Deciphering glycoprotein VI signalling in platelet activation

Impact

Thrombotic diseases are the cause of 40% of cardiovascular deaths in the EU, and cost the economy more than €200 billion annually.[1] Platelets play a major role in regulating haemostasis, while platelet activation at ruptured atherosclerotic plaque can result in pathological thrombus development. Currently, dual-antiplatelet therapy (DAPT) is used to prevent further platelet activation in arterial thrombosis, but numerous patients still develop thrombotic episodes, whereas treated individuals may also experience clinically significant and potentially fatal bleeding.[2] Therefore, new antiplatelet targets and drugs are needed. The collagen receptor GPVI and membrane phosphoinositides are potential new targets, which are deeply connected to platelet signalling, thrombosis and haemostasis. In this thesis, I combine experimental data and mathematical models to provide a better quantitative understanding of the interactions between platelet GPVI activation, phosphoinositide metabolism and Ca^{2+} mobilisation.

The isolation, identification and quantification of phospho-inositides are challenging steps, because of the low abundance of these lipids, and their amphipathic nature. Multiple mass-spectrometry-based methods have been developed to analyse phosphoinositides, but they lack either the sensitivity to measure the less abundant isomer PtdIns(3,4,5)P_3, or the resolution to separate the six PtdInsPs, PtdInsP_2 isomers.[3,4] Chapter 2 provides a novel label-free mass spectrometry-based method to accurately quantify these difficult phosphoinositide isomers. We demonstrated the reproducibility and efficiency of the new phospho-inositide extraction method, and optimised the ion chromatography gradient to achieve stable elution times and improve isomer resolution. Of particular interest was the agonist-induced change in PtdIns(3,4)P_2, which was previously overshadowed by PtdIns(4,5)P_2, present at a higher copy number.[5]

Most of the earlier studies on the GPVI-mediated phospho-inositide metabolism focused on single signalling routes, and did not combine several pathways or involve kinetic analyses. In chapter 3 we developed a new mathematical model to systematically study the phosphoinositide metabolism in platelets, based on novel and high density quantitative data of phosphoinositide isomers, protein tyrosine phosphorylation and Ca^{2+} mobilisation. The developed model is able to simulate the time

course changes of phosphoinositide isomers and $InsP_3$ in CRP-activated platelets. Although the model predicted certain effects of phosphoinositide-metabolising enzymes, it still had limitations related to the presence of subcellular lipid pools. This work yet serves as a basis for further exploration of specific phosphoinositide-modulating enzymes as targets for a novel anti-thrombotic medication.

Previous studies on platelet studies in flow conditions and light transmission aggregometry have shown the role of GPVI signalling, fibrinogen-GPVI, and fibrinogen-integrin αIIbβ3 interactions in thrombi and aggregates stabilisation.[6,7] To take this further, we investigated whether inhibitors of tyrosine kinases Src, Syk and Btk can impair GPVI signalling, and thereby lead to platelet disaggregation. We observed that the sustained tyrosine phosphorylation of a range of signalling proteins induced by CRP was reversed by tyrosine kinase inhibitors. On the other hand, the inhibitors did no more than partly reverse the platelet aggregation process. Overall, the findings imply that the reversal of platelet activation is well achievable, but also depends on the agonist, inhibitor and local flow conditions. The work also points to the suitability of whole-blood flow experiments in detecting platelet disaggregation.

In platelets, the receptors GPVI, CLEC-2 and PEAR1 show increased signalling through clustering, which increases the density of protein kinases to overcome the dephosphorylation of tyrosine phosphatases.[8,9] In Chapter 5, we used two models to show that the clustering of platelet glycoprotein receptors requires multivalent ligands, multimerised receptors or cytosolic crosslinkers to produce potent downstream signals. During the validation of the models, it appeared that a dimeric nanobody is a powerful GPVI antagonist, whereas a tetrameric nanobody serves as a GPVI agonist. This points to a new multivalent nanobody strategy, potentially also applicable to other related receptors,[10] for selectively activating or inhibiting platelets.

In the last experimental chapter, we used a high throughput method to assess the relative contributions of $InsP_3$-induced Ca^{2+} mobilisation and external Ca^{2+} entry in platelet GPVI and GPCR signalling. We found that the Ca^{2+} responses elicited by weaker stimulants were more dependent on secondary mediators, when compared to the stronger agonists CRP and thrombin. The findings offer novel quantitative information regarding

the extent of agonist-induced Ca^{2+} entry by different agonists in human platelets. We also determined that the ORAI1 channel and Na^{+}/Ca^{2+} exchangers contributed most to the Ca^{2+} entry process, which expanded previous results.[11,12] From the results it becomes attractive to find and develop new drugs that target these platelet channel proteins.

Overall, this thesis shows how combining functional data and mathematical modelling helped with hypothesis generation and validation and has resulted in a thorough understanding of the phosphoinositide turnover in platelets, GPVI activation and Ca^{2+} mobilisation. With the acquisition of a more complete dataset, the mathematical models developed in this thesis can be expanded to include all the elements of protein phosphorylation, Ca^{2+} mobilisation, and interactions with other platelet agonists. My expectation is that the obtained insights will provide by very helpful in the development of novel anti-platelet medications.

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