Characterization of platelet disorders using quantitative proteomics

Summary

Blood platelets are key mediators of hemostasis, being responsible of the formation of a blood clot after vessel wall injury. Platelet dysfunction or a low platelet count may thus lead to an increased bleeding risk. On the other hand, unwanted platelet reactivity in an atherosclerotic blood vessel may result in cardiovascular disease, such as heart infarction or stroke. Since platelets lack nuclei, they have a limited capacity of protein translation, implying that their protein content is relatively stable. Accordingly, proteomics approaches can be regarded as valuable tools to study the composition and activation state of platelets in health and disease. Usage of modern mass-spectrometry based proteomics, as presented in this thesis, allows full characterization of the platelet proteome, phosphoproteome and N-terminone. This technology now has the potential to elucidate new molecular mechanisms and novel biomarkers of platelet activation, which may serve for future diagnostic and therapeutic approaches.

Chapter 1 of this thesis provides a general introduction of established platelet functions and mechanisms in hemostasis. In addition, this chapter underscores the importance of studying platelets from healthy and diseased subjects using proteomics approaches, to gain insight into normal and affected platelet activation mechanisms. In **Chapter 2** a review is presented on important pitfalls in the performing and analysis of phosphoproteomics experiments. In this opinion article, major flaws and challenges of the technology are discussed, as well as strategies to address current problems in phosphoproteomics studies. In **Chapter 3** an improved Charged based Fractional Diagonal Chromatography (ChaFRADIC) approach is developed. Tools are described on how to: *(i)* assess the reproducibility of N-terminomics workflows using iTRAQ technology, *(ii)* evaluate the benefit of a multi-protease approach to increase the N-terminome coverage, and *(iii)* investigate the possibility to scale down sample amounts for N-terminomics experiments. For this work, *Arabidopsis thaliana* seedlings were divided into six aliquots, and per aliqout, the proteins were individually labeled with stable isotope iTRAQ reagents. After pooling, the sample was divided again into three parts, each of which was digested with different proteases. After ChaFRADIC, the enriched fractions containing N-terminal peptides were analyzed by LC-MS/MS. Altogether; we quantified 2,249 unique N-termini from 1,270 *Arabidopsis* proteins, in a way that sample amounts for N-terminomics workflows could be down-scaled to become applicable for biomedical research. In addition, the data showed the advantage of a multi-protease digestion approach. We observed low overlap between the quantified N-terminal peptides among the 3 digestions, in that 82% of the N-terminal peptides were quantified with just one protease. Given the low variation of the iTRAQ intensities in the three data sets, we

concluded that the reproducibility of this N-terminomics workflow was good. A relevant biological result was that our analysis could underscore the roles of known intracellular endoproteolytic pathways in plant cells, such as the N-terminal methionine excision and the N-end rule degradation pathways.

In **Chapter 4,** we performed a multipronged analysis of the proteome, phosphoproteome and N-terminome of platelets from control subjects and a Scott platelets in order to investigate mechanisms underlying the platelet procoagulant response. The Scott syndrome is a rare bleeding disorder, characterized by mutations in the ion channel/scramblase protein, anoctamin-6. Platelets from Scott patients show impairments in Ca^{2+}-dependent procoagulant phosphatidylserine exposure, protein cleavage and balloon formation (membrane blebbing). These impairments are seen, when Scott platelets are stimulated with strong, Ca^{2+}-elevating agonists, such as convulxin/thrombin or ionomycin. Using an iTRAQ-based proteomics workflow, we found minor changes at the quantitative proteome level in 2,278 proteins, except for anoctamin-6 and calpain-2 catalytic subunit which were down-regulated, and aquaporin-1 which was up-regulated in the patient´s platelets. By parallel reaction monitoring analyses, we verified that anoctamin-6 was absent and aquaporin-1 was present in the syndromatic platelets. Additional phosphoproteomic analysis, using the same samples, resulted in 1,566 quantified phosphopeptides, which showed major changes in phosphorylation patterns between control and Scott platelets, after stimulation with convulxin/thrombin or ionomycin.

In **Chapter 4**, we also assessed the intracellular proteolytic activity of the Ca^{2+}-dependent protease calpain in activated platelets. Firstly, calpain consensus motifs were defined using a separate proteomics workflow. Then, platelets from control subjects were stimulated in the presence or absence of a calpain or caspase inhibitor, and used for ChaFRADIC to enrich for newly formed N-terminal peptides. This resulted in a list of 180 calpain-regulated cleavage sites in platelets, while a distinct set of 23 cleavage sites appeared to be caspase-regulated. Subsequently, the convulxin/thrombin- or ionomycin-stimulated patient platelets were subjected to ChaFRADIC and neo-N-terminal peptide analysis, in order to assess the calpain substrates involved in the procoagulant response. This analysis indicated that multiple of the calpain-regulated cleavage sites were down-regulated in the activated Scott platelets. Phenotypic analysis, using microfluidics and Western blotting, confirmed that the patient's platelets were deficient in procoagulant response and were reduced in calpain activity.

In **Chapter 5,** we compared altered platelet function with quantitative phosphoproteomics analysis for platelets from Albright hereditary osteodystrophy (AHO) patients, carrying a mutation in the GNAS complex gene locus for Gsα. Such patients have impairments in the

inhibitory platelet signaling pathway, mediated by Gsα, adenylate cyclase, cAMP and protein kinase A (PKA). Under certain conditions, this hence leads to platelet hyperactivity. In AHO platelets, we observed a reduced ability of Gsα-signaling prostaglandins (prostaglandin E_1 and iloprost) to inhibit platelet aggregation, secretion and thrombus formation. This was confirmed by proteomics analyses, in that AHO platelets, in comparison to platelets from controls, showed hypo-responsiveness of iloprost-induced protein phosphorylation. Thus, from the 3,457 quantified phosphopeptides, 62% appeared to be upregulated in control platelets, but relatively decreased in the patient's platelets. Several of the regulated phosphorylation sites are known PKA targets, such as vasodilator-stimulated phosphoprotein and phospholipase C-β3, whilst 149 novel phosphorylation sites were elucidated.

In **Chapter 6**, we focussed on ADP-induced phosphorylation pathways in platelets from healthy subjects. The autacoid ADP, released from activated platelets, binds to the G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$, and triggers downstream signal cascades, leading to platelet aggregation. Drugs interfering with the $P2Y_{12}$ receptors are frequently prescribed to patients suffering from cardiovascular disease. We applied a quantitative phosphoproteomics approach to study the temporal phosphorylation events induced by ADP alone or in combination with the platelet-inhibiting prostacyclin analog, iloprost. This resulted in temporal profiles of 4,797 phosphopeptides, of which 608 showed a significant regulation by ADP/iloprost. The regulatory sites were linked to mostly to proteins involved in platelet degranulation, cytoskeletal re-organization and pathways involving ubiquitin ligases or GTPase exchange factors/GTPase-activating proteins. Overall, this resulted in a complete map of phosphorylation events induced by ADP alone or in combination with iloprost. Interestingly, certain phosphorylation sites were inversely regulated by ADP and iloprost, as confirmed by a developed assays using parallel-reaction monitoring, suggesting that these sites may act as central nodes in platelet homeostasis.

Chapter 8 presents a workflow to simultaneously analyzed cellular proteins, lipids and metabolites, named SImultaneous Metabolite, Protein and Lipid EXtraction procedure (SIMPLEX). The development of such a strategy allows the investigation of complex signaling networks that rely on protein-lipid and protein-metabolite interactions. In the SIMPLEX workflow, the separation of proteins, lipids and metabolites is done by differential extraction and centrifugation, to minimize contamination between these molecular classes. The method does not require higher amounts of starting material than any other proteomic measurements. As a proof-of-principle we conducted SIMPLEX using mesenchymal OP9 cells, in which we quantified 360 lipid species, 75 metabolites, 3327 proteins and 1846 phosphopeptides. We compared SIMPLEX to standard proteomics and metabolomics workflows, such as filter-aided sample

preparation and methanol extraction, respectively. In both comparisons, a good percentage of recovery was achieved with SIMPLEX. In addition, we investigated the peroxisomal proliferator-activated receptor-γ signaling pathway, where changes at the proteome level appeared to correlate well with changes at the lipidome level, particularly with respect to triacylglycerol turnover. Overall, SIMPLEX showed to be a robust, sensitive and reproducible protocol to analyze cross-talk between different molecular classes in a cell, with promising possibilities for the future analysis of platelets.