The functional roles of GPVI, GPIbα, and PARs in the generation of platelet-derived reactive oxygen species

A thesis submitted to the National University of Ireland for the Degree of Doctor in Philosophy

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Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree PhD is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Date

15.5.14

Student Number: 10114505
"It always seems impossible until it's done"

- Nelson Mandela
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Publications


Conferences

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**Young Life Scientists Ireland, March 2014** – *The role of reactive oxygen species formation in platelets and cardiovascular disease*

**Human Disease Mapping Conference, January 2014** – *Understanding reactive oxygen species formation in platelets and their role in cardiovascular disease*

Poster Presentations:

**UK Platelet Meeting, September 2013** – *A novel role for PAR4 in reactive oxygen species formation in platelets*

**RCSI Research Day, March 2013** – *GPIb-dependent reactive oxygen species formation in platelets*

**UK Platelet Meeting, October 2012** – *The role of FAK family members in GPVI-dependent reactive oxygen species formation*

**Young Life Scientists Ireland, November 2011** – *Pyk2 is prerequisite for GPVI-induced platelet ROS production, but not aggregation*

**Irish Platelet Symposium, November 2011** – *Pyk2 is prerequisite for GPVI-induced platelet ROS production, but not aggregation*

**ISTH Congress Meeting, July 2011** – *Pyk2 is prerequisite for GPVI-induced platelet ROS production, but not aggregation*
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<thead>
<tr>
<th>Abbreviations</th>
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<tr>
<td>12-HETE</td>
<td>12- hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>12-LOX</td>
<td>12-lipoxygenase</td>
</tr>
<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Activating peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSS</td>
<td>Bernard Soulier syndrome</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>Collagen related peptide</td>
</tr>
<tr>
<td>CVX</td>
<td>Convulxin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal related kinase</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FAT</td>
<td>Focal adhesion target</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>FcRγ</td>
<td>Fc receptor γ</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>Ferric chloride</td>
</tr>
<tr>
<td>FERM</td>
<td>Four point one ezrin radixin moesin homology</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>H₂DCFDA</td>
<td>Dihydropdichlorofluorescein diacetate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for T-cell activation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MTT</td>
<td>Methylthiazolyl diphenyl-tetrazolium</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NADPH/NOX</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OONO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PAK</td>
<td>P21 activating kinase</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>PGE₁</td>
<td>Prostaglandin E₁</td>
</tr>
<tr>
<td>PGl₂</td>
<td>Prostaglandin l₂</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
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<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP$_3$</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>Phospholipase A$_2$</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylerine</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Proline rich tyrosine kinase 2</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>SLP-76</td>
<td>Src homology 2-containing leukocyte protein 76</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TRAF4</td>
<td>Tumour necrosis factor receptor-associated factor 4</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor activating peptide</td>
</tr>
<tr>
<td>TxA/B$_2$</td>
<td>Thromboxane A/B$_2$</td>
</tr>
<tr>
<td>VWD</td>
<td>von Willebrand disease</td>
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VWF      von Willebrand factor
WT       Wild type
Abstract

Platelet receptor ligation initiates multiple signalling pathways leading to platelet-specific events such as shape change, secretion, integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation. In addition to these events, albeit less understood, platelets generate reactive oxygen species (ROS). In phagocytic cells, ROS act as a defence mechanism, while in non-phagocytic cells, such as platelets, the function of ROS has not been well described. Previous studies have shown that platelet-generated ROS serve to activate platelets and are involved in cellular signalling. This thesis focuses on the specific roles of two key platelet receptors pivotal in haemostasis and thrombosis, the glycoprotein (GP)Ib-IX-V complex and GPVI, and the signalling pathways induced through these receptors that lead to ROS production. An important role for focal adhesion kinase (FAK) in the GPVI pathway is described and it is shown that GPIb$\alpha$, like GPVI, plays a role in platelet-derived ROS generation. Signalling proteins involved in protease activated receptor (PAR)-induced ROS formation are identified, and a functional role for PAR4 is described that is independent of PAR1. Furthermore, a novel association between PAR4 and GPIb$\alpha$ in platelet-derived ROS generation is proposed. NOX1 is determined as the main source of ROS in platelets, regardless of the agonist employed, and is found to mediate thromboxane A$_2$ (TxA$_2$) generation, independent of platelet aggregation. In conclusion, ROS generation is an attractive potential target in cardiovascular disease. It remains to be established whether targeting ROS in cardiovascular disease provides an alternative, safer and more specific therapy than other anti-platelet drugs such as aspirin.
Chapter 1

Introduction
1.1 Platelets

The primary function of platelets is haemostasis, the limiting of bleeding upon vascular injury. However, in an intact vessel, platelet adhesion can manifest as a consequence of vascular damage, which leads to thrombosis and results in a heart attack or stroke (Andrews and Berndt, 2004). Circulating platelets are recruited to the damaged site, exposing key platelet receptors to components of the extracellular matrix culminating in platelet activation, aggregation and the formation of a haemostatic plug. In addition, activated platelets release thromboxane A₂ (TxA₂) and secrete dense granule contents such as adenosine di-phosphate (ADP) and serotonin that act as secondary messengers to amplify the activation process and recruit more platelets to the developing clot (Figure 1.1) (Stegner and Nieswandt, 2011b). To accomplish these functions, extensive signalling occurs within platelets, which tightly regulates haemostasis and thrombosis.

The platelet membrane contains a vast array of platelet receptors, which are involved in initiating platelet signalling and subsequently platelet activation. The glycoprotein (GP)Ib-IX-V complex that binds von Willebrand Factor (VWF) and GPVI that binds collagen are involved in the initial haemostatic response (Clemetson, 2012). Integrins, including the collagen (α₃β₁), laminin (α₄β₁) and fibronectin (α₅β₁) receptors also mediate platelet adhesion to the damaged site (Stegner and Nieswandt, 2011b). Platelet agonist receptors include G-protein coupled receptors (GPCRs) such as the protease activated receptors (PARs) 1 and 4, which are cleaved and activated by thrombin (De Candia, 2012). Other agonist GPCRs, P2Y₁ and P2Y₁₂ (ADP receptors) and the TxA₂ receptor, TP, are involved in amplifying the platelet activation response. Finally, the platelet specific integrin, α₉β₃, mediates platelet aggregation, allowing platelet to platelet cross-bridging by the multivalent ligands, fibrinogen and VWF (Clemetson, 2012).
Figure 1.1 Platelets in haemostasis and thrombosis

A. Circulating platelets adhere to von Willebrand factor (VWF) and collagen through the platelet receptors, GPIb-IX-V and GPVI, respectively. The platelets become activated, change shape, spread, secrete of secondary agonists, ultimately leading to integrin αIIbβ3 activation and platelet aggregation. B. As a result of occlusion of the blood vessel and damage to the sub-endothelial matrix, platelets are exposed to shear stress and become activated, culminating in platelet activation, aggregation and formation of a thrombus (Yip et al., 2005).
1.2 The GPIb-IX-V Complex

The GPIb-IX-V complex is a unique receptor specific to megakaryocytes and platelets (Andrews et al., 2003b). There are approximately 25,000 copies on the platelet membrane (Berndt et al., 1985). On binding VWF and other ligands, it propagates signalling events that lead to integrin αIIbβ3 activation and platelet aggregation (Ozaki et al., 2005). GPIb-IX-V is a member of the leucine-rich repeat superfamily and is comprised of one GPIbα subunit disulphide-linked to two GPIbβ subunits and non-covalently linked with GPIX and GPV in a ratio of 2:4:2:1 (Berndt and Andrews, 2011) (see Figure 1.2). A small fraction of the GPIb-IX-V complex is present in lipid rafts on the platelet membrane (Shrimpton et al., 2002), which can facilitate GPIb-IX-V-dependent platelet signalling (Ozaki et al., 2005). Genetic mutations in GPIbα, GPIbβ and GPIX, but not GPV, known as Bernard-Soulier Syndrome (BSS), result in dysfunction of the GPIb-IX-V complex (Andrews and Berndt, 2013). BSS patients have abnormally large platelets, prolonged bleeding time and thrombocytopenia. Their platelets do not adhere to VWF as they lack or have dysfunctional GPIbα (Andrews and Berndt, 2013). Mice genetically deficient in GPIbα replicate BSS (Ware et al., 2000). Re-engineering BSS mouse platelets with a chimaeric protein comprising the cytoplasmic domain of GPIbα and the ectodomain of the IL4 receptor mostly normalised the thrombocytopenia and platelet size but not the adhesive defect to VWF (Kanaji et al., 2002). Recently, BSS has been corrected in mouse models using gene therapy (Kanaji et al., 2012).
Figure 1.2 Structure and binding partners of GPIb-IX-V complex (Permission from Prof. Robert Andrews, ACBD, Melbourne, Australia)

The GPIb-IX-V complex is comprised of one GPIbα subunit disulphide-linked to two GPIbβ subunits and non-covalently linked with GPIX and GPV. Calmodulin (CaM) binds to the cytoplasmic domains of GPIbβ and GPV. 14-3-3ζ phosphorylation sites are located at Ser166 on GPIbβ and Ser609 and 590 on GPIbα. The p85 subunit of PI3-K and filamin bind to the cytoplasmic tail of GPIbα and TRAF4 binds to GPIbβ. HMWK: High molecular weight kinogen; F: Factor.
1.2.1 GPIb-IX-V Structure

All four subunits of the GPIb-IX-V complex contain one or more extracellular leucine-rich repeats, disulphide looped N- and C-terminal capping sequences and arise from distinct genes (Lopez et al., 1998). GPIIX has a single leucine-rich repeat and a short cytoplasmic tail of 5 amino acids (Hickey et al., 1989), whilst, GPV has 15 leucine-rich repeats and a cytoplasmic tail of 16 amino acids (Lanza et al., 1993). GPIbβ has a single leucine-rich repeat and a cytoplasmic sequence of 34 amino acids containing a Protein Kinase A (PKA) phosphorylation site at Ser166 (Wardell et al., 1989). It also contains a membrane proximal RRLRARARARA sequence within the cytoplasmic tail (Martin et al., 2003), which binds calmodulin (Andrews et al., 2001) and TRAF4 (Arthur et al., 2011). Interestingly, inhibition of this sequence attenuates GPIb-IX-V-dependent platelet activation including aggregation and adhesion (Martin et al., 2003). Furthermore, GPIbβ co-localises with GPIbα and GPIIX and links the two subunits (Lopez et al., 1994). GPIbα is the major ligand binding subunit of the GPIb-IX-V complex (605 residues long) and interacts with multiple ligands (Andrews et al., 2003b). It is comprised of a N-terminal leucine-rich repeat flanking sequence, seven leucine-rich repeats (each 24 amino acids long, residues 36-200), a C-terminal leucine-rich repeat flanking sequence (residues 201-268), an anionic sequence containing three sulphated tyrosines (276, 278 and 279) (Dong et al., 2001), a mucin macroglycopeptide region that that contains the two cysteines that form disulphide bonds with two GPIbβ subunits, and a cytoplasmic tail (96 amino acids long) (Lopez et al., 1987, Luo et al., 2007).
1.2.2 GPIb-IX-V Complex Ligands

1.2.2.1 von Willebrand Factor (VWF)

The initial step in platelet adhesion is the interaction between GPIb-IX-V and VWF (Kroll et al., 1991, Kroll et al., 1996). VWF is a multimeric protein existing as a series of oligomers. The precursor polypeptide is 2813 residues long consisting of a 22 residue signal peptide, a large 741 residue propeptide and the final mature subunit of 2050 residues (Ruggeri and Ware, 1993). Mature VWF consists of approximately 250 kDa subunits linked in large N-to N-terminal, and C- to C-terminal multimers up to 20,000 kDa in molecular weight (Ruggeri, 2001). It is stored in both Weibel Palade bodies in endothelial cells or α-granules in platelets. It consists of modular domains: D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2 (see Figure 1.3) (Ruggeri, 2001) and is found in an inactive conformation within the circulation through protection from the D'D3 domain (Ulrichts et al., 2006). VWF switches to an active form under high pathological shear following binding to exposed collagen, via its A3 domain, subsequently allowing the A1 domain of VWF to bind to GPIbα (Ruggeri, 2001). Blocking VWF-A3 domain binding to collagen inhibits thrombus formation under high shear conditions (Wu et al., 2002). Additionally, VWF can be cleaved by a disintegrin and metalloproteinase domain-containing protein thrombospondin motifs (ADAMTS)-13 within the A2 domain resulting in reduced multimer size (Lancellotti et al., 2013).
Figure 1.3 Representation of the VWF structure (Adapted from (Ruggeri, 2001))

The mature subunit (2050 residues) of VWF is found in an inactive conformation within the circulation through masking by the D’D3 domain. VWF switches to an active form following binding to exposed collagen, via its A3 domain, or under high pathological shear, subsequently allowing the A1 domain of VWF to bind to GPIIbα. An RGD sequence in the C1 domain mediates binding to α_{IIb}β₃. VWF is cleaved by ADAMTS-13 within the A2 domain. The A1 domain binds the modulators of VWF function, ristocetin and botrocetin, as well as heparin to further the interaction with collagen.
1.2.2.2 VWF Function

GPIbα interacts with the A1 domain of VWF through the N-terminal and C-terminal leucine-rich repeats of GPIbα (Kim et al., 2010). Leucine-rich repeats 2-4 on GPIbα regulate shear-dependent adhesion to VWF (Shen et al., 2006). Residues 60-128 (repeats 2-4) on GPIbα are net negatively charged and complement a positively charged patch within the VWF-A1 domain (Whisstock et al., 2002). The VWF-A1 domain also contains a negatively charged patch on the opposite side of the domain which may facilitate long range interactions between GPIbα and VWF (Huizinga et al., 2002). VWF-GPIbα interaction at high shear rate is required to slow platelets sufficiently to allow GPVI-collagen interaction and integrin αⅡbb3 activation (Reininger, 2008). VWF-A1 binding to GPIbα is a catch-slip bond mechanism (Ju et al., 2013, Yago et al., 2008) regulated by the N-terminal flanking region of VWF (Ju et al., 2013). The VWF-A1 domain also contains binding sites for the VWF function modulators, botrocetin and ristocetin, which allow VWF-GPIbα binding in vitro. Botrocetin is a C-type snake venom lectin which binds in a unique fashion to the VWF-A1 domain and the N-terminus of GPIbα (Fukuda et al., 2005). It contains an anionic sequence which extends a negative patch on GPIbα to facilitate VWF binding (Andrews et al., 2003a). Ristocetin from the bacteria Amycolatopsis lurida is a failed antibiotic related to vancomycin. Ristocetin-dependent binding of VWF to GPIbα is inhibited by antibodies against GPIbα that also inhibit shear-dependent interactions (Dong et al., 2001). Ristocetin binding of VWF to GPIbα mimics the behaviour of VWF-A1 binding to GPIbα and thus imitates a more physiological interaction in comparison to that of botrocetin (Kim et al., 2010). VWF contains an Arg-Gly-Asp (RGD) sequence within the C1 domain that mediates VWF binding to integrin αⅡbb3 and facilitates firm platelet adhesion and platelet aggregation (Reininger, 2008). Deficiency in VWF amount or function results in von Willebrand disease (VWD), classified as type I, II or III, which results in symptoms such as epistaxis, gingivitis or menorrhagia (Table 1.1) (James and Goedeve, 2011).
Table 1.1 Description of VWD types I, II and III and subtypes (Adapted from (Ky et al., 2006))

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Description</th>
<th>Inheritance</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Quantitative deficiency of VWF</td>
<td>Autosomal dominant</td>
<td>70-80%</td>
</tr>
<tr>
<td>Type IIA</td>
<td>Abnormal platelet-dependent function of VWF</td>
<td>Autosomal dominant</td>
<td>10-15%</td>
</tr>
<tr>
<td>Type IIB</td>
<td>Increased platelet-dependent functions of VWF</td>
<td>Autosomal dominant</td>
<td>~5%</td>
</tr>
<tr>
<td>Type IIM</td>
<td>Abnormal platelet-dependent function of VWF</td>
<td>Autosomal dominant</td>
<td>Rare</td>
</tr>
<tr>
<td>Type IIN</td>
<td>Decreased affinity of VWF for Factor VIII</td>
<td>Autosomal dominant</td>
<td>Rare</td>
</tr>
<tr>
<td>Type III</td>
<td>Near complete deficiency of VWF</td>
<td>Autosomal recessive</td>
<td>Rare</td>
</tr>
</tbody>
</table>
1.2.2.3 Thrombin

Thrombin is a serine protease activated during coagulation by cleavage of prothrombin via factor Xa (Di Cera, 2008). It binds to GPIbα, and binds and cleaves PAR1 and PAR4 (Coughlin, 2000), the latter of which will be further discussed in this chapter. Thrombin is the most potent platelet agonist leading to a wide range of downstream events including platelet shape change, initiating release of secondary mediators, activating the integrin αIIbβ3 and enabling additional generation of thrombin by promoting a procoagulant platelet surface (Coughlin, 1999a). Moreover, thrombin can initiate the release of VWF from endothelial cells (Hattori et al., 1989), induce phospholipase A2 (PLA2) activation (Purdon et al., 1987) and is also involved in regulating cytokine production (Colotta et al., 1994). Thrombin contains two main binding sequences within the active site region, exosite I and II, located in distinct and opposite locations (Di Cera, 2003). Exosite I is primarily involved in binding fibrinogen and the anticoagulant hirudin (from leech saliva) which blocks thrombin activity. Exosite II binds the anticoagulant heparin and thrombomodulin, a cofactor for thrombin-dependent protein C activation.

1.2.2.4 Mechanism of Action: Thrombin

The interaction between GPIb-IX-V and thrombin involves the anionic sulphated region on GPIbα and is inhibited by the murine monoclonal antibody, VM16d, against the C-terminal flanking sequence of GPIbα (Ward et al., 1996, Shen et al., 2002). Although the mechanism of how thrombin binds GPIbα has been extensively studied, it has yet to be fully elucidated. In 2003, two distinct models were proposed; both studies suggested that exosites I and II were involved in binding to GPIbα (Celikel et al., 2003, Dumas et al., 2003). Celikel and colleagues suggested that two thrombin molecules bind to one GPIbα promoting GPIbα clustering. However, Dumas et al. suggested that two GPIbα molecules bind to one thrombin resulting in GPIbα adhesive bridging. Both hypotheses provide interesting insights but the latter model would not allow for the simultaneous binding of VWF as well as thrombin to GPIbα (Adam et al., 2003a). Thrombin can induce intracellular signalling in vitro via GPIbα (Adam et al., 2003b). In addition, BSS patient platelets do not
respond to low dose thrombin (0.05 U/mL) (Berndt et al., 1989). Therefore, GPIbα may serve to localise thrombin, helping to trigger platelet activation.

Thrombin also cleaves GPV (Berndt and Phillips, 1981), an interaction which enhances thrombin binding to GPIbα (Dong et al., 1997). Anti-GPV antibodies that inhibit thrombin cleavage of GPV however do not inhibit thrombin-induced platelet aggregation, indicating that GPV is not essential for platelet activation through thrombin (Bienz et al., 1986). Similarly, GPV-deficient mice platelets aggregate normally to thrombin (Kahn et al., 1999a) or have potentiated thrombin-induced platelet activation (Ramakrishnan et al., 1999). However, adhesion to VWF is not impaired in GPV deficient mice, suggesting GPV deficiency is not a cause of BSS (Kahn et al., 1999a).

1.2.2.5 Other GPIb-IX-V Ligands

GPV also binds collagen although this does not appear to be as relevant to platelet function as binding to GPVI (Moog et al., 2001). GPIbα is an indirect collagen receptor as collagen binds to the A3 domain in VWF (Reininger, 2008). GPIb-IX-V can promote coagulant activity by binding kininogen (Joseph et al., 1999) and factors XI (Baglia et al., 2004) and XII (Joseph et al., 1999), and tissue factor through an indirect interaction with factor VIIa (Hoffman, 2008). The glycoprotein, thrombospondin-1, binds to GPIbα and can support platelet adhesion at high shear rates in the absence of VWF (Jurk et al., 2003). P-selectin, an inflammatory adhesion molecule, links endothelial cells and activated platelets and, like VWF, is found in Weibel Palade bodies and α-granules (Woollard and Chin-Dusting, 2007). P-selectin directly binds GPIbα and is co-localised with VWF (Romo et al., 1999). Furthermore, P-selectin is involved in anchoring VWF to the endothelium and is important for leukocyte rolling (Dole et al., 2005, Padilla et al., 2004). Leukocytes interact with platelets through binding of integrin αMβ2 to GPIbα and cleavage of GPIbα results in attenuation of this interaction (Simon et al., 2000). Snake venom proteins, C-type lectins and metalloproteinases, can bind the N-terminal portion of GPIbα and act directly as
agonists (e.g. alboaggregin A) or inhibit by cleavage of the N-terminus (e.g. mocarhagin) (Andrews et al., 2003a). GPIb-IX-V can also bind bacteria through GPIbα (Fitzgerald et al., 2006).

1.2.3 GPIb-IX-V Complex Cytoplasmic Binding Partners

The GPIb-IX-V complex is partially associated with the immunoreceptor tyrosine-based activation motif (ITAM)-containing Fc receptor γ (FcRγ)-chain through interaction with GPIbα (Wu et al., 2001). FcRγ-chain° mice display attenuated VWF/botrocetin-induced aggregation and a reduction in spleen tyrosine kinase (Syk) and phospholipase C (PLC)γ2 phosphorylation. The actin binding protein, filamin A, is associated with the cytoplasmic tail of GPIbα (Okita et al., 1985). Amino acid residues 535-610 on GPIbα cytoplasmic domain are essential for the filamin-GPIbα association (Andrews and Fox, 1992, Cunningham et al., 1996, Mistry et al., 2000, Williamson et al., 2002, Feng et al., 2005, Cranmer et al., 2005), with an important role for Phe568 and Trp570 in promoting GPIbα binding to filamin A (Cranmer et al., 2005). GPIbα interacts with the C-terminus of filamin A and binds in a groove formed between the C and D β strands of filamin A domain 17 (Nakamura et al., 2006). Mice platelets with GPIbα containing alanine substitutions at Phe568 and Trp570 have disrupted interaction with filamin A and demonstrate filamin A-GPIbα interaction is important for membrane integrity as well as platelet size (Cranmer et al., 2011). Furthermore, deletion of filamin A results in large platelets as seen in BSS (Jurak Begonja et al., 2011). The adapter protein, 14-3-3ζ, binds to the cytoplasmic tails of both GPIbα and GPIbβ (Du et al., 1994, Andrews et al., 1998). 14-3-3ζ binds to several sites on GPIbα: in the region between Asp596 and Leu610 (Du et al., 1996), residues 580-590 (specifically: Ser587/590) (Mangin et al., 2004), and residues 551-564 (Yuan et al., 2009). 14-3-3ζ plays a role in VWF-GPIbα interaction (Dai et al., 2005, Yuan et al., 2009) and activates Rho guanine triphosphate (GTP)ases Rac and Cdc42 (Bialkowska et al., 2003). Phosphorylation at Ser609 in the cytoplasmic domain of GPIbα is required for 14-3-3ζ binding to GPIbα (Bodnar et al., 1999). Chinese hamster ovary cells, expressing mutant GPIb-IX, replacing GPIbα Ser609 with alanine (S609A), in response to VWF-GPIbα binding,
have decreased phosphorylation of Src family kinases (SFKs) and protein kinase C (PKC) and attenuated cytoplasmic Ca\(^{2+}\) mobilisation (Zhang et al., 2012). The phosphoinositide 3-kinase (PI3-K) regulatory subunit p85 binds independently with 14-3-3\(\xi\) at the cytoplasmic tail of GPIb\(\alpha\) (Munday et al., 2000). Inhibiting the binding of PI3-K at residues 606-610 on GPIb\(\alpha\) inhibits GPIb\(\alpha\)-dependent spreading, independent of 14-3-3\(\xi\) (Mu et al., 2010).

The GPIb-IX-V complex interacts with calmodulin through association with the cytoplasmic tails of GPIb\(\beta\) (R149-L167) and GPV (K529-G544) (Andrews et al., 2001). Calmodulin inhibitors induce the ectodomain shedding of GPV and GPIb\(\alpha\) (Gardiner et al., 2007). ADAM10 and ADAM17 are members of the disintegrin and metalloproteinase family of sheddases expressed in platelets and both are involved in platelet receptor shedding (Gardiner et al., 2007). Shedding (of GPVI, GPIb, GPV) is believed to regulate membrane surface receptor expression levels and could potentially be involved in platelet clearance and aging (Bergmeier et al., 2003, Bergmeier et al., 2004, Best et al., 2003). The scaffold protein, tumour necrosis factor receptor associated factor (TRAF)4, binds to the cytoplasmic sequence of GPIb\(\beta\) through its MATH domain (residues 307-462) (Figure 1.3) (Arthur et al., 2011). In other cell types, TRAF4 is associated with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or NOX, subunit p47\(^{phox}\) (Xu et al., 2002). Similarly, in platelets p47\(^{phox}\) is associated, through its SH3 domain, with TRAF4. A complex involving TRAF4, p47\(^{phox}\), the paxillin homologue, Hic-5, and proline-rich tyrosine kinase (Pyk)2 are co-immunoprecipitated with antibodies against GPIb\(\alpha\) (Arthur et al., 2011).

### 1.2.4 GPIb-IX-V Complex Signalling

Following ligation, GPIb\(\alpha\) induces a cascade of signalling events required for platelet activation. The activation of SFKs and Syk are essential for platelet aggregation (Falati et al., 1999). The PI3-K effector, Akt, is required for early GPIb-IX mediated signalling (Yin et
Integrin $\alpha_{IIb}\beta_3$- and aggregation-independent TxA$_2$ production is elicited through VWF/botrocetin-dependent activation of Lyn, Syk, and the subsequent activation of the adaptor molecule Src homology 2-containing leukocyte protein-76 (SLP-76), PI3-K and PLCy2 (Liu et al., 2005). Bruton’s tyrosine kinase (Btk) is required for VWF/botrocetin-induced extracellular signal-related kinase (ERK)1/2 and PLCy2 activation, which in turn activates PKC, which is required for TxA$_2$ production, resulting in GPIb$\alpha$-dependent stable thrombus formation (Liu et al., 2006). Furthermore, PLCy2 catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce second messengers, inositol triphosphate (IP$_3$) and diacylglycerol (DAG) that induce Ca$^{2+}$ mobilisation and PKC activation respectively (Ozaki et al., 2005).

Rac1, involved in NOX complex formation (Bokoch and Diebold, 2002), is essential for GPIb$\alpha$-mediated platelet activation (Delaney et al., 2012). Activation of Rac1 is dependent on Lyn and occurs upstream of Akt phosphorylation (Delaney et al., 2012). GPIb-IX activity is dependent on the cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) pathway, which is mediated by Lyn and attenuated in the absence of Akt (Li et al., 2003, Yin et al., 2008). VWF-GPIb$\alpha$ interaction requires the PKG/P38 mitogen activated protein kinase (MAPK)/ERK pathway for integrin $\alpha_{IIb}\beta_3$ activation (Li et al., 2006) and Src/ERK-mediated TxA$_2$ generation is required for GPIb$\alpha$-VWF-mediated fibrinogen receptor activation (Garcia et al., 2005). LIM-1 kinase, an actin binding protein, is activated downstream of MAPK activation in the GPIb-IX-dependent pathway upstream of cytosolic PLA$_2$ activation and is required for arterial thrombosis but not haemostasis (Estevez et al., 2013).

1.3 Collagen Receptors

1.3.1 Integrin $\alpha_2\beta_1$

Collagen directly binds GPVI (Moroi et al., 1989), GPV (Moog et al., 2001) and the integrin $\alpha_2\beta_1$ (Nieuwenhuis et al., 1985) on platelets. Through the interaction with the A3 and A1
domain of VWF, collagen indirectly binds the integrin α\textsubscript{IIb}β\textsubscript{3} and GPIbα, respectively (Reininger, 2008). Integrin α\textsubscript{IIb}β\textsubscript{3} was the first collagen receptor identified (Nieuwenhuis et al., 1985) and is the most abundant collagen receptor on platelets (~2000-4000 copies) (Moebius et al., 2005). Interaction with α\textsubscript{IIb}β\textsubscript{3} is dependent on the type of collagen used (Savage et al., 1999), as it binds to fibrillar, but not soluble collagen (Nieswandt et al., 2001b). Although, GPVI is the main collagen receptor required to initiate platelet activation (Kehrel et al., 1998), α\textsubscript{IIb}β\textsubscript{3} can activate signalling molecules similar to those propagated by GPVI (Inoue et al., 2003). The specific binding sequence peptide ‘GFOGER’ from collagen can activate SFKs and PLCγ2 through an α\textsubscript{IIb}β\textsubscript{3}-dependent pathway.

1.3.2 GPVI

GPVI is a member of the immunoglobulin receptor superfamily, along with FcRγIIA and FcεRI (Clemetson et al., 1999). There are approximately 1000-2000 copies on the platelet membrane (Moebius et al., 2005). Composed of 339 amino acids, GPVI has two N-terminal immunoglobulin domains which encompass the binding domain for collagen (Dutting et al., 2012). Within the C-terminal of the immunoglobulin domains, GPVI contains a mucin-like core, a transmembrane domain containing a positively charged arginine residue, and a short cytoplasmic tail (Andrews and Berndt, 2004). GPVI is complexed with the FcRγ-chain (Tsuji et al., 1997), an interaction which is mediated by the arginine residue within the transmembrane domain and additional elements within the cytoplasmic tail (Berlanga et al., 2002, Gardiner et al., 2004, Calderwood et al., 2002). Expression of GPVI relies on this interaction, although expression of the FcRγ-chain does not require GPVI (Tsuji et al., 1997). Each FcRγ-chain contains an ITAM with the consensus amino acid sequence, YXXL/I\textsubscript{g-2}YXXL/I (Gibbins et al., 1996). The cytoplasmic tail of GPVI is composed of a proline-rich motif which selectively binds Fyn and Lyn through their SH3 domains (Suzuki-Inoue et al., 2002). Human GPVI has an intracellular tail of 51 amino acids (Clemetson et al., 1999). In contrast, murine GPVI has an intracellular tail of only 27 amino acids that lack the 24 amino acids that lie C-terminal to the proline-rich region in human GPVI (Jandrot-Perrus et al., 2000). Like GPIb-IX-V, GPVI is partly encompassed in lipid rafts (Ezumi et al.,
2002, Locke et al., 2002, Wonterow et al., 2002). Furthermore, GPVI is physically and functionally associated with the GPIb-IX-V complex (Arthur et al., 2005, Gardiner et al., 2010).

1.3.3 GPVI Ligands

1.3.3.1 Collagen

GPVI binds to collagen (Dutting et al., 2012), laminin (Ozaki et al., 2009), the snake venom C-type lectins, convulxin (CVX) (Polgar et al., 1997), alboagreggin-A (Asazuma et al., 2001), and a synthetic collagen-related peptide (CRP) (Morton et al., 1995). There are more than 20 forms of collagen in the human body, but types I, III and IV are the major isoforms involved in platelet activation (Nieswandt and Watson, 2003). Fibrillar collagen is cross-linked, usually containing more than one collagen type, and is the primary component of the extracellular matrix to which platelets bind (Nieswandt and Watson, 2003). Collagen interacts with GPVI at two distinct sites (Schulte et al., 2001). Monomeric GPVI (usually found on resting platelets) does not bind collagen (Asselin et al., 1999), whilst GPVI in dimeric form binds collagen with high affinity (Miura et al., 2002, Jung et al., 2012).

1.3.3.2 Collagen-related Peptide (CRP)

CRP contains the specific GPO (amino acid codes for glycine, proline and hydroxyproline, respectively) peptide sequence, which makes up approximately 10% of collagens types I and III (Morton et al., 1995). Additionally, CRP forms a triple helical structure, similarly to that found in collagen, and is further cross-linked through cysteine or lysine residues for stability (Morton et al., 1995). GPVI has at least 10-fold higher affinity for collagen than CRP (Miura et al., 2002), although CRP is about 30-fold more potent as an agonist than collagen (Asselin et al., 1997). Both collagen and CRP compete for binding to GPVI. Amino acids, Val34 and Leu36, in the hinge region of the receptor, are critical for their binding (Lecut et al., 2004).
1.3.3.3 Convulxin (CVX)

CVX is a C-type lectin snake venom which binds to and activates platelets through GPVI (Polgar et al., 1997). CVX activates GPVI by cross linking the receptor (Batuwangala et al., 2004). CVX and CRP bind to distinct but overlapping sites on GPVI (Asselin et al., 1999).

1.3.4 GPVI Function

GPVI is the major signalling receptor for collagen on platelets (Kehrel et al., 1998), which allows for stable adhesion to collagen (Savage et al., 1998, Penz et al., 2005, Cosemans et al., 2005, Reininger et al., 2010). GPVI deficient mice present with minimal prolongation of the bleeding times (Nieswandt et al., 2001c), however their platelets do not adhere to collagen (Nieswandt et al., 2001b), suggesting GPVI, but not \(\alpha_2\beta_1\), is essential for platelet interaction with collagen. Deficiency in humans is rare. Absence of GPVI in humans is generally characterised by normal platelet size and number, mildly prolonged bleeding time with normal coagulation, with absent or ablated aggregation in response to collagen or other GPVI agonists (Sugiyama et al., 1987). In 1999, Clemetson and colleagues cloned the cDNA for the GPVI gene (GP6) (Clemetson et al., 1999). Since then up to ten mutations have been identified in GPVI variants, which are associated with GPVI deficiency (Arthur et al., 2007). Platelets from patients with GPVI deficiency are unable to adhere to collagen under flow (Goto et al., 2002), although loss of GPVI can be overcome functionally by thrombin (Lecut et al, 2005, Mangin et al., 2006). Calmodulin binds the cytoplasmic tail of GPVI (Andrews et al., 2002) and is involved in regulating platelet receptor shedding of GPVI (Gardiner et al., 2005), which can occur through ADAM10 in human platelets and ADAM10 and 17 in mouse platelets (Bender et al., 2010) (see Figure 1.4). GPVI is the major platelet receptor described as involved in generating reactive oxygen species (ROS) in platelets (Bakdash and Williams, 2008, Arthur et al., 2012), an event which requires the activation of Syk (Arthur et al., 2012). Furthermore, the cytoplasmic tail of GPVI binds TRAF4 which is in complex with Hic-5, Pyk2 and p47\(^{phox}\) (Arthur et al., 2011), potentially allowing assemblage of NOX with the cytoplasmic face of GPVI.
GPVI is in complex with TRAF4, Hic-5, Pyk2 and p47phox and is functionally associated with the FcRγ-chain dimer, each subunit of which contains an ITAM sequence. The cytosolic tail of GPVI contains a calmodulin (CaM)-binding site and a proline-rich region that constitutively binds the SFKs, Fyn and Lyn. Fyn and Lyn doubly tyrosine phosphorylate the ITAM sequence, subsequently resulting in the activation of Syk. These early signalling responses initiate the activation of the LAT signalosome, which include the phosphorylation of the adaptor protein, SLP-76, Btk, Akt and the p85 subunit of PI3-K. Through interaction with tyrosine phosphorylated LAT, PI3-K is activated and leads to the activation of PLCγ2. The GPVI-dependent signalling pathway results in Ca^{2+} mobilisation, secretion, activation of the integrin αIβ3, and platelet aggregation. GPVI stimulation also results in ADAM10-mediated GPVI ectodomain shedding.
1.3.6 GPVI in Disease

Since GPVI deficiency generally causes only mild haemostatic defects, it has been proposed as an attractive therapeutic target in treating thrombosis and cardiovascular disease. GPVI inhibition in a mouse model of stroke (transient middle cerebral artery occlusion) significantly reduces infarct size (Stoll et al., 2010) and GPVI null mice display reduced pulmonary thromboembolism (Lockyer et al., 2006). Increased expression of platelet and plasma GPVI are observed in stroke in vivo (Al-Tamimi et al., 2010, Bigalke et al., 2010). Furthermore, GPVI also plays an important role in inflammation as rheumatoid arthritis is significantly reduced in GPVI<sup>−/−</sup> mice (Boilard et al., 2010). Finally, in the absence of GPVI, there is a 50% decrease in pulmonary metastasis, suggesting an involvement of platelet GPVI in tumour development (Jain et al., 2009).

1.3.5 GPVI Signalling

One of the earliest events in GPVI signalling is the phosphorylation of Fyn and Lyn, which then doubly tyrosine phosphorylate, the ITAM motif within the FcRγ-chain (Boulaftali et al., 2013). Double Fyn/Lyn KO mice display significant inhibition of shape change and aggregation in response to CRP stimulation (Quek et al., 2000). Subsequently, Syk is recruited (via its SH2 domain) to the doubly tyrosine-phosphorylated ITAM sequence in the FcRγ-chain and is autophosphorylated within its activation loop at Tyr525 and Tyr526, both of which are essential for full activation (Suzuki-Inoue et al., 2004). These early signalling responses initiate a series of downstream events including phosphorylation of the adaptor proteins, linker for T-cell (LAT) and SLP-76 (Pasquet et al., 1999b, Quek et al., 1998, Achison et al., 2001, Avraham et al., 2000). LAT activation is Syk-dependent and still occurs in SLP-76<sup>−/−</sup> mice and X-linked agammaglobulinemia patients lacking Btk (Pasquet et al., 1999b). Phosphorylated residues on LAT act as docking sites for recruitment of additional proteins, including the p85 regulatory subunit of PI3-K (Gibbins et al., 1998). PI3-K in turn activates other signalling molecules involved in the GPVI pathway such as Akt (Yin et al., 2008). Through interaction with tyrosine phosphorylated LAT, PI3-K catalyses the phosphorylation of PIP<sub>2</sub> to phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>) which acts
as a ligand for the pleckstrin homology domain of signal transducing proteins like PLCγ2 (Moroi and Jung, 2004). Although PI3-K inhibitors have no significant effect on GPVI-dependent PLCγ2 phosphorylation (Falet et al., 2000), there is evidence to suggest PI3-K may play a role in regulating PLCγ2 activity in the GPVI pathway via Btk (Pasquet et al., 1999a). PLCγ2 activation by GPVI signalling is dependent on phosphorylation of LAT at Tyr753 and Tyr759, which is partially mediated by Btk (Suzuki-Inoue et al., 2004). Moreover, collagen- and CVX-induced platelet activation is abrogated in PLCγ2 deficient platelets (Suzuki-Inoue et al., 2004).

1.4 Protease Activated Receptors (PARs)

PARs are 7-transmembrane G-protein coupled receptors (De Candia, 2012) found on a variety of vascular cells including platelets (Vu et al., 1991), endothelial cells (Ngaiza and Jaffe, 1991), neutrophils (Colotta et al., 1994), and fibroblasts (Rasmussen et al., 1991). Four PAR family members have been identified in humans and mice (Coughlin, 2000). PARs 1-3 are found on chromosome 5q13 (Bohm et al., 1996, Kahn et al., 1998a) and PAR4 is located on chromosome 19p12 (Xu et al., 1998). Human platelets express PAR1, PAR3 and PAR4, but not PAR2 (Lee and Hamilton, 2012). Around 1800 copies of PAR1 are expressed on human platelets (Brass et al., 1992) which is approximately 1.3 times higher than the number of PAR4 receptors (Kahn et al., 1999b).

1.4.1 PAR Activation

PAR activation occurs by proteolytic cleavage by a subset of serine proteases, principally thrombin, which unmask a new amino terminus that functions as a tethered ligand (see Figure 1.5) (Coughlin, 2000). PAR1 (Vu et al., 1991), PAR3 (Ishihara et al., 1997) and PAR4 (Xu et al., 1998) are cleaved by thrombin. PAR2 is cleaved by the proteases, trypsin (Nystedt et al., 1994), trypstatin (Molina et al., 1997), and factors VIIa and Xa (Camerer et al., 2000), but not thrombin. Alternatively, PARs can be non-proteolytically activated in vitro by the addition of activating peptides (APs) with sequences matching the tethered
ligand sequence. The PAR1 cleavage site is located between Arg41 and Ser42 (Loew et al., 2000) and the synthetic N-terminal activating peptide sequence is SFLLRN. Similarly, PAR4 is cleaved between Arg47 and Gly48 the synthetic N-terminal activating peptide sequence is GYPGKF (Xu et al., 1998).

PAR1 contains an amino-terminal exodomain (99 amino acids) which facilitates interaction with thrombin and enhances receptor recognition and cleavage (Vu et al., 1991). PAR4 has 33% amino acid sequence alignment with other PARs, although the extracellular amino-terminal and intracellular carboxyl terminus have little sequence similarity (Xu et al., 1998). Both PAR1 and PAR3 contain a hirudin-like sequence which allows binding to thrombin via exosite I (Coughlin, 1999a). In comparison, PAR4 requires a much higher concentration of thrombin for activation (Kahn et al., 1998b) and does not possess this hirudin-like sequence (Kahn et al., 1998b). PAR4 relies on substrate recognition alone for activation and can interact with thrombin through a unique cluster of anionic sites which slow the rate of thrombin dissociation (Jacques and Kuliopulos, 2003).

In platelets, PARs act both independently and synergistically. Human platelets are activated through the binding and cleavage of both PAR1 and PAR4 (Kahn et al., 1999b). In mouse platelets, PAR1 is absent (Kahn et al., 1998b) and PAR4 acts as the primary thrombin receptor (Hamilton et al., 2004). As a result, PAR4-deficient mice are completely unresponsive to thrombin (Hamilton et al., 2004). A dual receptor mechanism involving PAR3 and PAR4 is present in mouse platelets, whereby PAR3 acts a co-factor for PAR4 activation (Nakanishi-Matsui et al., 2000). PAR1 cleavage is facilitated by GPIbα which is also a receptor for thrombin (De Candia et al., 2001). In addition, through the α-thrombin-dependent formation of heterodimers, PAR1 enhances the cleavage of PAR4 (Arachiche et al., 2013). Additionally, PAR1 plays an important role in the generation of platelet procoagulant activity in the context of dual collagen and thrombin platelet activation (Keuren et al., 2005). PAR4, but not PAR1, supports thrombin-induced platelet aggregation via Ca2+ mobilisation and synergistic P2Y12 receptor activation (Holinstat et al., 2006).
Furthermore, PAR4 signalling is capable of overcoming PAR1 desensitisation in human platelets (Falker et al., 2011).
1.4.2 Signalling Through PARs

PAR1 and PAR4 both signal through Gα12 and Gαq, while PAR1 additionally signals through Gq/11 involving PI3-K (De Candia, 2012, Coughlin, 1999a, Faruqi et al., 2000, Macfarlane et al., 2001, Voss et al., 2007). Gαq-deficient mice are defective in platelet activation in response to thrombin, with IP3 production and Ca2+ mobilisation also absent, indicating a failure to activate PLCβ (Offermanns et al., 1997). PLC and SFKs are required to induce PKC phosphorylation downstream of thrombin activation (Hall et al., 2007). In this regard, the broad SFK inhibitor, PP2, partially inhibits thrombin-induced phosphatidylserine (PS) exposure (Topalov et al., 2012), but has no effect on PAR1- and PAR4-dependent platelet aggregation (Hughan et al., 2007, Xiang et al., 2012). Thrombin, through PAR1, leads to the activation of the small GTPase, Rac1 (Azim et al., 2000), and Rac1 is associated with and can be activated by PI3-K (Welch et al., 2003). PI3-K inhibition attenuates PAR1-induced aggregation (Nakanishi et al., 2010) but has no effect on PAR4-induced aggregation or ATP secretion (Ofose et al., 2009). However, inhibition of both PI3-K and PAR4 attenuate thrombin-induced aggregation (Wu et al., 2010). PKC negatively regulates Ca2+ release through PAR1, but not PAR4, and PKC inhibition potentiates thrombin-induced PS exposure (which requires a prolonged increase in Ca2+) and Ca2+ mobilisation (Harper and Poole, 2011). Interestingly, PARs demonstrate distinctive differences in generating intracellular Ca2+; PAR1 induces a response that is rapid and short-lived whereas PAR4 generates a steady prolonged Ca2+ influx following activation (Covic et al., 2000).

1.5 Reactive Oxygen Species (ROS)

ROS are highly reactive molecules derived as a by product of oxygen. Examples include superoxide anion (O2·−), hydrogen peroxide (H2O2), and hydroxyl radical (OH·) (Thannickal and Fanburg, 2000) (see Figure 1.6). The generation of ROS can occur via NOX activation (Krotz et al., 2002), xanthine oxidases (Miller et al., 1993), mitochondria (Hulsmans et al., 2012), endothelial nitric oxide synthase (Maron and Michel, 2012) and cytochrome p450 (Zangar et al., 2004). Physiologically, ROS are involved in the regulation of vascular tone,
cell growth, apoptosis and inflammation (Taniyama and Griendling, 2003). They can modify many biological molecules (e.g. lipids, proteins and DNA) and at high concentrations can result in oxidative stress which damages cells (Madamanchi et al., 2005). Oxidative stress is a well known characteristic of cardiovascular diseases such as atherosclerosis (Popolo et al., 2013), although ROS also play a role in hypertension (Dikalov and Ungvari, 2013), diabetes (Feng et al., 2013), cancer (Gorrini et al., 2013) and degenerative diseases (Zuo and Motherwell, 2013). In addition, ROS have been discovered to play a significant role in cellular signalling (Corcoran and Cotter, 2013, Finkel, 2000, Thannickal and Fanburg, 2000). ROS are commonly associated with phagocytic cells, where they provide a defence mechanism against invading microbes (Ray et al., 2012). Platelets are capable of producing ROS (Marcus et al., 1977), although the non-phagocytic nature of platelets suggests ROS are primarily involved in cellular signalling or amplification of platelet activation.
Figure 1.6 SOD and NO Compete in $O_2^-$ Scavenging

ROS ($O_2^-$) are produced by NADPH oxidase, xanthine oxidase, mitochondria and cytochrome p450. Both SOD and NO compete in $O_2^-$ scavenging. The rate of $O_2^-$ conversion to $H_2O_2$ catalysed by SOD is only one third of the reaction rate of $O_2^-$ with NO (Krotz et al., 2004). $H_2O_2$ undergoes the Fenton reaction to produce OH which subsequently leads to the production of $H_2O$, while NO produces peroxynitrite ($ONO_2^-$).
1.5.1 Sources of ROS

1.5.1.1 NADPH-oxidase (NOX)

The main source of ROS in platelets is through NOX (Begonja et al., 2005, Krotz et al., 2002, Leo et al., 1997, Walsh et al., 2014). The NOX complex, best characterised in phagocytes, consists of the membrane bound subunits gp91phox (catalytic subunit) and p22phox (regulatory subunit), and is activated by the cytosolic regulatory components, p47phox, p67phox, p40phox and Rac1/2 (GTP-binding protein) after their translocation to the membrane (Bedard and Krause, 2007). Deficiency in gp91phox, p47phox, p67phox and p22phox result in chronic granulomatous disease (CGD) (Holland, 2010). There are many isoforms of NOX (Bedard and Krause, 2007). In human platelets, both NOX1 and NOX2 have been identified (Dharmarajah et al., 2010, Pignatelli et al., 2004, Seno et al., 2001, Vara et al., 2013). In GPVI-dependent ROS formation, NOX1 has been identified as the main NOX isoform involved (Walsh et al., 2014). ROS are generated through the donation of an electron from NADPH to molecular oxygen (Babior, 2004). O$_2^-$ is a precursor of other ROS such as H$_2$O$_2$ and OH$^-$; catalysed through superoxide dismutase (SOD) and the Fenton reaction, respectively (Kamata and Hirata, 1999). Nitric oxide (NO) readily reacts with O$_2^-$, producing peroxynitrite (OONO$^-$) (Olas and Wachowicz, 2007). The rate of O$_2^-$ conversion to H$_2$O$_2$ catalysed by SOD is only one third of the reaction rate of O$_2^-$ with NO (Krotz et al., 2004). In platelets, NO and prostaglandin I$_2$ (PGI$_2$) function to keep platelets in a resting state by activating guanylyl cyclase and adenylate cyclase, respectively (de Graaf et al., 1992, Schafer et al., 1979). OONO$^-$ causes a decrease in bioavailability of NO (Essex, 2009), which subsequently results in increased platelet aggregation (Loscalzo, 2001). OONO$^-$ also induces oxidation of amino acids or nitration of tyrosine residues (Olas and Wachowicz, 2007) and can react with cysteinytl thiols to form S-nitrosothiol which can alter cellular signalling (Lima et al., 2010).
1.5.1.2 Arachidonic Acid Synthesis

An additional source of ROS production in platelets involves the arachidonic acid pathway (Caccese et al., 2000). PLA$_2$ activation catalyses the release of arachidonic acid from phospholipids and subsequently arachidonic acid is converted into 12-hydroxyeicosatetraenoic acid (HETE) via 12-lipoxygenase (12-LOX) (Brash, 2001). Additionally, arachidonic acid is converted into prostaglandins G$_2$ (PGG$_2$) and H$_2$ (PGH$_2$), via cyclooxygenase (COX), which is further converted into the unstable metabolite TxA$_2$ through thromboxane synthase (TxS) (see Figure 1.7) (Nakahata, 2008). It is not clear how arachidonic acid contributes to ROS formation in platelets but previous studies in other cell types have provided some insight. In a cell free system, arachidonic acid synergises with p47$^{phox}$ to directly activate NOX (Shiose and Sumimoto, 2000). In addition, the Ca$^{2+}$ and arachidonic acid binding protein, S100A8/A9, promotes NOX activation in phagocytes (Kerkhoff et al., 2005). In platelets, PLA$_2$ is required for O$_2^.$ generation through collagen and thrombin stimulation (Pignatelli et al., 2004). Separate pools of arachidonic acid are required for COX-dependent TxA$_2$ production and 12-LOX-dependent 12-HETE production in PAR-stimulated platelets (Holinstat et al., 2011). Inhibition of 12-LOX reduces thrombin-induced Ca$^{2+}$ mobilisation (Nyby et al., 1996). Further, PAR4-dependent aggregation and integrin $\alpha_{IIb}\beta_3$ activity is attenuated in 12-LOX$^{-/-}$ mice (Yeung et al., 2013). Previous reports have suggested that 12-HETE is required for NOX activation in the formation of platelet microparticles (Nardi et al., 2004). Additionally, hypertensive patients present with increased levels of 12-HETE (Gonzalez-Nunez et al., 2001). Recently, research from our laboratory has demonstrated NOX1 activation is required for GPVI-dependent TxA$_2$ formation in platelets (Walsh et al., 2014). COX is inhibited through the well known and affordable COX inhibitor, aspirin, which is used clinically worldwide for its analgesic, anti-inflammatory and anti-platelet properties (Catella-Lawson et al., 2001). However, aspirin can have allergic effects and exacerbate ulcers in the gut (Thiagarajan and Jankowski, 2012). Inhibitors of NOX1 may thus represent a novel alternate approach to anti-platelet therapy.
Figure 1.7 Arachidonic Acid Metabolism

PLA$_2$ is activated following platelet receptor ligation in a Ca$^{2+}$- and PKC-dependent manner. PLA$_2$ activation leads to arachidonic acid release resulting in the production of 12-HETE (via 12-LOX) (Brash, 2001) and prostaglandins G$_2$ (PGG$_2$) and H$_2$ (PGH$_2$) via COX. Arachidonic acid is further converted into the unstable metabolite thromboxane (Tx) A$_2$ through Tx synthase (TxS) (Nakahata, 2008). TxA$_2$ is involved in the secondary phase of platelet aggregation and in platelet recruitment to a growing thrombus. While the role of 12-HETE is not well known, it has been described as a marker for oxidative stress.
1.5.2 Platelet-derived ROS

Exogenous ROS can activate platelets and downstream signalling molecules (Del Principe et al., 1985, Pratico et al., 1991, Salvemini et al., 1989, Krotz et al., 2002). At present, GPVI is the main platelet receptor described in ROS formation, although \( \text{O}_2^\cdot \) can be generated through stimulation with either collagen or thrombin (Begonja et al., 2005). The role of ROS in platelet aggregation and thrombus formation is presently unclear with evidence both supporting either no role or a significant role. Initial platelet aggregation does not require \( \text{O}_2^\cdot \), but released \( \text{O}_2^\cdot \) enhances ADP-dependent recruitment of platelets when stimulated with collagen (Krotz et al., 2002). Additionally, GPVI-dependent ROS generation occurs independently of integrin \( \alpha_{IIb}\beta_3 \) activation (Arthur et al., 2012) and NOX1 inhibition has no inhibitory effect on CRP-induced platelet aggregation (Walsh et al., 2014). In contrast, CGD patients exhibit suppressed GPVI-dependent platelet recruitment (Pignatelli et al., 2010) and the ROS scavengers, diphenyleneiodonium and apocynin, prevent thrombus formation on collagen (Arthur et al., 2008). Further, SOD reduces secondary phase platelet aggregation and myocardial injury (Krotz et al., 2002, Arthur et al., 2008, Arthur et al., 2012) and similarly, the inhibition of NOX1 attenuates thrombus volume on collagen (Walsh et al., 2014). Additionally, natural dietary antioxidants also affect platelet activation. Polyphenols derived from olive oil reduce platelet adhesion and aggregation (Togna et al., 2003) and polyphenolic compounds including quercetin, apigenin and genistein inhibit thrombin-induced platelet signalling (Navarro-Nunez et al., 2009).

1.5.3 Signalling Molecules Involved in ROS Generation

In platelets, the signalling molecules involved in ROS generation are not well defined. TRAF4, an adaptor protein essential for endothelial cell ROS production in focal adhesions, is bound to the cytoplasmic tail of GPVI and is in a complex with \( \text{p47}^{\text{phox}} \), Hic-5 and Pyk2 (Arthur et al., 2011). Syk and PLC\( \gamma \)2 are activated prior to ROS generation in platelets activated by oxidised low density lipoproteins (Wraith et al., 2013). PI3-K has been described as a prerequisite for NOX activation in platelets stimulated with the PAR1-activating peptide, thrombin receptor activating peptide (TRAP) (Clutton et al., 2004).
Quercetin, a flavonoid antioxidant, found in green tea and red wine, inhibits GPVI-dependent platelet activation and FcRγ, Syk and PI3-K phosphorylation (Hubbard et al., 2003).

1.6 The Focal Adhesion Kinase (FAK) Family

Pyk2 (also known as related adhesion focal tyrosine kinase (RAFTK) (Avraham et al., 1995), cell adhesion kinase (CAK)β (Sasaki et al., 1995) or Ca\(^{2+}\)-dependent protein tyrosine kinase (CADTK) (Yu et al., 1996)) and focal adhesion kinase (FAK) (Schaller et al., 1994) are the only two known members of the FAK family and are non-receptor protein tyrosine kinases (PTKs) (Avraham et al., 2000). FAK is ubiquitously expressed and is commonly located within focal adhesions (Zheng et al., 1998). In contrast, Pyk2 is located mainly in hematopoietic cells and neuronal lineages (Avraham et al., 1995) and is diffuse throughout the cytoplasm (Zheng et al., 1998). Pyk2 (110 kDa) and FAK (125 kDa) share 45% identity and 65% homology (Avraham et al., 1995, Mitra et al., 2005) (see Figure 1.8). Pyk2 differs from FAK in that it is a Ca\(^{2+}\)-dependent kinase (Lev et al., 1995). Pyk2 and FAK are activated by a broad range of stimuli, including in response to platelet-derived growth factor, integrin activation, cytokine stimulation, through GPCRs, and by stress signals such as osmotic shock, UV light and tumour necrosis factor α (Avraham et al., 2000, Mitra et al., 2005). FAKs are involved in regulating the cytoskeleton (Schaller, 2010) and act as scaffolds for cellular signalling (Schlaepfer et al., 1999). In other cell systems than platelets, Pyk2 and FAK are known to be involved in intracellular ROS-dependent signalling (Gozin et al., 1998, Matsui et al., 2007).
Figure 1.8 Structures of the Focal Adhesion Kinase family members: FAK and Pyk2 (Adapted from Schlaepfer et al., 1999)

Pyk2 and FAK comprise a N-terminal containing FERM domain, flanked by a central kinase domain, a C-terminal containing a FAT domain and proline-rich regions. Phosphorylation occurs at Tyr397 on FAK and at Tyr402 on Pyk2. Phosphorylation allows the binding of SH2 domain containing proteins. The FAK FAT domain binds the cytoskeletal proteins Hic-5, paxillin and talin. Pyk2 does not bind talin. Sequence similarity is demonstrated through colour coding.
1.6.1 Structure of FAKs

Focal adhesion kinases lack SH domains but contain an N-terminal band four point one ezrin, radixin, moesin homology (FERM) domain, a central catalytic domain, a C-terminal domain containing proline-rich regions, and a focal adhesion target (FAT) domain which acts as a docking site and allows binding of signalling partners (Han et al., 2009). The FERM domain regulates catalytic activity (Frame et al., 2010), subcellular localisation, cell morphology and cellular migration (Dunty and Schaller, 2002). The N-terminal domain contains a 5-stranded anti-parallel β sheet and a single α-helix and the C-terminal lobe is mostly α-helical and contains an activation loop involved in substrate binding (Han et al., 2009). Within this activation loop lies an Asp-Phe-Gly (DFG) sequence which is required for enzyme activity and allows the binding of ATP. The C-terminal domains are responsible for differential regulation by integrins and soluble stimuli and for subcellular localisation (Zheng et al., 1998). The FAK C-terminal FAT domain mediates interaction with integrins and focal contacts (Mitra et al., 2005). Additionally, both FAK and Pyk2 C-terminal domains contain proline-rich regions which allow them to interact with SH2 domain containing proteins (Schlaepfer et al., 1999). Four major tyrosine sites have been identified for activation on FAK, Tyr397, Tyr407, Tyr576 and Tyr577, although maximal kinase activity requires both phosphorylation of Tyr576 and Tyr577 (Calalb et al., 1995). Tyr397 is required for FAK autophosphorylation and can be cis- or trans-acting (Zheng et al., 1998). The Pyk2 C-terminus is not required for autophosphorylation of Pyk2; instead the autophosphorylation site, Tyr402, is proximal to the kinase domain and is trans-acting (Park et al., 2004).

1.6.2 FAK Family Members and Their Substrates

Pyk2 contains a binding sequence for calmodulin, which is located within the α-helix (amino acid residues 135-260) in the FERM domain (Kohno et al., 2008). FAK has been previously described to associate with the SFKs, c-Src and Fyn, through Tyr397 (Cary et al., 1996, Cobb et al., 1994). Pyk2 activates Src through Tyr402 (Dikic et al., 1996). FAK Tyr397 binds the SH2 domains of the p85 subunit of PI3-K (Chen et al., 1996) and in thrombin-
stimulated platelets, Pyk2 is associated with PI3-K through interaction with Tyr402 (Sayed et al., 2000). In hepatic cells, PLCγ is recruited by FAK during adhesion (Carloni et al., 1997), whilst FAK promotes PLCγ1 activity in fibroblasts through a direct interaction between the autophosphorylation site on FAK and the SH2 domain of PLCγ1 (Zhang et al., 1999). Pyk2 directly associates with the SH2 domains of PLCγ2 in osteoclasts (Nakamura et al., 2001). FAK is well characterised for its role in the reorganisation of the cytoskeleton in platelets as a docking protein that associates with the cytoskeletal proteins, talin (Chen et al., 1995), paxillin (Salgia et al., 1996), Hic-5 (Fujita et al., 1998) and p130cas (Polte and Hanks, 1995). Pyk2 associates with both p130cas and paxillin (Keogh et al., 2002), although no association has been found for talin (Zheng et al., 1998). Hic-5 is also localised in focal adhesions and has been shown to associate with Pyk2 in platelets (Osada et al., 2001). Hic-5 translocates to the cytoskeleton upon platelet activation and is tyrosine phosphorylated dependent on integrin αIIbβ3-mediated platelet aggregation (Osada et al., 2001). Members of the GTPase Rho family, such as Rac and Cdc42, also play a prominent role in actin remodelling (Bishop and Hall, 2000) and in addition, FAK regulates the actin cytoskeleton through Rac1 (Hsia et al., 2003).

1.6.3 FAKs and Platelets

In platelets, FAK plays an important role in spreading, aggregation, intracellular Ca2+ mobilisation and dense granule release (Jones et al., 2009). The absence of FAK expression in a developing mouse embryo is lethal to embryonic development (Hitchcock et al., 2008). Mice deficient in platelet FAK exhibit increased bleeding times and exhibit poor spreading on fibrinogen and CRP (Hitchcock et al., 2008). Pyk2 is required for thrombus formation and fibrinogen binding in response to α4β1-induced platelet activation and thrombin stimulation (Consonni et al., 2012). Pyk2 is rapidly activated by the majority of physiological platelet agonists through an aggregation- and integrin αIIbβ3-independent mechanism (Ohmori et al., 2000, Raja et al., 1997, Sayed et al., 2000), and regulates integrin αIIbβ3 inside-out signalling (Cipolla et al., 2013). In comparison, FAK is described as an integrin αIIbβ3-dependent kinase (Lipfert et al., 1992, Shattil et al., 1994). Pyk2 KO mice
display normal behaviour, and are viable and fertile (Okigaki et al., 2003). Pyk2 deficient platelets display prolonged tail bleeding times, normal platelet count and no significant differences in platelet glycoprotein expression, although they display severely impaired aggregation in response to thrombin and PAR4-AP stimulation (Canobbio et al., 2013). Inside out activation of integrin α<sub>IIb</sub>β<sub>3</sub> is ablated in thrombin-stimulated Pyk2 KO platelets and Pyk2 is essential for TxA<sub>2</sub> generation (Canobbio et al., 2013).

1.6.4 FAKs and ROS

In endothelial cells, ROS regulates and activates FAK (Gozin et al., 1998, Usatyuk and Natarajan, 2005, Vepa et al., 1999) whilst Pyk2 is activated via ROS production through shear stress (Tai et al., 2002). Pyk2 can be activated by inducers of ROS such as diamide, an oxidising agent, and its activation is significantly inhibited by the antioxidant, N-acetyl-cysteine and the ROS scavengers, apocynin and ebselen (Tai et al., 2002, Arthur et al., 2011, Frank et al., 2000, Lysechko et al., 2010). Furthermore, Pyk2 KO mice display NO deficiency (Matsui et al., 2007). Recent studies have shown an association of TRAF4 with GPVI, in a novel potential mechanism for ROS production involving Hic-5 and Pyk2 (Arthur et al., 2011). TRAF4 specifically binds the membrane proximal cytoplasmic sequence of GPVI and is in a complex with Hic-5, Pyk2 and p47<sup>phox</sup>. In collagen stimulated platelets, FAK is activated upstream of the redox signalling molecule, p21 activated kinase (Jones et al., 2009). In this regard, platelet-derived ROS production may involve a role for FAK and/or Pyk2 downstream of GPVI activation.

1.7 Scope of Thesis

ROS production in platelets is well described although the overall relevance to platelet function is currently poorly defined. Based on available evidence, targeting ROS generation, or more specifically the signalling molecules that lead to ROS production in platelets, could potentially lead to novel therapies for the treatment of cardiovascular diseases such as heart attack and stroke. The complex involving TRAF4, p47<sup>phox</sup>, Hic-5 and
Pyk2 associated with GPVI suggests a role for either FAK and/or Pyk2 in GPVI-dependent ROS production. Furthermore, p47phox is indirectly associated with GPIbα, through TRAF4 and GPIbβ, suggesting that GPIbα signalling could potentially lead to platelet-derived ROS formation. Furthermore, the physiological agonist thrombin has been previously shown to induce ROS in platelets although it is unknown which of the three thrombin receptors on platelets (GPIbα, PAR1 and PAR4) are involved. The main aims for this thesis were therefore i) to distinguish the key signalling molecules involved in GPVI-, GPIbα-, and PAR-dependent ROS formation, ii) to determine the role of FAK family members in GPVI-dependent ROS formation, iii) to delineate where FAK lies within the GPVI signalling pathway, and iv) to determine the roles of GPIbα, PAR1 and PAR4 in thrombin-induced ROS formation.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 General Reagents

Ammonium persulphate, citric acid, D-glucose, fatty acid-free bovine serum albumin (BSA), glycine, HEPES, magnesium chloride (MgCl₂), monosodium phosphate (Na₃PO₄), sodium chloride (NaCl), sodium dodecyl sulphate (SDS), Tris base, tri-sodium citrate, and Tween-20 were all from Fisher Scientific (Dublin, Ireland).

Brilliant blue R, β-mercaptoethanol, calcium chloride (CaCl₂), dimethyl sulphoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), potassium bicarbonate (KHCO₃), sodium bicarbonate (NaHCO₃), sodium hydroxide (NaOH), tetramethylethlenediamine (TEMED), and Triton X-100 were from Sigma Aldrich (St. Louis, MO, USA).

Sodium phosphate was from Acros Organics (USA). Alexa fluor® 488 phalloidin was obtained from Invitrogen Corporation (Carlsbad, CA, USA). White 96-well plates and Poly-L-lysine slides were from Thermo Scientific (USA). Cover-slips were from VWR International (Dublin, Ireland). Polyvinylidene fluoride (PVDF) transfer membrane was from Millipore (Lake Placid, NJ, USA). Flat bottom aggregation tubes were obtained from BioData® Corporation (PA, USA).

RGD peptide was from Santa Cruz (CA, USA). N-acetyl cysteine (NAC) was from Calbiochem (UK). BAPTA (intracellular Ca²⁺ chelator), indomethacin and prostaglandin E₁ (PGE₁) were from Tocris Bioscience (R&D Systems Europe, UK).

All other reagents were of analytical grade or the best grade available commercially.
2.1.2 Antibodies

Mouse anti-FAK, rabbit anti-Pyk2, mouse anti-phosphotyrosine (clone 4G10), HRP-conjugated goat anti-mouse and mouse anti-rabbit light chain antibodies were all obtained from Millipore (Lake Placid, NJ, USA). Normal rabbit and mouse IgG were from Santa Cruz (CA, USA). Mouse anti-PLCγ2 was from Cell Signaling Technology, Inc. (Boston, MA, USA). Mouse anti-Rac1 was from Tebu-Bio (Peterborough, UK). Anti-GPⅠbα (AK2) and anti-VWF (5D2) were produced as previously described (Chong et al., 1989, De Luca et al., 2000); non-relevant isotype IgG2 was from BD Pharmingen (Oxford, UK). Rat anti-mouse GPⅠbα (Xia.G5) IgG2B and rat IgG2B isotype (both FITC conjugated) were obtained from Emfret (Würzburg, Germany).

2.1.3 Platelet Agonists and Antagonists

Cross-linked collagen related peptide (CRP) was purchased from Prof. Richard Farndale (Dept of Biochemistry, Cambridge University, UK). Thrombin was from Calbiochem (UK). PAR1 (TRAP: SFLLRN-NH$_2$) and PAR4 (PAR4-AP: AYPGKF-NH$_2$) agonists were from Abgent Europe (Oxfordshire, UK). Nk protease (a GPⅠbα-specific cleavage enzyme) was a generous gift from Prof. Robert Andrews (Australian Centre for Blood Disease, Monash University, Melbourne, Australia).
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2.2 Methods

2.2.1 Platelet Preparation

2.2.1.1 Isolation of Human Platelets from Whole Blood

Experiments performed at the Royal College of Surgeons in Ireland (RCSI) were approved by the institution’s Medical Research Ethics Committee (REC 676). Experiments performed at the Australian Centre for Blood Diseases, Monash University were approved by the institution’s Standing Committee on Ethics in Research Involving Humans, in accordance with the Declaration of Helsinki. Venous whole blood was drawn from drug-free healthy volunteers using 15% v/v acid citrate dextrose (ACD – 38 mM citric acid, 75 mM sodium citrate, 124 mM glucose) as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 190g for 20 minutes with no brake.

2.2.1.2 Preparation of Washed Human Platelets

Platelets were isolated from PRP by centrifugation for 8 minutes at 650g with no brake, resuspended gently (3x) in CGS buffer (123 mM NaCl, 33.3 mM glucose, 14.7 mM trisodium citrate, pH 7.0). Additionally, platelets to be used for immunoprecipitation studies were treated with 1 μM PGE$_3$ during preparation, pelleted by centrifugation and rested for 30 minutes prior to use. Platelets were resuspended to the required concentration in HEPES Tyrodes buffer (5 mM HEPES, 5.5 mM glucose, 138 mM NaCl, 12 mM NaHCO$_3$, 0.49 mM MgCl$_2$, 2.6 mM KCL, 0.36 mM NaH$_2$PO$_4$, pH 7.4) and supplemented with 1 mM CaCl$_2$ prior to experimentation.

2.2.1.3 Preparation of Washed Murine Platelets

Mouse blood was anticoagulated in ACD and prepared as previously described (Consonni et al., 2012). Briefly, approximately 900 μL of blood was drawn from the vena cava of CO$_2$ euthanised mice using a 23-gauge needle and 1 mL syringe containing 100 μL 15% ACD (v/v). Blood was further diluted with 200 μL of ACD and 800 μL of CGS buffer and
centrifuged at 190g for 10 minutes with no brake at room temperature. Platelet-rich plasma (PRP) was transferred to a new tube and the residual blood was diluted up to 1.8 mL with CGS buffer and re-centrifuged to obtain additional PRP. Platelets were isolated from PRP by centrifugation for 10 minutes at 650g, resuspended in modified HEPES Tyrodes buffer and maintained at 37°C before use.

Studies using platelets from Pyk2 knock out (KO) and PAR4 KO mice were performed at the University of Pavia (Dept. of Biochemistry, Italy) and at the Australian Centre for Blood Diseases (ACBD, Monash University, Melbourne, Australia), respectively. Blood was drawn by approved personnel, with approval from corresponding local Ethics Committees. Platelets were prepared as above with one noted exception: mice were euthanised with ether in an airtight glass jar at the University of Pavia, whilst mice were overdosed with pentobarbitone (60 mg/kg) at the ACBD prior to blood collection. The generation and characterisation of the Pyk2 KO and PAR4 KO mice have been previously described (Hamilton et al., 2004, Matsui et al., 2007). Age- and sex-matched wildtype (WT) littermates were used as controls.

Mice (WT, C57BL/6J background) and Btk KO used for experimentation at the RCSI were purchased from Harlan Laboratories (UK) and housed in a pathogen-free designated animal facility. Blood was drawn from the inferior vena cava of CO2 terminally-asphyxiated mice, by approved personnel, with approval from the local Ethics Committee.

2.2.2 Platelet Aggregation

Platelet aggregation experiments were performed using either a PAP 4-C aggregometer (BioData® Corporation, PA, USA) or a ChronoLog® lumiaggregometer (Havertown, PA, USA) using washed platelets (2.5 x 10^6/mL) under constant stirring at 900-1100 rpm at 37°C. For all inhibitor studies, platelets were pre-incubated with vehicle control or antagonists for 10 minutes at 37°C before the addition of an agonist.
2.2.3 Thromboxane (Tx) A₂ Detection

Washed human platelets (2.5 x 10⁸/mL) were incubated with antagonists for 15 minutes prior to stimulation with thrombin at 0.05 U/mL or 1 U/mL. Aggregation samples were stopped at 6 minutes with 5 mM EDTA and 200 µM indomethacin to prevent further TxA₂ formation. Samples were transferred to microcentrifuge tubes and centrifuged for 3 minutes at 13,000g, with the supernatant aliquoted and stored at -80°C until used. Platelet TxA₂, through measurement of its stable metabolite TxB₂, was analysed with a commercial ELISA kit (Enzo Life Sciences, Exeter, UK) according to the manufacturer’s instructions. Sample dilutions were 1:50 with HEPES Tyrodes.

2.2.4 Platelet Secretion

2.2.4.1 ATP Secretion

ATP release from dense granules was a modification of the method described by Lombardi et al. (Lombardi et al., 2012) and measured using Chrono-Lume® luciferin-luciferase reagent (Chrono-log corporations, Havertown, PA, US). In brief, washed platelets (5 x 10⁷/mL) were pre-incubated with antagonists for 10 minutes at 37°C. Platelets were then added to a 96-well plate containing agonist or buffer (HEPES Tyrodes) as a negative control and incubated for a further 10 minutes at 37°C. Chrono-Lume® luciferin-luciferase reagent was added (10 µL to 90 µL platelets/agonist/agonist mixture) and shaken for 2 minutes at medium speed with orbital shake. Luminescence was measured using a Wallac Victor® 1420 multilabel plate-reader.

2.2.4.2 P-selectin Expression

To measure α-granule release, washed platelets (2.5 x 10⁸/mL) in HEPES Tyrodes (0.1% BSA) were pre-treated with antagonists and PE-labelled mouse anti-human CD62P/mouse IgG1 Isotype (BD Biosciences, UK) to detect surface P-selectin exposure, then stimulated with 1 µg/mL CRP for 10 minutes. Reactions were diluted in 900 µL HEPES Tyrodes (0.1%
BSA) and analysed on a FACSCanto™ using 488 nm laser excitation and FL2 (575 nm band pass) emission for PE detection.

2.2.5 Fibrinogen Binding

Washed platelets (2.5 x 10⁸/mL) were stained with Oregon Green® 488 conjugated-fibrinogen (Biosciences, Ireland) for 10 minutes at 37°C in FACS tubes. CRP (1 µg/mL) was added and incubated for a further 10 minutes at 37°C. Reactions were diluted in 900 µL HEPES Tyrodes (0.1% BSA) and analysed on a FACSCanto™ using 488 nm laser excitation and a FL1 (525 nm band pass) emission filter.

2.2.6 Phosphatidylserine (PS) Exposure

To measure platelet pro-coagulant capacity, washed platelets (2.5 x 10⁸/mL) in HEPES Tyrodes (0.1% BSA) were pre-treated with antagonists and either FITC-labelled Annexin V (BD Pharmingen) to detect PS exposure, then stimulated with 1 µg/mL CRP for 10 minutes. Reactions were diluted in 900 µL HEPES Tyrodes (0.1% BSA) and analysed on a FACSCanto™ using 488 nm laser excitation and FL1 (525 nm band pass) for FITC detection.

2.2.7 Platelet Spreading

Poly-L-lysine slides were coated with 1 µg/mL CRP for 3 hours at room temperature, then blocked in 1% BSA for 1 hour with subsequent washing in Tris buffered saline (TBS – 10 mM Tris-HCl, 0.15 M NaCl, pH 7.4). Washed platelets (2 x 10⁷/mL), pre-incubated with vehicle control or antagonists, were allowed to adhere to pre-coated slides for 45 minutes at 37°C. Platelets were then washed, fixed in 3.7% paraformaldehyde for 10 minutes at room temperature, permeabilised with 0.2% Triton X-100 and stained with FITC-phalloidin (1 in 100 dilution of original commercial stock). Imaging was performed using a Zeiss 63x oil immersion 1.40 numerical aperture plan-apochromat lens on a Zeiss Axiovert 200M microscope with digital images captured by an Andor Xion cooled digital camera using
Metamorph 7.5 imaging software. Platelet spreading was quantified using the Image J® software package.

2.2.8 Confocal Microscopy

Sterile glass bottom culture dishes (Cruinn, Dublin, Ireland) were coated with 1 µg/mL CRP overnight at 4°C and then blocked in 1% BSA for 1 hour with subsequent washing in HEPES Tyrodes Ca²⁺ buffer. Washed platelets (2 x 10⁷/mL) were incubated for 20 minutes at 37°C with 10 µM of the cell-permeable dye, dihydrodichlorofluorescein diacetate (H₂DCFDA-Cambridge Bioscience, UK), and then allowed to adhere to pre-coated slides for 45 minutes at 37°C. Imaging was performed using a Zeiss 63x oil immersion 1.40 numerical aperture (NA) plan-apochromat lens on a Zeiss Axiovert 200M microscope with digital images captured by an Andor Xion cooled digital camera using Metamorph 7.5 imaging software. Zeiss LSM510 Meta confocal microscope. A 488 nm laser, at 1% intensity was employed for the analysis of platelets stained with H₂DCFDA (FITC). A 63x oil-immersion objective was used when obtaining confocal images. A bandpass 505-550 filter was used, along with a pinhole of 90 µm.

2.2.9 Measurement of Reactive Oxygen Species (ROS)

2.2.9.1 Intracellular ROS Measurement

Intracellular ROS measurement was quantified as previously described, with modifications (Arthur et al., 2012). Briefly, washed platelets (2.5 x 10⁸/mL) in HEPES Tyrodes were incubated for 30 minutes at 37°C with 10 µM of the cell-permeable dye, H₂DCFDA (Cambridge Bioscience, UK). Dye-loaded platelets (80 µL) were transferred to FACS tubes containing vehicle controls or antagonists and incubated for 10 min at 37°C prior to stimulation. Agonists were added for a further 10 minutes at 37°C and the reaction was then terminated by 10-fold dilution with buffer, HEPES Tyrodes (1% BSA + 10µM H₂DCFDA). Samples were covered with foil and read immediately on a FACS Canto™.
quantify ROS, fluorescent geomean values were normalised relative to unstimulated levels (ROS stimulation index). All ROS data in chapter 4 was measured using this ROS method.

2.2.9.2 Modified Intracellular ROS Measurement

Intracellular ROS measurement was quantified as previously described, with modifications (Arthur et al., 2012). Washed platelets (2.5 x 10^8/mL) in HEPES Tyrodes were incubated for 20 minutes at 37°C with 10 μM of the cell-permeable dye, H₂DCFDA (Cambridge Bioscience, UK). Dye-loaded platelets (100 μL) were transferred to eppendorf tubes in comparison to FACS tubes as described above and kept at a constant temperature of 37°C. Platelets were incubated with vehicle control or antagonists for 10 minutes at 37°C prior to stimulation. Ten μL of the sample was then removed and added to a FACS tube containing 500 μL HEPES Tyrodes (0.1% BSA + 10 μM H₂DCFDA) and read as time zero (i.e. unstimulated platelets). Ten μL of agonist was added to the remaining 90 μL of dye-loaded platelets and incubated for a desired period (30 seconds, 2 minutes, 5 minutes, 10 minutes). Following incubation with agonist, 10 μL of the reaction was diluted in 500 μL HEPES Tyrodes (1% BSA + 10 μM H₂DCFDA) in a FACS tube and analysed instantly on a FACSCanto™. To quantify ROS, fluorescent change fold values were normalised relative to unstimulated levels (ROS stimulation index). All ROS data in chapters 3, 5 and 6 were measured using this method.

N.B. In some cases, due to donor specificity, change fold values were normalised, where vehicle control was 100%, to show the effect of the drugs (% ROS production).

2.2.9.3 Extracellular ROS Measurement

Extracellular ROS measurement was quantified as previously described (Bakdash and Williams, 2008). Briefly, washed platelets (2.5 x 10^5/mL) in HEPES Tyrodes were incubated with a mixture of luminol (50 μM) and HRP (1 U/mL) in a 96-well plate. Following the
addition of agonists, luminescence was measured for 20 minutes, at 1 minute intervals, using a Wallac Victor® 1420 multilabel plate-reader (PerkinElmer, MS, USA).

2.2.10 Mitochondrial Potential/Uncoupling

To test for non-specific inhibitor-mediated alterations in the mitochondrial potential, platelets (2.5 x 10^8/mL) were incubated with the cationic, fluorescent dye, JC-1 (Millipore, Lake Placid, NJ, USA), at a final concentration of 1 µg/mL (1 minute at 37°C). Antagonists were then added for 5 minutes and the reaction was terminated by the addition of HEPES Tyrodes. Mitochondrial uncoupling was measured by FACSCanto™ by a decrease in the red/green fluorescence intensity ratio.

2.2.11 Nk Treatment of Platelets

Platelets (1 x 10^9/mL) were resuspended in HEPES Tyrodes buffer with Ca^{2+} and incubated with or without 10 µg/mL of Nk protease (a kind gift from Prof. Robert Andrews, Monash University) for 30 minutes at 37°C. Platelets were washed with CGS and centrifuged at 650g for 8 minutes with no brake and resuspended in HEPES Tyrodes buffer with Ca^{2+} at 2.5 x 10^8/mL.

2.2.12 Measurement of GPIbα Cleavage

Washed human platelets (2.5 x 10^9/mL) treated with or without Nk protease (10 µg/mL) were incubated with the PE-labelled GPIbα-specific antibody (2 µg/mL AN51) or isotype control (2 µg/mL) for 15 minutes at 37°C, then diluted 100-fold in HEPES Tyrodes and measured for intact GPIbα content on a FACSCanto™.
2.2.13 Analysis of COS-7 Cells Expressing VWF-A1 Domain

COS-7 cells stably expressing the VWF-A1 domain containing an R543W mutation (a gain-of-function mutation found in Type 2B von Willebrand's Disease), hereafter designated as R543W cells, and wild-type COS-7 cells (no mutation) were kindly provided by Prof. Robert Andrews (ACBD, Monash University). These cells have been described previously (Gardiner et al., 2010). Levels of VWF-A1/R543W on transfected COS-7 cells were assessed by flow cytometry. Cells were harvested using TBS containing 10 mM EDTA, pelleted, washed and resuspended (2.5 x 10^7 cells/mL) in HEPES Tyrodes buffer. Cells were pre-incubated with either 10 μg/mL of anti-VWF (5D2) or 20 μg/mL anti-GPlbα (AK2, negative control) for 1 hour at room temperature, washed, incubated for a further 30 minutes with anti-mouse FITC secondary antibody, washed, and resuspended in 200 μL HEPES Tyrodes for analysis on a FACSCalibur™. All COS-7 cell work was performed at the ACBD, Monash University, Melbourne, Australia.

2.2.14 Superoxide Anion (O2−)

This cell-free assay, adapted from Kunchandy et al. (Kunchandy E., 1990), was used to determine whether the pharmacological compounds employed in this study acted as O2− scavengers. All antagonists were diluted to final concentrations with 100% DMSO in a clear 96-well plate. Alkaline DMSO (900 μL DMSO + 1% 1 M NaOH in dH2O) and 0.25 mg/mL methylthiazolyldiphenyl-tetrazolium (MTT – Sigma Aldrich, St. Louis, MO, USA) were then added. Non-alkaline DMSO (100 μL) was immediately added to terminate the reaction and absorbance (570 nm) was measured using a Wallac Victor® 1420 multilabel plate-reader (PerkinElmer, MS, USA).

2.2.15 Immunoprecipitation

For detection of tyrosine phosphorylated FAK, PLCγ2, and Pyk2, stimulated platelets (1 x 10^9/mL) were lysed in 10X lysis buffer (final concentration: 1% Triton X-100, 20 mM Tris, 5
mM EGTA, pH 7.4) containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, IL, USA). Lysis proceeded for 30 minutes on ice, with subsequent clarification (10 minutes at 16,000g) and pre-clearance with either 15 µL Pansorbin® (10% cell suspension-Calbiochem) or 70 µL Protein A agarose beads (Millipore, Lake Placid, NJ, USA) for 1 hour at 4°C. Appropriate antibody/IgG (4 µg) control was added to the pre-cleared supernatant, which was left rotating at 4°C overnight. Then, 25 µL Pansorbin® or 100 µL Protein A agarose beads were added to each sample for 2 hour at 4°C. Antigen-absorbed Pansorbin® or Agarose beads were harvested (5 min at 1,000g), washed 3 times with 1X lysis buffer or 0.1% Triton X-100 in TBS and heated to 100°C in 2X SDS sample loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.125 M Tris, 0.1% (w/v) bromophenol blue + 5% (w/v) β-mercaptoethanol) for 10 minutes.

2.2.16 SDS Gel Electrophoresis SDS-PAGE

Proteins were resolved by SDS 5-20% polyacrylamide gel electrophoresis. Exponential gradient gels were prepared by mixing a 5% acrylamide gel solution (50 mM Tris (pH 8.8), 1% (w/v) SDS, 100 µL 10% (w/v) ammonium persulphate, 18 µL TEMED, 13.95 mL dH₂O and 3.75 mL 30% bis-acrylamide) and a 20% acrylamide gel solution (150 mM Tris (pH 8.8), 0.1% (w/v) SDS, 50 µL 10% (w/v) ammonium persulphate, 8 µL TEMED, 0.4 mL dH₂O and 6.7 mL 30% bis-acrylamide) in a 3:1 (vol:vol) ratio using a gradient former and a peristaltic pump as previously described (Walker, 2002). To level the acrylamide gels, water-saturated butanol was carefully placed on top and the gels were left to polymerise for 30 minutes. Butanol was then washed off using dH₂O and stacking acrylamide gel (0.5 M Tris (pH 6.8), 0.4% (w/v) SDS) was poured and left to polymerise for 30 minutes.

2.2.16.1 Protein Separation

Gradient gels (were placed into a Hoefer™ tank (Fisher Scientific, Ireland) containing 1X running buffer (25 mM Tris, 190 mM glycine and 0.1% (w/v) SDS pH 8.3) and protein samples were carefully loaded into each well. Page Ruler™ Plus Prestained protein ladder
(Thermo Scientific, USA) was used to monitor protein migration. Electrophoresis was performed overnight at room temperature at a constant voltage of 60 V (for two gels).

2.2.16.2 Protein Transfer and Western Blotting

Gels were removed from glass plates into transfer cassettes (Hoefer™, Fisher Scientific, Ireland) as per the manufacturer’s instructions and proteins transferred to a PVDF membrane. Protein transfer was performed at 65 V in 1X transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol) for 1.5 hours using a MultiTemp™ III cooling tank (Pharmacia Biotech). PVDF membranes were briefly rinsed in TBS and immediately blocked with 3% (w/v) BSA or 3% powder skimmed milk in TBS for 1 hour and then probed with appropriate antibodies diluted in 3% (w/v) BSA or powder skimmed milk in TBS, 0.1% Tween-20 (TBST) overnight at 4°C. The blots were then washed in TBS containing 0.2% Tween-20 (wash buffer), three times for 10 minutes on a rocker and then incubated with HRP-conjugated secondary antibodies diluted in 3% (w/v) BSA or powder skimmed milk in TBST for 1-2 hours at room temperature. Blots were rinsed three times in wash buffer and further washed 3 times for 15 minutes in wash buffer. BSA was used exclusively for detection of phosphorylated target proteins, while powder skimmed milk was utilised in all other Western blotting experiments.

2.2.16.3 Developing Western Blots

After probing with specific antibodies, washed PVDF membranes were incubated with enhanced chemiluminescence ECL substrate (SuperSignal West Pico, Thermo Scientific) for 5 minutes at room temperature and developed using Amersham Hyperfilm™ (GE Healthcare Ltd, UK) and Kodak® GBX developer and fixer solutions (Sigma Aldrich, St. Louis, MO, USA) in a dark room.
2.2.16.4 Densitometry of Blots

The ECL exposed film was scanned at high resolution (300dpi) for densitometry analysis using Image J® software. The intensity of each specific protein band was converted to density level (an arbitrary numerical unit value) and normalised to loading control signal of the same sample. The change in protein activity (e.g. phosphorylation) was determined by comparing normalised density level of target protein from stimulated and non-stimulated samples.

2.2.17 Statistical Significance

All statistical analysis was performed using GraphPad® Prism 5. Results are shown as mean ± SEM. Statistical significance of difference between means was determined using one-way ANOVA, with post-hoc analysis by the Bonferroni test or Student’s paired t-test as appropriate. A p-value ≤ 0.05 was considered to be statistically significant.
Chapter 3

Evaluating the signalling pathway involved in GPVI-dependent ROS formation
3.1 Introduction

GPVI is the main signalling collagen receptor on platelets (Kehrel et al., 1998) and is involved in stable thrombus formation (Savage et al., 1998, Penz et al., 2005, Cosemans et al., 2005). A complex cascade of signalling events follows ligation of GPVI which result in platelet aggregation, secretion, spreading and integrin $\alpha_{\text{IIb}}\beta_3$ activation (Watson et al., 2005). Another characteristic of platelet activation, although less described, is the generation of ROS (Krotz et al., 2004). Presently, GPVI is described as the main receptor involved in platelet-derived ROS production (Bakdash and Williams, 2008, Krotz et al., 2002, Pignatelli et al., 2004, Arthur et al., 2012, Vara et al., 2013). Whilst, the signalling events downstream of GPVI activation are well characterised (Dutting et al., 2012), the mechanism leading to ROS formation is poorly defined.

The main source of ROS in platelets is through the activation of NOX (Begonja et al., 2005, Pignatelli et al., 2010, Vara et al., 2013). While both NOX1 and NOX2 are expressed in platelets (Vara et al., 2013), previous work in our laboratory has shown that NOX1 is involved in GPVI-dependent ROS formation (Walsh et al., 2014). Patients with chronic granulomatous disease (CGD), who lack subunits of the NOX complex, display little or no ROS formation following collagen stimulation (Pignatelli et al., 2004). Similarly, inhibitors of the gp91$^{phox}$ subunit, involved in NOX complex formation, inhibit GPVI-dependent thrombus formation (Pignatelli et al., 2010). The NOX1-specific inhibitor, ML171, dramatically attenuates GPVI-dependent $\text{TxA}_2$ generation and thrombus formation (Walsh et al., 2014). In addition, ROS scavengers and antioxidants inhibit platelet aggregation (Pignatelli et al., 1998, Begonja et al., 2005, Arthur et al., 2011) and platelet-derived ROS generation is a prerequisite of integrin $\alpha_{\text{IIb}}\beta_3$ activation (Arthur et al., 2012, Begonja et al., 2005).

The GPVI signalling cascade is primarily mediated through the activation of the Src family kinases (SFKs), Fyn and Lyn, and consequent activation of Syk and PLC$\gamma_2$ (Figure 3.1.1)
(Suzuki-Inoue et al., 2002, Suzuki-Inoue et al., 2004). However, a multitude of signalling molecules are also involved in this complex signalling cascade, some of which have been previously implicated in ROS generation. Rac, a modulator of the NOX complex, is required for ROS production in neutrophils (Heyworth et al., 1993). In platelets, PI3-K (Clutton et al., 2004) and PLCγ2 (Wraith et al., 2013) are necessary for ROS generation and in collagen stimulated platelets, superoxide generation has been reported to require PLA₂ activation (Pignatelli et al., 2004). GPVI-dependent ROS formation can occur via both Syk-independent and dependent pathways (Arthur et al., 2012). In addition, antioxidants have been found to inhibit GPVI-dependent FcRγ, Syk and PI3-K phosphorylation, suggesting that these molecules are sensitive to ROS generation and that complexity might exist involving feedback activation pathways (Hubbard et al., 2003).

ROS amplifies platelet activation (Krotz et al., 2002, Bakdash and Williams, 2008, Begonja et al., 2005) and is implicated in vascular thrombosis (Freedman, 2008). The main aims of this chapter were to i) delineate the signalling molecules involved in GPVI-dependent ROS generation and ii) distinguish between signalling molecules required for ROS generation and those involved in platelet aggregation.
Figure 3.1.1 GPVI-dependent signalling pathway

GPVI activation with CRP triggers FcRγ-chain ITAM phosphorylation through Fyn and Lyn, which recruits and activates Syk, initiating a LAT signalosome involving LAT, SLP-76, PI3-K, Btk and PLCγ2. Activated PLCγ2 produces the secondary messengers, IP₃ and DAG, that induce Ca²⁺ mobilisation and PKC activation, respectively, ultimately allowing integrin α₅β₃ activation. PLCγ2 and subsequent Ca²⁺ mobilisation are required for intracellular ROS formation. The NOX1 modulator, Rac1, is a key component of NOX activation, which is upstream of P38 MAPK and TxA₂ generation. Activation of NOX1 results in ROS generation and platelet activation.
3.2 Results

3.2.1 CRP induces GPVI-dependent ROS formation which is NOX1-dependent

Firstly, the ability of CRP to induce ROS production in washed human platelets and the source of these ROS were determined. Incremental doses of CRP (0.1-5 µg/mL) were tested for their capacity to induce both platelet aggregation and ROS formation (Figure 3.2.1A and B). A dose of 1 µg/mL CRP was sufficient to induce maximal ROS production (Figure 3.2.1B). To distinguish if the ROS generated was dependent on NOX activity, a NOX1-specific antagonist, ML171, was used. CRP-induced ROS formation (1 µg/mL) was inhibited in the presence of ML171 (5 µM) (Figure 3.2.2A), whilst NOX1 inhibition had no effect on platelet aggregation (Figure 3.2.2B).

3.2.2 GPVI-dependent ROS generation and platelet aggregation are Rac1-dependent

Rac1 is essential for NOX complex formation and activity (Bokoch and Diebold, 2002). Its key role in platelet aggregation and spreading has been well documented (McCarty et al., 2005, Delaney et al., 2012). The Rac1 inhibitor, EHT-1864 (50 µM), significantly reduced CRP-induced (1 µg/mL) ROS formation and platelet aggregation (Figure 3.2.3A and B). These data are suggestive of a role for Rac1 in GPVI-dependent ROS generation.

3.2.3 GPVI-dependent ROS formation requires PLA$_2$

An alternative source of ROS in platelets is through arachidonic acid release by PLA$_2$ (Caccese et al., 2000). To address a role for PLA$_2$ in GPVI-dependent ROS formation, the specific PLA$_2$ inhibitor, AACOCF$_3$, was employed. In the presence of AACOCF$_3$ (10 µM), CRP-induced ROS formation (1 µg/mL) was significantly abrogated (Figure 3.2.4A) whilst platelet aggregation was unaffected (Figure 3.2.4B). These data indicate GPVI-dependent ROS generation is enhanced by the release of arachidonic acid.
Figure 3.2.1 Platelet-derived ROS production in response to CRP

Washed platelets (2.5 x 10^9/mL) preloaded with 10 µM H_2DCFDA (for ROS experiments only) were left untreated or treated with increasing concentrations of the GPVI-specific agonist CRP (0.1-5 µg/mL) and monitored for (A) aggregation by light transmission aggregometry and (B) ROS production by flow cytometry. To quantify ROS, fluorescent values were normalised relative to unstimulated levels (ROS stimulation index). Data are mean ± SEM, (n=7), *P≤0.01, **P≤0.001 vs. Unstimulated.
Figure 3.2.2 The effect of the NOX1 inhibitor, ML171, on CRP-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/ml) preloaded with 10 μM H₂DCFDA were pre-treated with the NOX1 inhibitor (5 μM ML171) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=5), aggregation data (n=8), ***P<0.001 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.3 The effect of the Rac1 inhibitor, EHT-1864, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H_2DCFDA were pre-treated with the Rac1 inhibitor (50 μM EHT-1864) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM (n=4), **P≤0.01 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.4 The effect of the PLA₂ inhibitor, AACOCF₃, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁷/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the PLA₂ inhibitor (10 μM AACOCF₃) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=7), *P≤0.05 vs. 0.1% v/v EtOH. Basal levels are indicative of non-stimulated platelets.
3.2.4 Early GPVI-dependent signalling events are required for ROS generation

The activation of the SFKs, Fyn and Lyn, is the earliest signalling event to occur upon ligand binding to GPVI (Quek et al., 2000). Therefore, it was reasoned that SFKs would play a significant role in the formation of ROS. Platelets were incubated with the broad SFK inhibitor, PP2 (25 μM), prior to stimulation with CRP (1 μg/mL) and analysed for both ROS generation and platelet aggregation. As expected, CRP-induced aggregation was ablated in the presence of PP2 (Figure 3.2.5B). Similarly, ROS generation stimulated by CRP was also abolished (Figure 3.2.5A). Following the activation of SFKs, the ITAM sequence of FcRγ is doubly tyrosine phosphorylated, allowing binding and phosphorylation of Syk, which is essential for GPVI-dependent platelet aggregation and spreading (Poole et al., 1997, Obergfell et al., 2002, Quek et al., 2000). A role for Syk in GPVI-dependent ROS formation was established using the Syk-specific inhibitor, BAY 61-3606 (5 μM). Inhibition of Syk dramatically reduced both CRP-induced ROS formation (Figure 3.2.6A) and platelet aggregation (Figure 3.2.6B).

3.2.3 GPVI-dependent ROS generation is PI3-K- and Btk-dependent

GPVI-dependent signalling requires the activation of PI3-K (Gibbins et al., 1998, Pasquet et al., 1999a) and subsequently the activation of Btk (Quek et al., 1998). To determine a role for PI3-K, washed platelets were pre-incubated with the PI3-K inhibitor, wortmannin (100 nM), and stimulated with CRP (1 μg/mL). Wortmannin significantly inhibited CRP-induced platelet aggregation (Figure 3.2.7A) and abolished CRP-induced ROS formation (Figure 3.2.7B), suggesting a significant role for PI3-K in GPVI-dependent ROS formation. In this study, Btk deficient mice were used to distinguish a role for Btk in platelet-derived ROS formation via GPVI. CRP (1 μg/mL) induced both platelet aggregation and ROS generation in WT mouse platelets. In comparison, Btk KO mouse platelets lacked GPVI-dependent ROS generation (Figure 3.2.8A) and aggregated poorly in response to CRP (Figure 3.2.8B), indicative of an important role for Btk downstream of GPVI activation.
Figure 3.2.5 The effect of the broad SFK inhibitor, PP2, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the SFK inhibitor (25 μM PP2) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=6), aggregation data (n=3), **P<0.01, ***P<0.001 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.6 The effect of the Syk inhibitor, BAY 61-3606, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were pre-treated with the Syk inhibitor (5 μM BAY 61-3606) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=5), aggregation data (n=7), ***P<0.001 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.7 The effect of the PI3-K inhibitor, Wortmannin, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets ($2.5 \times 10^8$/ml) preloaded with 10 $\mu$M H$_2$DCFDA were pre-treated with the PI3-K inhibitor (100 nM Wortmannin) for 10 minutes, then stimulated with CRP (1 $\mu$g/ml) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=6). ***P<0.001 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.8 CRP-induced ROS generation and platelet aggregation in Btk KO mice

Platelets ($2.5 \times 10^8$/mL) from WT and Btk KO mice were preloaded with 10 μM H$_2$DCFDA and stimulated with CRP (1 μg/mL) and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=4), **p≤0.01, ***p≤0.001 vs. WT littermates. Basal levels are indicative of non-stimulated platelets. Btk KO studies were performed in collaboration with Dr. Pat Methrom.
3.2.4 PLCγ2 and intracellular calcium are required for GPVI-dependent ROS generation and platelet aggregation

PLCγ2, downstream of GPVI ligation, is required to activate PKC and initiate Ca\(^{2+}\) mobilisation through the release of DAG and IP\(_3\), respectively (Stegner and Nieswandt, 2011a). Here the effects of a generic PLC inhibitor, U73122 (10 μM), and the intracellular Ca\(^{2+}\) chelator, BAPTA (10 μM), were assessed in CRP-induced (1 μg/ml) ROS formation and platelet aggregation. In the presence of U73122, both CRP-induced ROS formation (Figure 3.2.9A) and platelet aggregation (Figure 3.2.9B) were significantly decreased, whilst, CRP-induced ROS formation was ablated in the presence of BAPTA (Figure 3.2.10A) and platelet aggregation was significantly attenuated (Figure 3.2.10B). These findings indicate that PLCγ2 and intracellular calcium are required to elicit a maximal ROS response downstream of GPVI activation.

3.2.5 GPVI-dependent ROS formation does not require PKC activation

PKC is activated through the PLC-dependent generation of DAG (Stegner and Nieswandt, 2011a). To address the role of PKC in GPVI-dependent ROS formation the broad PKC inhibitor, GF109203X (10 μM), was used. Unlike PLC, PKC was not required for CRP-induced ROS generation (Figure 3.2.11A), but inhibition of PKC partially attenuated platelet aggregation (Figure 3.2.11B).

3.2.6 The role of secondary agonists in platelet-derived ROS formation

Secondary mediators can amplify and enhance platelet activation (Nieswandt et al., 2001a). To test if secondary mediators also induced platelet-derived ROS production ADP (10 μM) and the TxA\(_2\) mimetic, U46619 (10 μM), were used to stimulate washed platelets. Both agonists were ineffective in generating ROS (Figure 3.2.12).
Figure 3.2.9 The effect of the PLC inhibitor, U73122, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the PLC inhibitor (10 μM U73122) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3), *P<=0.05 vs. 0.2% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.10 The effect of calcium chelation on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/mL) preloaded with 10 µM H₂DCFDA were pre-treated with the intracellular Ca²⁺ chelator (10 µM BAPTA) for 10 minutes, then stimulated with CRP (1 µg/mL) for 5 minutes and monitored for ROS production by flow cytometry (A) and platelet aggregation by light transmission aggregometry (B). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=5), *P≤0.05, *** P≤0.001 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.11 The effect of the PKC inhibitor, GF109203X, on GPVI-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^8/mL) preloaded with 10 μM H$_2$DCFDA were pre-treated with the PKC inhibitor (10 μM GF109203X) for 10 minutes, then stimulated with CRP (1 μg/mL) for 5 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3). *P<0.05 vs. 0.1% v/v DMSO. Basal levels are indicative of non-stimulated platelets.
Figure 3.2.12 The effect of secondary agonists, ADP and U46619, on platelet-derived ROS production

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were stimuluted with (A) ADP (20 μM) and (B) U46619 (10 μM TxA2 mimetic) for 0-5 minutes and monitored for ROS production by flow cytometry. To quantify ROS, fluorescent geo-mean values were normalised relative to unstimulated levels (ROS stimulation index). Data are mean ± SEM (n=3).
Table 3.1 Pharmacological inhibitors targeting GPVI signalling molecules and their effects on CRP-induced platelet aggregation and ROS generation. All inhibition results are significant.

<table>
<thead>
<tr>
<th>Inhibitor Against</th>
<th>Aggregation</th>
<th>ROS Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>No effect</td>
<td>Inhibited</td>
</tr>
<tr>
<td>Rac1</td>
<td>Inhibited</td>
<td>Inhibited</td>
</tr>
<tr>
<td>PLA2</td>
<td>No effect</td>
<td>Partially Inhibited</td>
</tr>
<tr>
<td>SFKs</td>
<td>Inhibited</td>
<td>Inhibited</td>
</tr>
<tr>
<td>Syk</td>
<td>Inhibited</td>
<td>Inhibited</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Inhibited</td>
<td>Inhibited</td>
</tr>
<tr>
<td>PLC</td>
<td>Inhibited</td>
<td>Inhibited</td>
</tr>
<tr>
<td>PKC</td>
<td>Partially Inhibited</td>
<td>Partially Inhibited</td>
</tr>
<tr>
<td>Intracellular Ca^{2+}</td>
<td>Inhibited</td>
<td>Partially Inhibited</td>
</tr>
</tbody>
</table>
Figure 3.2.13 The GPVI-dependent signalling pathway and ROS generation

GPVI activation with CRP triggers FcRγ-chain ITAM phosphorylation through Fyn and Lyn, which recruits and activates Syk, initiating a LAT signalosome involving LAT, SLP-76, PI3-K, Btk and PLCy2. Activated PLCy2 produces the secondary messengers, IP₃ and DAG, that induce Ca²⁺ mobilisation and PKC activation, respectively, ultimately allowing integrin α₉β₃ activation. PLCy2 and subsequent Ca²⁺ mobilisation are required for intracellular ROS formation. The NOX1 modulator, Rac1, is a key component of NOX activation, which is upstream of P38 MAPK and TxA₂ generation. Activation of NOX1 results in ROS generation and platelet activation. Arachidonic acid release results in amplified ROS generation through a positive feedback loop mechanism. Molecules shaded in dark grey indicate proteins that have been investigated in this study.
3.4 Discussion

The generation of ROS can amplify platelet responses (Krotz et al., 2002, Bakdash and Williams, 2008, Begonja et al., 2005) and modulate various downstream signalling molecules leading to enhanced platelet activation (Hubbard et al., 2003). A role for GPVI in the formation of ROS has been well described (Bakdash and Williams, 2008, Krotz et al., 2002, Pignatelli et al., 2004, Arthur et al., 2012, Walsh et al., 2014) yet the precise molecular mechanisms underlying this event remain poorly defined. In this chapter, the key signalling events contributing to GPVI-dependent ROS generation were established using pharmacological inhibitors of known GPVI-associated downstream signalling molecules (Table 3.1).

Firstly, the optimal dose of CRP to induce ROS generation was established and the source of ROS was identified as NOX1 using the NOX1-specific inhibitor, ML171. Notably, the concentration of CRP required to generate ROS was 1 µg/mL compared to the 0.5 µg/mL dose necessary to induce maximal platelet aggregation. Therefore it is important to consider that the antagonists employed in this study may have had a more dramatic effect in platelet aggregation at lower doses of CRP. NOX2 KO mice platelets produce ROS at similar levels to that of WT (Walsh et al., 2014). These data suggest an important role for NOX1 and not NOX2 in GPVI-dependent ROS formation. Consistent with previous reports (Walsh et al., 2014), ML171 did not alter platelet aggregation downstream of GPVI activation, in contrast to other studies, which demonstrate an inhibitory effect of ROS scavengers and antioxidants on platelet aggregation (Arthur et al., 2011, Begonja et al., 2005, Pignatelli et al., 1998). The specificity of these antioxidants, such as apocynin, has previously been challenged based on their off-target effects (Dharmarajah et al., 2010), whereas ML171 targets NOX1 alone (Walsh et al., 2014).

In other cell types Rac1, along with p22phox, p67phox and p47phox, form the NOX complex (Bokoch and Diebold, 2002). The data presented in this study also suggest a role for Rac1.
in GPVI-dependent ROS generation, consistent with the involvement of NOX1. This is also consistent with Rac playing a vital role in platelet intracellular signalling (Aslan and McCarty, 2013). ROS generation can also occur through the release of arachidonic acid (Caccese et al., 2000), which is initiated through the activation of PLA₂ (Brash, 2001). In this regard, the PLA₂ inhibitor, AACOCF₃, significantly reduced CRP-induced ROS generation, in agreement with the results previously observed for collagen stimulated superoxide generation (Pignatelli et al., 2004). While, NOX1 was established as the main isoform involved in GPVI-dependent ROS formation, other sources of ROS may be available through PLA₂ activation and may provide an explanation as to why ML171 did not completely abolish ROS generation.

In this study, the role in ROS production of signalling molecules early in the GPVI-dependent pathway, including SFKs, Syk, PI3-K and Btk, were specifically examined. Both SFKs and Syk were essential for ROS formation, as well as CRP-induced platelet aggregation. Early signalling events downstream of GPVI activation are essential in initiating platelet activation through the collagen receptor. Knockout mice platelets, deficient in both Lyn and Fyn, have subsequent effective Syk phosphorylation and dramatically attenuated GPVI-dependent platelet aggregation (Quek et al., 2000). Following Syk activation, both PI3-K and Btk are activated (Gibbins et al., 1998, Quek et al., 1998). To examine the role of Btk in GPVI-dependent ROS formation, Btk KO mouse platelets were analysed for their capacity to generate ROS in comparison to WT littermates. CRP-induced ROS formation was completely absent with Btk deficiency. GPVI-dependent platelet aggregation was also attenuated in Btk KO mice, a finding which is also observed in X-linked agammaglobulinemia patients lacking Btk (Quek et al., 1998). PI3-K also contributes to early GPVI-dependent platelet activation (Gibbins et al., 1998, Pasquet et al., 1999a). In this regard, the PI3-K inhibitor, wortmannin, completely abolished both CRP-induced aggregation and ROS formation. In summary, early signalling molecules involved in GPVI-dependent functioning are also required for GPVI-dependent ROS formation.
Further downstream, PLCγ2 is activated (Pasquet et al., 1999b). Deficiency of PLCγ2 in mouse platelets results in complete inhibition of collagen-induced platelet aggregation (Suzuki-Inoue et al., 2003). In this study, the broad PLC antagonist, U73122, inhibited both CRP-induced ROS generation and platelet aggregation. Downstream of PLCγ2 activation, an increase in Ca^{2+} mobilisation and activation of PKC occur, due to the generation of IP_3 and DAG respectively (Stegner and Nieswandt, 2011a). The requirement for Ca^{2+} was therefore examined using the intracellular Ca^{2+} chelator, BAPTA. Intracellular Ca^{2+} is increased in platelets following agonist stimulation (Varga-Szabo et al., 2009). GPVI-dependent ROS formation was also dependent on increased intracellular Ca^{2+}. The effect of BAPTA was more dramatic than that of the PLC inhibitor as it functions to remove all intracellular Ca^{2+} available within platelets. In agreement with previous studies, the PKC antagonist, GF109203X, partially inhibited CRP-induced platelet aggregation (Gilio et al., 2010, Quinton et al., 2002). In contrast, PKC inhibition had no effect on GPVI-dependent ROS formation.

In line with previous evidence that NOX1 activity is required for CRP-induced TxA_2 generation (Walsh et al., 2014), the secondary platelet agonists ADP and TxA_2 mimetic, U46619, did not induce ROS in platelets. These secondary mediators are known to amplify other platelet responses including platelet aggregation (Nieswandt et al., 2001a), although NOX1 inhibition did not affect CRP-induced platelet aggregation. In contrast, collagen-induced aggregation is mainly dependent on these secondary responses (Atkinson et al., 2001) and Walsh et al. demonstrated that NOX1 inhibition did significantly reduce collagen-induced platelet aggregation (Walsh et al., 2014).

Overall, the findings in this chapter demonstrate, not unexpectedly, that the pathways downstream of GPVI involved in platelet activation leading to platelet aggregation and the pathway leading to NOX1 and ROS formation are the same at least until Ca^{2+} mobilisation downstream of PLCγ2 activation (Figure 3.2.13). Walsh et al. have provided evidence that NOX1 is upstream of the redox-sensitive kinase, P38 MAPK, on a pathway leading to
activation of PLA$_2$ and TxA$_2$ production (Walsh et al., 2014). Since in the present study, inhibition of PLA$_2$ by AACOCF$_3$ significantly inhibited ROS formation, it would appear that there is a complex positive feedback pathway involving arachidonic acid and NOX1 activation. This should prove a fruitful area for further investigation.
Chapter 4

The role of FAK family members in GPVI-dependent platelet activation and ROS formation
4.1 Introduction

In platelets, a complex involving TRAF4, p47phox, Hic5, and Pyk2 is found in association with the collagen receptor GPVI (Arthur et al., 2011). The occurrence of these proteins in a complex is consistent with a potential role in GPVI-dependent ROS formation. In other cell systems, Pyk2, a Ca\textsuperscript{2+}-dependent kinase, and its closely related family member FAK, are known to be involved in intracellular ROS-dependent signalling (Gozin et al., 1998, Matsui et al., 2007). Additionally, both protein tyrosine kinases (PTKs), FAK and Pyk2, are activated and phosphorylated downstream of ligand binding to GPVI (Achison et al., 2001, Raja et al., 1997), but an extensive characterisation of the relevance of these PTKs to GPVI-dependent signalling and ROS formation remains undetermined.

FAK and Pyk2 contain FERM and FAT domains which are essential for interaction with SH-domain containing proteins (Schlaepfer et al., 1999). Activation through the phosphorylation site Tyr402 on Pyk2 allows for interaction with Src (Park et al., 2004). Similarly, Tyr397 has been identified as the autophosphorylation site on FAK, which facilitates Src-mediated phosphorylation of Tyr576 and 577 (Calalb et al., 1995). These FAK family members have been implicated as essential regulators of cytoskeletal dynamics, particularly through modulation of the Rho family GTPase members, Rac and Cdc42 (Hsia et al., 2003, Moon and Zheng, 2003). Moreover, FAK and Pyk2 are also known to regulate PI3-K and PLC\textgamma isoforms (Choi et al., 2007, Guinebault et al., 1995, Sayed et al., 2000, Zhang et al., 1999).

Studies in recent years have described various functional roles for the FAK family in platelets. FAK deficient platelets exhibit increased bleeding times and spread poorly in response to CRP (Hitchcock et al., 2008). In addition, defects in GPVI-mediated calcium mobilisation and dense granule (ATP) secretion using the novel FAK inhibitor, PF-228, have also been reported (Jones et al., 2009). Interestingly, studies on Pyk2 deficient mice reveal no significant impairments in GPVI-dependent platelet activation, yet Pyk2 deficient mice
exhibit a marked reduction in thrombus formation over collagen, while GPCR-mediated platelet activation is substantially ablated (Canobbio et al., 2013, Consonni et al., 2012).

The aim of this study was to clarify the relative roles of Pyk2 and FAK in GPVI-dependent platelet activation, with particular emphasis on ROS formation, and the localisation of these PTKs within the GPVI pathway. We confirmed using pharmacological inhibitors and Pyk2 KO mice that FAK, and not Pyk2, is essential for GPVI-dependent ROS formation.
4.2 Results

4.2.1 FAK is a prerequisite for GPVI-mediated ROS generation and platelet aggregation

To examine the role of FAK and Pyk2 in GPVI-dependent platelet activation, platelets were pre-treated with 1 μM PF-228 (FAK inhibitor) and 10 μM tyrphostin A9 (Pyk2 inhibitor) and monitored for CRP-induced aggregation and ROS production. The Syk-specific inhibitor, BAY 61-3031 (5 μM), was used as a positive control, as it has been previously shown to abrogate CRP-induced ROS generation (Arthur et al., 2012). Interestingly, both FAK and Pyk2 (and Syk) inhibition significantly reduced CRP-induced ROS formation (Figure 4.2.1B), but only FAK was required for aggregation (Figure 4.2.1A). Maximum tyrosine phosphorylation of both FAK and Pyk2 was achieved at 3 minutes (3') stimulation with CRP (1 μg/mL) (Figure 4.2.1C). To confirm FAK inhibitor specificity, washed platelets were pre-treated with PF-228 (1 μM) and stimulated with CRP (1 μg/mL). Immunoprecipitation and phosphotyrosine analyses of FAK and Pyk2 confirmed the specificity of PF-228 for FAK and not Pyk2 (Figure 4.2.1D).

4.2.2 GPVI-dependent ROS and platelet aggregation does not involve Pyk2

Tyrphostin A9 has been previously described as a Pyk2 specific inhibitor in platelets (Anand et al., 2009, Evangelista et al., 2007, Fuortes et al., 1999, Lakkakorpi et al., 2001, Sagara et al., 2002). In order to confirm this, the inhibitor was tested in Pyk2 deficient platelets. Interestingly, washed platelets from WT and Pyk2 KO mice produced comparable levels of ROS (and platelet aggregation) following stimulation with CRP, while pre-treatment with tyrphostin A9 (10 μM) completely blocked ROS production in both genotypes (Figure 4.2.2B). These data indicate that tyrphostin A9 has off-target effects and more importantly that Pyk2 does not play a role in ROS production or aggregation in response to GPVI activation. Inhibition of FAK with PF-228 was equally effective at blocking CRP-dependent ROS formation and platelet aggregation in both WT and Pyk2 KO platelets, confirming a crucial role for this signalling molecule in GPVI-mediated platelet activation (Figure 4.2.2A and B). Given the off-target effects of tyrphostin A9, a cell free
superoxide generation assay was employed to determine if it was acting as a ROS scavenger. The well known ROS scavenger, N-acetyl cysteine (NAC; 1 mM), served as a positive control. The results indicated that neither tyrphostin A9 nor PF-228 or BAY 61-3606 were acting as superoxide scavengers (Figure 4.2.3A). While the current literature describes tyrphostin A9 as a Pyk2 inhibitor, one previous study suggested it may act as a mitochondrial un-coupler (Sagara et al., 2002). To assess this, tyrphostin A9 was incubated with platelets that had been incubated with the membrane-permeable dye JC-1, and analysed for loss of mitochondrial membrane potential by flow cytometry. A membrane shift was observed with tyrphostin A9 compared to 0.1% DMSO, confirming that tyrphostin A9 was acting as a mitochondrial un-coupler (Figure 4.2.3B). The combined data indicate that tyrphostin A9 cannot be used as a pharmacologically selective inhibitor of Pyk2 function.

4.2.3 GPVI-dependent α and dense granule release, PS exposure, fibrinogen binding and spreading require FAK

Previous studies have implicated key roles for FAK in GPVI-dependent platelet aggregation and platelet spreading (Hitchcock et al., 2008, Jones et al., 2009). We therefore evaluated the role of FAK with respect to additional parameters of GPVI-mediated platelet activation. Fibrinogen binding, which provides a sensitive readout of integrin α1β3 inside-out activation, was significantly blocked by PF-228 (1 μM) following CRP (1 μg/mL) stimulation, similar to the inhibitory effects of the SFK inhibitor, PP2 (25 μM), included as a positive control (Figure 4.2.4A). There was also a marked reduction in the percentage of annexin-V positive platelets, a marker of PS exposure, following CRP (1 μg/mL) stimulation and treatment with PF-228 (1 μM) and BAY 61-3606 (5 μM) (Figure 4.2.4B). Similarly, platelets demonstrated a significant decrease in P-selectin surface expression (α-granule release) under these conditions (Figure 4.2.5A). Consistent with a previous study (Jones et al., 2009), CRP-mediated secretion of ATP from dense granules was significantly blocked on FAK inhibition (Figure 4.2.5B), as was CRP-induced spreading (Figure 4.2.6). Together, these results demonstrate that FAK plays a major role in GPVI-induced fibrinogen binding,
PS exposure, alpha and dense granule release, as well as on platelet aggregation, spreading and ROS formation.

4.2.4 GPVI-dependent FAK activation and ROS production is integrin αIIbβ3-independent

To investigate whether GPVI-dependent FAK phosphorylation was integrin αIIbβ3-independent or -dependent, washed platelets were pre-incubated with RGD peptide (50-150 µg/mL), which dose-dependently blocked platelet CRP-induced (1 µg/mL) aggregation (Figure 4.2.7A). RGD-treated platelets also showed similar FAK phosphorylation on CRP-dependent activation as compared to the vehicle control (Figure 4.2.7B). Consistent with FAK activation being upstream of integrin αIIbβ3 activation, GPVI-dependent ROS formation was also found to be integrin αIIbβ3-independent. These findings place GPVI-dependent ROS formation and FAK phosphorylation as key signalling events upstream of integrin αIIbβ3 activation (Figure 4.2.7C).

4.2.5 FAK is activated within the GPVI-dependent LAT signalosome

Following GPVI stimulation, one of the earliest signalling events to occur is the activation of Syk by Src family kinases (SFKs), Lyn and Fyn, which involves a well characterised LAT signalosome (Watson S.P., 2005). To investigate the location of FAK within the GPVI signalling pathway, we monitored CRP-mediated (1 µg/mL) FAK phosphorylation in the presence of pharmacological inhibitors against SFKs (25 µM PP2), Syk (5 µM BAY 61-3606), and PI3-K (100 nM Wortmannin), all of which potently inhibited FAK tyrosine phosphorylation following GPVI activation (Figure 4.2.8A). Importantly, it was also confirmed through studies involving Btk KO mice that CRP-dependent FAK activation is downstream of Btk signalling (Figure 4.2.8B). It was further demonstrated that FAK activation is also partially dependent on intracellular Ca2+, using the Ca2+ chelator, BAPTA (10 µM), which reduced FAK tyrosine phosphorylation following CRP stimulation (Figure 4.2.8C). Similarly, the generic PKC inhibitor, GF109302X (10 µM) also partially reduced FAK phosphorylation (Figure 4.2.8C). In other cell types, FAK has been shown to regulate PLC
activation and consistent with these reports, PF-228 significantly blocked CRP-induced PLCγ2 tyrosine phosphorylation over time (Figure 4.2.8D). Consistent with this, a generic PLC inhibitor, U73122 (10 μM), did not inhibit FAK tyrosine phosphorylation (Figure 4.2.8E). To further establish a link between FAK and GPVI-dependent ROS formation, the effect of PF-228 on Rac1 activation was analysed. Rac1 is an essential precursor in the assemblage of an active NOX complex, and its activation following CRP stimulation was completely inhibited by PF-228 (1 μM), similar to that with the Rac1-specific inhibitor, EHT-1864 (50 μM), employed as a control (Figure 4.2.8F). Finally, there was no detectable decrease in CRP-mediated FAK phosphorylation in the presence of a NOX1 specific inhibitor, ML171, suggesting FAK activation precedes NOX1 complex formation (Figure 4.2.8E). Western blot densitometry analyses are shown in Figure 4.2.9.
Figure 4.2.1A-B FAK is a prerequisite for GPVI-mediated ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^6/mL) preloaded with 10 μM H₂DCFDA (for ROS experiments only) were pre-treated with vehicle control (0.1% DMSO), FAK inhibitor (1 μM PF-228), Pyk2 inhibitor (10 μM Tyrophostin A9) or Syk inhibitor (5 μM BAY 61-3606) for 10 minutes, then stimulated with 1 μg/mL CRP and monitored for (A) aggregation by light transmission aggregometry and (B) ROS production by flow cytometry. Data are mean ± SEM, (n=6), ***P<0.0001 vs. 0.1% v/v DMSO. Aggregation traces in A are representative of 6 independent experiments.
C. To monitor GPVI-dependent FAK/Pyk2 activation, washed platelets (1 x 10^9/mL) were stimulated with 1 μg/mL CRP (with stirring) for 30 seconds (30'), 1 minute (1'), 3' and 6'. Samples were lysed, immunoprecipitated with anti-phosphotyrosine (4G10 and PY20), analysed by SDS 5-20% polyacrylamide gel electrophoresis, and immunoblotted with anti-FAK or Pyk2. Blots are representative of three independent experiments. D. To assess inhibitor specificity, platelets (1 x 10^9/mL) pre-treated with or without 1 μM PF-228 for 10 minutes, were then stimulated with 1 μg/mL CRP and immunoprecipitated with either anti-FAK or anti-Pyk2 and blotted for phosphotyrosine (4G10). Blots are representative of three independent experiments. P.L., platelet lysate; IB, immunoblot.
Figure 4.2.2 GPVI-dependent ROS and platelet aggregation does not involve Pyk2

Washed platelets (2.5 x 10^5/mL) from WT and Pyk2 KO mice were pre-treated with 0.1% DMSO, 1 μM PF-228 or 10 μM tyrphostin A9 for 10 minutes and assessed for (A) aggregation by light transmission aggregometry and (B) ROS production by flow cytometry following stimulation with 1 μg/mL CRP. Data are mean ± SEM, (n=6), ***P≤0.01, ***P≤0.001 vs. 0.1% v/v DMSO. Aggregation traces are representative of 4 independent experiments. Pyk2 KO studies were performed in collaboration with Dr. Pat Metharom.
A. Using a cell-free superoxide anion (O$_2^-$) generation assay, pharmacological inhibitors used throughout this study were tested for their capacity to scavenge ROS (NAC was included as positive control). Data are mean ± SEM, (n=3), ** P<0.002 vs. 0.1% v/v DMSO. B. To clarify the non-specific effects of the Pyk2 inhibitor, tyrphostin A9, washed platelets (2.5 x 10$^8$/mL) loaded with the mitochondrial membrane-permeable dye JC-1 were pre-treated with vehicle control (0.1% DMSO) or 10 μM tyrphostin A9 for 10 minutes and monitored for alterations in the mitochondrial membrane potential. Changes in mitochondrial membrane potential, which are based on a decrease FL2/FL1 intensity ratios, were quantified by flow cytometry. Results are representative of three independent experiments.
Figure 4.2.4 GPVI-dependent fibrinogen binding and PS exposure require FAK

A. Washed platelets (2.5 x 10^9/mL) mixed with Oregon Green® fibrinogen were pre-treated with vehicle control (0.1% DMSO) and antagonists; PF-228 (1 µM) and PP2 (25 µM) for 10 minutes, stimulated with CRP (1 µg/mL) for 10 minutes. Platelets were then analysed by flow cytometry. Data are mean ± SEM, (n=3), **P≤0.001, ***P≤0.0001 vs. 0.1% v/v DMSO. B. Washed platelets (2.5 x 10^9/mL) pre-treated with vehicle control (0.1% DMSO) and antagonists; PF-228 (1 µM) and BAY 61-3606 (5 µM) for 10 minutes at 37°C were treated with 1 µg/mL CRP for 10 minutes and monitored by flow cytometry for PS exposure (FITC-labelled Annexin-V). Data are mean ± SEM, (n=3), ***P≤0.0001 vs. 0.1% v/v DMSO.
Figure 4.2.5 GPVI-dependent alpha and dense granule release require FAK

A. Washed platelets (2.5 x 10^6/mL) pre-treated with vehicle control or pharmacological inhibitors (10 minutes at 37°C) were treated with 1 μg/mL CRP for 10 minutes and monitored by flow cytometry for P-selectin surface expression (using PE-labelled anti-CD62P antibody or isotype control). Data are mean ± SEM, (n=3), ***P<0.0001 vs. 0.1% v/v DMSO. B. Washed platelets (3 x 10^6/mL) were pre-incubated with antagonists; PF-228 (0.5 μM) or BAY 61-3606 (5 μM) or vehicle control (0.1% DMSO) for 15 minutes and then stimulated with CRP (1 μg/mL) for 10 minutes in a 96-well plate. Chronolume® containing luciferase was added to each reaction and luminescence was measured using a Wallac Victor™ 1420 multilabel counter. Data are mean ± SEM (n=6), *P<0.05 vs. 0.1% v/v DMSO.
Figure 4.2.6 GPVI-dependent spreading requires FAK

Washed platelets (2 x 10^7/mL) were pre-treated with vehicle control or pharmacological inhibitors; 1 μM PF-228 (FAK) or 5 μM BAY 61-3606 (Syk), for 10 minutes at 37°C. Platelets were then placed onto coverslips for 45 minutes at 37°C (that had been pre-coated with 1 μg/mL CRP), fixed, permeabilised, stained with FITC-labelled phalloidin and visualised by fluorescence microscopy. Quantified values represent total surface coverage (μm^2) per field of view. Scale bar = 10 μm. Data are mean ± SEM, (n=3), ***P≤0.001 vs. 0.1% v/v DMSO. CRP-induced spreading was performed in collaboration with Dr. Tony Walsh.
Figure 4.2.7A-B GPVI-dependent ROS production and FAK activation are integrin \( \alpha_{\text{IIb}}\beta_3 \)-independent

Washed platelets \((2.5 \times 10^9$/mL\) were pre-treated with various concentrations of RGD peptide \((50-150 \mu$g$/mL\) were stimulated with $1 \mu$g$/mL$ CRP and monitored for (A) aggregation by light transmission aggregometry and (B) ROS production by flow cytometry. Aggregation traces are representative of three independent experiments. Data are mean $\pm$ SEM, \( (n=3) \), *$P<0.02$ vs. Unstimulated.
Figure 4.2.7C GPVI-dependent ROS production and FAK activation are integrin αIIbβ3-independent

C. Washed platelets (1 x 10⁹/mL) pre-incubated with RGD peptide (150 μg/mL) and stimulated with 1 μg/mL CRP for 3 minutes (with stirring), were lysed, immunoprecipitated with anti-FAK (4 μg) analysed by SDS 5-20% polyacrylamide gel electrophoresis, and immunoblotted for phospho-tyrosine (4G10) or FAK. Blots are representative of three independent experiments. P.L.: platelet lysate; IB: Immunoblot.
Figure 4.2.8A-C FAK is activated within the GPVI-dependent LAT signalosome

A. Washed human platelets pre-incubated with vehicle control (0.1% DMSO) or inhibitors for 10 minutes; 25 μM PP2, 5 μM BAY 61-3606, 100 nM Wortmannin (final concentrations) were stimulated with 1 μg/mL CRP for 3 minutes (3') (with stirring), lysed, immunoprecipitated with anti-FAK and immunoblotted for phosphotyrosine (4G10) and FAK. Blots are representative of three independent experiments. B. Washed murine platelets (1 x 10^8/mL) were stimulated with 1 μg/mL CRP (with stirring) for 3 minutes, lysed, immunoprecipitated with anti-FAK, analysed by SDS 5-20% polyacrylamide gel electrophoresis, and immunoblotted for pTyr (4G10) and FAK. Blots are representative of two independent experiments. Btk IP was performed in collaboration with Dr. Pat Metharom. C. Washed human platelets pre-incubated with vehicle control (0.1% DMSO) or inhibitors for 10 minutes; 10 μM GF109302X (GF-10X) or 10 μM BAPTA (final concentrations) were stimulated with 1 μg/mL CRP for 3 minutes (with stirring), lysed, immunoprecipitated with anti-FAK and immunoblotted for phosphotyrosine (4G10) and FAK. Blots are representative of three independent experiments. IB: immunoblot.
Figure 4.2.8D-F FAK is activated within the GPVI-dependent LAT signalosome

**D.** Washed platelets pre-incubated with 0.1% DMSO or PF-228 (1 μM) for 10 minutes, were stimulated with 1 μg/mL CRP for up to 3 minutes (with stirring), lysed, immunoprecipitated with anti-phosphotyrosine (4G10) and immunoblotted for PLCγ2. Blots are representative of three independent experiments. 

**E.** Washed human platelets pre-incubated with vehicle control (0.1% DMSO) or inhibitor(s) for 10 minutes; 10 μM U73122 or 5 μM ML171 (final concentrations) were stimulated with 1 μg/mL CRP for 3 minutes (with stirring), lysed, immunoprecipitated with anti-FAK and immunoblotted for phosphotyrosine (4G10) and FAK. Blots are representative of three independent experiments. 

**F.** Washed platelets pre-incubated with 0.1% DMSO, PF-228 (1 μM) or Rac-1 inhibitor, EHT-1864 (50 μM) for 10 minutes, were stimulated with 1 μg/mL CRP for up to 1 minute (with stirring), lysed, subjected to Rac1 GTP ‘pulldown’ analysis and immunoblotted for Rac1 to detect active ‘GTP’ loaded Rac1. Loading controls for total Rac1 levels were subsequently performed using equal sample volumes. The Rac1 pull down was performed in collaboration with Dr. Tony Walsh. IB: Immunoblot.
Figure 4.2.9 Densitometry of Western blots shown in figure 4.2.8

Following immunoprecipitation and western blot analysis, densitometry was performed using Image J®. Results correspond to the blots in Figure 4.2.8. Data are mean ± SEM, (n=3), *P<0.05, **P<0.01, ***P<0.001 vs. 0.1% v/v DMSO.
Figure 4.2.10 Representation of the GPVI signalling pathway

GPVI activation with CRP triggers Fcγ-chain ITAM phosphorylation through Fyn and Lyn, which recruits and activates Syk, initiating a LAT signalosome involving LAT, SLP-76, PI3-K, Btk, Rac1 and PLCγ2. FAK appears to be present within this signalosome as it is downstream of SFKs, Syk, PI3-K and Btk but upstream of Rac1 and PLCγ2 following GPVI activation. Activated PLCγ2 produces secondary messengers, IP₃ and DAG that induce Ca²⁺ mobilisation and PKC activation, respectively, allowing maximal activation of FAK and integrin α₅β₃ activation. FAK also regulates the NOX1 modulator, Rac1, which along with Hic-5 leads to the activation of NOX1, resulting in ROS generation and platelet activation. Key proteins investigated in this section are shaded grey.
4.3 Discussion

In this study, the FAK family members, Pyk2 and FAK, were investigated for their involvement in GPVI-dependent ROS formation and platelet activation. A novel role for FAK, but not Pyk2, was demonstrated as a prerequisite for GPVI-dependent ROS formation, as well as PS exposure, fibrinogen binding and α-granule secretion. Despite Pyk2 activation occurring downstream of GPVI, no effect on in vitro GPVI-mediated functional assays was observed with Pyk2 deficiency in mouse platelets. In contrast FAK was demonstrated as important in platelet-derived ROS generation, confirming its role as a proximal signalling molecule in the GPVI pathway.

In platelets, ROS generation is a downstream consequence of receptor/ligand interaction, as are platelet aggregation and spreading. There has been much emphasis on the involvement of ROS in platelet activation, and more recently in cellular signalling (Arthur et al., 2012, Begenja et al., 2005, Pignatelli et al., 2010). Both FAK family inhibitors employed, PF-228 (FAK) and tyrphostin A9 (Pyk2), ablated ROS production in human platelets suggesting that both FAK and Pyk2 play a significant role in ROS production. In contrast, platelet aggregation was only inhibited by the FAK inhibitor, PF-228. Pyk2 KO mice displayed similar ROS generation (and platelet aggregation) to that of WT mice. However, tyrphostin A9 inhibited ROS generation in both WT and Pyk2 KO, indicating that the inhibitor was acting off target. These results were surprising considering that in platelets, Pyk2 is in complex with Hic5, TRAF4 and p47εphox, a subunit of the NOX2 complex, and additionally, endothelial cells deficient in Pyk2 lack ROS-mediated pro-inflammatory reactions (Katsume et al., 2011, Zhao and Bokoch, 2005). However, as demonstrated here and previously (Walsh et al., 2014), platelets employ NOX1 and not NOX2 downstream of GPVI to generate ROS, as shown with the NOX1-specific inhibitor ML171. It is possible that Pyk2 (and the NOX2 complex) exerts no measurable influence in GPVI-mediated platelet activation, as suggested by studies using NOX2 KO mice (Walsh et al., 2014). Importantly though, PF-228 inhibited ROS production (and aggregation) in WT and Pyk2 KO platelets, suggesting a fundamental role for FAK in the GPVI pathway controlling ROS production.
Tyrphostin A9, but not PF-228, was confirmed to cause mitochondrial membrane depolarisation; an effect which decreases cellular ATP levels (Park et al., 2011). Therefore, tyrphostin A9 may perturb the signalling mechanisms necessary to induce NOX-mediated ROS production following GPVI ligation.

As no notable differences were observed in GPVI-dependent platelet activation associated with Pyk2 deficiency, we then focused on further understanding the role of FAK in the GPVI-mediated signalling pathway. FAK has been previously described as of importance in mediating platelet spreading (Hitchcock et al., 2008), and more recently in platelet aggregation, dense granule secretion and Ca²⁺ mobilisation following GPVI stimulation (Jones et al., 2009). We therefore examined the relevance of FAK to other downstream consequences involving the GPVI pathway. Using PF-228, FAK was also shown to be important in regard to PS exposure, α-granule secretion and integrin α₁bβ₃ activation. Contrary to evidence in the literature suggesting FAK is an integrin α₁bβ₃-dependent kinase (Lipfert et al., 1992, Shattil et al., 1994), our findings indicate that RGD peptide did not have any effect on FAK phosphorylation in the GPVI pathway. These results are consistent with another report on the effect of an RGD α₁bβ₃ antagonist on collagen- and CRP-induced FAK phosphorylation (Achison et al., 2001). Further, the result is not surprising given that FAK is essential for fibrinogen binding (and aggregation) and is upstream of integrin α₁bβ₃ signalling. Additionally, it has also been demonstrated that GPVI-dependent ROS formation is also integrin α₁bβ₃-independent (Arthur et al., 2012).

Following ligand interaction, GPVI signals through an ITAM mediated pathway involving activation of the SFKs, Lyn and Fyn, which allows the assemblage of Syk. Subsequent signalling initiates a series of downstream events including activation of PI3-K, recruitment of Btk and activation of PLCy2 by tyrosine phosphorylation, which facilitate Ca²⁺ mobilisation and PKC activation (see Figure 4.2.10) (Pasquet et al., 1999b, Quek et al., 1998, Suzuki-Inoue et al., 2002, Watson S.P., 2005). To investigate the regulation and positioning of FAK within this pathway, a pharmacological and genetic approach was
adopted to target a number of these key signalling molecules. Pharmacological inhibition of SFKs, Syk and PI3-K completely blocked CRP-induced FAK phosphorylation. Consistent with these observations, Jones et al. reported that PF-228 did not affect CRP-induced Syk phosphorylation (Jones et al., 2009). In contrast, a previous study demonstrated that thrombin-mediated PI3-K activity required FAK; an effect which is dependent on integrin α1β3 signalling highlighting differences between different signalling pathways (Guinebault et al., 1995).

Recruitment of Btk to the plasma membrane is regulated through its PH domain, which binds the PI3-K product, PIP3. Here, it was confirmed, using Btk deficient mouse platelets, that GPVI-mediated FAK phosphorylation is downstream of Btk. In contrast, PLCγ2 activation was shown to be FAK-dependent as the generic PLC inhibitor, U73122, did not block CRP-induced FAK activation and reciprocally PF-228 inhibited PLCγ2 phosphorylation. FAK has been shown to bind the PLCγ1 isoform in fibroblasts, an interaction mediated by the SH2 domain of PLCγ1 and Tyr397 on FAK (Zhang et al., 1999). While a regulatory role for FAK regarding GPVI-dependent PLCγ2 activation was demonstrated, a physical association between the two signalling proteins via co-immunoprecipitation could not be detected (data not shown). Consistent with previous observations, FAK phosphorylation is partially attenuated by Ca2+ chelation and PKC inhibition; similarly, FAK activation is enhanced by the addition of Ca2+ (Haimovich et al., 1996, Shattil et al., 1994). Although these data conflict with previous studies where PF-228 inhibits Ca2+ mobilisation (Jones et al., 2009), it is possible that a positive feedback loop facilitates FAK activation following Ca2+ mobilisation and subsequent PKC activation.

Both FAK activation and ROS generation were found to occur rapidly following ligand binding to GPVI. The C-terminal focal adhesion targeting domain and proline-rich region on FAK provide docking sites for binding partners and signalling molecules and additionally allow for the regulation of small Rho GTPases such as Rac1 (Schlaepfer and Mitra, 2004). Rac1 is essential for GPVI-mediated platelet activation and has been shown to regulate
PLCγ2 (Pleines et al., 2009). Our results indicate that Rac1 activation is dependent on FAK, which is consistent with the report showing that activation of the Rac1 effector, p21 activated kinase (PAK1), is FAK-dependent following GPVI stimulation (Jones et al., 2009). Furthermore, NOX1-mediated ROS generation has been shown to be regulated by Rac1, providing further evidence for a potential regulatory role of FAK in NOX-mediated ROS production, via Rac1 (Cheng et al., 2006). In this study it was demonstrated that NOX1 inhibition did not affect GPVI-mediated FAK activation.

Previous studies have identified compensatory roles for Pyk2 in FAK−/− endothelial cells, while evidence in fibroblasts suggests otherwise (Klingbeil et al., 2001, Weis et al., 2008, Ueki et al., 1998). Whilst mice specifically deficient in the megakaryocyte lineage for FAK (Pf4-Cre/FAK-floxed) displayed an excessive amount of megakaryocyte progenitor cells and mature bone marrow megakaryocytes (Hitchcock et al., 2008), they showed no difference in Pyk2 expression in comparison to WT. Interestingly, the NH2-terminal and kinase domain of Pyk2 can functionally substitute for FAK in promoting signalling and motility events in fibronectin-stimulated hapotactic cells (Klingbeil et al., 2001). In this regard, FAK−/− megakaryocytes exhibited a significant increase of Pyk2 phosphorylation, implying a compensatory role between these two non-receptor tyrosine kinases.

Notably, whilst this thesis was in preparation, Roh et al. provided evidence for off target effects of PF-228 (Roh et al., 2013). They explored collagen-induced aggregation in WT and FAK−/− murine platelets and showed no significant difference. Moreover, the addition of PF-228 (1 μM) completely abrogated collagen-induced aggregation in both WT and FAK−/− platelets. In contrast, FAK−/− platelets were previously demonstrated to show defective spreading in response to CRP (Hitchcock et al., 2008). In this regard, Roh et al. did not explore the effects of CRP-induced aggregation in this recent study. Furthermore, they observed an increase in Pyk2 tyrosine phosphorylation in FAK−/− platelets which could possibly compensate for the loss of FAK. Although we show that PF-228 does not inhibit
Pyk2 phosphorylation, it is possible that in the absence of FAK that PF-228 could target Pyk2 as it binds to the ATP binding pockets of these tyrosine kinases.

As the only two known members of the FAK family and with significant structural homology, one could assume that FAK and Pyk2 could play a similar role in certain cell types. This study describes a novel role for FAK in GPVI-mediated ROS formation and demonstrates a novel position for FAK in the GPVI pathway, which precedes Rac1, PLCγ2, NOX1 and integrin αIIbβ3 activation. However, the role of Pyk2 in GPVI-mediated platelet function appears to be dispensable. We therefore demonstrate key functional differences between these two closely related FAK family members following specific stimulation of the GPVI pathway.
Chapter 5

Thrombin-induced reactive oxygen species
generation: A novel role for PAR4 and GPIbα
5.1 Introduction

Thrombin is a potent platelet activator and signals through platelet receptors, GPIbα, PAR1 and PAR4 on human platelets (De Candia, 2012). Recently, thrombin has been implicated in the generation of ROS as well as leading to integrin αIIbβ3 activation (Bakdash and Williams, 2008, Begonja et al., 2005). While ROS are known to have key roles in intraplatelet signalling (Finkel, 2000) and subsequent platelet activation (Krotz et al., 2002), the exact receptors and signalling pathways involved in thrombin-induced ROS generation have yet to be fully elucidated.

Thrombin contains two anion binding sites: exosites I and II (Di Cera, 2008). The latter binds the anticoagulant heparin, while exosite I allows for binding to a unique hirudin-like binding site on both PAR1 and PAR3. Inhibition of this site abrogates PAR-induced responses (Liu et al., 1991, Di Cera, 2008). PAR activation is initiated by thrombin cleavage at its extracellular N-terminal region (Vu et al., 1991). Cleavage generates a tethered ligand that folds back onto the PAR itself and triggers auto-activation. In human platelets, PAR1 and PAR4 act as a dual system involved in platelet aggregation and downstream signalling (Kahn et al., 1999b, Kahn et al., 1998b). PAR1 is a high affinity thrombin receptor found on human platelets and signals through G_{α12}, G_{aq} and G_{i/o} (Coughlin, 1999b, Macfarlane et al., 2001, Voss et al., 2007). In contrast to PAR1, PAR4 requires higher concentrations of thrombin due to the absence of the hirudin-like binding sequence, and signals through G_{α12} and G_{aq} only (De Candia, 2012, Faruqi et al., 2000). In mouse platelets, PAR1 is not expressed and PAR4 is the major receptor required for thrombin signalling (Kahn et al., 1998b). Efficient functioning of PAR4 in murine platelets requires thrombin cleavage and activation of PAR3 (Nakanishi-Matsui et al., 2000). Although cleavage of PAR3 does not elicit a signalling response, it is essential for optimal PAR4 activation as it presents the bound thrombin to PAR4.
Distinct differences have been reported between PAR1 and PAR4 in human platelets. Calcium responses elicited through PAR1 are short and rapid but prolonged and sustained following PAR4 stimulation (Covic et al., 2000). PAR4, but not PAR1, is regulated by P2Y_{12}-stimulated feedback (Holinstat et al., 2006). Desensitisation of PAR1 platelets, where aggregation no longer occurs through PAR1 stimulation, is overcome by signalling through PAR4 (Falker et al., 2011). Furthermore, PAR4 is required for thrombin-induced PS exposure, whereas PAR1 is not (personal communication; Hamilton JR, Monash University).

Along with PARs, thrombin also cleaves GPV and binds GPIbα of the GPIb-IX-V complex (Calverley et al., 1995). At present, no signalling cascade is known to occur upon thrombin cleavage of GPV, although the presence of a conserved regulatory protein, 14-3-3ζ, bound to the cytoplasmic tail of GPV may suggest a potential for signalling (Andrews et al., 1998). Compared to WT, thrombin activation is potentiated in GPV^{−/−} platelets which consequently form larger thrombi upon injury (Ramakrishnan et al., 1999), although this has not been confirmed by others (Kahn et al., 1999a). Therefore, GPV may act as a negative regulator of thrombin-induced platelet activation.

Thrombin binds to high affinity sites on GPIbα, involving the anionic sulphated region of the N-terminal domain (De Marco et al., 1994). At present, two mechanisms to how this occurs have been proposed (Dumas et al., 2003, Celikel et al., 2003). Thrombin can signal through GPIbα and induce the phosphorylation of signalling molecules required for integrin α_{IIb}β_{3} activation (Adam et al., 2003b, Dubois et al., 2004). Such signalling cascades can be inhibited experimentally by the use of specific enzymes derived from snake venoms such as the metalloproteinases, moccarragin and Nk protease (Andrews et al., 2003a). These metalloproteinases cleave the extracellular portion of GPIbα N-terminal 282-283, downstream of the sulphated tyrosine sequence that binds thrombin (Ravanat et al., 2010, Lova et al., 2010).
Both thrombin and TRAP stimulate ROS production, thus implicating PAR1, PAR4 and GPIbα in generating ROS in platelets (Begonja et al., 2005, Bakdash and Williams, 2008), although GPIbα- and PAR4-specific agonists have not been previously evaluated for their capacity to generate ROS. To investigate what receptors are involved in thrombin-induced ROS generation, highly specific antagonists were used in this study targeting PAR1 and PAR4. To address the role of GPIbα in thrombin-induced ROS formation, the GPIbα-cleaving enzyme, Nk protease, was used. The findings of this chapter demonstrate specific roles for the different platelet thrombin receptors in ROS generation.
5.2 Results

5.2.1 Thrombin-induced ROS generation requires higher concentrations of thrombin than needed for aggregation

In this study, various concentrations of thrombin were used to assess ROS production. Figure 5.2.1A shows aggregation profiles of platelets stimulated with incremental thrombin doses (0.2-2 U/ml). There was no significant difference between the varying concentrations as the lowest concentration of thrombin used in this study was sufficient to induce a maximal aggregation response. In contrast, thrombin-induced ROS generation was undetectable at 0.2 U/ml but was evident at 1-2 U/ml (Figure 5.2.1B). Therefore, we used a concentration of 2 U/ml throughout.

5.2.2 PAR1 and PAR4 agonist-induced ROS production is dose dependent

Although it is known that thrombin signals through PARs to induce platelet activation, the generation of ROS via this pathway has yet to be fully explored. Previous studies have shown TRAP-induced ROS production through PAR1 in platelets (Begonja et al., 2005), but a role for PAR4 in platelet generated ROS remains unknown. The PAR1-specific agonist, TRAP, which contains the amino acid sequence SFLLRN, induced aggregation at concentrations as low as 10 µM but required higher doses (20-50 µM) to generate ROS (Figure 5.2.2A). In contrast, the PAR4 specific agonist, PAR4-AP, comprising the amino acid sequence AYPGKF, elicited a significant ROS response with the same doses (150-250 µM) that were required for optimum aggregation (Figure 5.2.2B). Additionally, the combination of both TRAP and PAR4-AP generated higher ROS in comparison to TRAP or PAR4-AP alone (Figure 5.2.2B), whilst thrombin elicited a much higher response in comparison to both TRAP and PAR4-AP alone, suggesting a possible role for GPIIbα in thrombin-induced ROS production (Figure 5.2.2C).
Figure 5.2.1 Thrombin-induced ROS generation requires higher concentrations of thrombin than needed for aggregation.

Washed human platelets preloaded with 10 μM H$_2$DCFDA (for ROS experiments only) were stimulated with increasing concentrations of thrombin (0.2-2 U/mL) and monitored for (A) platelet aggregation by light transmission aggregometry and (B) ROS production by flow cytometry. To quantify ROS, fluorescent values (geo-mean) were expressed as a ratio relative to unstimulated platelets (i.e. resting platelets have an index of 1). Data are mean ± SEM (n=3).
Figure 5.2.2A-B TRAP and PAR4-induced platelet aggregation and ROS generation

Washed platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA (for ROS experiments only) were left untreated or treated with increasing concentrations of (A) TRAP (SFLLRN; 10-50 μM) and (B) PAR4-AP (AYPGKF; 100-250 μM) for 2 minutes. ROS production was measured by flow cytometry and platelet aggregation by light transmission aggregometry. To quantify ROS, fluorescent values were normalised relative to unstimulated levels (ROS stimulation index). Data are mean ± SEM (n=3), *P≤0.05 vs. Unstimulated. PAR4-AP-induced ROS Production PAR4+TRAP bar indicates that PAR4 (250 μM) and TRAP (50 μM) were used simultaneously (n=2).
Figure 5.2.2C ROS production through PAR1 and PAR4

C. To compare ROS production through PARs, platelets were stimulated with TRAP (50 μM), PAR4-AP (250 μM) and thrombin (2 U/mL) for 2 minutes and ROS production was measured by flow cytometry. To quantify ROS, fluorescent values were normalised relative to unstimulated platelets, as described in the legend to figure 4.2.1 (ROS stimulation index). Data are mean ± SEM (n=3) ***P<0.001 vs. Unstimulated.
5.2.3 Thrombin-induced ROS production requires PAR4 but not PAR1

To further delineate the contribution of PAR1 and PAR4 in thrombin-induced ROS formation, PAR-specific antagonists were examined for their effect on thrombin-stimulated ROS formation. Interestingly, in comparison to vehicle control (0.1% DMSO), the PAR1 antagonist, SCH79797 (1 µM), had no inhibitory effect on thrombin-induced ROS at 2 U/mL, but significantly potentiated the level of ROS detected (Figure 5.2.3). An additional PAR1 antagonist, BMS200261 (1 µM) exhibited the same results (Figure 5.2.4). However, the PAR4 antagonist, tcY-NH₂ (400 µM), significantly inhibited thrombin-induced ROS production (Figure 5.2.3). These data suggest a novel role for PAR4 in thrombin-induced ROS generation in platelets, independent of PAR1. The specificity of these antagonists is demonstrated in figure 5.2.4.

5.2.4 PAR4-deficient mouse platelets lack thrombin-induced ROS production

To confirm the role of PAR4 in thrombin-induced ROS formation, ROS production was monitored in WT and PAR4 KO mice. PAR4 KO mice do not aggregate in response to thrombin (Hamilton et al., 2004). When stimulated with thrombin (2 U/mL), ROS formation was absent in PAR4 KO mice in comparison to WT littermates (stimulated) and was not comparable to that of unstimulated WT and PAR4 KO platelets (Figure 5.2.5A). Platelets derived from PAR4 KO mice were not completely devoid of ROS producing capability as they generated ROS in response to CRP similarly to WT (Figure 5.2.5B). Thus, although thrombin can bind and potentially signal through GPIIbα, PAR4 is absolutely required in the signalling pathway for thrombin-induced ROS production in mice as well as for platelet aggregation.
Figure 5.2.3 Thrombin-induced ROS production is PAR4-dependent but PAR1-independent

Washed platelets were pre-treated with the PAR1 antagonist (1 μM SCH79797), the PAR4 antagonist (400 μM tcY-NH₂) or vehicle control (0.1% DMSO), then stimulated with high dose thrombin (2 U/mL) for 2 minutes. ROS production was measured by flow cytometry. Fluorescent values were normalised to vehicle control (% ROS production) to show the effect of the drugs. Data are mean ± SEM (n=3), *P<0.05, **P<0.01 vs. 0.1% v/v DMSO.
Figure 5.2.4 Specificity of PAR antagonists

A. Washed platelets (2.5 x 10^5/mL) preloaded with 10 μM H_2DCFDA were left untreated or treated with PAR1 antagonist BMS200261 (1 μM), then stimulated with thrombin (1 U/mL) for 2 minutes and monitored for ROS production by flow cytometry. To quantify ROS, fluorescent values were normalised relative to unstimulated levels (ROS stimulation index). Data are mean ± SEM (n=3), *P<0.05 vs. vehicle control. Washed platelets (2.5 x 10^5/mL) preloaded with 10 μM H_2DCFDA were left untreated or treated with PAR1 antagonists: BMS200261 (1 μM) or SCH79797 (1 μM), then stimulated with (B) TRAP (50 μM) or (C) PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry. To quantify ROS, fluorescent geo-mean values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n=3), **P<0.001 vs. 0.1% v/v DMSO. D Washed platelets (2.5 x 10^5/mL) preloaded with 10 μM H_2DCFDA were left untreated, or treated with PAR4 antagonist tcY-NH₂ (400 μM), then stimulated with TRAP (50 μM) or PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry. To quantify ROS, fluorescent values were normalised relative to unstimulated levels (ROS stimulation index). Data are mean ± SEM (n=2) for TRAP vs. PAR4 antagonist (tcY-NH₂). Data are mean ± SEM (n=6), *P<0.05 vs. PAR4-AP.
Figure 5.2.5 PAR4-deficient mice lack thrombin-induced ROS production

Washed platelets from WT (black bars) and PAR4 KO (shaded bars) mice (2.5 x 10⁶/mL) were preloaded with 10 μM H₂DCFDA and left unstimulated, or stimulated with (A) thrombin (2 U/mL) or (B) the GPVI agonist, CRP (1 μg/mL), for 2 minutes and ROS production was measured by flow cytometry. To quantify ROS, fluorescent geo-mean values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n = 3). **P<0.01 vs. WT littermates.
5.2.5 Thrombin-induced ROS production is GPIbα-dependent

To investigate the role of GPIbα in thrombin-induced ROS generation, Nk protease, an enzyme isolated from the snake venom of the cobra *Naja kaouthia* was used to treat human platelets. This metalloproteinase has been shown to be specific to GPIbα, cleaving between amino acid residues 282 and 283 and thus removing the GPIbα thrombin binding site (Wijeyewickrema et al., 2007). Nk protease (10 μg/mL) consistently removed ~90% of intact GPIbα as measured by flow cytometry with the GPIbα (CD42b)-specific antibody, AN51 (Figure 5.2.6A) and completely abolished thrombin-induced ROS production (Figure 5.2.6B). In contrast, GPVI-dependent ROS production remained unaffected (Figure 5.2.6B). Thrombin has been proposed to bind in two different ways within the GPIbα N-terminal domain (Celikel et al., 2003, Dumas et al., 2004). Here, we demonstrate that without these GPIbα ligand-binding sites, thrombin no longer elicits ROS generation. This suggests that GPIbα is required, along with PAR4 for thrombin-induced ROS production in human platelets. To investigate whether the removal of thrombin-binding sites on GPIbα also affected PAR-induced ROS formation, Nk protease-treated platelets were stimulated with either TRAP (50 μM) or PAR4-AP (250 μM). The absence of the GPIbα N-terminal region did not inhibit PAR1-induced ROS formation, but PAR4-AP-induced ROS generation was significantly reduced (Figure 5.2.6C). These results suggest that GPIbα is not essential for PAR4-dependent ROS formation but is required for maximal response.

5.2.6 GPIbα is required for maximal PAR4-dependent aggregation

To further investigate the relationship between PAR4 and GPIbα, Nk (10 μg/mL) treated platelets were stimulated with PAR4-AP (250 μM) and monitored for their aggregation response. Analysis of overall aggregation, as measured by the area under the curve, showed a small but significant reduction due to the absence of intact GPIbα (Figure 5.2.7B), suggesting a possible functional association between PAR4 and GPIbα on the platelet membrane.
Figure 5.2.6A-B Thrombin-induced ROS production is GPIbα-dependent

Washed human platelets (2.5 x 10⁸/mL) were pre-treated with vehicle control or Nk protease (10 µg/mL) for 30 minutes. A. Platelets were then labelled with the GPIbα-specific antibody (2 µg/mL AN51) or isotype control (2 µg/mL) and intact GPIbα levels were measured by flow cytometry. Data is represented as flow cytometry readout of CD42b positive platelets and is representative of 4 independent experiments. B. Untreated platelets (black bars) and Nk treated platelets (shaded bars) were stimulated with 2 U/mL thrombin (left) and 1 µg/mL CRP (right) for 2 minutes and then ROS production was measured by flow cytometry. To quantify ROS, fluorescent geo-mean values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n=4), ***P<0.001 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 5.2.6C Thrombin-induced ROS production is GPIbα-dependent

Untreated platelets (black bars) and Nk treated platelets (shaded bars) were stimulated with 250 μM PAR4-AP (above) or 50 μM TRAP (below) for 2 minutes, and then ROS production was measured by flow cytometry. To quantify ROS, fluorescent geo-mean values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n=4), *P<0.05, vs. PAR4-AP. Basal levels are indicative of non-stimulated platelets.
Figure 5.2.7 PAR4-induced platelet aggregation is reduced after Nk protease treatment

Washed human platelets were pre-treated with 10 µg/mL Nk protease, for 30 minutes, and then stimulated with 250 µM PAR4-AP for 3 minutes and monitored for platelet aggregation by light transmission aggregometry using a PAP4 platelet aggregometer. Data are mean ± SEM (n=4) **P<0.002 vs. PAR4-AP. Aggregation displayed in a trace (A) and as area under the curve (B).
5.2.7 GPIbα-dependent ROS formation in human platelets

To further understand the role of GPIbα in ROS formation, we used COS-7 cells expressing the VWF-A1 domain containing a gain-of-function mutation R543W as a GPIbα specific agonist (these cells are hereafter referred to as R543W cells). R543W cells express the A1 domain of VWF in a constitutively open and active conformation on the cell membrane as a GPI-linked construct (Figure 5.2.8A) (Gardiner et al., 2010). The VWF-specific antibody, 5D2, confirmed A1 domain surface expression on the R543W cells in comparison to no addition of antibody or non-transfected COS-7 cells (WT: Figure 5.2.8B and 5.2.8C). The non-relevant antibody, AK2 (20 μg/mL), directed against GPIbα was used as a negative control (Figure 5.2.8D).

Whilst a ratio of 1 R543W cell to 400 platelets was sufficient to induce an aggregation response (Figure 5.2.9A), a 1:50 (cell:platelet) ratio was required to elicit a ROS response in washed platelets (Figure 5.2.10A). As expected, non-transfected COS-7 cells (described as WT) at a 1:50 (cell:platelet) ratio were not capable of eliciting ROS production in platelets (Figure 5.2.10B). Although these studies confirm that specific stimulation of GPIbα can inherently induce platelet ROS generation, sub-maximal concentration of R543W cells elicited significantly lower ROS when compared to ROS produced via the CRP and GPVI signalling pathway (Figure 5.2.10B). Pre-incubation with the specific VWF blocking antibody 5D2 (10 μg/mL) significantly inhibited the R543W cell-induced ROS response, confirming that the event was agonist-dependent (Figure 5.2.10C). To confirm that the ROS response was GPIbα-specific, platelets were pre-incubated with the GPIbα-specific antibody AK2 (20 μg/mL) and stimulated with R543W cells. AK2, which binds to the VWF-binding site on GPIbα, significantly blocked both aggregation (Figure 5.2.10D) and ROS formation (Figure 5.2.10C) as stimulated by R543W cells, at 1:400 and 1:50 (cell:platelet ratio), respectively, confirming the direct interaction of GPIbα-VWF A1 domain in platelet derived ROS formation.
Figure 5.2.8 Expression of VWF-A1 domain on R543W cells compared to WT cells

A. A schematic of the recombinant VWF-A1 (R543W mutation) transfected COS-7 cells (Gardiner et al., 2010). Washed R543W and WT cells (2.5 x 10^7/mL) were left (B) untreated or (C) pre-incubated for 1 hour with either the VWF-specific antibody SD2 (10 μg/mL) or (D) the irrelevant antibody AK2 (20 μg/mL). Cells were then labelled with FITC antibody for 30 minutes in the dark. Data are representative of 3 independent experiments.
Figure 5.2.9 R543W-induced ROS production and platelet aggregation at 1:400 (cell:platelet ratio)

Washed human platelets (2.5 x 10⁶/mL) were pre-loaded with 10 μM H₂DCFDA (for ROS experiments only) and stimulated with R543W cells (1:400, cell:platelet ratio) for 2 minutes and monitored for (A) ROS production by flow cytometry and (B) platelet aggregation (1:400, cell:platelet ratio) by light transmission aggregometry. Fluorescent geo-mean values were normalised relative to unstimulated platelets (ROS stimulation index). Data are mean ± SEM (n = 3).
Figure 5.2.10 R543W cells induce VWF-dependent ROS generation

A. Washed human platelets (2.5 x 10⁶/mL) were pre-loaded with 10 μM H₂DCFDA and stimulated with R543W cells at 1:100, 1:50, or 1:20 (cell:platelet) ratio for 2 minutes. ROS production was measured by flow cytometry. Fluorescent values were normalised relative to unstimulated platelets (ROS stimulation index). B. To compare GPIbα-dependent ROS to GPVI-dependent washed human platelets (2.5 x 10⁶/mL) were stimulated with CRP (1 μg/mL) or R543W cells (1:50, cell:platelet ratio) or WT cells (1:50, cell:platelet ratio) for 2 minutes. To quantify ROS, fluorescent values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n = 3), ***P<0.001 vs. CRP, WT cells. C. Washed human platelets (2.5 x 10⁶/mL) were pre-loaded with 10 μM H₂DCFDA and left untreated, or treated with the GPIbα-specific antibody, AK2 (20 μg/mL) or the VWF-specific blocking antibody SD2 (10 μg/mL), and were stimulated with R543W cells (1:50, cell:platelet ratio). Fluorescent geo-mean values were normalised relative to unstimulated platelets. Data are mean ± SEM (n = 3), ***P<0.001 vs. R543W cells. D. Washed human platelets (2.5 x 10⁶/mL) left untreated, or treated with the GPIbα-specific antibody, AK2 (20 μg/mL), which is against the N-terminal domain of GPIbα, were stimulated with R543W cells (1:400, cell:platelet ratio). Platelet aggregation was monitored by light transmission aggregometry (Data are mean ± SEM (n = 3), ***P<0.001 vs. R543W cells.
5.2.8 GPIbα facilitates PAR4-dependent thrombin-induced ROS generation

From the above data it is determined that the two main receptors involved in thrombin-induced ROS generation are PAR4 and GPIbα. A potential association between these two receptors was further investigated by using WT mice, which contain PAR3 and PAR4 but not PAR1 on the platelet membrane. WT platelets were treated with Nk protease (15 µg/mL) to remove any signalling of thrombin through GPIbα. GPIbα cleavage was quantitated by flow cytometry using a GPIbα-specific antibody, Xia.G5 (Figure 5.2.11A). Thrombin-induced ROS generation was inhibited following Nk treatment in WT platelets (Figure 5.2.11B). These results indicate that GPIbα may be acting as an accessory receptor for PAR4 during thrombin activation in murine platelets as well as in human platelets.
Figure 5.2.11 Thrombin-induced ROS after Nk protease treatment of murine platelets

Washed murine platelets (2.5 x 10^7/mL) were pre-treated with (shaded bars) or without (black bars) Nk protease (15 μg/mL) for 45 minutes. (A) GPIbα levels were measured by flow cytometry using PE-labelled GPIbα-specific antibody (Xia.G5) or isotype control. Data are mean ± SEM (n = 3), *P<0.05 vs. GPIbα antibody. (B) H2DCFDA (10 μM)-stained platelets were stimulated with thrombin (2 U/mL) for 10 minutes and ROS production was monitored by flow cytometry. To quantify ROS, fluorescent values were normalised relative to stimulated values (% ROS production). Data are mean ± SEM (n = 3), **P<0.01 vs. thrombin.
5.3 Discussion

In this chapter the roles of thrombin receptors were investigated with key roles for thrombin-induced ROS formation for PAR4 and GPIbα, but not PAR1. The capacity of GPIbα to signal ROS formation was confirmed using the GPIbα specific agonist, R543W cells. Removal of the GPIbα thrombin binding site with Nk protease attenuated PAR4-induced ROS generation and completely inhibited thrombin-induced ROS formation. Similarly, the blockade and absence of PAR4 abolished thrombin-induced ROS generation. These data combined suggest a potentially novel association between GPIbα and PAR4 in this signalling pathway.

Thrombin has previously been implicated in the generation of ROS in platelets but the exact receptors and their respective roles remain unclear (Bakdash and Williams, 2008, Begonja et al., 2005). High concentrations of thrombin are typically required for PAR4 activation, whilst low dose thrombin activates platelets through both GPIbα and PAR1 (De Candia, 2012). Here, washed human platelets were stimulated with increasing doses of thrombin (0.2-2 U/mL). While all thrombin concentrations induced maximal platelet aggregation, only thrombin concentrations of 1 U/mL and higher stimulated ROS formation consistent with an important role for PAR4. In line with previous studies (Begonja et al., 2005), maximum concentrations of TRAP (20-50 μM) were required to induce ROS formation. In comparison, threshold PAR4-AP concentrations (150-250 μM) required for maximum aggregation response induced significant ROS generation. While, a recent study associated platelet activation via PAR4 with the redox signalling molecule 12-LOX (Yeung et al., 2013), to the best of our knowledge the present results are the first report of a role for PAR4 in platelet-derived ROS generation. Not surprisingly, thrombin-induced ROS levels were higher than when PAR1 and PAR4 were activated individually, suggesting that two or more receptors may be involved following stimulation with thrombin.
This issue was addressed using pharmacological antagonists against PAR1 (SCH79797) and PAR4 (tCY-NH$_2$). Interestingly, SCH79797 potentiated thrombin-induced ROS generation, and this response was confirmed using another PAR1 inhibitor, BMS200261. These data suggest that in the absence of PAR1, the ROS response may be increased due to more thrombin being available to bind to PAR4. This data further suggests that PAR1 is not essentially involved in thrombin-induced ROS generation. In contrast, the PAR4 antagonist tCY-NH$_2$ completely abolished thrombin-induced ROS generation, suggesting PAR4 as the main receptor involved in thrombin-dependent ROS production. This role of PAR4 was confirmed using PAR4-deficient platelets which showed a negligible ROS response to thrombin. Overall, these findings indicate a novel and significant functional role for PAR4, independent of PAR1, in thrombin-induced ROS generation in human platelets.

Recent studies show a physical association of the GPIb-IX-V complex with the other important adhesion receptor signalling surface receptor on platelets, GPVI (Arthur et al., 2005, Baker et al., 2004, Gardiner et al., 2010). While GPVI-dependent signalling results in ROS formation (Arthur et al., 2008, Arthur et al., 2012), the role of GPIb$\alpha$ in platelet-derived ROS generation is not clear. In this study, when the N-terminal domain of GPIb$\alpha$ was removed by Nk protease, thrombin-induced ROS response was completely abolished. Interestingly, this decreased response to thrombin was also observed when PAR4, but not PAR1, were inhibited with PAR antagonists. Additionally, in the absence of the thrombin binding sites on GPIb$\alpha$ (via Nk protease cleavage), the platelets also responded poorly to PAR4-AP. These results suggest a possible functional relationship between GPIb$\alpha$ and PAR4 on the platelet membrane. Further investigation demonstrated that PAR4-AP-induced aggregation was also significantly reduced after GPIb$\alpha$ removal, but not abolished, suggesting GPIb$\alpha$ is required for optimal PAR4 activation, further supporting a potential link between these two receptors on the platelet membrane. To date, however, there is no reported connection between PAR4 and GPIb$\alpha$. In contrast, GPIb$\alpha$ is known to act as a co-factor for PAR1 hydrolysis, but not PAR4 (De Candia et al., 2001), and moreover GPIb$\alpha$ amplifies PAR1 responses (Adam et al., 2003c). A possible explanation is that the association with PAR4 is masked by that with PAR1 with respect to platelet aggregation.
but revealed at higher thrombin concentrations with ROS generation, which does not involve PAR1.

*In vivo,* GPIbα interacts with the A1 domain on VWF to activate platelets and initiate adhesion at sites of injury (Kroll et al., 1991). To expand our understanding of the role of GPIbα in ROS formation we used COS-7 cells expressing the VWF-A1 domain with a gain-of-function mutation (R543W) (Gardiner et al., 2010). R543W cells induced a dose-dependent ROS response; however, while 1:400 (cell to platelet ratio) was sufficient to induce platelet aggregation, higher cell to platelet ratios, such as 1:50, were required to induce ROS generation. In addition, the GPIbα-specific response was significantly less robust than GPVI-induced ROS generation induced by CRP, even though the number of copies of GPVI on the platelet membrane is much lower than GPIbα (~1 copy of GPVI for every 6 GPIbα) (Moebius et al., 2005). It was, however, confirmed that VWF initiated platelet ROS production was specifically mediated by the interaction between GPIbα and VWF-A1 domain as monoclonal antibodies against both the VWF-binding site on GPIbα (AK2) and the VWF-A1 domain (5D2) inhibited platelet ROS production.

It is clear from this study that both GPIbα and PAR4 play a prominent role in thrombin-derived platelet ROS generation. In the functional absence of either receptor on human platelets, thrombin cannot elicit ROS production, implying an activation process that involves both proteins. As it is known that upon platelet activation thrombin cleaves GPV to promote GPIb-IX-V receptor complex function (Ramakrishnan et al., 2001), this might also explain why high doses of thrombin are required to generate ROS. Future studies using GPV−/− mice would be beneficial to investigate its role in thrombin-induced ROS formation. In a recent publication, Lova and colleagues showed high dose thrombin (1 U/ml) could induce aggregation in desensitised PARs and GPIbα-cleaved platelets, consistent with the presence of another thrombin receptor (Lova et al., 2010). GPV involvement was ruled out as an anti-GPV blocking antibody did not inhibit thrombin-induced aggregation, therefore the authors speculated to an additional interaction with
thrombin. Indicating that there are possibly other ways to which thrombin can elicit a response, although in our study residual amounts of GPIbα were present (~10%) and concentrations of PAR antagonists used could possibly have been overwhelmed with 2 U/mL thrombin.

At present, PAR4 in human platelets has been established as a low affinity thrombin receptor initiating downstream signalling events following primary activation of platelets through PAR1. In this study, a novel role for PAR4, but not PAR1, was identified in thrombin-induced ROS production. Additionally, the involvement of GPIbα in platelet derived ROS generation was confirmed with a GPIbα-specific agonist. The combined data suggest GPIbα is required to facilitate the interaction of thrombin with PAR4 on the platelet membrane, implicating an association between these two receptors in human and mouse platelets.
Chapter 6

GPIbα, PAR1 and PAR4 platelet-derived ROS generation
6.1 Introduction

Secondary agonists, ADP and TxA₂, do not generate platelet-derived ROS (Begonja et al., 2005). However, thrombin and the PAR1 agonist, TRAP, have previously been implicated in platelet-derived ROS production (Bakdash and Williams, 2008, Pignatelli et al., 2004). In chapter 5, it was demonstrated that thrombin-induced ROS generation requires both GPIbα and PAR4, but the transduction mechanism through which these receptors signal to produce ROS remains unexplored. In this chapter we identify key signalling molecules required for thrombin-, PAR- and GPIbα- dependent ROS generation, and demonstrate where similarities exist with the GPVI-dependent ROS pathway.

Both NOX1 and NOX2 are expressed in human platelets (Dharmarajah et al., 2010, Vara et al., 2013) and notably NOX1 is activated downstream of GPVI-dependent activation (Walsh et al., 2014). Rac1 plays a prominent role in GPIbα-dependent platelet activation and is activated downstream of PAR1 cleavage (Delaney et al., 2012, Azim et al., 2000). NOX complex formation is dependent on Rac1 activation (Bokoch and Diebold, 2002) and in phagocytic cells, where the formation of the NOX complex is well defined, Rac activates NOX, along with other subunits: p47phox, p67phox and p22phox (Krotz et al., 2004, Heyworth et al., 1993). An alternative source of ROS in platelets involves arachidonic acid production, which is a consequence of PLA₂ activation through thrombin stimulation (Caccese et al., 2000, Brash, 2001, Purdon et al., 1987). Arachidonic acid is converted into TxA₂ via COX, or 12-HETE via 12-LOX, resulting in the formation of ROS (Brash, 2001). Additionally, inhibitors of both LOX and COX attenuate platelet responses after stimulation with physiological agonists (Ozeki et al., 1998, Nyby et al., 1996, Chen et al., 1997).

Ligand binding to GPIbα initiates downstream signalling similar to that of the GPVI pathway (Gardiner et al., 2010), involving the activation of Src family kinases (SFKs) and Syk, leading to PI3-K and PKC activation (Falati et al., 1999, Liu et al., 2005, Mu et al., 2010,
Liu et al., 2006, Adam et al., 2003b). PI3-K and SFKs have been implicated in PAR-dependent signalling but a functional role for Syk has not been defined (Hughan et al., 2007, Xiang et al., 2012). PLCβ, specific to PAR signalling, and PLCγ2, specific to signalling through GPVI and the GPIb-IX-V complex, are the major isoforms of PLC found in platelets (Lian et al., 2005, Suzuki-Inoue et al., 2003, Lee et al., 1996, Suzuki-Inoue et al., 2004). PLCβ is activated through receptor-associated Gαq and, similar to PLCγ2, results in the hydrolysis of PIP2 (Lian et al., 2005). These events lead to the formation of IP3 and DAG, subsequently increasing Ca2+ mobilisation and activating PKC, respectively (Offermanns et al., 1997). At the same time, DAG induction of PKC activation also leads to an increase in Ca2+ entry (Rosado and Sage, 2000), ultimately leading to platelet activation.

To further understand the roles of PAR4 and GPIbα in thrombin-induced signalling leading to ROS generation, specific pharmacological antagonists were used to target platelet signalling molecules. The main objectives for this study were i) to determine what signalling molecules are involved in thrombin-, GPIbα-, and PAR-dependent ROS formation and ii) to identify similarities between these signalling molecules and those employed for GPVI-dependent ROS formation.
6.2 Results

6.2.1 NOX1 is the main source of ROS downstream of GPIbα and PAR activation

In chapter 3 it was shown that NOX1 was the main isoform involved in GPVI-dependent ROS generation. Here, we explored the role of NOX1 in ROS formation induced by other platelet receptors using the NOX1 inhibitor, ML171. Thrombin (1 U/mL – Figure 6.2.1A)-, R543W cells (1:50, cell to platelet ratio – Figure 6.2.1B)-, TRAP- (50 μM) and PAR4-AP-(250 μM) induced ROS generation (Figure 6.2.1C), but not platelet aggregation, were abolished by the NOX1-specific inhibitor, ML171 (5 μM). These results suggest that platelet-derived ROS generation is NOX1-dependent regardless of the agonist employed and furthermore demonstrate that NOX1 is not required for maximal platelet aggregation.

6.2.2 Rac1 is required for PAR-induced ROS formation stimulated by thrombin

As NOX1 was required for thrombin-induced ROS generation, it was investigated if Rac1, an essential component for NOX complex assemblage, was also required. Washed platelets were pre-treated with the Rac1 inhibitor, EHT-1864 (50 μM), stimulated with 1 U/mL thrombin and monitored for platelet aggregation and ROS production. Figure 6.2.2A shows a significant inhibition by EHT-1864 (~50%) for both thrombin-induced platelet aggregation and ROS generation. Notably, EHT-1864 completely abolished both TRAP- (50 μM) and PAR4-AP- (250 μM) induced ROS generation, and significantly reduced PAR1-dependent and PAR4-dependent platelet aggregation by about 20% and 50%, respectively (Figure 6.2.2B).

6.2.3 Thrombin-induced ROS production does not require PLA₂

PLA₂ is required for arachidonic acid synthesis (Brash, 2001). Therefore, inhibition studies were performed to determine if arachidonic acid production contributed to the thrombin-induced ROS response. Washed platelets were pre-treated with the PLA₂-specific inhibitor, AACOCF₃ (10 μM), and stimulated with 1 U/mL thrombin. ROS generation and platelet
aggregation stimulated by thrombin were not significantly inhibited, although a trend towards a reduction was observed (Figure 6.2.3A). In contrast, the PLA₂ antagonist potentiated PAR4-AP-induced ROS generation (250 µM) significantly, with no effect on aggregation (Figure 6.2.3B). No effect was evident for TRAP-induced ROS generation or platelet aggregation (50 µM) (Figure 6.2.3B). These data indicate that PLA₂ is not required for thrombin-induced ROS production.

6.2.4 Thrombin-induced ROS generation requires activation of SFKs

SFKs are phosphorylated after stimulation of platelets with thrombin and have previously been implicated in thrombin-induced platelet activation (Topalov et al., 2012, Xiang et al., 2012, Cho et al., 2002). To investigate whether SFKs are involved in ROS formation downstream of thrombin stimulation, washed platelets were pre-incubated with the broad SFK inhibitor, PP2 (25 µM). Thrombin-induced ROS production (1 U/mL) was significantly abrogated in the presence of PP2, but platelet aggregation was unaffected (Figure 6.2.4A). SFKs have been implicated downstream of PAR activation, although their exact role remains unclear (Hughan et al., 2007, Xiang et al., 2012). Washed platelets were pre-incubated with PP2 and stimulated with R543W cells (1:50, cell:platelet ratio), TRAP (50 µM) or PAR4-AP (250 µM). PP2 had no effect on PAR-induced ROS generation nor did it effect PAR4-AP-induced platelet aggregation (Figure 6.2.4B). It did, however, significantly reduce TRAP-induced platelet aggregation (Figure 6.2.4B). Previous studies have shown that activation of SFKs is an early signalling event in GPIbα-dependent signalling and are vital for platelet function (Falati et al., 1999). The SFK inhibitor completely abrogated both GPIbα-dependent platelet responses (ROS and platelet aggregation – Figure 6.2.4C), implying a prominent role for SFKs in GPIbα-mediated ROS production.
Figure 6.2.1A Effect of the NOX1 inhibitor, ML171, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the NOX1 inhibitor (5 μM ML171) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3), ***P<0.001 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.1B Effect of the NOX1 inhibitor, ML171, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/mL) preloaded with 10 μM DCFDA were pre-treated with the NOX1 inhibitor (5 μM ML171) for 10 minutes, then stimulated with TRAP (50 μM) or PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=4), aggregation data (n=3), ***P<0.01 vs. PAR4-AP, **P<0.001 vs. TRAP. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.1C Effect of the NOX1 inhibitor, ML171, on GPIbα-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 × 10⁶/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the NOX1 inhibitor (5 μM ML171) for 10 minutes, then stimulated with R543W cells (1:50) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3), **P≤0.01 vs. R543W cells. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.2A Effect of the Rac1 inhibitor, EHT-1864, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 × 10^5/mL) preloaded with 10 μM H$_2$DCFDA were pre-treated with the Rac1 inhibitor (50 μM EHT) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM (n=5), **P<0.01 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.2B Effect of the Rac1 inhibitor, EHT-1864, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁷/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the Rac1 inhibitor (50 μM EHT) for 10 minutes, then stimulated with TRAP (50 μM) and PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM (n=5), *P<0.05 vs. PAR4-AP, ***P<0.001 vs. TRAP. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.3A Effect of the PLA₂ inhibitor, AACOCF₃, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁹/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the PLA₂ inhibitor (10 μM AACOCF₃) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=6), aggregation data (n=4). Basal levels are indicative of non-stimulated platelets.
Figure 6.2.3B Effect of the PLA₂ inhibitor, AACOCF₃, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the PLA₂ inhibitor (10 μM AACOCF₃) for 10 minutes, then stimulated with TRAP (50 μM) or PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=5), *P<0.05 vs. PAR4-AP. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.4A Effect of the SFK inhibitor, PP2, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were pre-treated with the SFK inhibitor (25 μM PP2) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=6), aggregation data (n=3). **P<0.01 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.4B Effect of the SFK inhibitor, PP2, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were pre-treated with the SFK inhibitor (25 μM PP2) for 10 minutes, then stimulated with TRAP (50 μM) and PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=7), aggregation data (r=3), *P<0.05 vs. TRAP. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.4C Effect of the SFK inhibitor, PP2, on GPIbα-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁹/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the SFK inhibitor (25 μM PP2) for 10 minutes, then stimulated with R543W cells (1:50) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3), **P<0.01 vs. R543W cells. Basal levels are indicative of non-stimulated platelets.
6.2.5 GPIbα-dependent ROS generation requires Syk

Syk plays a prominent role in GPVI-dependent ROS formation as demonstrated in chapter 3. Syk phosphorylation is induced through stimulation with thrombin (Clark et al., 1994, Hughan et al., 2007, Taniguchi et al., 1993), although a functional role has yet to be characterised. Here, the Syk-specific inhibitor, BAY 61-3606 (5 μM) had no inhibitory effect on either thrombin-induced platelet aggregation or ROS generation (Figure 6.2.5A). Similarly, there was no difference on stimulation with PAR agonists (TRAP; 50 μM, and PAR4-AP; 250 μM) between BAY 61-3606-treated and untreated platelets (Figure 6.4.5B).

In contrast, GPIbα-dependent ROS generation (induced with R543W cells, 1:50, cell:platelet ratio) was abolished in the presence of BAY 61-3606 and platelet aggregation was significantly reduced (Figure 6.2.5C). Combined, these results suggest that Syk plays an important role in GPIbα-mediated signalling leading to ROS production, but is not essential when thrombin is employed as a stimulus.

6.2.6 Thrombin (0.05 U/mL)-induced TxA2 generation is Syk- and NOX-dependent

Previous studies have shown Syk is involved in GPIbα-dependent TxA2 production after stimulation with VWF and the modulator botrocetin (Liu et al., 2005). Therefore, to fully elucidate the role of Syk in thrombin-induced platelet activation, we assessed the effect of BAY 61-3606 on TxA2 production. Walsh et al. have shown NOX1 is required for TxA2 generation downstream of GPVI activation (Walsh et al., 2014) and similarly, here ML171 was used to investigate the role of NOX1 in thrombin-induced TxA2 production. Washed platelets were pre-incubated with the Syk inhibitor, BAY 61-3606 (5 μM), and the NOX1 inhibitor, ML171 (5 μM), stimulated with 0.05 U/mL or 1 U/mL of thrombin, and assessed for their ability to aggregate and generate TxA2. Thrombin-induced platelet aggregation at 0.05 U/mL was slightly reduced and TxA2 production was completely abolished in the presence of BAY 61-3606 (Figure 6.2.6A). In contrast, 1 U/mL thrombin-induced platelet aggregation and TxA2 production were not affected by the Syk inhibitor (Figure 6.2.6B). The NOX1 inhibitor, ML171, completely abolished TxA2 production, but not platelet
aggregation, in response to high and low dose thrombin (Figures 6.2.6A and B), suggesting thrombin-induced TxA₂ generation is downstream of NOX1-derived ROS production.

6.2.7 FAK is a prerequisite for thrombin-induced ROS production

As described in chapter 4, FAK plays a prominent role in GPVI-dependent ROS formation and has previously been implicated in downstream signalling following thrombin stimulation (Shattil et al., 1994). To assess whether FAK functions as a downstream mediator of thrombin-induced ROS formation, washed platelets were pre-incubated with the FAK inhibitor, PF-228 (1 μM), stimulated with thrombin (1 U/mL) and monitored for both platelet aggregation and ROS formation. While thrombin-induced ROS production was significantly inhibited by PF-228, there was no significant impact on platelet aggregation (Figure 6.2.7A). R543W cell- (1:50), TRAP- (50 μM) and PAR4-AP- (250 μM) induced ROS formation and platelet aggregation were inhibited by PF-228 (1 μM) (Figures 6.2.7B and C), suggesting that FAK plays a functional role downstream of GPIbα and PARs, following thrombin-induced ROS production.

6.2.8 PI3-K is involved in PAR4- but not PAR1-dependent ROS formation

PI3-K is well known as a downstream mediator of GPIbα and PAR signalling (Voss et al., 2007, Wu et al., 2010, Nakanishi et al., 2010, Mu et al., 2010). To investigate the role of PI3-K in thrombin-induced ROS generation, we used the PI3-K inhibitor, wortmannin. Washed platelets pre-treated with wortmannin (100 nM) and stimulated with thrombin (1 U/mL) had no inhibitory effect in ROS production and platelet aggregation compared to untreated platelets (Figure 6.2.8A). Wortmannin did not inhibit TRAP-induced ROS production (50 μM) but did significantly abrogate TRAP-dependent platelet aggregation (Figure 6.2.8B). In comparison, PAR4-AP-induced ROS production (250 μM) was significantly abrogated in the presence of wortmannin but platelet aggregation was unaffected (Figure 6.2.8B).
Figure 6.2.5A Effect of the Syk inhibitor, BAY 61-3606, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^8/mL) preloaded with 10 μM H2DCFDA were pre-treated with the Syk inhibitor (5 μM BAY 61-3606) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=6). Basal levels are indicative of non-stimulated platelets.
Figure 6.2.5B Effect of the Syk inhibitor, BAY 61-3606, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 µM H2DCFDA were pre-treated with the Syk inhibitor (5 µM BAY 61-3606) for 10 minutes, then stimulated with TRAP (50 µM) and PAR4-AP (250 µM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=4). Basal levels are indicative of non-stimulated platelets.
Figure 6.2.5C Effect of the Syk inhibitor, BAY 61-3606, on GPIbα-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were pre-treated with the Syk inhibitor (5 μM BAY 61-3606) for 10 minutes, then stimulated with R543W cells (1:50) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=3), **P<0.01 vs. R543W cells. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.6A Low dose thrombin-induced TxA2 generation (0.05 U/mL) is Syk-dependent

Washed human platelets (2.5 x 10^9/mL) pre-treated with the Syk inhibitor (5 μM BAY 61–3606) or the NOX1 inhibitor (5 μM ML171) for 10 minutes were stimulated with 0.05 U/mL thrombin and monitored for platelet aggregation by light transmission aggregometry (above) and TxA2 generation (below) using a TxB2 ELISA. Data are mean ± SEM (n=4), **P<0.01, ***P<0.001 vs. thrombin.
Figure 6.2.6B High-dose thrombin-induced TxA₂ generation (1 U/mL) is Syk-independent

Washed human platelets (2.5 x 10⁵/mL) pre-treated with the Syk inhibitor (5 μM BAY 61-3606) or the NOX1 inhibitor (5 μM ML171) for 10 minutes were stimulated with 1 U/mL thrombin and monitored for platelet aggregation by light transmission aggregometry (above) and TxA₂ generation (below) using a TxB₂ ELISA. Data are mean ± SEM (n=4), *P<0.05 vs. thrombin.
Figure 6.2.7A Effect of the FAK inhibitor, PF-228, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^8/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the FAK inhibitor (1 μM PF-228) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=6), aggregation data (n=3), ***P<0.001 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
**Figure 6.2.7B Effect of the FAK inhibitor, PF-228, on PAR-dependent ROS generation and platelet aggregation**

Washed human platelets (2.5 x 10^8/mL) preloaded with 10 μM H2DCFDA were pre-treated with the FAK inhibitor (1 μM PF-228) for 10 minutes, then stimulated with TRAP (50 μM) and PAR4-AP (250 μM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=4). **P≤0.01 vs. PAR agonists, ***P≤0.01 vs. TRAP, **P≤0.001 vs. PAR4-AP. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.7C Effect of the FAK inhibitor, PF-228, on GPIbα-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁶/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the FAK inhibitor (1 μM PF-228) for 10 minutes, then stimulated with R543W cells (1:50) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=3), **P<0.01 vs. R543W cells. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.8A Effect of the PI3-K inhibitor, Wortmannin, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) pre-loaded with 10 μM H2DCFDA were pre-treated with the PI3-K inhibitor (100 nM Wortmannin) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent gene-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=5). Basal levels are indicative of non-stimulated platelets.
Figure 6.2.8B Effect of the PI3-K inhibitor, Wortmannin, on PAR-dependent ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁹/mL) preloaded with 10 µM H₂DCFDA were pre-treated with the PI3-K inhibitor (100 nM Wortmannin) for 10 minutes, then stimulated with TRAP (50 µM) or PAR4-AP (250 µM) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, (n=5), *P<0.05 vs. TRAP, **P<0.01 vs. PAR4-AP. Basal levels are indicative of non-stimulated platelets.
6.2.9 PLC and PKC are non-essential for thrombin-induced ROS generation

PLCγ2 and PLCβ are involved in GP Ibα- and PAR-dependent signalling, respectively, resulting in the subsequent activation of PKC (Lian et al., 2005, Suzuki-Inoue et al., 2004). In this study, generic inhibitors were employed to determine whether PLC and PKC play significant roles in thrombin-induced ROS formation. Washed platelets pre-treated with inhibitors, were stimulated with 1 U/mL thrombin and assessed for platelet aggregation and ROS production. The PLC inhibitor, U73122 (10 μM), had no effect on either ROS generation or platelet aggregation elicited by thrombin (Figure 6.2.9A). In contrast, while the PKC inhibitor, GF109203X (20 μM), did not exert any effect on thrombin-induced ROS generation, it significantly reduced platelet aggregation (Figure 6.2.9B).

6.2.10 Intracellular Ca^{2+} is not required for thrombin-induced ROS production

Following receptor ligation on platelets, PLC activation induces Ca^{2+} mobilisation and subsequent intracellular calcium influx (Varga-Szabo et al., 2009). To investigate the role of Ca^{2+} in thrombin-stimulated platelets, we used the intracellular Ca^{2+} chelator, BAPTA (10 μM), and the extracellular Ca^{2+} chelator, EGTA (2.5 mM). BAPTA alone was not sufficient to reduce thrombin-induced ROS generation or platelet aggregation (Figure 6.4.10A). Treatment with EGTA reduced ROS production significantly and inhibited platelet aggregation (Figure 6.4.10A). Combined treatment with BAPTA and EGTA completely ablated the thrombin-induced ROS and platelet aggregation responses (Figure 6.4.10A). In comparison, the effect of BAPTA on TRAP- (50 μM) and PAR4-AP- (250 μM) induced ROS was more robust (Figure 6.2.10B). BAPTA slightly reduced platelet aggregation in response to PAR1 activation, and significantly reduced PAR4-dependent platelet aggregation.
Figure 6.2.9A Effect of the PLC inhibitor, U73343, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H2DCFDA were pre-treated with the PLC inhibitor (10 μM U73343) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=6), aggregation data (n=7). Basal levels are indicative of non-stimulated platelets.
Figure 6.2.9B Effect of the PKC inhibitor, GF109203X, on thrombin-induced ROS generation and platelet aggregation

Washed human platelets (2.5 x 10⁵/mL) preloaded with 10 μM H₂DCFDA were pre-treated with the PKC inhibitor (20 μM GF109203X) for 10 minutes, then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=6) *P<0.05 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.10A Intracellular Ca\(^{2+}\) is not required for thrombin-induced ROS production

Washed human platelets (2.5 x 10^9/mL) preloaded with 10 μM H\(_2\)DCFDA were pre-treated with the intracellular Ca\(^{2+}\) chelator (10 μM BAPTA) for 10 minutes or the extracellular Ca\(^{2+}\) chelator (2.5 mM EGTA), then stimulated with 1 U/mL thrombin for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=5), aggregation data (n=4), *P≤0.05 vs. thrombin. Basal levels are indicative of non-stimulated platelets.
Figure 6.2.10B Intracellular Ca\(^{2+}\) is required for PAR-dependent ROS production

Washed human platelets (2.5 \(\times\) 10\(^5\)/mL) preloaded with 10 \(\mu\)M H\(_2\)DCFDA were pre-treated with the intracellular Ca\(^{2+}\) chelator (10 \(\mu\)M BAPTA) for 10 minutes, then stimulated with TRAP (50 \(\mu\)M) or PAR4-AP (250 \(\mu\)M) for 2 minutes and monitored for ROS production by flow cytometry (above) and platelet aggregation by light transmission aggregometry (below). To quantify ROS, fluorescent geo-mean values were normalised relative to 100% stimulated levels (% ROS Production). Data are mean ± SEM, ROS data (n=3), aggregation data (n=4), *P≤0.05 vs. PAR4-AP, **P≤0.01 vs. PAR agonists. Basal levels are indicative of non-stimulated platelets.
Table 6.1 A summary of the inhibitors used in this chapter and in chapter 3: Inhibitors against signalling molecules involved in GPVI-dependent ROS generation and their effect on platelet-derived ROS generation stimulated through CRP, thrombin, TRAP, PAR4-AP and R543W cells

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6.3 Discussion

In platelets, ROS are generated through thrombin-stimulation, PAR and GPIbα activation, as demonstrated in chapter 6. The underlying pathway(s) behind platelet-derived ROS generation remains unclear. In this study, pharmacological inhibitors were used to delineate the signalling molecules downstream of thrombin-, GPIbα- and PAR-dependent ROS generation.

The findings of this study support a role for NOX1 as the main source of ROS in platelets. As platelet aggregation was affected but not inhibited in the presence of the NOX1 inhibitor we speculate that ultimately divergent pathways are required for platelet aggregation and ROS generation. Furthermore, the fact that NOX1 lies upstream of GPVI-dependent (Walsh et al., 2014) and thrombin-induced TxA₂ generation implicates ROS in intracellular signalling in platelets. Inhibition of the GTPase activity of Rac1 caused a partial reduction in thrombin- and PAR-induced ROS formation. A feasible explanation for the incomplete inhibition in thrombin-induced ROS production may be due to the presence of residual activity of other components of the NOX complex, including p22phox, p47phox and p67phox (Bokoch and Diebold, 2002). In this regard, Rac1 has been demonstrated to translocate independently of p47phox and p67phox in human neutrophils (Heyworth et al., 1994). Previous reports have implicated PLA₂ in thrombin-induced platelet aggregation and ROS formation (McNicol and Nickolaychuk, 1995, Pignatelli et al., 2004). In our hands AACOCF₃ did not inhibit thrombin-induced ROS formation. However, a different assay was employed previously to measure superoxide production in comparison to that used here and the concentration of thrombin used in this study is 10-fold higher than in the previous study. Here, inhibition of PLA₂ potentiated PAR4-induced ROS generation, thereby suggesting an arachidonic acid-dependent negative feedback loop.

One of the main objectives of this study was to determine similarities between GPVI-dependent ROS formation and ROS production by other agonists. SFKs are critical for
downstream signalling through GPVI and the GPIb-IX-V complex (Falati et al., 1999, Suzuki-Inoue et al., 2002), while their role in PAR-dependent signalling is not well defined. Here, we show SFKs are required for thrombin-induced ROS generation. To determine what receptor(s) SFKs were signalling through, both GPIbα- and PAR-induced ROS formation were further analysed in the presence of PP2. Previous studies have detected SFK activation downstream of PAR1 activation in comparison to its receptor proximal role with GPIbα (Xiang et al., 2012). Consistent with these studies, SFKs were not required for PAR-dependent ROS formation, possibly because ROS generation is an early signalling event independent of integrin α<sub>IIb</sub>β<sub>3</sub> activation (Arthur et al., 2012). However, the variability of ROS generation between donors may also explain why the broad SFK inhibitor reduced PAR-dependent ROS generation but had no statistical significance. SFKs were essential, however, for both GPIbα-dependent ROS generation and platelet aggregation. It is interesting to speculate that thrombin-induced ROS production is therefore mediated through a GPIbα-dependent pathway, which results in the activation of SFKs. Furthermore, PP2 had no effect on thrombin-induced aggregation consistent with previous reports (Hughan et al., 2007). In summary, whilst SFKs are essential for GPVI- and GPIbα-dependent signalling, they play only a minor role downstream of thrombin stimulation.

Syk has previously been shown to play a prominent role in GPVI-dependent ROS formation (Arthur et al., 2012). Similarly, in chapter 3 inhibition of Syk dramatically attenuated all CRP-induced responses. The importance of Syk was further established in GPIbα-dependent ROS generation. In contrast, thrombin- and PAR-induced ROS production did not require Syk. Stimulation with thrombin can result in the activation of Syk (Clark et al., 1994, Hughan et al., 2007, Taniguchi et al., 1993); however, in agreement with a previous report, our findings suggest that Syk is non-essential for thrombin-induced platelet aggregation (Hughan et al., 2007) and TxA<sub>2</sub> generation. However, at low concentrations of thrombin, BAY 61-3606 reduced platelet aggregation and TxA<sub>2</sub> production, suggesting that at 1 U/mL thrombin, other signalling pathways eliminate a partial role for Syk. In
conclusion, Syk is not required for thrombin-induced ROS generation, but is essential in GPVI- and GPIbα-dependent ROS production.

FAK has been implicated as a key signalling kinase in thrombin-induced platelet activation and is involved in PAR4-dependent platelet spreading (Guinebault et al., 1995, Hitchcock et al., 2008, Shattil et al., 1994). In chapter 4, a novel role for FAK in GPVI-dependent ROS formation was described. Similarly, in this study, the FAK inhibitor, PF-228, dramatically abolished thrombin-, GPIbα- and PAR-dependent ROS generation and platelet aggregation. In contrast, FAK was not required for thrombin-induced platelet aggregation, suggesting that pathways leading to ROS generation and platelet aggregation diverge and that alternate pathways can be activated that in some instances bypass the requirement for signalling proteins such as FAK.

PI3-K activation occurs downstream of Rac1 during PAR1-induced platelet activation (Azim et al., 2000). Similarly, in this study PI3-K inhibition attenuated PAR1-induced platelet aggregation. PAR4 and PI3-K have been previously reported to work in synergy to maintain thrombin-induced aggregation and integrin αIIbβ3 activation (Wu et al., 2010). Interestingly, the PI3-K antagonist, wortmannin, inhibited PAR4-dependent ROS formation with no effect on PAR1-dependent ROS formation. These findings demonstrate a reverse role for PI3-K in ROS generation and aggregation downstream of PAR1 and PAR4 activation further confirming the unique and distinct differences between PARs. However, neither thrombin-induced ROS generation nor platelet aggregation were inhibited by wortmannin. Overall, the combined results do not support a crucial role for PI3-K in thrombin-induced ROS formation, although it appears to play a more significant role downstream of PAR4 activation.

PI3-K catalyses the phosphorylation of PIP2 toPIP3, which acts as a substrate for PLC, generating DAG (which activates PKC) and IP3 (which mobilises Ca2+) (Moroi and Jung,
In this study, the generic PLC inhibitor, U73122, was used to target all isoforms of PLC and likewise, the broad acting PKC antagonist, GF109203X, was used to inhibit PKC. Neither PKC nor PLC were required for thrombin-induced ROS formation. Downstream of thrombin stimulation, PLCβ appears to play a role in Ca$^{2+}$ mobilisation but plays no role in platelet aggregation or secretion (Lee et al., 1996, Lian et al., 2005). Importantly, these data strongly suggest that intracellular calcium release is not essential for ROS generation following platelet stimulation with thrombin, consistent with the lack of effect of the PLC inhibitor. In contrast, under the same experimental conditions, EGTA and BAPTA together dramatically inhibited ROS production indicating that extracellular calcium influx is a key event in eliciting this response. Additionally, intracellular Ca$^{2+}$ mobilisation was required for PAR- and GPVI- dependent ROS formation. In this study, PKC inhibition had no effect on thrombin-induced platelet aggregation or ROS generation. In contrast, thrombin-induced platelet aggregation in the presence of both PKC and PAR4 antagonists is significantly reduced (Wu et al., 2010), indicative of a role for PAR4 that might be relevant at higher thrombin concentrations. PAR4 is required for a prolonged Ca$^{2+}$ response (Covic et al., 2000) and therefore higher amounts of thrombin may sustain platelet aggregation through PAR4, but not PAR1, and subsequently a requirement of PKC may be dependent on PAR4 activation. Furthermore, different isoforms of PKC are involved in thrombin-dependent platelet functions (Harper and Poole, 2010), which prevents a clear interpretation of the data presented here as only a general PKC agonist was employed.

This study, using thrombin at 1 U/mL, has for the first time identified distinct signalling proteins downstream of PARs and GPIIb$\alpha$ activation involved in ROS production. A limitation of the present study is that lower concentrations of thrombin were not examined as the primary query was determining the signalling proteins involved in ROS generation. While the effects of the chosen inhibitors on thrombin-induced ROS and aggregation are quite clear the small sample sizes and high concentrations of thrombin may impact on the true results. For example, this higher concentration of thrombin might overwhelm the inhibitory effects of antagonists and mask the role of specific signalling protein involvement for platelet aggregation. For example, a role for PI3-K is difficult to
establish with such high concentrations of thrombin, although it inhibits PAR1-dependent aggregation. Future studies employing a range of thrombin concentrations in platelet aggregation are needed to address this issue. Nevertheless, the results presented here have identified key signalling proteins required for GP Ibα, PAR1, PAR4 and thrombin responses. Distinct differences were found in comparison to GPVI (Table 6.1). Delineating the molecular mechanisms behind platelet-derived ROS generation is vital to further understand this less described functional response of platelets. As demonstrated here, inhibition of NOX1 targets secondary platelet functions with less effect on platelet aggregation. This observation suggests that targeting ROS generation may be an important therapeutic target in cardiovascular disease. As demonstrated in previous studies NOX is an attractive candidate to target during cardiovascular disease (Vara et al., 2013, Walsh et al., 2014). Inhibition of NOX1 attenuated platelet thrombus volume but had no effect on platelet thrombus coverage over collagen, in comparison to aspirin which significantly disrupted both (Walsh et al., 2014). Therefore targeting NOX may inhibit secondary events and effect thrombosis without disrupting haemostasis. However, it still remains to be established whether targeting NOX in vivo provides an alternative to other anti-platelet therapy.
There is considerable evidence in the literature to suggest ROS play a prominent role in the pathogenesis of cardiovascular disease (Kim et al., 2013, Landmesser et al., 2002, Madamanchi et al., 2005). Hypertensive patients present with increased levels of 12-HETE (Gonzalez-Nunez et al., 2001) and SOD2 KO mice exhibit increased infarct size (Murakami et al., 1998). ROS may also be a consequence of cardiovascular disease and further amplify the causative effects (Juranek and Bezek, 2005). Moreover, CGD patients (lacking the p91phox subunit of the NOX complex) exhibit platelet dysfunction in response to various stimuli (Pignatelli et al., 2010). Further, naturally occurring antioxidants, found in garlic, onions, red wine and olive oil, inhibit platelet activation (Navarro-Nunez et al., 2009, Togna et al., 2003). In comparison to phagocytic cells, which release ROS to fight invading bacteria, ROS production in platelets is intracellular, suggesting a role in signalling. Thus, it is of importance to understand how exactly ROS are regulated in platelets.

GPVI is the main receptor involved in platelet-derived ROS generation (Arthur et al., 2012, Bakdash and Williams, 2008, Begonja et al., 2005, Pignatelli et al., 2010, Vara et al., 2013). In chapter 3, the key signalling proteins leading to GPVI-dependent ROS formation were identified. Consistent with previous studies (Walsh et al., 2014), NOX1 was identified as important for CRP-induced ROS formation, but not platelet aggregation, suggesting a clear bifurcation between the pathways. In addition, the data shown in chapter 4 demonstrate that GPVI-dependent ROS formation was also integrin $\alpha_{\text{IIb}}\beta_3$-independent and therefore precedes aggregation. These findings are in accordance with previous reports (Arthur et al., 2012), and similarly CGD patient platelets exhibit partial activation of integrin $\alpha_{\text{IIb}}\beta_3$ (Pignatelli et al., 2010). A role for PLA$_2$ was also described consistent with ROS generation downstream of arachidonic acid mobilisation. This observation is in agreement with the finding that NOX1 activation is required for P38 MAPK activation, which results in the activation of PLA$_2$ and TxA$_2$ generation (Walsh et al., 2014) and indicative of a possible feedback role for arachidonic acid release on NOX1 activation.
A complex, comprised of Pyk2, Hic-5, TRAF4 and p47\textsuperscript{phox}, associated with GPVI, has been previously described in platelets (Arthur et al., 2011). In chapter 4, the functional roles of the FAK family members, Pyk2 and FAK, were defined in GPVI-dependent platelet activation. Through the use of pharmacological inhibitors and Pyk2 KO mice, a novel role for FAK in GPVI-dependent ROS formation was identified, while Pyk2 appeared to be non-essential. Additionally, the location of FAK within the GPVI signalling pathway was determined through the use of pharmacological inhibitors and by immunoprecipitation studies. Although they are useful tools in molecular biology, pharmacological inhibitors are not always target specific, as demonstrated with the purported Pyk2 inhibitor, tyrphostin A9, which attenuated ROS generation in Pyk2 KO mice. Nevertheless, functional differences were determined for both FAK and Pyk2 providing novel insights into the divergent molecular mechanisms downstream of GPVI activation.

In chapter 5, it was demonstrated that PAR4 and GPIb\(\alpha\) were required to facilitate thrombin-induced ROS generation. Through the use of Nk protease to remove the thrombin binding site on GPIb\(\alpha\), it was hypothesised that GPIb\(\alpha\) may facilitate the functional role of PAR4 in platelet-derived ROS generation induced by thrombin. Synergistic functions of PARs with other platelet receptors are not uncommon (De Candia et al., 2001, Graff et al., 2004, Holinstat et al., 2006, Keuren et al., 2005). PAR1 cleavage is accelerated in the presence of GPIb\(\alpha\) (De Candia et al., 2001) and thrombin- and collagen-induced Ca\(^{2+}\) mobilisation and procoagulant activity are sustained through PAR1 (Keuren et al., 2005). In contrast, PAR4, but not PAR1, functions synergistically with GPVI to activate the integrin, \(\alpha_{IIb}\beta_3\) (Graff et al., 2004), and the P2Y\textsubscript{12} ADP receptor is required for PAR4-mediated platelet aggregation (Holinstat et al., 2006). Previous studies using mouse platelets and COS-7 cells expressing GPIb\(\alpha\) (including the binding site for thrombin) found no association between GPIb\(\alpha\) and PAR4 (Nakanishi-Matsui et al., 2000). However, on the mouse platelet membrane, the functional role of PAR3 should be taken into consideration as it functions to enhance PAR4 activation (Nakanishi-Matsui et al., 2000). Therefore, future work requires investigation of PAR4-dependent ROS production in PAR3 KO mice.
with and without GPIbα cleavage. There is also the possibility that Nk protease could be cleaving PAR4, which is unlikely but was not formally investigated.

The work carried out in this thesis demonstrates the direct involvement of GPIbα in ROS production through the use of a GPIbα-specific agonist, R543W cells (COS cells expressing VWF-A1 domain with a gain-of-function mutation). GPIbα and GPVI are physically and functionally associated on the platelet membrane (Arthur et al., 2005, Gardiner et al., 2010) and in vitro studies have determined that GPVI mediates platelet activation at low shear stress while GPIbα plays a unique role in platelet adhesion at high shear stress (Goto et al., 2002, Siljander et al., 2004). It was therefore surprising that binding of R543W cells to platelets resulted in less ROS production compared to that with CRP. This difference, however, may simply reflect the amount of TRAF4 (and hence p47phox) associated with each receptor. In a physiological setting, GPIbα would presumably bind both VWF and thrombin and would work in synergy with GPVI-collagen activation. Notably, previous findings have shown that VWF-GPIbα interactions are hindered in the presence of thrombin (George and Torres, 1988) and a thrombin mutant (WE-thrombin; mutations at Trp215Ala and Glu217Ala) that only allows for interaction with GPIbα and not PARs (Berny et al., 2008). Similarly, the Dumas model of thrombin binding to GPIbα (Dumas et al., 2004) is inconsistent with the simultaneous binding of thrombin and VWF (Adam et al., 2003a). Recently, however, Zarpellon and colleagues employed an elegant approach to understanding the mechanism of binding of thrombin to GPIbα. Using genetically modified GPIbα, they established that the inhibitory or enhanced platelet response depends on which thrombin exosite is bound to GPIbα (Zarpellon et al., 2011). Thus, physiologically GPIbα could potentially produce ROS through interaction with both thrombin and VWF and with synergistic effects on platelet activation.

In chapter 6, the role of key signalling molecules downstream of PAR- and GPIbα-dependent ROS formation were established through the use of pharmacological inhibitors
and specific agonists; R543W cells (GPIbα), TRAP (PAR1) and PAR4-AP (PAR4). SFKs, essential for GPIbα- and GPVI-dependent signalling (Falati et al., 1999, Schmaier et al., 2009), were also important for thrombin-induced ROS formation, consistent with a role for GPIbα in thrombin-induced ROS formation. Syk was required for both GPVI- and GPIbα-dependent ROS generation. Interestingly, the role of Syk in thrombin- and PAR-induced ROS formation was dispensible. TxA2 generation was Syk-dependent and independent at low and high concentrations of thrombin, respectively. Interestingly, a role for NOX1 upstream of thrombin-induced TxA2 generation was described. These results are consistent with the findings in chapter 3 in that the secondary agonists ADP and the thromboxane analogue, U46619, did not induce ROS in platelets. Furthermore, NOX1-dependent TxA2 generation suggests that ROS are involved in intracellular signalling, although the mechanism at a molecular level for how this occurs remains to be defined.

The main aim of chapter 6 was to identify and compare the key signalling proteins involved in platelet-derived ROS formation. Differences between PAR1- and PAR4-induced ROS formation were clearly apparent and there is emerging evidence that these two receptors have distinct functional roles. PAR4, but not PAR1, is required for actin-polymerisation-mediated thrombin-induced Ca2+ signalling (Harper and Sage, 2006). Activation of PAR4 results in prolonged platelet-procoagulant-dependent thrombin generation that is more rapid than that through activation of PAR1 (Duverney et al., 2013). Further, PAR1, but not PAR4, mediates PS exposure through PKC activation and Ca2+ mobilisation (Harper and Poole, 2011). In addition, PAR1 signals through a Gi/o-mediated pathway, specific to PI3-K activation, but PAR4 does not utilise this signalling pathway (Voss et al., 2007). In chapter 6, PAR1- and PAR4-dependent ROS generation in part required distinct signalling proteins downstream of receptor ligation, providing further evidence for distinct activation pathways. Consistent with previous reports (Voss et al., 2007), TRAP-induced platelet aggregation was attenuated in the presence of the PI3-K inhibitor, wortmannin, but ROS formation was unaffected. In contrast, wortmannin inhibited PAR4-dependent ROS generation but not platelet aggregation. Furthermore, SFKs were involved in PAR1- but not PAR4-dependent platelet aggregation. Thus this study
further emphasises that PAR4-dependent signalling is distinct from that of PAR1, although downstream signalling leads to ROS generation through both receptors.

Studies reported in this thesis have established that ROS can be produced through ligand binding to GPVI, GPIbα, PAR1 and PAR4. It is evident that the signalling pathways downstream of platelet receptor ligation are diverse and agonist-dependent. A synergistic role for GPIbα and PAR4 was hypothesised and important signalling proteins leading to ROS generation were identified downstream of these receptors. Minor limitations within these antagonist studies include the higher concentrations of agonists required to induce ROS formation, with particular regard to 1 U/mL thrombin in chapter 6. Therefore future studies using full dose response curves with and without inhibitors may detect alternative and more accurate results, especially in platelet aggregation. Future research using CHO constructs involving GPIbα and the PAR4 sequence, GYPKGF, or modified thrombin (which can only bind to GPIbα) may provide insight into the synergistic role between GPIbα and PAR4. The role of NOX1-derived TxA2 generation demonstrates that ROS are not only a consequence of platelet receptor ligation but that they also play a role in intracellular signalling. ROS generation is therefore an attractive potential target in cardiovascular disease as ROS modulate secondary platelet functions (TxA2 generation) but do not dramatically affect platelet aggregation, at least in vitro.

To further evaluate the role of ROS as a therapeutic target in cardiovascular disease, it would be interesting to examine the use of NOX1 inhibitors in NOX1 KO mice in laser injury models of thrombosis. In doing so we would determine any off-target inhibitory effects of the NOX1 antagonist in the NOX1 KO mice. Furthermore, understanding the inhibition of NOX1 in a whole system (i.e. a mouse model) would prove most useful as to whether targeting NOX1 is of any benefit. Previous results have already demonstrated that targeting NOX1 does not affect neutrophil-derived ROS generation (Walsh et al., 2014) and therefore will not affect normal neutrophil phagocytosis. In addition, the widely used anti-platelet drug, aspirin, had more of an additive effect in vitro than the NOX1
inhibitor (Walsh et al., 2014), suggesting that NOX1 is a more specific protein to target. As ROS generation are a consequence of platelet activation and may play a prominent role in amplifying the platelet response inhibiting NOX1 activation could potentially affect the thrombotic effect of platelets without disrupting normal haemostasis. Nevertheless, it remains to be established whether targeting ROS in cardiovascular disease provides an alternative, safer and more specific therapy to other anti-platelet drugs such as aspirin.
References


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