosine phosphorylation partly through Syk and entirely downstream of Src kinases, yet neither kinase is critical for thrombin-induced platelet aggregation. Lastly and most interestingly, our studies reveal the existence of Syk- and Src kinase-independent signals generated by the GPVI in combination with thrombin(PAR4), which raises interesting issues for the clinical management of thrombosis. 

Acknowledgments
The authors thank Prof Shaun Jackson (ACBD, Monash University, Australia) for valuable discussions.

Sources of Funding
S.C.H. holds a National Health and Medical Research Council (Aust.) CJ Martin Fellowship. S.P.W. holds a British Heart Foundation Chair. This work was supported by the Wellcome Trust. V.L.J.T. and E.S. were funded by the Medical Research Council, and V.L.J.T. has received a Seminar Honoraria. J.I.W. is funded by the National Institute of Health (HL50545). O.J.T.M. is currently funded by the American Heart Association Beginning Grant-in-Aid.

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2007;27:422-429; originally published online November 16, 2006;
doi: 10.1161/01.ATV.0000252826.96134.21

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTARY MATERIAL

Materials and Methods

Materials

PE-labeled rat anti-mouse integrin αIIbβ3 monoclonal JON/A antibody was from Emfret Analytics (Wurzburg, Germany). Von Willebrand Factor (VWF) was kindly provided by Prof Berndt (Monash University, Clayton, Victoria, Australia). Precision plus protein standards were from BioRad (Hercules, CA, U.S.A). Oregon-Green fibrinogen (OG-FGN) was from Molecular Probes (Invitrogen, Eugene, OR, U.S.A). ARC69931MX was kindly provided by AstraZeneca (Leicestershire, UK) and MRS2179 was obtained from Tocris Bioscience (Bristol, UK). All other reagents were from Sigma (Poole, UK) or have been described. 

Protease Activation Receptor (PAR) activation peptides TRAP (SLLRN) (for PAR1) and AYPGKF (for PAR4) were synthesized by Dr Phil Thompson, (Medicinal Chemistry, Victorian Collage Pharmacy, Monash University, Melbourne, Australia).

Mouse strains

Syk−/− and wild-type radiation chimeras were generated as described. Briefly, wild type mice, (Balb/cxB10D2) F1, were irradiated using a 137-Cs source with 2x500 rads, 3 h apart. Hemopoietic progenitor cells (1-5 million cells/recipient) were obtained from freshly harvested fetal liver from wild type or Syk deficient 15-16 day embryos (B10.D2), or frozen cell suspensions, and injected intravenously into the lethally-irradiated mice. After injections, mice were maintained on water containing neomycin for four weeks and cells were analyzed 6-8 weeks post-injection. Mice were maintained for up to 14 weeks after bone marrow reconstitution. FcRγ chain deficient (−) mice and GPVI deficient (−) mice have been described previously. Briefly, FcRγ chain knockout mice (C57BL/6J) were used with age-matched wild type C57BL/6J mice. GPVI knockout mice were generated after six backcrossings with C57BL/6J wild type mice.
Preparation of mouse platelets

Murine platelets were obtained and prepared as previously described \textsuperscript{12}, in accordance with the Animals (Scientific Procedures) Act 1986, United Kingdom. Briefly, whole murine blood was collected in acid-citrate-dextrose (90mM sodium citrate, 7mM citric acid, 140mM D-glucose) from the hepatic portal vein of terminally anesthetized mice. Following platelet isolation \textsuperscript{12}, platelets were resuspended in Tyrode’s buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na\textsubscript{2}HPO\textsubscript{4}, 12H\textsubscript{2}O, 12mM NaHCO\textsubscript{3}, 20mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), pH 7.3, 1mM MgCl\textsubscript{2}, 5mM glucose) at 2 to 5x10\textsuperscript{8}/ml. Human platelets were prepared according to Goncalves et al \textsuperscript{13}. All Donors had provided informed written consent, according to the Declaration of Helsinki.

Platelet aggregation and stimulation studies

Washed murine platelets (2x10\textsuperscript{8}/ml) were stimulated with thrombin (0.01 or 3.0U/ml, 90 sec) or Collagen Related Peptide (CRP) (3μg/ml, 2.5 min) and aggregation determined by maximum change in light transmission. For synergy aggregation experiments, wild type or FcR g chain knockout platelets were aggregated to thrombin (0.0325 – 0.08 U/ml) or CRP (0.74 – 2.0 μg/ml), alone or in combination for 10 minutes and aggregation was determined by a change in light transmission. For some studies, platelets were treated with the integrin α\textsubscript{IIb}β\textsubscript{3} inhibitor, lotrafiban (10μM), prior to stimulation with thrombin (1.0 U/ml, 90 sec) in the presence or absence of PP2 (20μM), or CRP (3μg/ml, 90 sec). Platelets were lysed using 2xlysis buffer (300mM NaCl\textsubscript{2}, 20mM Tris, 2mM EGTA, 2mM EDTA, pH 7.5, 2 % NP-40 detergent (v/v), 5mM Na\textsubscript{3}VO\textsubscript{4}, 2mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin) and the whole cell lysates were used in tyrosine phosphorylation studies. For control studies using human platelets, washed platelets (3x10\textsuperscript{8}/ml) were aggregated with increasing concentrations of thrombin (0.005-1.0U/ml), in the presence or absence of PP2 (10μM) for 10 min. For the sub-threshold concentrations of thrombin, exogenous fibrinogen (500μg/ml) was added to the aggregation. For studies using the PAR activation peptides,
TRAP and PAR4AP, sub-threshold concentrations of each agonist were detected, prior to combination of the agonist with CRP, in the presence of exogenous fibrinogen (500μg/ml). These concentrations varied with different donors and were over the following range; TRAP (0.375-0.75μM), PAR4AP (37.5-67.5μM) and CRP (0.15-1.0μg/ml). In some studies, platelets were treated with vehicle control (DMSO) or with PP2 (10μM) and/or ADP receptor antagonists ARC69931MX (10μM) and MRS2179 (100μM) and the Thromboxane A2 (TxA2) inhibitor, indomethacin (10μM), then aggregated with CRP for 10 sec, followed by the addition of thrombin/PAR activation peptides. The extent of aggregation was recorded for a maximal of 10 min, as the maximal change in light transmission.

**Platelet spreading and imaging**

Static adhesion assays were performed using a modified method of Inoue *et al* 14 and Yuan *et al* 15. Briefly, glass coverslips were coated with Von Willebrand Factor (VWF) plus botrocetin (VWF, 10 μg/ml; botrocetin, 5μg/ml), Horm collagen (100μg/ml), fibrinogen (200μg/ml), Collagen Related Peptide (CRP) (100μg/ml) or fibronectin (100μg/ml) for 2h at room temperature or overnight at 4°C. Non-immobilized protein was removed and coverslips were blocked with fatty-acid free BSA (5mg/ml) for 60 min at room temperature, and washed prior to addition of platelets. Washed murine (3-5x10^7/ml) platelets were incubated on the coverslips for 45 min at 37°C. In some studies, thrombin (1.0U/ml) or ADP (10μM) were added to the platelet suspension five minutes before addition to the adhesive matrix. After 45 min, non-adherent platelets were removed with gentle washing and adherent platelets were fixed (3.7% formaldehyde, 10 min) and imaged using Differential Interference Contrast (DIC) microscopy 16 (Zeiss 63xoil immersion 1.40NA plan-apochromat lens, Zeiss Axiovert 200M microscope). In control human studies, coverslips were prepared as described in Yuan *et al* 15, and platelets (3x10^7/ml) were allowed to spread on the thrombogenic matrix in the presence or absence of PP2 (10μM), in combination with Tyrode’s buffer containing thrombin (1.0 U/ml) or ADP (10μM).
Western blotting

Aggregated platelet samples were lysed by addition of 2x reducing buffer and whole cell lysates were reduced and denatured through boiling for 10 min. Proteins were resolved on 4 to 12% precast gels and electrically transferred onto PVDF membranes. Membranes were blocked in 10 % (w/v) BSA dissolved in Tris Buffered Saline-Tween (TBS-T), for 1 h at room temperature or overnight at 4°C. An anti-phosphotyrosine monoclonal antibody (4G10) was incubated with the membranes for 1 to 2h at room temperature. Membranes were washed thrice with TBS-T and incubated with Mouse-Horseradish peroxidase secondary antibody. In all studies, membranes were developed using the Enhanced Chemiluminescence system (ECL®, Amersham Pharmacia Biotech, Cardiff, U.K).

Flow cytometric measurement of integrin $\alpha_{\text{IIb}}\beta_3$ activation.

Suspensions of murine platelets (wild type and Syk deficient) (25μl of $5\times10^6$ platelets) were prepared in Tyrode’s buffer containing 1mM Ca$^{2+}$. Platelets were activated with thrombin (0.01–1.0U/ml), CRP (2-10μg/ml), ADP (10μM), thrombin in combination with CRP (thrombin 0.01U/ml and CRP 10μg/ml) or ADP in combination with CRP (ADP 10 μM and CRP 10 μg/ml), for 10 min at 37°C in the absence of stirring. The PE-conjugated JON/A antibody, which binds selectively activated murine $\alpha_{\text{IIb}}\beta_3$, was added to the platelet suspension, and allowed to incubate for 15 min at room temperature. The reaction was terminated by addition of Tyrode’s buffer and the samples were read in FL2 within 30 min. Control samples containing unstimulated platelets, treated with lotrafiban, PE-labelled Rat IgG or vehicle were also tested. Washed human platelets ($5\times10^7$/ml) or washed murine platelets (wild type, FcR γ chain deficient and GPVI deficient) were stimulated with thrombin (0.01-0.05U/ml) alone, CRP alone (1.0-8.0μg/ml) or thrombin in combination with CRP, for 5 min, in the presence or absence of PP2 (10μM), prior to addition of Oregon-green fibrinogen (OG-FGN, 20μg/ml) for an additional 5 min at room temperature. In some studies,
platelets were fixed with 2% paraformaldehyde for 30 min, before centrifugation at 2000g for 2min. The supernatant was removed and the platelet pellet was resuspended in 500μl filtered 1xPBS. Alternatively, filtered PBS was added to the reaction suspension straight after the OG-FGN incubation to terminate the reaction. Finally fluorescence was detected in the FL1 channel using a FACs machine (Becton Dickinson). To demonstrate the specificity of OG-FGN binding, control platelets were incubated with EGTA (5 mM)/Mg^{2+} (1 mM) or 7E3 (40 μg/ml) prior to stimulation with thrombin and detection of OG-FGN binding.

**Statistical analysis**

Statistical significance of results was determined using One-way analysis of variance, using the Prism Software program (GraphPAD Software for Science, San Diego, USA.) The p values are indicated where appropriate.

**References**

Figure S1. The requirement for Src kinases in spreading on different matrix proteins is bypassed by thrombin. Glass coverslips were coated with Von Willebrand Factor (VWF) (10µg/ml), fibrinogen (FGN, 200µg/ml) or collagen (100µg/ml). Non-immobilized protein was removed and coverslips were blocked with 2% human serum in the presence of PMSF, and washed. Washed human platelets were incubated with DMSO or PP2 (10µM) prior to exposure to the coverslips for 45 min at 37°C in the absence or presence of thrombin (1.0U/ml). Non-adherent platelets were removed and adherent platelets were fixed with 3.7% formaldehyde and imaged using Differential Interference Contrast microscopy. Scale bar=20µm.
### Supplementary Table S1

<table>
<thead>
<tr>
<th></th>
<th>WT platelet morphology</th>
<th></th>
<th>Syk&lt;sup&gt;−/−&lt;/sup&gt; platelet morphology</th>
<th></th>
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<tr>
<td></td>
<td>% lamelli</td>
<td>% filo</td>
<td>% lamelli</td>
<td>% filo</td>
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<tr>
<td>VWF Con</td>
<td>77.3 ± 7.6</td>
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<td>+thr</td>
<td>81.9 ± 1.0</td>
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<td>+ADP</td>
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<td>% filo</td>
<td>% lamelli</td>
<td>% filo</td>
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<tr>
<td>FGN Con</td>
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<td>% filo</td>
<td>% lamelli</td>
<td>% filo</td>
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<td>CRP Con</td>
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<td></td>
<td>% lamelli/filo</td>
<td>% lamelli/filo</td>
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<td>Coll Con</td>
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<td>+thr</td>
<td>88.0 ± 1.3</td>
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<tr>
<td>+ADP</td>
<td>72.0 ± 4.4</td>
<td>19.5 ± 4.4</td>
<td>- 19.5 ± 4.4</td>
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**Table 1.** Murine platelet morphology in wild type and Syk<sup>−/−</sup> platelets, exposed to Von Willebrand Factor (VWF), Fibrinogen, Collagen Related Peptide (CRP) and collagen.

Washed murine wild type (WT) and Syk deficient (Syk<sup>−/−</sup>) platelets were allowed to adhere and spread on immobilized matrix proteins, VWF, fibrinogen (FGN), Collagen Related Peptide (CRP) and Collagen (Coll) in the presence or absence of thrombin (1.0 U/ml, +thr) or
ADP (10 μM, +ADP). Platelets were fixed and imaged using Differential Interference
Contrast optics. Platelets were then scored as exhibiting filopodia (filo), lamellipodia (lamelli)
or a resting, discoid morphology (not shown) and these were expressed as a percentage of the
total number of platelets within a field. Note that for platelets adherent to collagen, platelets
have been scored as either resting or exhibiting filopodia/lamellipodia (%Lamelli/filo).
Values are mean ± SEM, n=2-3 experiments performed in duplicate, with five fields taken per
sample.
**Supplementary Table S2**

<table>
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<th>Matrix</th>
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<th>PP2/Thrombin</th>
<th>PP2/ADP</th>
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<td>VWF</td>
<td>96.8 ± 1.1</td>
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<td>64.5 ± 2.8</td>
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<td>Fibrinogen</td>
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<td>97.2 ± 0.6</td>
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<td>Collagen</td>
<td>97.7 ± 0.8</td>
<td>17.0 ± 2.7</td>
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<td>73.5 ± 4.0</td>
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</table>

**Table 2.** Analysis of PP2-treated platelets following adhesion to immobilized proteins in the presence of thrombin or ADP.

Washed human platelets, treated with vehicle control (control) or PP2 (PP2, 10 μM), were allowed to adhere and spread on immobilized matrix proteins, Von Willebrand Factor (VWF), fibrinogen and Collagen in the presence or absence of thrombin (1.0 U/ml, PP2/Thrombin) or ADP (10 μM, PP2/ADP). Platelets were fixed and imaged using Differential Interference Contrast optics. The percentage of platelets demonstrating filopodia and/or lamellipodia were calculated and expressed as a percentage of the total number of platelets within a field. Values are mean ± SEM, n=3 experiments performed in duplicate.