Deciphering glycoprotein VI signalling in platelet activation

Summary

Cardiovascular diseases are the leading causes of mortality worldwide, and platelets are critical in their pathophysiology. Current antiplatelet therapies come with the risk of bleeding, so treatments targeting other platelet pathways that preserve haemostasis are needed. Two potential inter-related candidates being investigated in this thesis are the collagen and fibrinogen receptor glycoprotein VI (GPVI), which has a major role in thrombosis but a minor one in haemostasis; and phosphoinositides and their associated kinase/phosphatases, which are involved in Ca^{2+} mobilisation and regulation of pleckstrin homology domain-containing proteins. **Chapter 1** provides a general introduction to the knowledge of how platelets contribute to thrombosis and haemostasis, focusing on the roles of GPVI, GPVI-related signalling pathways, and the platelet phosphoinositide metabolism.

Current mass spectrometry-based methods to analyse phosphoinositides provide insufficient resolution of lipid isomers. In **Chapter 2**, we developed a sensitive ion chromatography-mass spectrometry (IC-MS)-based approach, and validated this from an analytical perspective. We demonstrate the reproducibility and efficiency of the used phosphoinositide extraction method by spiking in a phosphoinositide standard. We also optimised the IC gradient to achieve stable elution time and to improve isomer resolution, particularly for the six major positional isomers of phosphatidylinositol phosphate (PtdInsP) and phosphatidylinositol bisphosphate ($PtdInsP_2$). The method has a linear range from 0.3-10 pmol, with a limit for detection for $PtdIns(3,4,5)P_3$ at 0.3 pmol, comparable with the literature. Application of the method showed adequate phosphoinositide detection in human platelets and other cells. Analysis of platelets using showed that treatment with the GPVI agonist, collagen-related peptide (CRP), leads to increased levels of (PtdIns4P), $PtdIns(4,5)P_2$, $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$. This is consistent with previous findings.

To better understand the platelet phosphoinositide metabolism, in

Chapter 3, I developed mathematical models for prediction of molecular changes in platelets upon GPVI signalling. This concerned the CRP-induced changes of tyrosine phosphorylation of the membrane protein LAT and PLCγ2 (phospholipase Cγ2), of altered Ca^{2+} mobilisation, and changes in the levels of PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Based on prior experimental data, each of the two mathematical models could simulate the changes over time of the phosphoinositide isomers. This model was able to predict the effects of inhibition of phosphatidylinositol 4-kinase A (PI4KA), but not of inositol polyphosphate-5-phosphatase (OCRL). The latter limitation we attributed to the specific localisation of OCRL in the Golgi system, thereby making it unable to convert all substrates. The Ca^{2+} mobilisation prediction indicated that not only PtdIns(4,5)P_2 and Ins(3,4,5)P_3 regulate this process, but also other factors like extracellular Ca^{2+} entry and Ca^{2+} back-pumping by Ca^{2+}-ATPases.

It has been debated to which extent the GPVI-induced platelet integrin αIIbβ3 activation is persistent or reversible. **Chapter 4** investigated the ability of small molecule inhibitors of the protein tyrosine kinases Src, Syk and Btk can disrupt GPVI signalling and cause platelet disaggregation. The results show a reversal of the CRP-induced phosphorylation of Syk, LAT, Btk and PLCγ2 by the secondary addition of Src or Syk inhibitor. However, the platelet aggregation process was only reversible upon inhibition of both GPVI signalling and secondary mediator (thromboxane A_2 and ADP) formation. Further, partial aggregation reversal was observed by combined inhibition of Src and integrin αIIbβ3, indicating that sustained aggregation requires continued αIIbβ3 outside-in signalling. Overall, the results suggest that the reversibility of platelet activation is a matter of experimental design and method.

Chapter 5 describes a novel ordinary differential equation (ODE) based model and an agent-based model (ABM) to predict how the binding of platelet stimuli can lead to the clustering of receptors and the ensuing

signals generation. The model simulations suggest that the clustering of receptors like GPVI on the cell surface can be achieved through at least three mechanisms, including multimerisation of receptors, multivalent ligands and cytosolic crosslinkers, all of which can induce powerful downstream signals. To validate the two models, we used a set of novel GPVI ligands, next to the recently identified nanobody 2 (Nb2), namely the dimer Nb2-2 and the tetramer Nb2-4. We found that Nb2-2 is more potent than Nb2 in blocking collagen and CRP-induced platelet aggregation. On the other hand, Nb2-4 acts as a GPVI agonist, causing platelet aggregation and tyrosine phosphorylation, which processes could be antagonised by blockage of GPVI or by inhibition of tyrosine kinases. In addition, we observed that the Syk inhibitor PRT-060318 suppressed the agonist-induced clustering of CLEC-2. The same compound also synergised with threshold concentrations of other GPVI- or CLEC-2-blocking molecules. Together, this demonstrates the key role of Syk kinase in the GPVI and CLEC-2 clustering and activation.

Elevated cytosolic Ca^{2+} is involved in all functional responses of platelets. In **Chapter 6**, we compared the platelet Ca^{2+} rises in the presence of extracellular EGTA or $CaCl_2$ to determine the relative contribution of $InsP_3$-induced Ca^{2+} mobilisation on one hand, and the extracellular Ca^{2+} entry on the other hand. In comparison to the stronger platelet agonists CRP and thrombin, we observed that the Ca^{2+} rises induced by the weaker agonists collagen and TRAP-6 were more dependent on secondary mediators ADP and thromboxane A_2. Especially when back-pumping Ca^{2+}-ATPases were blocked, we measured a very high Ca^{2+} entry ratio. Experiments to identify the major Ca^{2+} entry mechanisms identified key roles of the ORAI1 ion channel and Na^{+}/Ca^{2+} exchange proteins, in response to GPVI and the PAR receptors for thrombin.

The conclusive **Chapter 7** discusses the findings and limitations of this thesis, and highlights the need for obtaining advanced computational models, which allow for the testing of promising anti-platelet drugs.