Platelets and Blood Cells

Streptococcus sanguis-induced platelet activation involves two waves of tyrosine phosphorylation mediated by FcγRIIA and αIibβ3

Caroline Pampilina, Archibald McNicol
Departments of Oral Biology and Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada

Summary
The low-affinity IgG receptor, FcγRIIA, has been implicated in Streptococcus sanguis-induced platelet aggregation. Therefore, it is likely that signal transduction is at least partly mediated by FcγRIIA activation and a tyrosine kinase-dependent pathway. In this study the signal transduction mechanisms associated with platelet activation in response to the oral bacterium, S. sanguis were characterised. In the presence of IgG, S. sanguis strain 2017–78 caused the tyrosine phosphorylation of FcγRIIA 30s following stimulation, which led to the phosphorylation of Syk, LAT, and PLCγ2. These early events were dependent on Src family kinases but independent of either TxA2 or the engagement of the αIibβ3 integrin. During the lag phase prior to platelet aggregation, FcγRIIA, Syk, LAT, and PLCγ2 were each dephosphorylated, but were re-phosphorylated as aggregation occurred. Platelet stimulation by 2017–78 also induced the tyrosine phosphorylation of PECAM-1, an ITIM-containing receptor that recruits protein tyrosine phosphatases. PECAM-1 co-precipitated with the protein tyrosine phosphatase SHP-1 in the lag phase. SHP-1 was also maximally tyrosine phosphorylated during this phase, suggesting a possible role for SHP-1 in the observed dephosphorylation events. As aggregation occurred, SHP-1 was dephosphorylated, while FcγRIIA, Syk, LAT, and PLCγ2 were rephosphorylated in an RGDS-sensitive, and therefore αIibβ3-dependent, manner. Additionally, TxA2 release, 5-hydroxytryptamine secretion and phosphatidic acid formation were all blocked by RGDS. Aspirin also abolished these events, but only partially inhibited αIibβ3-mediated re-phosphorylation. Therefore, S. sanguis-bound IgG cross links FcγRIIA and initiates a signaling pathway that is down-regulated by PECAM-1-bound SHP-1. Subsequent engagement of αIibβ3 leads to SHP-1 dephosphorylation permitting a second wave of signaling leading toTxA2 release and consequent platelet aggregation.

Keywords
FcγRIIA, platelet, PECAM-1, SHP-1, Streptococcus sanguis

Introduction
Several studies have suggested that oral infections, most commonly manifested in periodontal disease, are related to the incidence of certain cardiovascular diseases including atherosclerosis and myocardial infarction (1–3). In addition, the incidence of myocardial infarction and stroke increases during acute infections (4), and several viral and bacterial infections are positive risk factors in susceptible individuals (5–7).

Streptococcus sanguis (S. sanguis), a gram-positive oral bacterium, binds to and activates platelets in vitro (8–10) and has been shown to precipitate myocardial infarction, thrombocytopenia and pulmonary platelet accumulation in rabbits (11–13). The mechanisms underlying S. sanguis-induced platelet activation have been partially characterized. For example, the collagen receptor αIbβ3 (14), the von Willebrand factor receptor subunit GPⅠbα (15, 16), complement proteins on the bacterial surface (17), the interaction of plasma-derived IgG with the platelet Fc receptor (18), and the cyclooxygenase/thromboxane A2 (TXA2) pathway (9, 10) have each been suggested to mediate platelet-S. sanguis interaction and/or S. sanguis-induced platelet aggregation.

Of particular interest is the potential role played by FcγRIIA, the platelet low-affinity IgG receptor, in the platelet response to S. sanguis. Specific antibodies in plasma bind to bacterial surfaces, and the Fc portion of these antibodies interact with FcγRIIA to initiate platelet activation (19). Indeed, removal of IgG from plasma or pre-treatment of platelets with IV3, a monoclonal antibody that blocks IgG binding to FcγRIIA, prevents platelet aggregation to S. sanguis (9, 18), demonstrating a requirement for FcγRIIA activation.

Direct cross-linking and activation of FcγRIIA using an anti-FcγRIIA antibody and F(ab')2 fragments causes rapid platelet activation and aggregation (20). Intracellular signalling initially

Correspondence to:
Dr. A. McNicol
Department of Oral Biology
780 Bannatyne Avenue, Winnipeg
Manitoba, Canada, R3E 0V2
Tel: +1 204 789-3527, Fax: +1 204 789-3913
E-mail: mcnicol@ms.umanitoba.ca

Received August 6, 2004
Accepted after resubmission February 7, 2005

Grant support:
These studies were funded by grants from the Manitoba Medical Service Foundation, the Dr. Paul H.T. Thorlakson Foundation, the Heart and Stroke Foundation of Canada and the Canadian Institutes of Health Research.

Prepublished online April 14, 2005 DOI: 10.1160/TH04–08–0482
involves the Src family kinase-mediated phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain of FcγRIIA (21, 22). This allows for the non-covalent engagement of SH2 domain-containing proteins such as the tyrosine kinase Syk (23), which is crucial for events that lead to phospholipase Cγ2 (PLCγ2) phosphorylation (24–26). PLCγ2 is a central enzyme in early FcγRIIA signaling as it induces the production of the second messengers inositol (1,4,5)-trisphosphate (IP₃) and diacylglycerol (DAG) (27), leading to secretion and aggregation responses.

Unlike direct FcγRIIA cross-linking, platelet aggregation induced by S. sanguis is typified by a long lag phase prior to rapid aggregation (8–10). Therefore, inhibitory mechanisms may be activated that oppose the FcγRIIA-mediated response. Studies in immune cells suggest that ITAM-containing receptor tyrosine kinase pathways may be regulated by immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors, which can recruit inhibitory phosphatases such as the protein tyrosine phosphatases (PTP) SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2, and the lipid phosphatases SH2 domain-containing inositol phosphatase-1 (SHIP-1) and SHIP-2 (28). Platelets possess the platelet-endothelial cell adhesion molecule-1 (PECAM-1), a 130-kD membrane glycoprotein that is currently the only known ITIM-containing receptor on the platelet surface (29). Its direct activation by cross-linking agents induces the association of SHP-1 and/or SHP-2 and inhibits platelet aggregation in response to collagen and FcγRIIA activation (30–33).

In the present study, we have investigated signaling pathways triggered in platelets by the S. sanguis strain, 2017–78. FcγRIIA cross-linking and activation initiates an early signaling pathway involving the tyrosine phosphorylation of several platelet proteins that is negatively regulated by PECAM-1 and its associated PTP SHP-1. α(IIb)β₃ activation mediates a second wave of tyrosine phosphorylation that leads to the production of secondary messengers necessary for aggregation.

Materials and methods

S. sanguis strain 2017–78 was a gift from Dr. M. Herzberg (University of Minnesota, Minneapolis, Minnesota). Aspirin, staurosporine, Triton X-100, Igepal CA-630, BSA, and RGDS were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). ECL reagents, Hyperfilm, 5-hydroxy-[G-3H]-tryptamine creatine sulphate ([3H]-5-HT) and [32P]-ortho-phosphate were purchased from Amersham Pharmacia Biotechnology (Baie D’Urfe, PQ). Protein A/G PLUS agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). EDTA-free protease inhibitor cocktail tablets were obtained from Boehringer Mannheim (Laval, PQ). PIP1 from Biomol Research Laboratories Inc. (Plymouth Meeting, PA) and the TxB2 enzyme immunoassay kit from Oxford Biomedical Research Inc. (Oxford, MI). All other chemicals were of the highest grade available.

Antibodies

Monoclonal anti-phosphotyrosine Ab clone 4G10 and polyclonal anti-LAT Ab were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal Abs anti-Syk (LR and N-19), anti-PLCγ2, anti-PECAM-1 (M-20 and C-20), and anti-SHP-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-FcγRIIA Ab, IV.3, was purchased from Medarex Inc. (Annandale, NJ). Polyclonal anti-FcγRIIA, 260, was a gift from Dr. J. L. Teillaud (Université Pierre and Marie Curie, Paris, France).

Preparation of bacterial suspensions

S. sanguis strain 2017–78 was grown on blood agar plates (18 h, in candle jars at 37°C), and cells were harvested and washed twice with Heps Tyrodes buffer. Cells were briefly sonicated (10 s on ice) to disperse any bacterial clumps. The absorbance at 660 nm was recorded and the number of bacteria in the suspension was calculated according to a standard curve of absorbance versus bacteria/mL, with bacterial counts obtained using a Petroff-Hauser counting chamber.

Preparation of platelet-rich plasma (PRP)

The study was approved by the Research Ethics Board of the University of Manitoba. Blood was drawn by venipuncture from healthy, consenting adults who had denied taking any medications known to interfere with platelet function within the previous two weeks. It was mixed with 0.1x volume of 3.8% sodium citrate and centrifuged at 200 x g for 20 min at room temperature to obtain PRP. Platelets were allowed to equilibrate for 30 min at 37°C prior to use. Presented results are representative of 2–3 similar experiments using blood from different donors.

Preparation of IgG-free PRP

IgG was depleted from plasma by affinity chromatography using Protein A coated-sepharose beads. The column was initially eluted with wash buffer (100 mM Tris, pH 7.5, 1.5 mM NaCl), followed by Heps Tyrodes buffer. Platelet-poor plasma (PPP), which was prepared by centrifugation of 1 mL aliquots of citrated PRP at 2000 x g for 3 min, was subsequently applied to the column. The first 0.5 mL of eluate was discarded and the next 1.5–2 mL collected into an Eppendorf tube and stored at 37°C until use.

0.5 mL aliquots of acidified PRP were centrifuged (2000 x g, 3 min) to obtain platelet pellets. Plasma was aspirated, platelets were resuspended in 0.5 mL of IgG-depleted PPP and allowed to equilibrate at 37°C for 30 min prior to use.

Platelet aggregation

Aggregation of platelets (2.5 x 10⁸ platelets/mL) was carried out in a Payton dual channel aggregometer as previously described (34). S. sanguis strain 2017–78 was added to PRP at a 1:2 platelet to bacteria ratio.

Immunoprecipitation

Reactions were stopped by the addition of an equal volume of 2x stopping solution (20 mM EDTA, 6 mM Na₃VO₄, 20 μM staurosporine) (35). Platelets were pelleted by centrifugation at 8500 x g for 30 s, sonicated 4 x 10 s in cold 2x RIPA buffer (36) or 1x lysis buffer (37) (for PECAM-1 immunoprecipitation) and lysed for 30 min at 4°C. The lysate was centrifuged at 12 000 x g for 10 min at 4°C, and the supernatant was precleared with Protein A/G
PLUS agarose beads. Lysates were incubated overnight at 4°C with anti-PLCγ2 Ab (2.5 μg/mL), anti-LAT Ab (2.5 μg/mL), anti-PECAM-1 Ab (10 μg/mL), or anti-SHP-1 Ab (2.5 μg/mL), or for 90 min at 4°C with anti-Syk (LR) Ab (2.5 μg/mL) or IV3 mAb (2.5 μg/mL), then agarose beads were added for a further 60 min. The beads were isolated by centrifugation and washed 3 times with 1x RIPA wash buffer or PBS (for PECAM-1 immunoprecipitations). The proteins were eluted by boiling with reducing buffer for 10 minutes, separated by 7.5 or 10% SDS-polyacrylamide gels and immunoblotted for phosphorytosine with the mAb 4G10 according to the method of Clark and Brugge (36). Proteins were visualized with a commercial ECL kit.

Blots were subsequently stripped with 0.1 M NaCl and 0.1 M glycine (pH 2.2), washed with TBS-Tween, and re-probed with the appropriate antibody to ensure even loading among lanes or to detect for co-precipitation.

**Thromboxane B2 release**

2017–78 was added to PRP and reactions terminated at the point of full aggregation by the addition of an equal volume of ice cold acid/citrate/dextrose anticoagulant (ACD) (34). Platelets were pelleted by centrifugation at 8 500 x g for 30 s and the supernatant from each sample was collected into separate Eppendorf tubes. Levels of released TxB2 were detected using a TxB2 enzyme immunoassay kit according to the manufacturer’s instructions.

**[3H]-5-hydroxytryptamine (HT) secretion**

Dense granule secretion was measured by [3H]-5-HT release from prelabeled platelets as previously described (34). Briefly, PRP was incubated with [3H]-5-HT for 60 min and aggregation carried out in response to S. sanguis strain 2017–78 as outlined above. Release was terminated at the point of full aggregation by addition of an equal volume of 0.1% glutaraldehyde in White’s saline, samples were centrifuged at 12 000 x g for 2 min, and [3H]-5-HT in the supernatants and in the formic acid-digested pellets were determined by liquid scintillation counting.

**Phosphatidic acid formation**

Equal quantities of blood were drawn into 0.1x volume of 3.8% sodium citrate and 0.18x volume of acid-citrate-dextrose. PRP was obtained as outlined above. Platelets were pelleted by further centrifugation at 800 x g for 15 min at room temperature. Citrated PPP was stored at 37°C until used. The platelet pellet from acidified PRP was resuspended in 1–2 mL of PO4-free Hepes Tyrodes buffer (150 mM NaCl, 4 mM KCl, 1 mM MgCl2, 5 mM HEPES, 10 mM dextrose, 0.3% bovine serum albumin; pH 7.4) and incubated with 100 - 120 μCi [32P]-ortho-phosphate for 1 hour at 37°C. Platelets were isolated by centrifugation, resuspended in citrated PPP and allowed to equilibrate for 30 min at 37°C prior to use. Under these labelling conditions, the formation of [32P]-phosphatidic acid is regarded as an index of phosphoinositide-specific phospholipase C activity (38).

Platelet aggregation in response to S. sanguis was carried out as outlined above, terminated, phospholipids extracted and separated by thin-layer chromatography as described previously (39). [32P]-phosphatidic acid was identified by comparison with unlabelled standard and the levels of [32P]-phosphatidic acid and total [32P]-labelled phospholipid in each sample were quantified by liquid scintillation counting.

**Results**

2017–78 induces platelet aggregation and FcγRIIA tyrosine phosphorylation in an IgG-dependent manner

Human platelets aggregate following challenge by S. sanguis strain 2017–78, as has been shown previously (8). There is a donor dependent lag phase prior to the onset of aggregation with maximum aggregation occurring at 12.1±1.6 min (range from 5 to 19 min; n=10). As previously demonstrated (9, 10), S. sanguis-induced platelet aggregation is sensitive to pre-treatment with aspirin (100 μM; 30 min) or RGDS (1 mM; 5 min) and is therefore dependent on TXA2 production and fibrinogen binding to α5β3 (data not shown). Due to the significant donor variability in the lag phase prior to the onset of aggregation, in the subsequent studies protein phosphorylation status was determined at points corresponding to the lag phase, the start of aggregation, mid-aggregation and full aggregation for that specific donor (Fig. 1).

As outlined above, IgG and the activation of its receptor, FcγRIIA, are essential for platelet aggregation in response to viridans group streptococci (18). In the present study, pre-incubation of platelets with the anti-FcγRIIA mAb IV3 (2.5 μg/mL; 1 min) or depletion of plasma IgG inhibits 2017–78-induced aggregation, confirming that aggregation in response to this strain requires FcγRIIA cross-linking by IgG (data not shown). The addition of 2017–78, in the presence of IgG, causes the tyrosine phosphorylation of FcγRIIA. Phosphorylation occurs
within 30 s of the addition of 2017–78, decreases in the lag phase prior to aggregation and subsequently increases as aggregation progresses (Fig. 2A).

Tyrosine phosphorylation of FcγRIIA occurs in the intracellular ITAM domain, likely due to the actions of Src family tyrosine kinases, such as Src and Lyn (21). Pre-treatment of platelets with the Src family kinase inhibitor PP1 (40), which blocks the activities of Lck, Lyn, Src and Hck, inhibits platelet aggregation in response to 2017–78 in a dose-dependent manner (Fig. 2B).

2017–78 induces the tyrosine phosphorylation of several proteins

Direct FcγRIIA cross-linking and activation is associated with the recruitment, phosphorylation and activation of a variety of signaling molecules, including Syk, LAT, PI3-K and PLCγ2 (41). In the present study, platelet activation following the addition of 2017–78 involves Syk, LAT, and PLCγ2 tyrosine phosphorylation (Fig. 3A) with similar time courses to that seen for FcγRIIA phosphorylation (Fig. 2A).

Depletion of IgG from plasma blocks 2017–78-induced phosphorylation of PLCγ2 (Fig. 3B) and LAT (Fig. 3C), consistent with FcγRIIA cross-linking initiating the signaling pathway, as has previously been reported for IgG crosslinking.

Figure 3: Activation of human platelets with 2017–78 induces the tyrosine phosphorylation of several proteins. A). Platelets were stirred with 2017–78 for the times indicated and reactions terminated. Syk (i), LAT (ii), and PLCγ2 (iii) were isolated by immunoprecipitation, and tyrosine phosphorylation was monitored by immunoblotting using 4G10. Blots were stripped and re-probed with the appropriate antibody to confirm equal loading (lower panel). Labels below the upper panel refer to aggregation status. Experiments are representative of three similar experiments using different donors. B). Platelets were untreated or i) depleted of IgG, ii) treated with PP1 (5 min; 30 μM), iii) RGD (5 min; 1 mM), or iv) aspirin (20 min; 100 μM) prior to stimulation with 2017–78 for 1 min. PLCγ2 was isolated by immunoprecipitation, and tyrosine phosphorylation was monitored by immunoblotting using 4G10 (upper panels). The blot was stripped and re-probed with anti-PLCγ2 antibody to confirm equal loading (lower panels). Experiments are representative of two to three similar experiments using different donors. C). Platelets were untreated or i) depleted of IgG prior to stimulation with 2017–78 for 1 min. LAT was isolated by immunoprecipitation, and tyrosine phosphorylation was monitored by immunoblotting using 4G10 (upper panel). The blot was stripped and re-probed with anti-LAT antibody to confirm equal loading (lower panel). Experiment is representative of three similar experiments using different donors.
Pretreatment of platelets with PPI (Fig. 3B), but with neither aspirin (Fig. 3B) nor RGDS (Fig. 3B), inhibited the early phosphorylation of PLCγ2. Taken together these data suggest that Src family kinases play a role in the activation pathway, and that the pathway is activated prior to, or independently of, TxA2 synthesis or aggregation.

2017–78 induces PECAM-1 and SHP-1 phosphorylation

As outlined above, platelet activation following the addition of 2017–78 is associated with a triphasic phosphorylation time course of FcγRIIA, Syk, LAT and PLCγ2. In each case two waves of phosphorylation separated by a dephosphorylation phase is observed, suggesting the actions of both kinases and phosphatases. The inhibitory effect of PECAM-1 on ITAM-containing receptor signaling pathways (32) and the recruitment of protein tyrosine phosphatases, SHP-1 and SHP-2, to phosphorylated PECAM-1 (42–44) suggest the possibility that PECAM-1 plays a role in this dephosphorylation phase.

In the current study, 2017–78 causes the rapid tyrosine phosphorylation of PECAM-1 in an IgG-dependent manner (Fig. 4A). Therefore, PECAM-1 activation likely occurs downstream of FcγRIIA cross-linking. PECAM-1 also becomes dephos-
phorylated, however in contrast to FcγRIIA, Syk, LAT, and PLCγ2, PECAM-1 remains phosphorylated longer within the lag phase (Fig. 4A). PECAM-1 rephosphorylation is inhibited by either depletion of IgG or pre-treatment with RGDS (Fig. 4A), and is therefore dependent on αιβ3 engagement.

The protein tyrosine phosphatase SHP-1 co-precipitates with PECAM-1 within the lag phase (Fig. 4B). In contrast there is no detectable co-immunoprecipitation of SHP-2 with PECAM-1 (data not shown), which suggests that SHP-1 is the primary tyrosine phosphatase that associates with PECAM-1 following platelet activation by 2017–78 and bound IgG.

SHP-1 is also tyrosine phosphorylated; maximal phosphorylation occurs during the lag phase followed by dephosphorylation during aggregation (Fig. 4B). Thus, this phosphorylation time course is opposite to that observed for FcγRIIA, Syk, LAT, and PLCγ2. Blocking aggregation with RGDS prevents SHP-1 dephosphorylation at the timepoint corresponding to aggregation, confirming a role for αιβ3 and subsequent outside-in signaling in SHP-1 dephosphorylation (Fig. 4B). There was no detectable phosphorylation of SHP-2 at any time point (data not shown).

\( \alpha\text{ιβ}3 \) mediates protein re-phosphorylation, \( TXA_2 \) release, dense granule secretion and phosphatidic acid formation

As shown above, the re-phosphorylation of FcγRIIA, Syk, LAT, and PLCγ2 occurs simultaneously with 2017–78-induced platelet aggregation. To establish a role for \( \alpha\text{ιβ}3 \)-mediated outside-in signaling in this process, platelets were pre-incubated with RGDS. Pretreatment with RGDS abolished the re-phosphorylation of FcγRIIA, Syk, LAT, and PLCγ2 (Fig. 5A), as well as \( TXA_2 \) release, dense granule secretion and PLC activation (Table 1 and Fig. 5C), suggesting that all of these events occur downstream of \( \alpha\text{ιβ}3 \) engagement.

Pre-incubation of platelets with aspirin decreases, but does not abolish, FcγRIIA, Syk, LAT, and PLCγ2 phosphorylation (Fig. 5B). However, dense granule secretion and PLC activation are abolished in the presence of aspirin (Fig. 5C). Taken together, these results suggest that \( \alpha\text{ιβ}3 \)-mediated outside-in signaling leads to the dephosphorylation of SHP-1, as mentioned above, which may permit re-phosphorylation of several proteins in a signaling pathway that induces \( TXA_2 \) release, which in turn potentiates the aggregation response by promoting dense granule secretion and PLC activity.

Discussion

There is increasing evidence for an association between certain cardiovascular diseases and infectious organisms. For example it has been estimated that conventional risk factors (hypertension, smoking, obesity, hypercholesterolemia, genetic predisposition) predict the development of less than half of the observed atherosclerotic plaques (45). Consequently, several novel risk factors for atherosclerosis have been proposed, including platelet hyperactivity (45) and infectious organisms (45, 46). Similarly, it has been suggested that low-level bacteremia in a host responses altering coagulability, endothelial and vessel wall integrity and platelet function, and subsequently result in atherothrombotic and thrombotic events (47). Most studies have focused on Gram-negative organism-derived lipopolysaccharide (LPS) acting to promote the atherosclerotic process (48), however the ability of \( S. \text{sanguis} \) to activate platelets has been cited as a significant contributing factor to coronary heart disease (49).

Specific strains of oral bacteria in the viridans group of streptococci induce platelet activation, both in vitro (8–10) and, in rabbits, in vivo (12, 13), supporting a thrombogenic role for these strains. While several studies have addressed possible bacterial ligands and corresponding platelet receptors mediating the interaction (10, 14, 50, 51), the specific signaling pathways triggered in platelets by bacterial challenge have not been characterised.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{TXB2 released (ng/mL)} & \\
\hline
\textbf{Control} & 0.071 ± 0.029 \\
\textbf{2017–78} & 21.5 ± 0.07 \\
\textbf{RGDS + 2017–78} & 0.375 ± 0.130 \\
\textbf{Aspirin + 2017–78} & 0.0207 ± 0.0048 \\
\hline
\end{tabular}
\caption{TXA2 release is dependent on \( \alpha\text{ιβ}3 \) engagement. Platelets were untreated, or treated with RGDS (5 min, 1 mM) or aspirin (20 min, 100 μM) prior to the addition of 2017–78. Concentration of TXB2 released (ng/mL) into the supernatant was measured by ELISA. Results are the mean ± SEM of three experiments each with two observations.}
\end{table}

In the present study, \( S. \text{sanguis} \) strain 2017–78-induced platelet activation was associated with the tyrosine phosphorylation of the low affinity IgG receptor on platelets, FcγRIIA. This correlates with previous studies where platelets failed to respond to \( S. \text{sanguis} \) strain NCTC 7863 when plasma is depleted of IgG (18) and is consistent with FcγRIIA playing a fundamental role in \( S. \text{sanguis} \)-induced platelet aggregation. FcγRIIA phosphorylation initiated a pathway involving Src family tyrosine kinases, Syk, and LAT, leading to the phosphorylation of PLCγ2. Activation of this pathway is independent of \( \alpha\text{ιβ}3 \) and thromboxane A2, demonstrating that it is an early signaling event. Not surprisingly, this pathway is similar to that evoked by direct stimulation of FcγRIIA with anti-FcγRIIA antibody and secondary cross-linking antibodies (23, 41, 52).

However, in the current studies, using optimal platelet to bacteria ratios, FcγRIIA, Syk, LAT and PLCγ2 are each subsequently dephosphorylated prior to the engagement of \( \alpha\text{ιβ}3 \) activation. This contrasts with direct FcγRIIA cross-linking where, again using optimal concentrations of crosslinking secondary antibody (20, 26), the dephosphorylation of PLCγ2 (26) and LAT (53) occurs downstream of \( \alpha\text{ιβ}3 \) engagement. Taken together these data suggest that, although the early signalling pathways evoked by 2017–78 are likely mediated by the action of IgG on FcγRIIA, there are significant differences in the mechanisms of platelet activation by \( S. \text{sanguis} \) and by direct FcγRIIA crosslinking.

Several studies have suggested that PECAM-1 negatively regulates platelet responses to ITAM-mediated signaling pathways, such as those stimulated by collagen, FcγRIIA and glycoprotein Ib/V/IX (30–33, 54). In the present study, PECAM-1 is rapidly tyrosine phosphorylated in response to 2017–78 and remains phosphorylated within the lag phase. The phosphorylation
of PECAM-1 in the lag phase results in its association with the phosphatase SHP-1. Moreover, SHP-1 is maximally tyrosine phosphorylated, which enhances its enzymatic activity (55), during this lag phase and this phosphorylation is subsequently decreased in an α<sub>INHβ<sub>3</sub>-dependent manner. These observations are consistent with a role for the PECAM-1 recruitment of SHP-1 in the dephosphorylation of FcγRIIA, Syk, LAT, and PLCγ2, which may explain the protracted lag phase seen in response to S. <i>sanguis</i>. It has been shown previously that FcγRIIA and PECAM-1 colocalise in the platelet membrane (33), therefore such a role for PECAM-1 on FcγRIIA-mediated signaling is reasonable. However, in platelets directly activated by FcγRIIA cross-linking, the dephosphorylation of LAT occurs by PTP1B (53), again supporting potentially subtle differences in the mechanism of IgG- and <i>S. sanguis</i>-induced platelet activation.

Interestingly PECAM-1 recruits SHP-1, but not SHP-2, to the membrane, which contrasts previous studies which suggest that SHP-2 is the preferred PECAM-1-associated phosphatase (42, 56–58). The preferential recruitment of SHP-1 observed in this study is, however, plausible. Firstly, previous studies have recognized SHP-1 as a negative influence on signaling in haematopoietic cells (59, 60), whereas SHP-2 is believed to enhance cell signaling. Secondly, direct FcγRIIA activation in myeloid cells (61) using cross-linking antibodies leads to both SHP-1 phosphorylation and its association with tyrosine phosphorylated FcγRIIA. Finally, substrate specificity may dictate which protein tyrosine phosphatase associates with PECAM-1. Frank et al recently demonstrated that Src substrates are efficiently dephosphorylated by SHP-1 (62). The inhibition of the <i>S. sanguis</i>-induced early signaling pathway by Src family kinase inhibitors implicates a central role for these kinases, and the substrates of Src and other related kinases may therefore be targets of SHP-1. Syk is also a substrate of SHP-1 in B and T cells (63), and may also be a SHP-1 substrate in platelets since it co-precipitates with SHP-1 (64).

The function of PECAM-1 dephosphorylation prior to aggregation is unknown, however it is likely that it attenuates SHP-1 recruitment thereby allowing the proaggregatory signaling to proceed. The rephosphorylation of PECAM-1 during aggregation is interesting and may be a result of increased homophilic binding, since cell-cell contact induced by stirring alone leads to a low degree of PECAM-1 phosphorylation (37). Alternatively, aggregation leads to release of granule contents, and since PECAM-1 is present in the membranes of α-granules (65), increased surface expression of PECAM-1 may contribute to its increased activation.

RGDS blocked the rephosphorylation of FcγRIIA, Syk, LAT, and PLCγ2, and the dephosphorylation of SHP-1, consistent with a vital role for α<sub>INHβ<sub>3</sub>-mediated outside-in signalling. TxA<sub>2</sub> potentiates outside-in signaling as aspirin decreases, but does not abolish, the late phosphorylation events. However the precise mechanism leading to α<sub>INHβ<sub>3</sub> activation in response to <i>S. sanguis</i> is unknown and requires further study.

The current study supports a requirement for IgG in the platelet aggregation response to <i>S. sanguis</i> (18). However there are differences between signalling pathways engaged by <i>S. sanguis</i> and by the direct crosslinking of FcγRIIA, and these may result from the involvement of other pathways. For example Ford et al have shown that inhibition, or deletion, of complement proteins prevents aggregation in response to <i>S. sanguis</i> strain NCTC 7863 (17). Furthermore roles for GP Ib, which has been shown to be essential for platelet-<i>S. sanguis</i> adhesion (16), and the platelet aggregation-associated protein (PAAP), which contains a collagen-like domain and is found on the surface of aggregation-inducing strains of <i>S. sanguis</i> (66, 67), are also possible contributing factors to the observed α<sub>INHβ<sub>3</sub> activation. Interestingly, there is precedence for a dual activation process. In platelet activation induced by anti-streptokinase antibodies, both Fc receptor engagement and cleavage of the protease-activated receptor, PAR-1, are involved (68) in a similar manner to that outlined in the current study.

The activation of platelets by <i>S. sanguis</i> strain 2017–78 is therefore dependent on a number of sequential biochemical events. IgG bound to <i>S. sanguis</i> interact with FcγRIIA stimulating a signal transduction cascade including activation of the receptor itself, Src-family kinases, Syk, LAT, and PLCγ2. PECAM-1 phosphorylation and its association with SHP-1 appear to play a negative regulatory role. Subsequent activation of α<sub>INHβ<sub>3</sub> by an as yet undetermined mechanism induces SHP-1 dephosphorylation to overcome this negative effect, allowing a second wave of tyrosine phosphorylation that induces TxA<sub>2</sub> release, which is essential for secretion and aggregation.

Acknowledgments

The authors would like to thank Dr. George Bowden for the use of microbiological facilities and helpful discussions, Dr. Mark Herzberg for providing <i>S. sanguis</i> strain 2017–78, Ms. Elke Jackson for excellent technical assistance and Dr. Sara Israels for helpful discussions and critical reading of this manuscript. CP is the recipient of a Manitoba Health Research Council Studentship.

References


12. Herzberg MC, Meyer MW. Dental plaque, pla-
13. Meyer MW, Gong K, Herzberg MC. Streptococcus
genus-induced platelet clotting in rabbits and he-
mamals: platelet aggregation and cardiopulmonary consequences. In:
14. Soberay AH, Herzberg MC, Rudney JD, et al. Re-
sponses of platelets to strains of Streptococcus sanguis:
findings in healthy subjects, Bernard-Soulier, Glanz-
mann’s, and collagen-unresponsive patients. Thromb
Haemost 1987; 57: 5–5.
15. Douglas CW, Brown PR, Preston FE. Platelet ag-
gregation by oral streptococci. FEMS Microbiol Lett 1990;
glycoprotein Ib in Streptococcus sanguis-induced pla-
the involvement of complement proteins in platelet aggre-
gation by Streptococcus sanguis NCTC 7863. Br J Haem-
18. Ford I, Douglas CW, Cox D, et al. The role of immu-
noglobulin G and fibrinogen in platelet aggregation by
19. Sjoberg U, Ringdahl U, Ruggieri ZM. Induction of
platelet thrombi by bacteria and antibodies. Blood
2002; 100: 4470–7.
20. Anderson GP, Anderson CL. Signal transduction by
21. Daion M. Structural bases of Fc gamma R func-
FcγRII induces tyrosine phosphorylation of multiple
proteins including FcγRII. J Biol Chem 1992; 267:
5467–73.
Clustering of the platelet Fcγ receptor induces nonval-
ent association with the tyrosine kinase p72syk. J
24. Keel PJ, Parise LV. The ε2β1 integrin is a nec-
essary co-receptor for collagen-induced activation of Syk
and the subsequent phosphorylation of phospholipase C.
tyrosine-phosphorylated proteins in human pla-
etles activated by collagen and cross-linking of the Fc
dy 3,4,5-trisphosphate-dependent stimulation of
phospholipase C-2 is an early key event in FcγRIIA-
mediated activation of human platelets. J Biol Chem
stimulates [3H]inositol trisphosphate formation in
28. Gergely J, Pecht I, Sarmay G. Immunoreceptor tyro-
sine-based inhibition motif-bearing receptors regul-
ate the immunoreceptor tyrosine-based activation motif-induced activation of immune competent cells.
29. Gibbons JM. The negative regulation of platelet
function: extending the role of the ITIM. Trends Cardi-
30. Patil S, Newman DK, Newman PJ. Platelet en-
dothelial cell adhesion molecule-1 serves as an
inhibitory receptor that modulates platelet responses to
adhesion molecule-1 is a negative regu-
lator of platelet-collagen interactions. Blood 2001;
98: 1456–63.