Glycoprotein Ibα signalling in platelet apoptosis and clearance

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Glycoprotein Ibα signalling in platelet apoptosis and clearance

Glycoproteine Ibα signallering in apoptose en klaring van bloedplaatjes
(met een samenvatting in het Nederlands)

Proefschrift

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Evertdina van der Wal

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Promotor: Prof. dr. J.W.N. Akkerman

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Ter ere van mijn allerliefste oma

For the life of a creature is in the blood

(Lev 17:11)

CONTENTS

I see genius in everybody,
perceive it in yourself is the difficulty

(Faithless)

Chapter 1

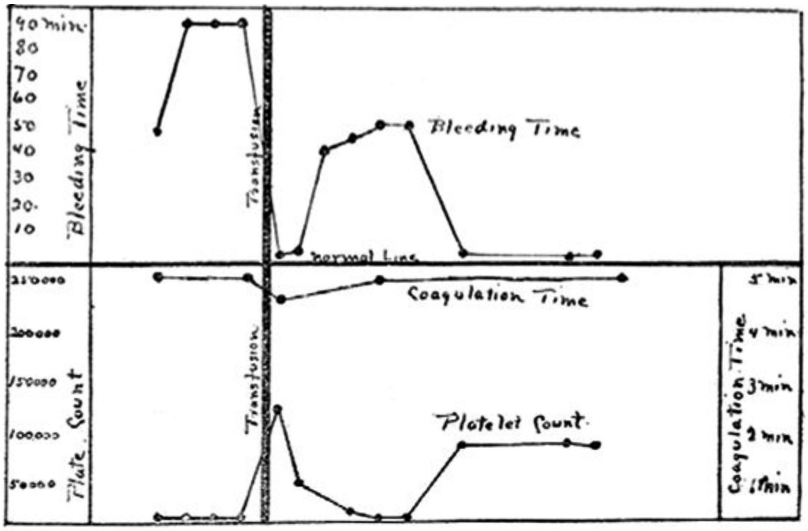
General introduction

1.1 PLATELET TRANSFUSION

1.1.1 History and clinical studies

The role of platelets in haemostasis was for the first time demonstrated by Dr. William W. Duke in 1910 in a study published in JAMA. His report is now considered as an outstanding contribution to medical science. He demonstrated in a patient that the bleeding time was increased in proportion to the severity of the thrombocytopenia and showed that both bleeding time and thrombocytopenia were corrected after transfusion of fresh blood. (Figure 1 [1]).

Figure 1: Transfusion of blood platelets relieves hemorrhagic disease



A follow-up study with more than 400 acute leukaemia patients included over a period of 10 years (1954-1963) showed that fatal haemorrhages were reduced by 30% by regular blood transfusions compared with the period before 1954 [2]. In another study, 57 patients were examined post-mortem between 1963 and 1965 which led to the conclusion that after the introduction of platelet transfusion in 1964 about 50% less patients had died of bleeding than before [3]. Today, the transfusion of platelets is a life-saving procedure, especially for haemato-oncology patients, patients with immune thrombocytopenia and patients undergoing invasive surgery procedures. Platelet transfusions are applied as prophylaxis and for the treatment of bleeding. The threshold for platelet transfusion is a platelet count of < $10x10^9$/L, but this number varies strongly depending on clinical criteria.

There is debate about the effectiveness of platelet transfusions. The question has been raised whether platelet prophylaxis in haematology patients is really effective in reducing bleeding. This question is not easy to answer. A study addressing this question would involve administration of potentially harmful and certainly costly platelet transfusions to a large number of patients, many of whom might not bleed without transfusions. Nevertheless, a few clinical trials have been performed, measuring platelet transfusion requirements, duration of the thrombocytopenia and clinical bleeding as clinical outcomes.
The PLADO (Prophylactic PLAtelet DOse) [4] study was designed to determine the optimal prophylactic platelet dose. The outcome was somewhat surprising: there was no effect of the number of platelets transfused (1.1-4.4x10^{11} platelets per square meter of body surface area) on the incidence of bleeding. In contrast, administration of low dose platelet transfusion regimen as a prophylactically transfusion, led to significantly more transfusion episodes and a shorter interval between transfusions [4].
In the SToP study (Strategies for the Transfusion of Platelets), the effects of low (150-299x10^{9}/L) *versus* standard (300-600x10^{9}/L) platelet doses were compared. The StoP-study was stopped by the Data Safety Monitoring Board because of higher bleeding incidence in the group which received the low dose platelet transfusion regime [5].
The TOPPs study (Trial of Prophylactic Platelets Study) was designed to determine whether no prophylaxis is as clinically effective and safe as prophylaxis. Unfortunately, the study is not completed and conclusions cannot yet be drawn [6].

The effectiveness of the platelet transfusion is generally evaluated by determining platelet number before, 1 hour and 24 hours after the transfusion. From these follow-up values, the *'corrected count increment'* (CCI) is calculated according to the equation:

$$\text{CCI} = \frac{\text{Post-transfusion} - \text{pre-transfusion platelet number}}{\text{Number of transfused platelets (x}10^{11}\text{)}} \times \text{body surface (m}^2\text{)}$$

A platelet transfusion is considered to be effective when the CCI ≥ 7.5 at 1 hour after transfusion and when the CCI ≥ 2.5 at 24 hours after transfusion. A low 1-hour follow-up value is seen with sepsis, serious Graft Versus Host Disease, splenomegaly and obvious haemorrhages.

1.1.2 Quality

Before platelet concentrates are administered, pH, lactate production, the presence of large aggregates and "swirling" have to be determined. During the storage of platelet concentrates (PCs), pH decreases due to accumulation of lactate produced in anaerobic metabolism of glucose and free fatty acids. When the pH falls to 6.0, platelets change their shape from disc to sphere; at a pH below 6.0 shape change is irreversible [7]. The swirling phenomenon can be observed when platelets are manually resuspended and held up to a strong light and reflects the presence of discoid platelets [8].

1.1.3 Platelet concentrates

Today, there are three methods to prepare PCs. First, the platelet-rich plasma (PRP) method which is mainly used in the United States. Whole blood is collected in disposable plastic bags, centrifuged at slow speed to sediment red and white cells and to concentrate most platelets in the supernatant plasma. The PRP is centrifuged at a higher speed to sediment the platelets. Most plasma is removed and platelets are resuspended in a small volume of plasma or synthetic medium and stored at room temperature with mild rotation [9, 10]. Second, the buffy coat method, which is mainly used in Europe and Canada. Whole blood is centrifuged at a high speed and separated in red blood cells (RBCs), plasma and a buffy coat using an automated blood component extractor. Buffy coats are stored overnight at 22°C. Then, 4 units are pooled with 4 units of ABO-matched plasma and centrifuged. Third, the aphaeresis method is used when patients are at risk for transfusion-related infectious diseases. Blood from the donor is processed with a cell separator with an in-line centrifuge for platelet collection. Platelets are collected in citrate in plastic bags, while the other blood cells and most of the plasma are returned to the donor. Aphaeresis machines apply leukocyte reduction during the donation [9].
There is a continuing debate about the best source of PCs and the relative benefits and risks of whole blood-derived versus aphaeresis-derived PCs. The lower donor exposure associated with aphaeresis is an important advantage of this method. In contrast, whole blood-derived PCs are much cheaper than aphaeresis-derived PCs.
Platelets, obtained from PRP or buffy coat method, from whole blood withdrawn from 4-8 donors, need to be pooled to PCs before transfusion to achieve the desired platelet number. For adults the standard administration dose is a total of 300-600x10^9 platelets or 50x10^9 platelets/kg body weight [8].

1.1.4 Platelet dose

Guidelines have been developed for the threshold level of the minimal platelet count in patients for transfusion of PCs: 5x10^9/L for patients without fever of bleeding, 10x10^9/L for patients with such signs and 20x10^9/L for patients on heparin-treatment, before bone marrow biopsy or for patients with coagulation disorders [11]. In general, increasing platelet numbers to 40-50x10^9/L is sufficient to stop major bleeding episodes.

1.1.5 Transfusion-related acute lung injury

In about 20-30% of platelet transfusions, adverse febrile and allergic reactions occur caused by cytokine-producing leukocytes and plasma proteins [8]. Most of these reactions are mild and consist of fever, chills, rigor and allergic reactions. Febrile reactions are more likely to occur following transfusion with stored than with fresh platelets, suggesting that bioactive lipids and interleukins-1β and -6 are produced during storage. About 1 in 1500 to 1 in 10 000 transfusions of plasma containing blood products causes severe pulmonary reactions, known as transfusion related acute lung injury (TRALI). In general, this occurs at 1-6 hours after transfusion and 5-

15% of these reactions are fatal. TRALI is now the leading cause of transfusion related deaths. The main causes of the injury are neutrophil-specific antibodies, lipids and cytokines from the donor. A second major clinical outcome of TRALI is continued respiratory distress [12].

1.1.6 Pathogen inactivation

During blood collection the needle is entering the vein through the skin and bacteria may enter the blood when disinfection is inadequate. Since PCs are stored in 30% of plasma, they possess a greater risk of transfusion-transmitted bacteraemia than RBCs. Methods used to screen for bacteria-contaminated PCs include analysis of a fall in pH, glucose levels, oxygen tension and a loss of swirling [8]. Pathogens can be inactivated by amotosalen HCl (S-59) which inactivates bacteria upon irradiation with ultraviolet light by forming cross-links between nucleic acids, preventing replication. Unfortunately, PCs treated with amotosalen have a reduced CCI which leads to shorter transfusion intervals [9].

1.1.7 Anticoagulants

Generally, whole blood is drawn in solutions which contain citric acid, trisodium citrate and dextrose (ACD). The concentration of citrate is usually about 20 mM. Dextrose provides a source of energy. ACD maintains pH levels and concentrations of Ca^{2+}-ions leading to less platelet activation during storage.

1.1.8 Platelet additive solutions

Synthetic media such as the platelet additive solutions (PAS) T-sol, InterSol, Composol and PAS-IIIM (SSP+) have been developed to reduce adverse transfusion reactions. They contain the following components to reduce lactate production. First, acetate which has an alkalizing effect and therefore buffers the hydrogen ions produced in anaerobic glycolysis. Acetate is oxidized in the tricarboxylic acid cycle before it is metabolized as acetyl-Co-A. Second, phosphate, which has two effects: it acts as a buffer, however, it also stimulates glycolysis [9, 13]. PAS always consists of 30% plasma, because it contains bicarbonate which buffers the liberated CO_2 [9].

1.1.9 Leukocyte reduction

The major cause of refractoriness to platelet transfusions is the production of antibodies against alloantigens on donor platelets in response to HLA class I antigens on leukocytes and platelets. Therefore, in many countries PCs are leukoreduced by the use of a filter composed of charged polyester or noncharged polyurethane. Removal of leukocytes is performed by several mechanisms, including sieving/mechanical retention based on cell size, direct adhesion to fibres and indirect adhesion through platelets [14]. Another method is gamma-irradiation with 5000 cGy which is sufficient to damage nuclear DNA of the leukocytes. In 1997, Dr. Sherril Slichter conducted a trial to determine which method of leukocyte reduction was most effective in

preventing the alloantibody-mediated refractoriness to platelets during chemotherapy for acute myeloid leukaemia [15]. Patients received PCs in which leukocytes were reduced by a filter or irradiated with ultraviolet B or aphaeresis-derived PCs from a single donor. There were no significant differences between the three groups; all reduced the development of alloantibodies to the same extent [15].

1.2 STORAGE OF PCS

1.2.1 Blood bank conditions

Currently, platelets are being stored for a maximum of 7 days at 22-24°C, with slight agitation. Agitation facilitates the exchange of O_2 and CO_2 and helps to preserve the pH [16]. Nevertheless, platelets stored under these conditions undergo changes known as the platelet storage lesion (PSL), which hamper their haemostasis effectiveness post-transfusion [17]. The PSL can be defined as the sum of all deterioration processes of the quality of platelets that arise from the time of blood withdrawal to the time of platelet transfusion. PSL can be monitored by changes in morphology, activation, metabolism, senescence and apoptosis. The PSL is accompanied by loss of disc shape, change in mean platelet volume (MPV), altered glycoprotein (GP) expression and the release of factors stored in α-granules, like β-thromboglobulin (β-TG) and platelet factor 4 [17].

1.2.2 Cold-storage of platelets

Originally, PCs were stored at 4°C but it was soon discovered that platelets had shorter *in vivo* survival when stored at low temperature compared with RT [18]. Platelet morphology changed from discoid to an irregular spherical shape and channels of the open canalicular system (OCS) appeared dilated [19, 20]. When the discoid shape was preserved by addition of the actin-inhibitor cytochalasin B and the membrane-permeable Ca^{2+}-chelator EGTA-AM, still these platelets were cleared rapidly, indicating no major role for shape change in cold-induced platelet clearance [21, 22].

Early studies described that at 15-18°C the membrane undergoes a phase transition through a liquid-crystalline to gel phase, which is accompanied by secretion of α-granules and lysosomes [23, 24]. Antifreeze glycoproteins (AFGPs) isolated from the serum of Antarctic fishes prevented this cold-activation [25]. Also, addition of the sugar trehalose was used to stabilize platelet membranes in a liquid crystalline phase and could therefore be used to better preserve platelets at low temperatures [26].

Low temperature triggered an increase in the cytosolic Ca^{2+}-concentration, triggering the activation of gelsolin followed by its binding to the cytoskeleton [27]. Similar as the liquid to gel phase transition in the membrane after chilling, the removal of cholesterol from the membrane induced these changes, suggesting that cholesterol plays an important role in stability of the membrane. This was explained by changes in the order of phospholipids [23]. Also, aggregation of rafts was observed [28]. These cold-induced rafts were enriched in oligomers of the scavenger receptor CD36 and colocalized with the activated tyrosine kinases Src and Syk and with phospholipase (PL) Cγ2, whereas Lyn remainded in its inactive form [29].

Platelets stored at low temperature seem to be less activated than platelets stored at RT, as illustrated by the absence of secretion of β-TG and P-selectin [30]. Survival of cold-stored

platelets is shorter than of RT-stored platelets, which was explained by a change in the receptor for von Willebrand factor (VWF), GPIbα, a member of the GPIb-V-IX complex [22]. Upon chilling of platelets, this receptor changed its localization which was recognized by the integrin $\alpha_M\beta_2$/MAC-1 on macrophages, promoting platelet clearance from the circulation. Transmission Electron Microscopy-studies of murine platelets stored for 2 hrs at RT showed GPIbα in linear arrays; while after chilling GPIbα formed so-called 'clusters'. Chilled platelets were mainly cleared at the sinusoidal macrophage-rich areas in the liver, while RT-stored platelets were cleared by the spleen. In mice deficient for $\alpha_M\beta_2$, no rapid clearance of cold-stored platelets was found, indicating a major role for this receptor [22]. In the same year, this group published an explanation for the rapid clearance of chilled platelets. Chilling promoted deglycosylation of GPIbα, resulting in exposed β-*N*-Acetyl-*D*-glucosamine (β-GN) residues which are recognized by the α_M-lectin domain of $\alpha_M\beta_2$, as shown in Sf9 insect cells expressing different domains of α_M [31]. Exposure of β-GN and β-GN together with sialic acid (SA) residues was deduced from the respective binding to the fluorochrome-conjugated sugar-binding lectins succinyl-wheat germ agglutinin (sWGA) and *Ricinus communis* agglutin 1 (RCA-1) [32]. Addition of β-GN and UDP-Galactose inhibited phagocytosis by matured THP-1 monocytic cells [32].

Interestingly, 4°C-stored human platelets stimulated by thrombin or VWF/ristocetin showed better aggregation/agglutination responses than RT-stored platelets. Galactosylation of cold-stored platelets by addition of UDP-Galactose, further improved VWF/ristocetin-induced agglutination suggesting that galactosylation specifically affects the GPIbα-receptor. Also, static adhesion of cold-stored platelets showed better lamellae and filopodia formation than RT-stored platelets [33]. Unfortunately, after 5 days, P-selectin expression was 2-fold higher after refrigeration, than after RT-storage, independent of the extent of galactosylation. However, this did not result in increased phagocytosis by matured THP-1 monocytic cells [33].

Galactosylation of platelets, stored for 14 days, suppressed the *in vitro* phagocytosis by matured monocytic THP-1 cells. Also, galactosylation of platelets, during storage for 2 hrs at 0°C, decreased the clearance of platelets in mice. Unfortunately, it did not prevent the enhanced clearance in human subjects after 48-72 hrs platelet storage at 4°C [34]. These findings reveal different clearance mechanisms for short-term (≤ 4 hrs 0°C) and long-term (≥ 48 hrs) refrigerated platelets. Long-term refrigeration promoted clearance by the Ashwell-Morell receptor of hepatocytes through galactose exposure on GPIbα [35].

After storage for 18 hours at 4°C, platelets showed loss of swirling and a 75% lower RANTES (regulated upon activation, normal T-cell expressed and secretion) concentration, compared with platelets stored at RT. The release of the extracellular part of GPIbα, called glycocalicin, by ADAM17-mediated cleavage after 7 days was twice as high after storage at low temperature than at RT and accompanied by enhanced microvesicle formation [36].

The possibility that the disaccharide trehalose might act as a cryoprotectant for platelets has long been investigated. It was recently demonstrated that trehalose inhibited phagocytosis of platelets chilled for 9 days by preventing the cold-induced membrane transition through the liquid-crystalline to gel phase [37].

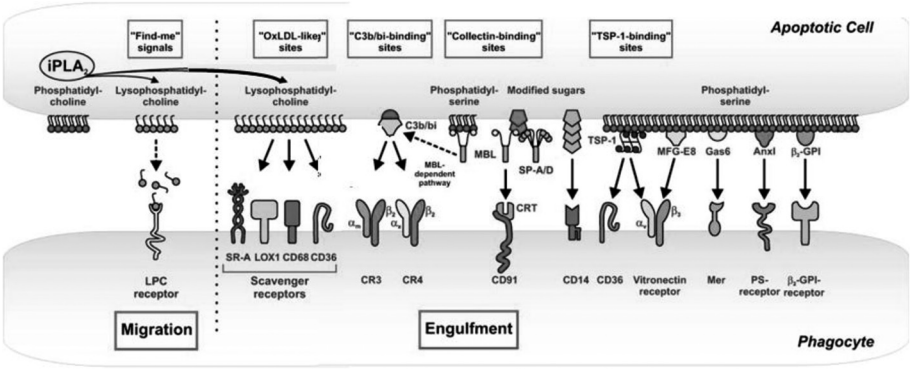
New attempts to prevent platelet activation during storage are based on raising cAMP and cGMP levels or affect pH regulation [38]. ThromboSol consists of amiloride, an inhibitor of the plasma membrane Na^+/H^+-pump [39]. Sodium nitroprusside which activates guanylate cyclase and increases cGMP by releasing nitric oxide (NO). Combinations of these inhibitors lead to preservation of GPIbα and decrease cell lysis [40].

1.3 PHAGOCYTOSIS OF APOPTOTIC CELLS

1.3.1 Phagocyte attraction, recognition and removal

The interaction between apoptotic cells and phagocytes can be divided into the following phases: recognition, tethering, engulfment and intracellular degradation. First, recognition of apoptotic cells by the phagocytes starts with the secretion or externalization of various chemoattractants. On the apoptotic cell, phospholipase A_2 ($iPLA_2$) is cleaved and activated by caspase-3 during apoptosis. $iPLA_2$ hydrolyzes phosphatidylcholine (PC) from the membrane yielding arachidonic acid and lysophosphatidylcholine (LPC) which is externalized and secreted by unknown mechanisms. Consequently, LPC can bind to its counter receptor on the phagocyte, triggering tethering of the apoptotic cell (Figure 2, adapted from [41, 42]).

Figure 2: Overview of engulfment of apoptotic cells [41]



Second, tethering of the apoptotic cell to the phagocyte involves 'Eat-me' signals which are markers for phagocytes to initiate engulfment of dying cells. An example of a direct "eat me" signal is the appearance of PS on the cell surface of apoptotic cells. Indirect 'eat-me' signals can appear through interaction of phospholipids on the apoptotic cell surface with extracellular bridging molecules, such as milk fat glycoprotein (GP) lactadherin (MFG-E8), β_2GPI and growth arrest-specific gene 6 (Gas-6). Surface-bound MFG-E8 is recognized by $\alpha_v\beta_3$-integrins on the phagocyte through its RGD-motif. Other bridging molecules such as surfactant proteins-A and -D (SP-A/D), mannose-binding lectin (MBL) and collectin-related first component of the classical complement cascade (C1q) recognize altered sugars on the surface of apoptotic cells. On apoptotic cells, the extracellular matrix GP thrombospondin-1 (TSP-1) binds through an interaction with phagocyte $\alpha_v\beta_3$ and CD36 [43].

By presenting "don't-eat-me" signals on cells, engulfment by phagocytes can be prevented. One example is the homophilic interaction between platelet endothelial cell adhesion molecule-1

(PECAM-1/CD31) molecules, detaching leukocytes from phagocytic macrophages. Self/nonself signal CD47 functions as a "don't-eat-me" signal through interaction with its counter receptor signal-regulatory-proteinα (SIRPα) on the phagocyte [44]. CD47 inhibits FcγR-mediated phagocytosis of thymocytes, but promotes phagocytosis of unopsonized cells by non-activated macrophages. After induction of apoptosis in thymocytes, clustered patches are formed promoting binding to phagocytic macrophages [45]. Platelets deficient in SIRPα are more rapidly cleared than Wild-Type (WT)-platelets [46].
Third, after engulfment of apoptotic cells, the phagocyte cytoskeleton is rearranged, linking its receptors to actin-filaments. GTP-ases, such as Rac-1 and Cdc42 are important in the maturation of phagosomes. Phagosomes are fused with endosomes and lysosomes. Fourth, the engulfed apoptotic cells are intracellular degraded by the phagolysosome which contains acid hydrolases and has a low pH [42, 47].

1.3.2 PS-exposure

Normally, phospholipids are distributed asymmetrically in the plasma membrane. PS and phosphatidylethanolamine (PE) are present in the inner leaflet; PC and sphingomyelin are concentrated in the outer leaflet of the membrane. Apoptosis induction leads to a loss of asymmetry and exposure of PS. PS-exposure is rapid and achieved by lipid transport across the bilayer by a calcium-dependent scramblase. In contrast, an ATP-dependent aminophospholipid translocatase/flippase transports PS from the outer to the inner leaflet of the membrane. PS-exposure is now used as a characteristic of apoptosis cells and measured by FACS using fluorochrome-labelled Annexin V, which binds PS or lactadherin in a calcium-dependent or independent manner respectively [48]. Lactadherin binding and is a more sensitive technique for measuring exposed PS than Annexin-V binding, which has a threshold of about 2.5 - 8% surface PS to bind [49, 50]. In platelets, surface expression of PS is thought to be the result of activation of caspases. Whether the effect of caspases is by cleavage/activation of a component of PS scramblase or the cleavage/destruction of an inhibitory protein, is unclear [51].

1.3.3 P-selectin

P-selectin is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells. Following platelet activation, P-selectin becomes surface-expressed where it is rapidly shed and appears in the circulation as in a soluble form. The main counter receptor for P-selectin is P-selectin protein ligand-1 (PSGL-1), expressed abundantly in leukocytes. PSGL-1 shares structural homology with GPIbα. The engagement of P-selectin with PSGL-1 triggers the upregulation of $\alpha_M\beta_2$, mediating tethering and rolling and finally firm adhesion of the leukocytes [52]. PSGL-1 is also present on macrophages and has been demonstrated that binding of stored platelets is for 50% dependent on P-selectin/PSGL-1 interaction [53].

1.4 PLATELET SIGNALLING

1.4.1 Haemostatic plug formation

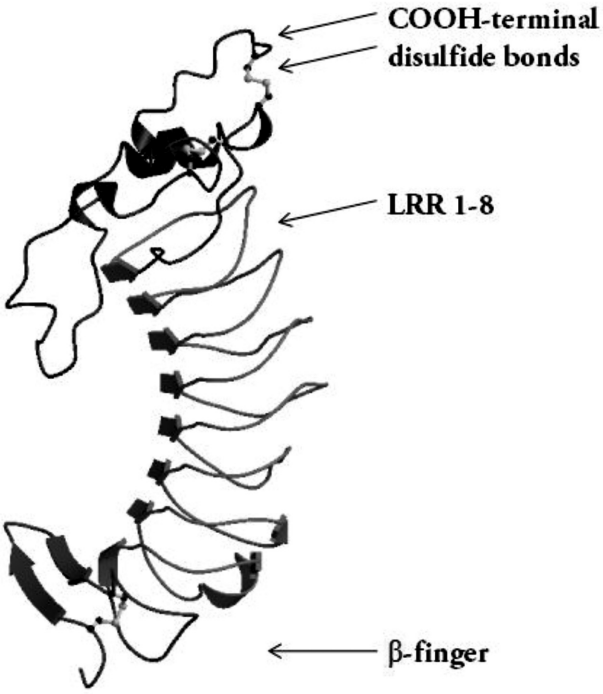
Haemostasis, the arrest of bleeding by vascular damage, requires recruitment of circulating platelets to develop a haemostatic plug. Normally, the vessel wall is covered with endothelium, which releases the platelet inhibitors NO and prostacyclin and expresses the ectonucleotidase CD394 which neutralizes the platelet activator ADP. Together, they provide a defence against thrombus formation. When the vessel wall is injured or the endothelium is disrupted, collagen and tissue factor become exposed to the flowing blood, initiating formation of the platelet plug. Exposed collagen triggers adhesion and activation of platelets, whereas exposed tissue factor initiates generation of thrombin which activates platelets and converts fibrinogen into fibrin. Thrombin cleaves the protease-activated receptors 1 and -4 (PAR-1/4) on the platelet surface, activating the cells and inducing release of ADP, serotonin and thromboxane (Tx)A_2 [54]. These agonists activate adjacent platelets and amplify platelet aggregation. The activated integrin $\alpha_{IIb}\beta_3$ mediates recruitment of platelets, as well as platelet-platelet interactions by binding fibrinogen or VWF which act as bridging molecules. Protein disulfide isomerases are involved in the activation of $\alpha_{IIb}\beta_3$. Activation of platelets bound to the wall of the injured vessel causes a conformational transition in $\alpha_{IIb}\beta_3$ increasing the affinity for its soluble ligands fibrinogen and VWF. At low shear rates, fibrinogen is the predominant ligand, whereas VWF plays an important role at high shear rates. During platelet activation, late signalling events enhance platelet-platelet interaction. Gas6, CD40-ligand and ephrin-Eph interactions contribute to stabilization of the plug [55, 56]. Platelet activation leads to release of α-granules and dense granules. α- Granules contain platelet factor 4, platelet derived growth factor, fibronectin, β-TG, VWF, TSP-1, fibrinogen and coagulation factor V, while dense granules contain ADP, ATP, serotonin and Ca^{2+}-ions [54]. The release of ADP stimulates platelet activation through the P2Y1 and P2Y12 receptors. In addition, upon collagen binding, GPVI, $\alpha_2\beta_1$ and the GPIb-V-IX receptor complex are involved. The GPIb-V-IX receptor complex can bind to VWF when the A1-domain of VWF is activated by VWF binding top collagen through its A3-domain. Binding of VWF to GPIb-V-IX induces a signalling cascade in the platelet resulting in activation of $\alpha_{IIb}\beta_3$.

1.4.2 GPIbα-structure

The crystal structure of GPIbα, published by Huizinga *et al.,* displays an elongated, curved shape which is typically for receptors rich in leucine-rich repeats (LRR) (Figure 3).
The central region of GPIbα consists of eight LRRs. The NH_2-terminal forms the so-called 'β-hairpin' of 14-residues and is bordered by a conserved disulfide bond. The tip of the β-hairpin, which is referred as the β-finger, protrudes from the surface of the protein. Also, conserved disulfide bonds stabilize the COOH-terminal flank. Residues 227-241 project from the concave face and form a highly flexible loop [57].

Figure 3: Crystal structure of GPIbα

adapted from Huizinga *et al.* [57]



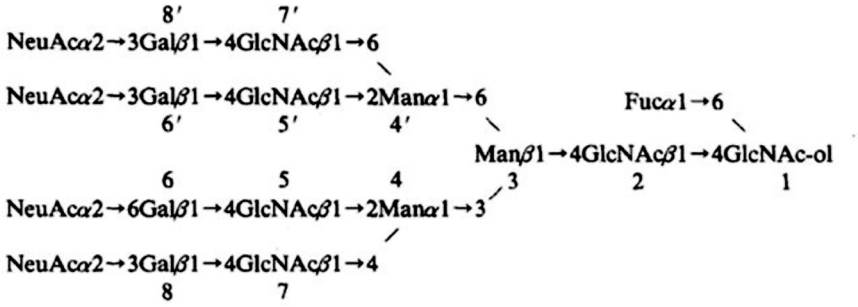
1.4.3 GPIbα glycosylation

The extracellular part of GPIbα, called glycocalicin, can be divided in (i) the ligand binding (LB) domain which includes the *N*-terminal flank (NTF), LRR 1-7, the *C*-terminal flank and the sulfated region, and (ii) the *C*-terminal macroglycopeptide region [58]. GPIbα is heavily glycosylated and contains *O*-linked and *N*-linked complex-type branched and di-, tri- and tetra-antenary carbohydrate chains covalently attached to proteins via asparagine residues. When completely assembled, they are capped by sialic acid. The *N*-linked glycosylation sites are located within the LB-domain of GPIbα, LRR 1 and 6 (Figure 4 and [59, 60]).

Neuraminidase (NA) cleaves sialic acid from the platelet surface *in vitro*, leading to shedding of GPIbα together with GPV. Shedding was suggested to cause the enhanced platelet clearance resulting from NA-treatment. Using a specific inhibitor 2, 3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) or the substrate for NA, fetuin, shedding was prevented. Also, platelet survival was rescued when DANA was added [61].

Figure 4: *N*-linked carbohydrate chain present on GPIbα [60]

The position of the -OH group is depicted by α or β. The α-position is defined as the -OH being on the opposite/axial side of the ring (downward), whereas the β-position is on the same/equatorial side of the ring (horizontal); NeuAcα (α-*N*-Acetylneuraminic acid), Gal (β-*D*-Galactose), GlcNAc (β-*D*-*N*-Acetylglucosamine), Man (β-*N*-Mannose) and Fuc (α-L-Fucose).

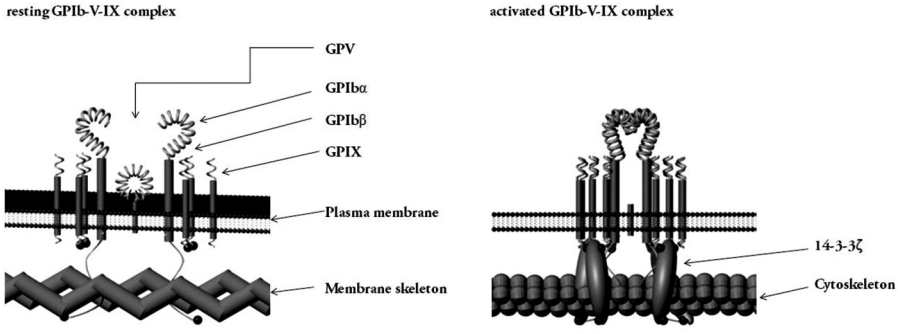


1.4.4 GPIbα-signaling

Under high shear conditions, platelets adhere to the site of vascular damage by interaction with subendothelial matrix-bound VWF and its platelet receptor GPIb-V-IX. GPIb-V-IX is a complex and consists of GPIbα, GPIbβ, GPV and GPIX, in a 2:4:1:2 stoichiometry (Figure 5 and [62]). The α-chain of GPIb is disulfide-linked to the β-chain and noncovalently associated with GPV and GPIX (Figure 5). Apart from binding sites for VWF, GPIbα contains bindings sites

Figure 5: GPIb-V-IX complex and translocation of GPIbα from membrane skeleton to cytoskeleton after activation by its ligand, VWF

(Courtesy of Arjan Barendrecht).

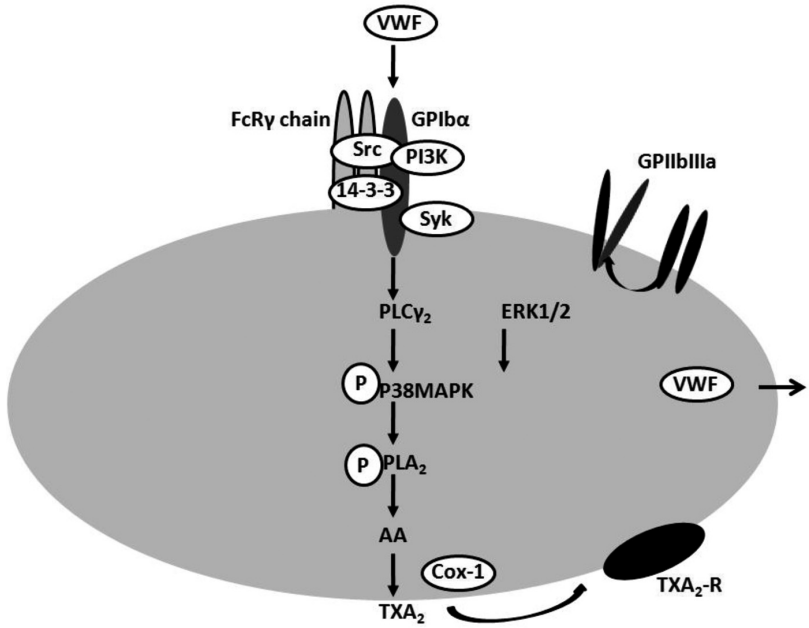


for P-selectin, $\alpha_M\beta_2$, α-thrombin, high molecular weight kininogen, coagulation factors XII and VII, TSP-1 and β_2GPI [63, 64, 65]. GPIbα can be phosphorylated at Ser-609 and Ser-587/590 and GPIbβ at Ser-166 [66]. The protein kinase A (PKA) - dependent phosphorylation status of the cytoplasmic domain of GPIbα controls the binding with adaptor molecule 14-3-3ζ. In the resting state, GPIbα is bound to filamin A in the membrane skeleton. However, after activation by VWF, GPIbα is coupled to the cytoskeleton via 14-3-3ζ (Figure 5 and [67, 68]).

In stirred suspensions, platelet aggregation by VWF occurs in two waves. The first wave represents the VWF-mediated crosslinking of platelets called agglutination; the second wave is triggered by the secretion of granules and results in integrin-dependent irreversible platelet aggregation. Following VWF-stimulation, GPIbα becomes associated with the members of the tyrosine kinase family c-Src and Lyn (Figure 6). This interaction requires phosphoinositide 3-kinase (PI3K) [69]. Activation of the immunoreceptor tyrosine-based activation motif (ITAM) receptors, the Fc receptors FcRγ and FcγRIIA play an important role. The dual tyrosine-phosphorylated ITAM-domain interacts with Syk. The complex signals through the adaptor proteins SLP-76 and LAT, leading to activation of PLCγ2, Ca^{2+}-mobilization and protein kinase C (PKC) activation, providing an important amplification mechanism promoting GPIbα-induced secretion [64].

Many intracellular signalling processes contribute, including phosphorylation by mitogen-activated protein (MAP) kinases, mitogen-activated protein kinase kinases (MEK) and

Figure 6: GPIbα-signalling after stimulation by VWF



extracellular signal-regulated kinase (ERK). P38MAPK activates cytosolic phospholipase A_2 ($cPLA_2$), leading to release of arachidonic acid (AA) from membrane phospholipids. Conversion of AA to TxA_2 is mediated by cyclooxygenase-1 (COX-1) and Tx synthase. The released TxA_2 then binds to the G-protein–coupled receptor TPα, resulting in $\alpha_{IIb}\beta_3$ activation and platelet aggregation (Figure 6 and [70]). Other studies describe that shear stress and GPIbα-mediated TxA_2-formation promotes the release of soluble CD40-ligand [71, 72].

1.4.5 GPIbα mobility

The GPIb-complex is anchored in the plasma membrane via its cytoplasmic domain, restrained by Filamin A. About 75-90% of GPIbα is bound to the membrane skeleton and 25% appears to be free. The actin polymerization inhibitor cytochalasin D increases the amount of free GPIbα and enhances VWF-induced platelet aggregation. GPIbα is recruited to the actin cytoskeleton (Figure 5 and [68]. Activation of GPIbα enhances receptor mobility, which is initiated by a release from the membrane skeleton (Figure 5 and [67, 73]). Previous studies showed that truncations of the *C*-terminal residues in GPIbα resulted in increased mobility [74].

1.4.6 GPIbα-deficiency

A deficiency or dysfunction of the GPIb-V-IX complex is known as Bernard-Soulier Syndrome (BSS). It is an inherited platelet disorder characterized by an increased bleeding tendency, giant platelets and low platelet counts (20 to $100x10^{10}$/L). The syndrome is extremely rare. Clinical manifestations include purpura, epistaxis, menorrhagia, gingival and gastrointestinal bleeding [75]. In the laboratory, platelets fail to aggregate with VWF/ristocetin and show a slow response to low-dose thrombin, while aggregations with epinephrine, ADP, collagen and AA are normal. Electron microscopy show platelets with abundant cytoplasmic vacuoles and an abnormal presence of membrane complexes concentrated in discrete zones. In addition, absent binding of FXI to GPIbα is observed [76]. In addition, bone marrow megakaryocytes (MK) from BSS-patients do not express the GPIb-V-IX complex and have increased cell volume, cytoplasmic granularity and increased ploidy. The thrombocytopenia is thought to be caused by both decreased platelet survival and ineffective platelet production [77]. Enhanced platelet size is supposed to be correlated with increased PS-exposure, as well as microparticle formation, similar as in the giant platelets in MYH9-related-disorder patients [78].

To date, 47 different genetic defects have been associated with BSS. There are 20 mutations in *GPIBA* including nonsense mutations leading to truncation or loss of the transmembrane domain and missense mutations interfering with trafficking. Also mutations in *GPIBB* and in *GP9* have been identified [www.bernardsoulier.org and 79]. Therapeutic approaches include prophylaxis and treatment of uncontrolled bleeding with platelet/blood transfusions.

A murine model of the human BSS has been described showing the phenotype of the human syndrome, including bleeding, macrothrombocytopenia and abnormal megakaryocyte maturation [80]. To address the question if the presence of the cytoplasmic tail of GPIbα plays

a role in platelet size, a mouse model was engineered expressing a fusion protein composed of a part of the human α-subunit of GPIb fused to the transmembrane and cytoplasmic tail of the human interleukin-4 receptor (IL-4Rα). Platelets from these mice revealed a 2-fold increase in circulating platelets and a 50% reduction in platelet size compared with platelets from BSS mice, suggesting that platelet size and consequent survival depend on the cytoplasmic domain of GPIbα [81].

1.4.7 Other roles for GPIbα

The number of new roles for the GPIbα-receptor is rapidly expanding. First, the gene encoding GPIbα is a transcriptional target for the c-myc oncogene, mediating tetraploidy [82]. GPIbα itself can function as a classical oncoprotein mediating transformation, proliferation, genomic instability, DNA damage in human cancer cell lines and p53-dependent senescence in primary cell strains [83, 84]. Second, GPIbα functions in experimental metastasis. A functional absence of GPIbα correlates with a 15-fold reduction in the number of lung metastatic foci in melanoma cells [85]. Third, GPIbα plays a role in apoptosis of MKs during megakaryocytopoiesis. The cytoplasmic tail of GPIbα sequesters signaling proteins, like 14-3-3ζ. However, when GPIbα lacked the 14-3-3ζ bindings site, thrombopoietin-mediated phosphorylation of Akt/PKB was increased, leading to aberrant apoptosis and therefore abnormal proliferation and polyploidization of MKs [86]. Fourth, GPIbα expression is associated with decreased proliferation of the megakaryocytic DAMI cell line and apoptosis, implying that GPIbα regulates growth of megakaryocytes [87]. Fifth, VWF-stimulation triggers apoptosis markers in platelets like caspase-activation and PS-exposure. Chinese hamster ovary (CHO) cells expressing a mutant GPIb-V-IX complex which lacked the 14-3-3 binding site had diminished apoptosis [88].

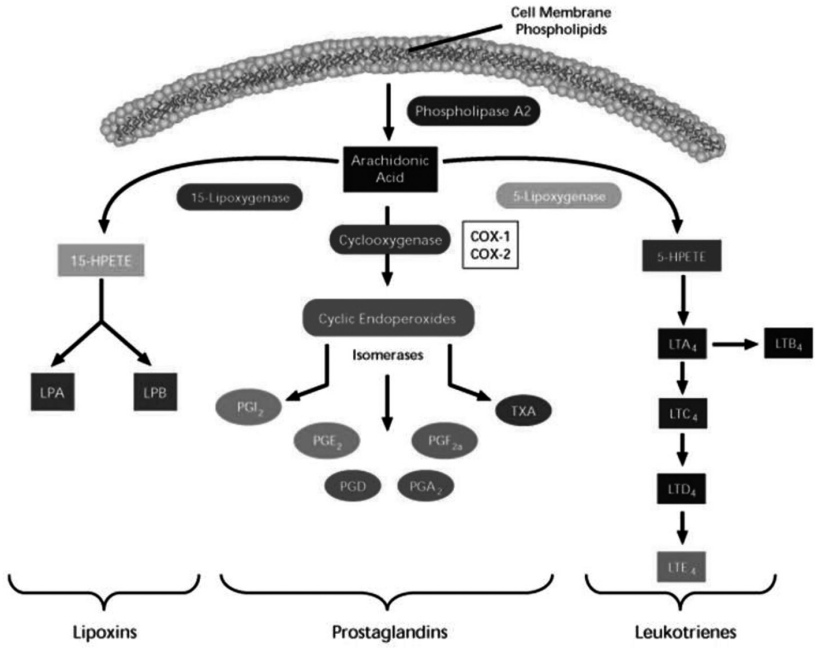
5.5 ARACHIDONIC ACID CASCADE

1.5.1 Phospholipase A_2 signalling

Phospholipase A_2 (PLA_2) releases polyunsaturated fatty acids such as AA from the *sn*-2 position of membrane phospholipids. AA is then metabolized to eicosanoids including prostaglandins and leukotrienes and related bioactive lipid mediators (Figure 7).

Figure 7: Arachidonate pathway

(from Sigma-Aldrich)

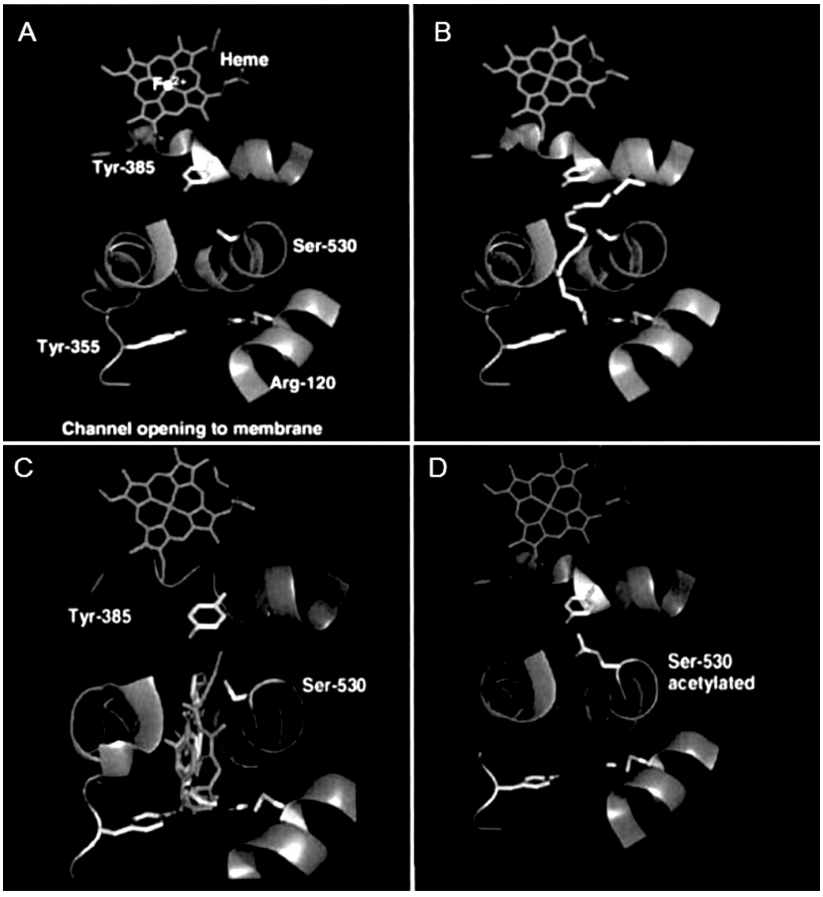


PLA_2 is known as a major component of snake venoms. PLA_2s are divided in four main subgroups: secreted $sPLA_2$s, cytosolic $cPLA_2$s, calcium-independent $iPLA_2$s and platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated- (Lp) PLA_2s [89]. $cPLA_2$ is composed of a Ca^{2+}-dependent lipid binding C2-domain, which is critical for association with phospholipid membranes and a catalytic α/β-hydrolase domain. Both domains are required for full activity. Activity of the enzyme is also regulated through phosphorylation on Ser-505, -515 and -727 by MAPKs [90].

Platelets also synthesize long-chain fatty acids, such as hydro (pero)xy-eicosatetraenoic acids (H(p)ETEs) by 12-lipoxygenase and to 14, 15-epoxyeicosatrienoic acids (EETs) by cytochrome P450 monooxygenase [91, 92].

Figure 8: Regulation of COX-1

The substrate channel and active site of COX-1 (A) and binding of AA (B) NSAIDs prevent binding of AA by mechanical channel blockade (C) whereas aspirin (D) blocks the access of AA through acetylation of Ser-530.



1.5.2 Cyclooxygenases

Cyclooxygenase (COX) is a glycosylated, bifunctional membrane-bound enzyme and is primarily found in the ER. It catalyzes the conversion of AA to PGG_2, as well as the conversion of PGG_2 to PGH_2 by peroxidase activity. COX exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed, while COX-2 expression is inducible. COX is integrated in only one leaflet of the lipid bilayer. Due to the hydrophobicity, AA remains associated with the bilayer in the vicinity of COX, once it is released from membrane phospholipids [93]. COX is the primary target of non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin, which acetylates Ser-530. Although, aspirin does not bind in the active site of COX-1 (Arg120-Tyr385), it blocks the access of AA to Tyr-385 inside the protein, located at the end of the hydrophobic channel that AA has to pass through to reach the catalytic site. Aspirin introduces a bulky compound into the channel that causes steric hindrance of access of AA to the catalytic site (Figure 8 and [93, 94, 95]).

The active site of COX consists of a long and narrow hydrophobic channel extending from the outer surface of the membrane-binding-motif into the centre of the catalytic domain [96]. AA binds in close proximity of Tyr-385, with the carboxylate group interacting with Arg-120 [97]. Tyr-355 determines the binding of 2-phenylpropionic acid class of NSAIDs, like ibuprofen, naproxen and flurbiprofen. Moreover, Tyr-355 is also the binding site for indomethacin, but Arg-120 is also required for the inhibition.

NSAIDs can be divided in two major functional groups. Indomethacin causes a time-dependent inhibition and is slowly reversible, while ibuprofen inhibits in a freely reversible competitive manner [96]. Aspirin however, binds covalently to COX in an irreversible manner. The time-dependent inhibition appears to depend on the presence of a halogen atom and a free carboxylic acid group in the molecular structure [98]. Because the molecular structure of indomethacin does contain a halogen atom, indomethacin inhibits irreversibly in many different cells. One exception is the slow reversible action of indomethacin in human platelets [99].

1.5.3 Thromboxane receptor signaling

PGH_2 produced by COX, is converted to PGI_2 and TxA_2 by prostaglandin and TxA_2 synthase respectively. PGI_2 and TxA_2 have opposing roles in vascular haemostasis when they bind to their respective seven-membrane/G-protein coupled receptors IP and TP. PGI_2 inhibits while TxA_2 stimulates platelet aggregation. Platelet stimulation with TxA_2 triggers shape change, aggregation and secretion, promoting haemostasis plug formation. In humans there are two TxA_2-receptors, TPα and TPβ. TPβ is present in endothelial cells, while TPα is the isoform present in platelets. TPα-mediated platelet shape change depends mainly on $G_{12/13}$ whereas aggregation depends primarily on G_q [100, 101].

1.6 APOPTOSIS

1.6.1 Apoptosis in nucleated cells

Apoptosis is often referred to as programmed cell death. Apoptosis inducing events (e.g. stress, cold, UV radiation or virus infections) trigger programmed cell death leading and clearance by macrophages. Apoptosis cells have typical characteristics, including cell shrinkage, membrane blebbing, nuclear fragmentation and the formation of apoptosis bodies. Apoptosis markers, linked to the mitochondria are cytochrome *c* release and depolarization of the inner mitochondrial membrane ($\Delta\psi_m$-change). All apoptosis pathways depend on the activation of caspases, which are cysteine aspartatic proteases. Caspases are synthesized as inactive zymogens containing a prodomain and a large p20 and small p10-subunit. They are activated by proteolytic cleavage [102]. The family of caspases can be divided in initiator- and effector-caspases. Initiator-caspases, like caspase-9, consist of long prodomains, containing either the death effector (DED) motif or the caspase recruitment domain (CARD). The effector-caspases, including caspase-3 and -7 have short prodomains [102].

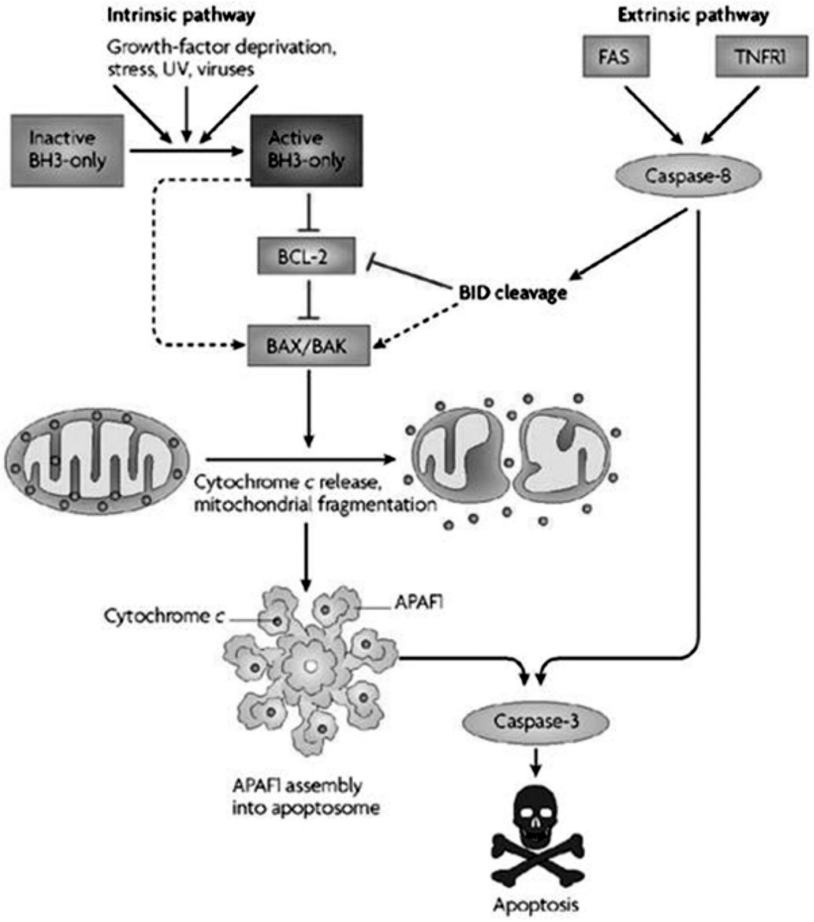
Two cell death pathways can be distinguished (Figure 9).

First, there is the intrinsic pathway controlled by the Bcl-2 (B-cell lymphoma) family proteins. This pathway leads predominantly to the activation of caspase-9 by apoptosis protease-activating factor-1 (apaf-1) [103, 104]. Second, there is the extrinsic (death receptor) pathway, which is triggered by the ligation of death receptors. Death receptors are members of the tumor necrosis factor (TNF) receptor family, such as Fas or TNF receptor-1 (TNFR-1), containing an intracellular death domain, which activates caspase-8. Interaction of both intrinsic and extrinsic pathways occurs via caspase-8 mediated cleavage of the pro-apoptosis protein Bid. The *C*-terminal truncated form of Bid (tBid) translocates to mitochondria and induces further activation of caspase-9 and effector-caspase-3, -6 and -7 through the intrinsic pathway [105, 106]. Active caspase-3 is the most important effector-caspase. Some members of the Bcl-2 family proteins have pro-apoptosis activities while others are pro-survival. There are three classes. First, pro-survival proteins like Bcl-2 and Bcl-x_L. Second, pro-apoptosis proteins like Bax and Bak. Third, BH3-only proteins like Bad and Bid, contain a conserved BH3-domain, which binds and regulates pro-survival Bcl-2 proteins promoting apoptosis. Both Bax and Bak can form pores in the membrane. Permeabilization of the outer mitochondrial membrane results in cytochrome *c* release, thereby activating caspases by binding to apaf-1. This leads to the assembly of the so-called apoptosome, which binds pro-caspase-9 and induces activation by conformational change (Figure 9).

Recent findings show that BH3-only proteins suppress Bax and Bak by direct binding and inhibition of Bcl-2 and other pro-survival family members. On the other hand, direct activation of Bax and Bak by BH3-only proteins is possible [106].

The pro-survival protein Bcl-2 is embedded in the Endoplasmatic Reticulum (ER), the nuclear envelope and the outer mitochondrial membrane. Bax exists as a monomer in the cytosol.

Figure 9: Apoptosis pathways [106]



After apoptosis induction, Bax translocates specifically to the mitochondria where it inserts itself into the outer mitochondrial membrane. The pro-apoptosis protein Bak already resides in the outer mitochondrial membrane. Both pro-apoptosis and pro-survival Bcl-2 family members can undergo conformational changes during apoptosis to unfold and insert deeply into the lipid bilayer. Bax changes its conformation to reveal a hidden epitope in the *N*-terminus. Consequently, Bax shows increased proteolytic sensitivity and forms oligomeric complexes. In addition, Bcl-2 changes its conformation during apoptosis after binding of BH3-only proteins. Bcl-2 and Bcl-x_L may bind Bax and Bak upon their translocation to the membranes [106].

1.6.2 14-3-3 proteins and Bad

14-3-3 proteins are specific phosphoserine/threonine-binding proteins and play a key role in apoptosis by interacting with pro-apoptosis protein Bad. In a pro-survival situation, 14-3-3ζ is coupled to the Serine-phosphorylated form of Bad in HEK293, HeLa and COS-7 cells [107, 108]. In mice, phosphorylation at Ser-112 is a prerequisite for phosphoryation at Ser-136, which correspond with the human orthologues Ser-75 and Ser-99 respectively. Ser-99 phosphorylation is dependent on PKB/Akt and this site is found to be essential in binding to 14-3-3ζ. It has been shown that Bad can bind to 14-3-3ζ when either singly or doubly phosphorylated, but phosphorylation of both Ser-75 and Ser-99 increases binding [108, 109, 110, 111, 112].

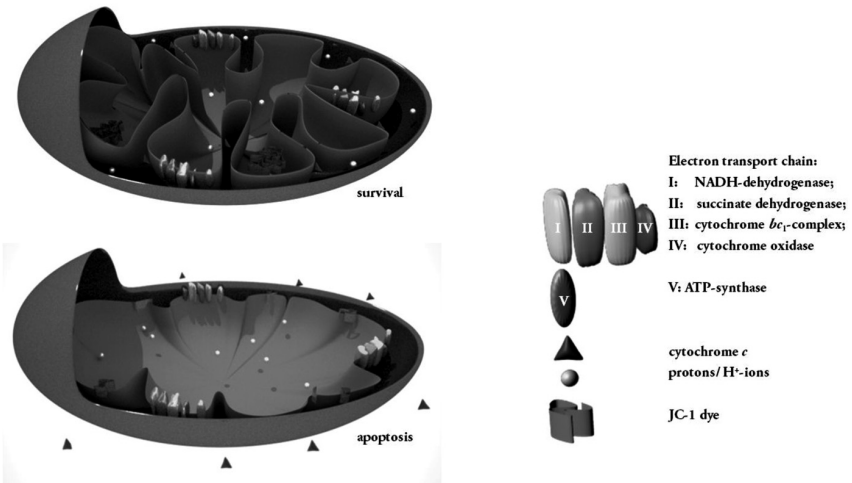
1.6.3 Mitochondrial regulation of apoptosis

The mitochondrial permeability transition pore (MPTP) is a multiprotein complex in between the inner and outer mitochondrial membranes. The MPTP regulates Ca^{2+}-concentration, pH, mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial volume. The open channel allows passage of solutes with molar masses up to 1.5 kDa. The increase in permeability by the MPTP allows the respiratory chain to create $\Delta\psi_m$-driven ATP-synthesis. The electron transport chain is composed of the multi-protein complexes I, III, and IV, which are the main proton pumps (Figure 10).

The proton gradient, which generates both a $\Delta\psi_m$ and a pH-gradient, is coupled to the activity of the ATP-synthase F0-F1 (Complex V). ATP-synthase uses protons to generate ATP from ADP. The gradient created by high $[H^+]$ in the intermembrane space and low $[H^+]$ in the matrix, causes H^+ to flow through the ATP-synthase (Figure 10). Energy released in the oxidative respiratory

Figure 10: Polarization of the inner membrane in healthy and apoptosis mitochondria

(Courtesy of Arjan Barendrecht)



chain is stored as an electrochemical gradient $\Delta\psi_m$ of -180-200 mV. After the onset of apoptosis, the membrane potential will decrease 80-100 mV. Uncouplers inhibit phosphorylation of ADP and create ion channels where H^+ can leak through without concurrent ATP-generation.
The cationic, membrane permeable dye, 5,5',6,6'- tetrachloro -1,1',3,3'- tetraethyl benzimidazol carbocyanine iodide (JC-1) can be used to measure $\Delta\psi_m$ by FACS. In healthy cells, reversible formation of JC-1 aggregates upon membrane polarization causes shifts in emitted light from 530 nm (emission of JC-1 in monomeric form) to 590 nm (*i.e.*, emission of J-aggregates) when excited at 490 nm. Thus, the colour of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized. In healthy cells, there is uptake of lipophillic JC-1 molecules by $\Delta\psi_m$ into the aqueous matrix space (negative charge inside mitochondria) which results in reversible aggregates, maintained and formed by $\Delta\psi_m$ (Figure 10). The formation of JC-1 aggregates depends strongly on dye concentration, intramitochondrial pH range (8-8.2) and ionic strength [113].

1.6.4 Apoptosis in platelets

Platelets are formed by megakaryocytes through release of proplatelets. This process may involve features resembling apoptosis. There is activation of caspase-3 and -9, depolarization of the mitochondrial membrane (fall in $\Delta\psi_m$) and cytochrome *c* release. Pro-platelet formation was diminished by overexpression of the prosurvival protein Bcl-2 [114]. Ligation of pro-apoptosis Fas-ligand increased proplatelet production in the human megakaryocytic cell line Meg01 [115]. Cytochrome *c* plays a critical role in apoptosis. A Gly41Ser mutation in the gene encoding cytochrome *c* (*CYCS*) caused thrombocytopenia by premature platelet release, without a reduction in platelet life span. The thrombocytopenia was caused by increased binding of apaf-1 to cytochrome *c* and caspase-3 activation [116].
The weak platelet activators ADP, epinephrine and the TxA_2-mimetic U46619 induce apoptosis features like a fall in $\Delta\psi_m$ without concurrent PS exposure [117]. Apoptosis is also triggered by the potent platelet activator thrombin via processes involving formation of reactive oxygen species (ROS) resulting in Bid, Bax and Bak-translocation to the mitochondria, cytochrome *c* release and PS-exposure [118]. Moreover, activation with VWF initiates apoptosis features such as elevation of Bax/Bak, a change in $\Delta\psi_m$, gelsolin cleavage by caspase-3 and PS-exposure [88]. There were indications that 14-3-3ζ was involved in triggering the GPIbα-induced apoptosis.
Platelet-storage under blood bank conditions for more than 12 days lead to fall in $\Delta\psi_m$, release of microparticles, caspase-3 activation and PS exposure [119]. Aging of platelets in plasma at 37°C induced Bax expression, while Bcl-x_L expression declined [120]. Platelets stored for 24 hours at this temperature, showed release of cytochrome *c* and caspase-9 activation, together with calpain-mediated proteolysis of Src and gelsolin followed by its translocation to the cytoskeleton [121]. Other studies demonstrated in platelets stored for 24-48 hours at 37°C more caspase-3 activation, decrease in Bcl-x_L and cytochrome *c* release compared with RT-storage [122]. Prolonged storage of PCs at normal blood bank conditions, without agitation, increased apoptosis markers such as ROS-production, a fall in $\Delta\psi_m$ and PS-exposure [123].

Although platelets lack a nucleus, they do contain mRNA. However, analysis of protein synthesis revealed no evidence for expression of the death ligands and death receptors TNFR1, DR4, 5, TRADD [124, 125]. In contrast, pro-apoptosis TNF-related apoptosis-inducing ligand (TRAIL) protein was upregulated after thrombin-stimulation [126]. Expression of DcR2, the decoy receptor of TRAIL together with pro-apoptosis Diablo/Smac and Bim release was increased after 5-day storage of PCs at blood bank conditions [124]. These findings suggest roles for death receptor and ligands in platelets.

When mice were injected with the BH3-mimetic ABT-737, an antagonist of pro-survival Bcl-2 and Bcl-x_L, acute thrombocytopenia followed. Also, a transient increase in reticulated platelets was found, a sign of increased platelet production. In Bak-deficient mice, these effects were absent. Mice with mutations in Bcl-x_L and Bcl-x_L knockout mice were also thrombocytopenic. These findings indicate that platelet survival is determined by the intrinsic apoptosis program, in which a balance between pro-survival Bcl-x_L and pro-apoptosis Bak functions as a molecular clock [127].

Mice deficient in the BH3-only pro-apoptosis protein Bad had elevated platelet numbers compared with WT-mice. This was caused by a prolonged platelet life span rather than increased production, suggesting an important role for Bad in platelet survival [128].

One popular hypothesis for the brief life span of platelets is the "multiple hit" model. This model is based on damage by external hits, finally triggering platelet clearance. This model was refuted by a mathematical approach. Also, a novel labelling technique which distinguished internal and external control of platelet fate did not support the multiple hit model. Platelets from Bcl-x_L or Bak-knockout mice were stored in diluted platelet-poor-plasma at 37°C for 28 hours. Young born platelets exhibited a delay in clearance, when compared with the other population. Moreover, platelets deficient in Bcl-x_L could withstand more hits than WT-platelets. Earlier results showed a major role for the spleen in platelet life span: dogs which had a splenectomy had a higher platelet survival than the control dogs [129]. To test if the spleen indeed played a major role here, splenectomy was performed, but this had only small effects on platelet survival, also shedding doubts on the model [130].

Two distinct pathways regulating PS-exposure in platelets have been described. Treatment with ABT-737 triggered PS-exposure in the presence of thrombin-generation, which was dependent on Bak/Bax and caspase-activity. Platelet activation inhibitors did not affect this response. Stimulation of platelets from Bak/Bax-deficient mice showed the same PS-exposure as WT-mice, but this response was inhibited by platelet inhibitors, indicating that different mechanisms control the procoagulant function of platelets [131]. In a recent review by the same authors, different mechanisms for inducing platelet death are proposed. Necrosis is characterized by metabolic failure or ATP-depletion, a loss of plasma membrane integrity, swelling of mitochondria, other organelles and plasma membrane, leading to lysis. Ca^{2+}-overload and ROS-production are supposed to play key roles in changing $\Delta\psi_m$ and calpain-, but not caspase-activation. After necrosis, immune responses are triggered and platelets become procoagulant and 'balloon'-shaped [132]. In contrast, apoptosis is characterized by absence of inflammatory responses, membrane blebbing, caspase-activity and is dependent on energy.

1.7 AIM OF THE THESIS

The aim of the studies described in this thesis was to investigate platelet functions after cold-storage to find ways to decrease cold-storage induced platelet damage. We used an incubation scheme of 4 hours storage at 0°C followed by 1 hour incubation at 37°C to mimic conditions when cold-stored platelets are transfused to patients.

We addressed the following research questions:

1. Does cold-storage damage the haemostasis properties of platelets?
2. How does GPIbα contribute to apoptosis regulation in platelets?
3. Does arachidonate play a role in platelet apoptosis?
4. What is the cause of the increased apoptosis in a patient with spontaneous platelet activation?

We have summarized current methodology for blood collection, preparation of PCs and storage conditions (Chapter 1). A recent modification to reduce the damage inflicted to platelets during storage at RT is to apply storage at low-temperature [22]. The impact on the haemostasis properties of cold-stored platelets has been addressed in a number of *in vitro* tests (Chapter 2). During these studies, it became evident that incubation at 0°C could affect platelet survival since those conditions triggered the initiation of apoptosis (Chapter 3). Apoptosis induction was initiated by changes in the GPIbα-receptor possibly caused by the formation of clusters. However, this relatively minor event could not fully explain the strong induction of caspase-9 activity, exposure of PS and platelet binding followed by phagocytosis by a macrophage cell line. We searched for other pathways for apoptosis induction that might work in parallel or in synergy with GPIbα-induced apoptosis (Chapter 4). Apart from finding tools to improve cold-storage conditions, these studies greatly increased our insight in the regulation of the intrinsic pathway for apoptosis in platelets in general and the role of GPIbα in this pathway in particular. This insight was used to understand the cause of spontaneous platelet apoptosis in a patient whose platelets aggregated during blood collection (Chapter 5). The results were evaluated in relation to recent developments in the field of platelet storage for transfusion, the control of the intrinsic pathway for apoptosis and the multiple functions of GPIbα (Chapter 6).

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You can only take what you can carry

(Snow Patrol)

Chapter 2

Role of glycoprotein Ibα mobility in platelet function

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2.1 SUMMARY

Incubation at 0°C is known to expose β-*N*-acetyl-*D*-glucosamine residues on glycoprotein (GP) Ibα inducing receptor clustering and $\alpha_M\beta_2$-mediated platelet destruction by macrophages. Here we show that incubation at 0/37°C (4 hrs at 0°C, followed by 1 hr at 37°C to mimic cold-storage and post-transfusion conditions) triggers a conformational change in the *N*-terminal flank (NTF, amino acids, aa 1-35) but not in aa 36-282 of GPIbα, as detected by antibody binding. Addition of the sugar *N*-acetyl-*D*-glucosamine (GN) inhibits responses induced by 0/37°C. Incubation at 0°C shifts GPIbα from the membrane skeleton to the cytoskeleton. Different GPIbα conformations have little effect on VWF/ristocetin-induced aggregation, but arrest of NTF change by GN interferes with agglutination and spreading on a VWF-coated surface under flow. Strikingly, incubation at 0/37°C initiates thromboxane A_2 formation through a VWF-independent and GPIbα-dependent mechanism, as confirmed in VWF- and GPIbα-deficient platelets. We conclude that the NTF change induced by 0/37°C incubation reflects clustering of GPIbα, supports VWF/ristocetin-induced agglutination and spreading and is sufficient to initiate platelet activation in the absence of VWF.

2.2 INTRODUCTION

Platelet glycoprotein (GP) Ibα is a component of the $[GPIb\alpha]_2[GPIb\beta]_4$ GPV$[GPIX]_2$ complex and serves as a receptor for multiple ligands such as von Willebrand Factor (VWF), P-selectin, α-thrombin, coagulation factors XI and XIIa, high-molecular-weight kininogen, thrombospondin-1 and β_2-glycoprotein I [1, 2]. The highly glycosylated GPIbα is a class-I transmembrane receptor containing an extracellular region with an *N*-terminal flank, seven leucine rich repeats, a *C*-terminal flank, a sulphated region and a *C*-terminal macroglycopeptide region. A major function of GPIbα is binding to the A-1 domain of VWF, which in the disturbed vessel wall binds to collagen fibres. Binding induces a conformational change and VWF becomes a docking site for platelets in flowing blood. The rapid and reversible GPIbα-VWF interaction slows down the platelets and enables firm attachment and further platelet activation through the collagen receptors integrin $\alpha_2\beta_1$, GPVI and the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$. GPIbα mutations in Bernard-Soulier Syndrome (BSS) patients and studies with antibodies against different parts of the receptor have identified amino acids (aa) 1-81 and 104-128 as binding sites for VWF and aa 60-128, aa 201-268 and aa 251-279 as binding sites for the VWF/ristocetin complex [3].

In resting platelets, 70-90% of the GPIb-V-IX complex is anchored to the membrane skeleton through interaction with filamin A and F-actin filaments [4]. Treatments that release GPIbα enhance VWF-binding and platelet activation, suggesting that restrains in lateral mobility reduce receptor binding of multimeric VWF [5]. Following platelet activation by VWF/ristocetin, a large part of GPIbα together with adapter protein 14-3-3ζ, associates with the cytoskeleton [6].

The mechanism by which GPIbα activates platelets is poorly understood. A first step following receptor occupancy by VWF/ristocetin is the association of c-Src and Lyn and further signaling through phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinases (MAPKs). Activation of PI3-K requires recruitment of Syk and the adapter protein SLP76 and initiates downstream signaling to phospholipase Cγ2, Ca^{2+} rises, protein kinase C and activation of integrin $\alpha_{IIb}\beta_3$ inducing aggregation. Concomitant release of ADP together with p38MAPK-mediated generation of thromboxane A_2 (TxA_2) enhances signaling to $\alpha_{IIb}\beta_3$ [7]. Interactions with the cytoskeleton through filamin (GPIbα), the adapter protein 14-3-3-ζ (GPIbα, GPIbβ and GPIX) and calmodulin (GPIbβ and GPV) further contribute to VWF-induced platelet activation [8].

In an attempt to improve storage conditions, Hoffmeister *et al.* [9] found that incubation at 0°C made platelets prone to phagocytosis by liver macrophages. The cause was sought in damage of the glycosylation of GPIbα, exposing β-*N*-acetyl-*D*-glucosamine (βGN) residues on *N*-linked GPIbα glycans normally capped by sialic acid and galactose [9]. The damage triggers formation of GPIbα clusters, which are recognized by the lectin binding domain of $\alpha_M\beta_2$ on macrophages initiating platelet destruction. GN inhibits platelet phagocytosis by matured monocytic THP-1 cells, strongly implicating *N*-glycans in the recognition mechanism of $\alpha_M\beta_2$ [10].

Recently, our laboratory demonstrated that cold-induced changes in GPIbα reduce its accessibility for the anti-GPIbα antibody AN51 directed against amino acids (aa) 1-35 of the *N*-terminal flank (NTF). Interestingly, GN inhibits the change in AN51 binding and reduces platelet destruction, suggesting that affinity changes for AN51 binding reflect changes in GPIbα that accompany the formation of clusters [11].

In the present study, we used a combination of incubation at 0°C (4 hrs) and at 37°C (1 hr) to mimic cold storage and post-transfusion conditions and investigated how changes in the GPIbα-NTF affected the haemostatic properties of the receptor.

2.3 MATERIAL AND METHODS

2.3.1 Patients

The patient with von Willebrand Disease (VWD) type 3 had 1-3% plasma Factor VIII, < 10% plasma VWF antigen, < 15% VWF/ristocetin-induced aggregation and normal platelet count and volume [12]; the patient with BSS had 40 x 10^9 platelets/l, a mean platelet volume of 17 fl, absent VWF/ristocetin-induced aggregation and absent GPIbα on FACS.

2.3.2 Chemicals

We purchased anti-human GPIbα antibodies AN51 from DakoCytomation (Glusdorp, Denmark), AK2 from Genetex (Irvine, CA), VM16D from Sanbio BV (Uden, the Netherlands), SZ2 from Immunotech (Marseille, France) and HIP1 and anti-human P-selectin antibody from BD Pharmingen (San Diego, CA). Tapi-2 was from Peptides International (Louisville, KY), TxA_2 Enzyme Immuno Assay (EIA) kit from Assay Designs (Ann Arbor, MI); *N*-acetyl-*D*-glucosamine (GN) from Merck (Darmstadt, Germany), PP1 from Alexis Biochemicals (San Diego, CA), Bapta-AM from Calbiochem (La Jolla, CA), thrombin receptor (PAR1)-activating peptide (trap, SFLLRN) and RGDS (Arg-Gly-Asp-Ser) from Bachem (Switzerland); O-sialoglycoprotein endopeptidase (osge) from Cedarlane Laboratories (Hornby, Ontario, Canada), phalloidin-Alexa 568 from Invitrogen (Carlsbad, CA), cytochalasin D, latrinculin A, A3P5PS and indomethacin from Sigma-Aldrich (St Louis, MO), fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse (GAM) antibody from Jackson Immunoresearch Laboratories (West Grove, PA) and ristocetin (diaRistin) from Diamed AG (Cressier s/Morat, Switzerland). Human VWF was purified as described [13]. Anti-GPIbα 6.30 was an inhouse mouse monoclonal antibody [2]. PGI_2 analog iloprost (Ilomedine) was a kind gift of Schering AG (Berlin, Germany). Recombinant A1 domain of VWF (aa 498-705) was purified from *Pichia Pastoris* [13]. AR-C69931MX (AR-C) was a kind gift from Astra Zeneca (Loughborough, UK). Llama derived anti-VWF antibody fragment 12B6 was produced as described (control nanobody in [14]).

2.3.3 Platelet isolation and incubations

Washed platelets were prepared as described [15]. Platelets from the BSS patient were isolated after prior sedimentation (2 hrs, 22°C) and depletion of WBCs by CD45-beads (Miltenyi Biotec, Germany). Final concentrations were 2 x 10^{11} cells /l (normal platelets) and 4 x 10^{10} cells /l (BSS platelets). Incubations were during 4 hrs at 0°C, followed by 1 hr at 37°C (defined as 0/37°C incubation) to mimic conditions when cold-stored platelets are transfused to patients. In a few experiments, incubations at 37°C varied between 0.5 and 2 hrs. GN (100 mM) was present as indicated. In control studies, fresh platelets were stimulated with VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C). This suboptimal concentration was chosen to make VWF

signalling maximal responsive to changes in GPIbα. Other incubations with fresh platelet were with cytochalasin D and latrinculin A (5 μM, 5 min, 37°C, each) and anti-VWF nanobody 12B6 (5 μg/ml, 15 min, 22°C). Before 0°C-treatment, platelets were incubated (15 min, 22°C) with the following metabolic inhibitors: PP1 (10 μM), iloprost (1 μg/ml), AR-C (250 nM), A3P5PS (500 μM), Bapta-AM (10 μM) and indomethacin (30 μM). All incubations were without stirring except in experiments where stirring is part of VWF/ristocetin induced responses (the change in GPIbα anchorage, aggregation, agglutination). A few experiments were performed with the ADAM17 inhibitor Tapi-2 (20 μM) to clarify possible interference by GPIbα shedding.

2.3.4 Analysis of GPIbα binding to AN51-PE

Antibody AN51 is directed against aa 1-35 of the NTF of GPIbα [16, 17]. We found earlier that at a strictly standardized platelet concentration, incubation time with antibody and antibody concentration that occupies about 90% of GPIbα copies, AN51 binding decreases when platelet suspensions are cooled/rewarmed, a condition known to induce GPIbα clusters that mediate platelet destruction by macrophages [9]. The fall in AN51 binding was inhibited by GN (and to a lesser extent by galactose, but not by glucose or mannose, ([17] and data not shown), which also inhibits destruction of cold-rewarmed platelets by macrophages. These observations suggest that the GN-sensitive fall in AN51 binding observed when platelets are cold-rewarmed is caused by clustering of GPIbα (For details, [17]). AN51 binding was measured by incubating platelet suspensions with PE-conjugated AN51 (2 μg/ml, 15 min, 37°C) and analyzing 10.000 platelets on a FACS Calibur (BD Biosciences, San Jose, CA). For each fresh platelet suspension, settings were adjusted to include 90% of total platelets and defined as M1 fraction. Cold-rewarmed platelets showed a decrease in antibody binding, defined as M2 fraction. Incubations with fresh platelets (without stirring to prevent aggregation, 37°C) were with: VWF/ristocetin (3 μg/ml VWF, 0.3 mg/ml ristocetin, 5 min), osge (80 μg/ml, 30 min), A1 domain of VWF (20 μg/ml with 0.3 mg/ml ristocetin, 5 min), trap (10 μM, 5 min) and the GPIbα antibodies AK2 (directed against aa 35-59), HIP1 (against aa 59-81), VM16D (against aa 200-268) and SZ2 (against aa 276-282, 2 μg/ml each, 15 min). Binding of unconjugated antibodies AK2 and VM16D was measured by adding a secundary GAM-FITC antibody (45 min, 22°C). Binding of FITC-conjugated HIP1 and SZ2 (1:20 and 1:50 dilution respectively) was measured as described for AN51. P-selectin expression was measured with P-selectin-FITC antibody (1:20 dilution).

2.3.5 Analysis of GPIbα-anchorage to the cyto- and membraneskeleton

Platelet suspensions were lysed [2] and the actin cytoskeleton was isolated by centrifugation (15.600 x g, 4 min, 4°C). The supernatant was used to isolate the membrane skeleton (100.000 x g, 2.5 hrs, 4°C [18]). Pellets from both fractions obtained from the same donor were resuspended in 50 μl sample buffer. Samples of 15 μl each were used for protein separation by SDS-PAGE and immunoblotted with anti-GPIbα antibody 6.30 (0.25 μg/ml). Blots were developed with

enhanced chemiluminescence reagent plus (Perkin Elmer Life Sciences, Boston, MA) and quantified with Image-J software (NIH, Bethesda, MD).

2.3.6 Platelet interaction with VWF

Platelet aggregation/agglutination was measured in a Chronolog aggregometer at 37°C with stirring at 900 rpm (Chronolog Corporation, Haverford, PA). Following stimulation with VWF/ristocetin, aggregation was measured in the absence and agglutination in the presence of 100 μM RGDS to block ligand binding to $\alpha_{IIb}\beta_3$. Platelet adhesion and spreading on surface-coated VWF was investigated in triplicate in a single passage perfusion chamber [19] at 37°C and shear rates of 800 and 1600 s^{-1}. Coverslips were coated for 1 hr at 22°C with a solution of 15 μg VWF/ml (4.5 ng/mm^2 coverslip) and blocked with 1% human albumin (4°C). Coverslips were fixed and stained with May-Grünwald-Giemsa. Adhesion was evaluated with a light microscope equipped with a JAI-CCD camera coupled to a Matrox frame grabber using Optimas 6.2 software (Optimas Inc., Seattle, WA) for image analysis and expressed as percentage surface coverage. Spreading was calculated by dividing surface coverage by number of adhered platelets, as described [20].

2.3.7 Stress fibres and formation of TxA_2

For visualization of stress fibres, platelets were fixed with 0.5% (v/v) glutaraldehyde immediately after perfusion. Samples were treated with ammoniumchloride (50 mM, 10 min), permeabilized (0.2% Triton X-100, 2% BSA, 10 min), and stained for 1 hour with Phalloidin-Alexa 568. Coverslips were analysed with a TCS4D confocal laser microscope (Leica Lasertechnik, Heidelberg, Germany).

For analysis of TxA_2 formation, suspensions were incubated without stirring as indicated. At the end of incubations, samples were rapidly mixed with indomethacin (30 μM, final concentration) to halt COX-1 activity. Platelets were pelleted (15.000 x g, 30 sec, 22°C) and TxA_2 was measured in the supernatant by EIA. Data were expressed as ng TxA_2 formed/min/10^{11} platelets.

2.3.8 Statistics

Data are means ± SEM of three experiments or more, as indicated. Statistical analysis was performed using GraphPad InStat (San Diego, CA) software. Differences between untreated and treated samples were considered significant at $p < 0.05$ (*) or $p < 0.01$ (**).

2.4 RESULTS

*2.4.1 Changes in the GPIb*α *N–terminal flank*

GPIbα antibody AN51 is directed against aa 1-35 of the NTF [16, 17] and binds GPIbα with characteristics that change in cold-rewarmed platelets. The change is accompanied by induction of platelet phagocytosis by macrophages and inhibited by GN, suggesting that it reflects the receptor redistribution that serves in macrophage recognition [9, 17]. To explore this possibility

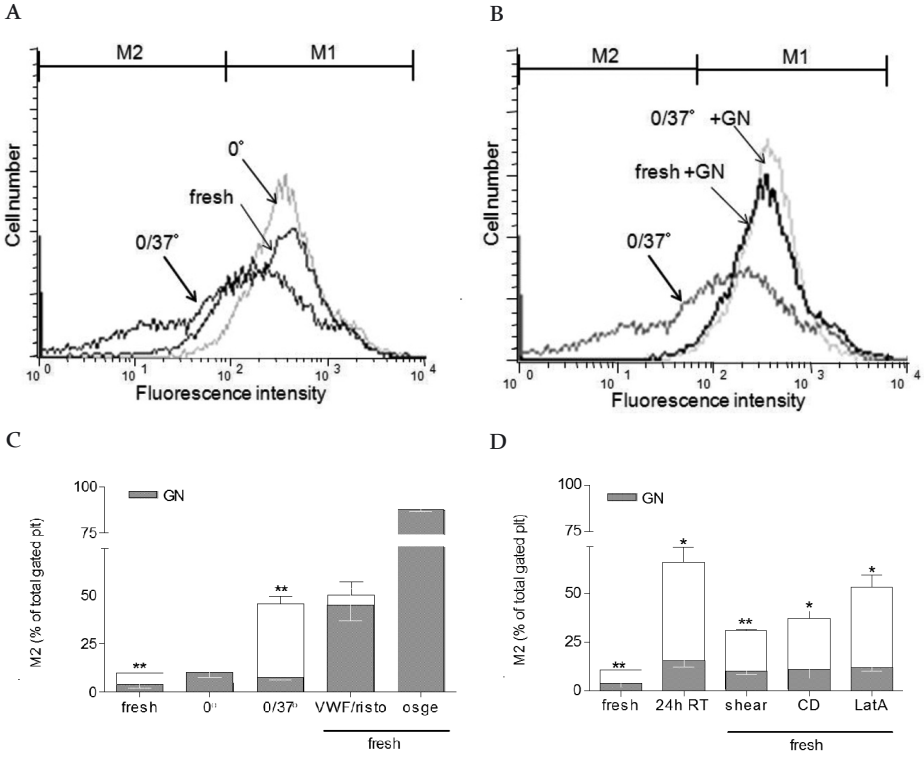
Figure 1: Effect of 0/37°C-incubation on binding of AN51 antibody to GPIbα

A, B) Fresh, 0°C (4 hrs)- and 0/37°C (4 hrs/1 hr)-treated platelets were incubated with anti-GPIbα antibody AN51-PE and binding was measured by FACS. GN depicts the presence of 100 mM *N*-acetyl-*D*-glucosamine, added at the start of incubations.

C) Percentage of total platelets in the low affinity M2 range in a fresh suspension (set at 10%) and in platelets incubated at 0°C (4 hrs) and at 0°C followed by 37°C (1 hr). The effect of VWF on AN51 binding was evaluated by stimulating fresh platelets with VWF/ristocetin (3 μg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C, without stirring) and the effect of GPIbα-depletion by treatment with osge (80 μg/ml, 30 min, 37°C).

D) Percentage of total platelets in the low affinity M2 range in fresh platelets, platelets stored for 24 hours at room temperature (24h RT), fresh platelets after a single passage through a perfusion chamber (shear) and in fresh platelets treated with cytochalasin D (CD) and latrinculin A (LatA, 5 μM, 5 min, 37°C each).

Platelets were incubated without (open bars) and with GN (grey bars). Data are means ± SEM, n = 4. Differences between untreated and GN-treated samples were significant at $p < 0.05$ (*) or $p < 0.01$ (**).



in detail, AN51-PE binding was measured in fresh and cold-stored platelets (4 hrs, 0°C) and in cold-stored platelets that were rewarmed (1 hr, 37°C) to mimic conditions when platelets are transfused in recipients. AN51-PE binding to fresh and cold-stored platelets was similar, but cold followed by rewarming induced a shift from the high affinity (M1) to the low affinity (M2) binding range (Figure 1A). Cold-storage is known to damage GPIbα by removing sialic acid and galactose residues [10]. The result is exposure of βGN residues and formation of GPIbα clusters that bind $\alpha_M\beta_2$ on macrophages in the absence but not in the presence of GN. GN did not change AN51 binding to fresh and cold-stored platelets but inhibited the fall in AN51 binding in cold-rewarmed platelets (Figure 1B). AN51-PE binding to fresh platelets was set to include 90% of total platelets in M1. After cold-storage, the distribution was similar but rewarming induced a shift of 46 ± 4% of total platelets to M2 (Figure 1C). GN induced a slight shift to the M1 fraction in fresh platelets, had no effect on cold-stored platelets and strongly interfered with the affinity decrease seen in cold-rewarmed platelets, preserving about 90% of total platelets in M1. Apparently, interference with GPIbα-mediated platelet destruction also prevents the fall in AN51 binding. When fresh platelets were stimulated with VWF/ristocetin, a similar shift to M2 was observed. This shift was not inhibited by GN. Osge removes the *N*-terminal part of GPIbα [21] and, as expected, induced complete loss of AN51-PE binding.

To search other conditions that induce a similar, GN-sensitive, change in AN51 binding, we found that prolonged incubation at room temperature (24 hrs) and a single perfusion through a laminar flow chamber at a shear of 1600 s^{-1} induced a similar change in GPIbα (Figure 1D). GPIbα is anchored to a loose network of actin fibres known as the membrane skeleton [18]. Interference with this network with cytochalasin D, an inhibitor of *de novo* actin polymerisation, and latrunculin A, which sequesters G-actin and prevents F-actin assembly, also shifted AN51-PE binding to the M2 range and again GN prevented the shift. Together, these findings reveal different conditions that decrease antibody binding to aa 1-35 in GPIbα through a mechanism that is inhibited by GN.

Cooling-rewarming is known to induce secretion of granule content [22]. We found little α-granule secretion in cold-treated platelets but cold-rewarming induced 15 ± 3% P-selectin positive platelets. This is in the range of fresh platelets stimulated with a suboptimal concentration of VWF-ristocetin (without stirring). The P-selectin expression was completely abolished by $P2Y_{1/12}$ blockade, indicating that is was induced by ADP (Figure 2A). To investigate whether the secretion response leads to release of VWF and interference with AN51 binding to GPIbα, platelets were incubated with anti-VWF nanobody 12B6, which inhibits GPIbα-VWF interaction by binding to the VWF-A1 domain [14]. The nanobody reduced GN-sensitive AN51 binding (defined as ΔM2) by about 30%, indicating that the major part was caused by VWF-independent changes in GPIbα induced by 0/37°C-incubation (Figure 2B). A control confirmed that the GPIbα change by added VWF was inhibited by this antibody. The GPIbα change induced by multimeric VWF was also induced by the monomeric VWF-A1 domain and insensitive to interference by GN. Platelet activation with trap also induced the M2 shift and showed no significant inhibition by VWF nanobody and GN.

Since inhibitors of actin polymerization induced a similar, GN-sensitive, fall in AN51-PE binding as 0/37°C incubation, we investigated whether the GPIbα change induced signalling to mechanisms that affect membrane/cytoskeleton re-arrangements. An inhibitor of Src-family kinases (PP1) inhibited the GPIbα change by almost 100% (Figure 2C). The stable prostacyclin analog iloprost raises cAMP and inhibited the shift by 40%. Blockade of $P2Y_{1/12}$ signalling induced about 20% inhibition, confirming the contribution by a minor ADP secretion also observed at the level of P-selectin expression. Inhibitors of Ca^{2+} increases (bapta-AM), COX-1 mediated TxA_2 formation (indomethacin), p38MAPK activity (SB203580) and ERK2 activity (the MEK inhibitor PD098059) left the GPIbα change intact (Figure 2C and data not shown). Thus, the mechanism that controls a decrease in AN51 binding to GPIbα is under control of Src family kinases and inhibited by a rise in cAMP. PP1 also interfered with the fall in AN51-binding induced by VWF and VWF-A1 indicating that also the ligand-induced GPIbα change was under metabolic control. Changes in AN51 binding remained unchanged in the presence of RGDS indicating that the shift to low affinity AN51 binding was independent of $\alpha_{IIb}\beta_3$ (data not shown).

Sequential analysis of AN51-PE binding showed that 0/37°C incubation induced a maximal shift to low affinity binding after about 60 min. Thereafter, antibody binding returned to the range of fresh platelets, suggesting that the GPIbα change is reversible in nature (Figure 2D). To investigate whether the change in aa 1-35 of GPIbα that affects AN51 binding is also seen with antibodies against more membrane proximal regions of the receptor, binding studies were repeated with antibodies AK2 (directed against aa 35-59), HIP1 (against aa 59-81), VM16D (against aa 200-268) and SZ2 (against aa 276-282). None of these antibodies revealed a shift to low affinity binding induced by 0/37°C incubation (Figure 2E). The decrease in AN51 binding and interference by GN was unchanged in the presence of tapi-2, indicating that under these conditions receptor shedding was absent (data not shown).

Figure 2: Characteristics of low affinity binding of AN51 antibody to GPIbα

A) P-selectin expression of fresh platelets and platelets incubated at 0°C and at 0°C followed by 37°C without and with $P2Y_{1/12}$ blockade by AR-C/A3P5PS. For comparison, P-selectin expression was measured in fresh platelets following stimulation with VWF/ristocetin without stirring.

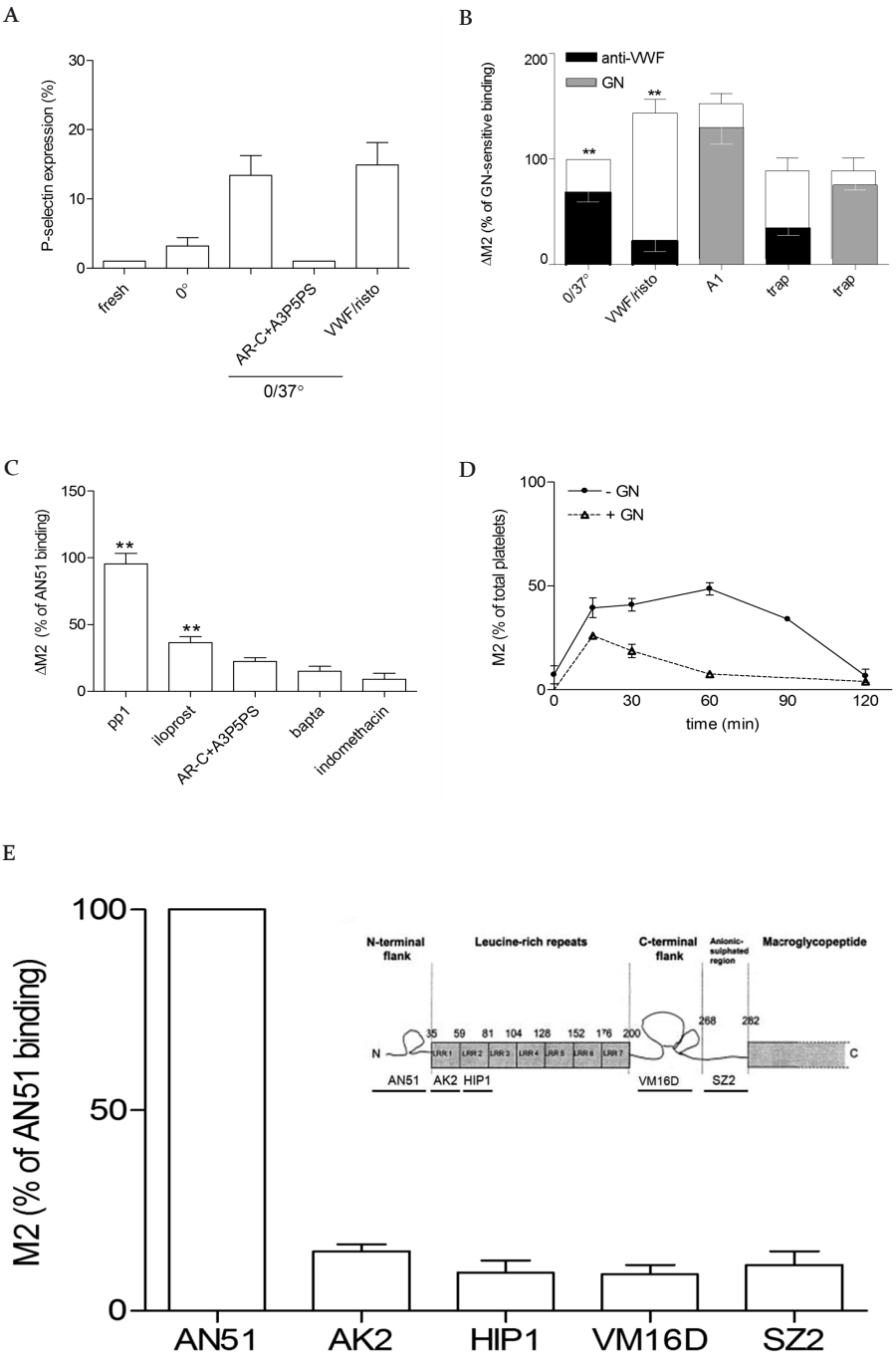
B) Effect of anti-VWF nanobody and GN on GN-sensitive AN51 binding (defined as ΔM2) of 0/37°C-treated platelets and fresh platelets treated with VWF/ristocetin, VWF-A1 domain and trap.

C) Inhibition of 0/37°C-induced ΔM2 binding by receptor- and metabolic inhibitors: PP1 (Src kinase inhibitor), iloprost (cAMP raising agent), A3P5PS ($P2Y_1$ blocker), AR-C69931MX ($P2Y_{12}$ blocker), bapta-AM (chelator of cytosolic Ca^{2+}) and indomethacin (inhibitor of COX-1 mediated TxA_2 formation).

D) Reversibility of low affinity AN51 binding. Time course of low affinity AN51 binding (M2) in 0°C-treated platelets during subsequent incubation at 37°C.

E) Binding of antibodies against epitopes between residues 36-282 is not changed by 0/37°C incubation. The insert shows the different binding epitopes of the antibodies (modified from Cauwenbergh *et al.* [34]). ** $p < 0.01$.

Figure 2:

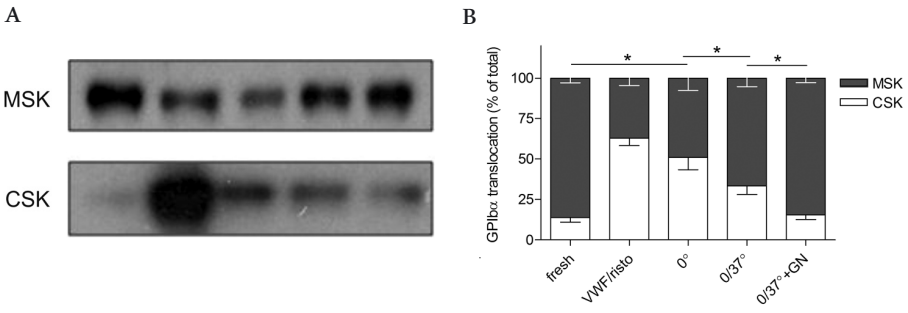

A
P-selectin expression (%)
30
20
10
0
fresh
0°
AR-C+A3P5PS
VWF/risto
0/37°
B
ΔM2 (% of GN-sensitive binding)
200
100
0
anti-VWF
GN
**
**
0/37°
VWF/risto
A1
trap
trap
C
ΔM2 (% of AN51 binding)
150
100
50
0
**
**
pp1
iloprost
AR-C+A3P5PS
bapta
indomethacin
D
M2 (% of total platelets)
100
50
0
- GN
+ GN
0
30
60
90
120
time (min)
E
M2 (% of AN51 binding)
100
50
0
AN51
AK2
HIP1
VM16D
SZ2
N-terminal flank
Leucine-rich repeats
C-terminal flank
Anionic-sulphated region
Macroglycopeptide
35
59
81
104
128
152
176
200
268
282
N
C
LRR 1
LRR 2
LRR 3
LRR 4
LRR 5
LRR 6
LRR 7
AN51
AK2
HIP1
VM16D
SZ2

2.4.2 Anchorage of GPIbα to membrane- and cytoskeleton

Since 0/37°C incubation caused a similar fall in AN51 binding as inhibitors of actin polymerization, we investigated whether 0/37°C incubation interfered with anchorage of GPIbα to the membrane- and cytoskeleton. Triton X-100 insoluble fractions were isolated by low-speed (cytoskeleton) and high-speed (membrane skeleton) centrifugations [18]. VWF/ristocetin is known to release GPIbα from the membrane skeleton [23] and to initiate anchorage to the cytoskeleton [24]. and these fractions were taken as an internal control. Incubation at 0°C induced a fall in membrane skeleton-bound GPIbα and an increase in cytoskeleton-bound GPIbα (Figure 3A, B). Subsequent incubation at 37°C slightly restored the coupling of GPIbα to the membrane skeleton. Importantly, GN inhibited the GPIbα translocation. These data indicate that incubation at 0°C releases a major part of anchored GPIbα from the membrane skeleton which then binds to the cytoskeleton.

Figure 3: Treatments that affect AN51-GPIbα interaction, change GPIbα anchorage to the membrane/cytoskeleton

GPIbα associations with the membrane skeleton (MSK, black bars) and the cytoskeleton (CSK, white bars) in fresh platelets (without stirring), platelets stimulated with VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C, with stirring) and platelets incubated at 0°C (4 hrs), 0/37°C (4 hrs/1 hr) and at 0/37°C in the presence on GN (without stirring). A) Blots representative of 3 experiments with similar results. B) Semi-quantification of GPIbα re-distribution between membrane- and cytoskeleton fractions. Means ± SEM, n = 3; * $p < 0.05$.



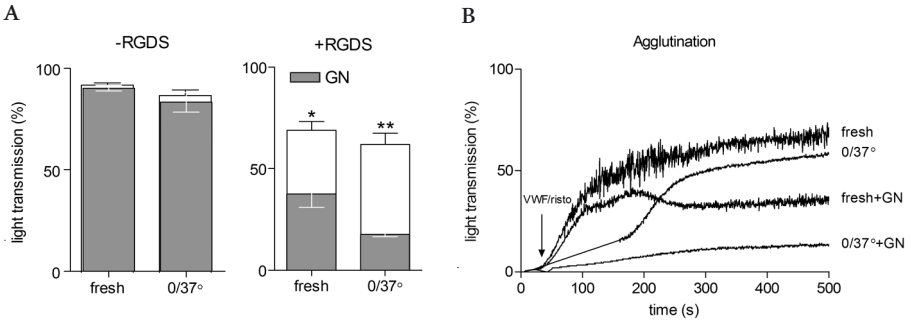
2.4.3 Changes in GPIbα N–terminal flank and VWF/ristocetin-induced aggregation and agglutination

The finding that 0/37°C incubation reduces AN51-PE binding and disrupts GPIbα anchorage to the membrane skeleton is best explained by assuming that these treatments remove lateral constrains in GPIbα mobility and triggers formation of GPIbα clusters. This concept predicts that GN has no effect on fresh platelets, unless changes in free and clustered GPIbα take place that escape detection by AN51. To address this possibility, the effect of GN on VWF/ristocetin-induced platelet functions was investigated. Aggregation by fresh and 0/37°C incubated platelets was similar and not affected by GN (Figure 4A). In aggregations induced by trap, interference by

GN was <10%. Earlier findings in fresh platelets identified an agglutination phase which initiates TxA_2 production followed by an aggregation phase accompanied by an increase in Ca^{2+} [24]. When aggregation was prevented by inhibiting fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ with RGDS, the remaining agglutination by fresh platelets (73% ± 4%) was 30% lower in the presence of GN (46% ± 5%, Figure 4B). Strikingly, in 0/37°C-treated platelets GN inhibited agglutination almost completely. These findings suggest that changes in GPIbα that are sensitive to GN contribute to the agglutination response by fresh and cold-rewarmed platelets.

Figure 4: Effect of *N*-acetyl-*D*-glucosamine on aggregation/agglutination induced by VWF/ristocetin

A) Aggregation (without RGDS) and agglutination (with the $\alpha_{IIb}\beta_3$ blocker RGDS) induced by VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C with stirring) by fresh platelets and platelets incubated at 0/37°C without (white bars) and with (grey bars) GN. Means ± SEM, n = 3. Further details as in Figure 1.



2.4.4 Platelet adhesion to VWF under flow

When perfused over surface-coated VWF at 1600 s^{-1}, 0/37°C incubation preserved normal adhesion and spreading (Figure 5). Surprisingly, GN increased adhesion by cold-rewarmed platelets by 35%. In the presence of GN there was a decline in spreading of 25% due to strong interference with the formation of stress fibres. Similar data were obtained when platelets were perfused at 800 s^{-1}, but the number of adhered platelets was about 25% lower.

2.4.5 TxA_2 formation through GPIbα

As expected, there was little TxA_2 formation in fresh and cold-stored platelets without and with GN, the minor production (106 ± 8.3 ng/min/10^{11} platelets, n = 3) due to preparation of platelets, as in other studies [25]. In contrast, cooling-rewarming induced a more than 200% increase in TxA_2 formation which is more than induced by VWF/ristocetin in fresh platelets ([26] and Figure 6A). Treatment with osge completely blocked TxA_2 formation, confirming that it was initiated through GPIbα. The 0/37°C-induced TxA_2 production was completely blocked by GN, such in contrast with VWF/ristocetin induced TxA_2 formation. Interference with inhibitors of

Figure 5: *N*-acetyl-*D*-glucosamine (GN) inhibits spreading and formation of stress fibres by 0/37°C-treated platelets

Platelets were perfused over immobilized VWF at 1600 s^{-1}. The number of adhered platelets was counted per field (A) and spreading (B) was calculated by dividing surface coverage by number of adhered platelets. Stress fibre formation (C, D) and surface coverage (E, F) are shown in the absence and presence of GN. Means ± SEM, n = 3; * $p < 0.05$.

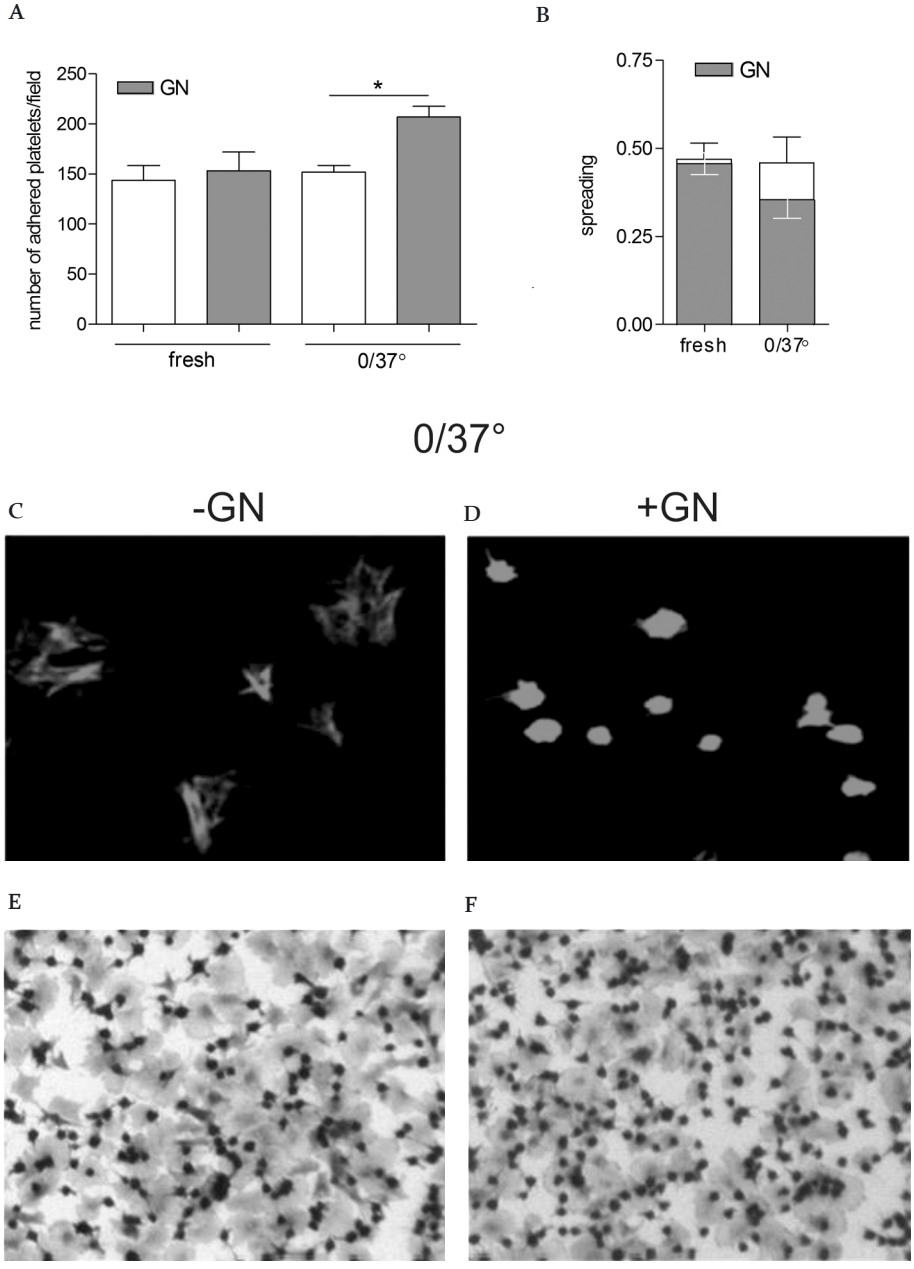


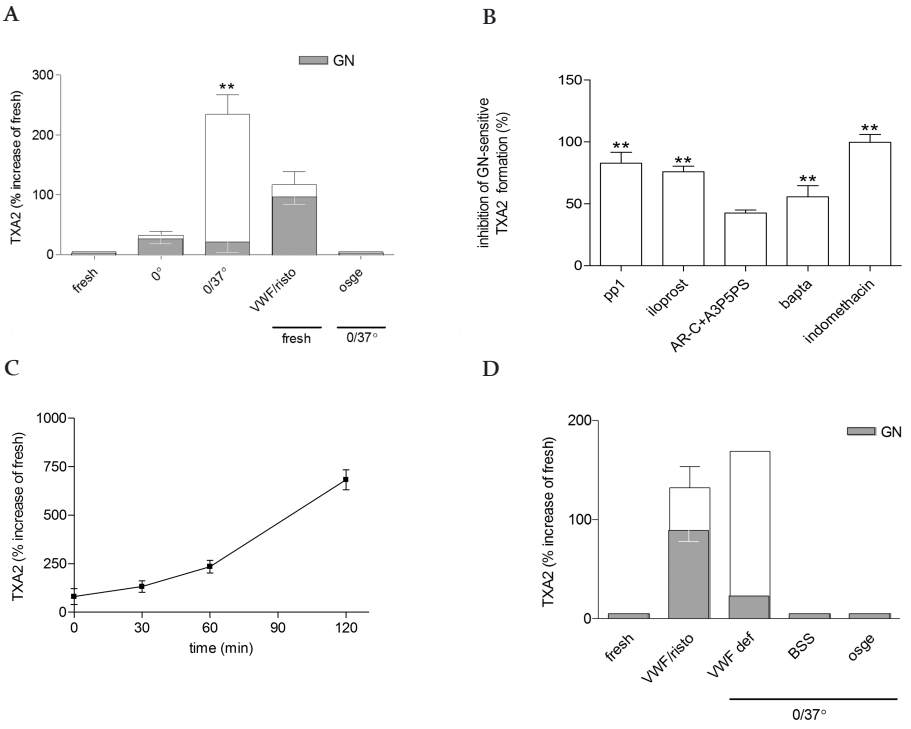
Figure 6: Effect of 0/37°C-incubation on TxA_2 formation

A) TxA_2-formation in fresh platelets and in platelets incubated at 0°C (4 hrs) and at 0°C followed by 37°C (1 hr). VWF-induced TxA_2-formation was evaluated by stimulating fresh platelets with VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C without stirring) and the effect of GPIbα-depletion by treatment with osge (80 µg/ml, 30 min, 37°C) prior to the 0/37°C incubation. TxA_2 formation in fresh platelets was 106 ± 8 ng/min/10^{11} platelets. Platelets were incubated without (open bars) and with GN (grey bars).

B) Inhibition of 0/37°C-induced TxA_2 formation by receptor- and metabolic inhibitors: PP1 (Src kinase inhibitor), iloprost (cAMP raising agent), A3P5PS ($P2Y_1$ blocker), AR-C69931MX ($P2Y_{12}$ blocker), bapta-AM (chelator of cytosolic Ca^{2+}) and indomethacin (inhibitor of COX-1 mediated TxA_2 formation).

C) Irreversibility of TxA_2 formation by 0°C-treated platelets during subsequent incubation at 37°C. Increase in TxA_2 formation was measured after 0°C incubation and rewarming at 37°C at indicated times.

D) TxA_2 formation by VWF-deficient (VWF-def) and GPIbα-deficient (BSS- and osge-treated) platelets after 0/37°C incubation without and with GN. Resting VWF-deficient and BSS platelets produced 74 and 340 ng/min/10^{11} platelets respectively, which were set at 100% (n = 4 for normal platelets and n = 1 for patient platelets). Means ± SEM, n = 3. Further details as in Fig.1.



signalling pathways revealed a major inhibition by PP1 (about 80%), iloprost, (75%), $P2Y_{1/12}$ blockade (45%), bapta-AM (55%) and, as expected, indomethacin (100%; Figure 6B). Blockers of p38MAPK- (SB203580) and ERK2 (PD098059) signalling induced 90% and 60% inhibition respectively (data not shown). Thus, the change in GPIbα induced by 0/37°C-incubation starts signalling to TxA_2 formation through pathways known to be induced by VWF/ristocetin.

Cold-stored platelet kept at 37°C showed a persistent increase in TxA_2 formation for 2 hrs (Figure 6C). This contrasts with the transient nature of the fall in AN51-PE binding (Figure 2C)

and suggests that a short change in GPIbα conformation is sufficient to induce persistent TxA_2 production. Since α-granules are a storage site for VWF [27], release of platelet-VWF might be the cause of TxA_2 production in suspensions without added VWF/ristocetin. To address this possibility, studies were repeated with platelets from a patient with VWD type 3, which contain VWF below detection limits (< 10%). These platelets responded to VWF/ristocetin addition with normal TxA_2 formation. Strikingly, without addition VWF/ristocetin, 0/37°C-incubated VWF-deficient platelets produced normal amounts of TxA_2 and again GN inhibited the response (Figure 6D). Thus, cold-stored platelets subsequently incubated at 37°C produce TxA_2 in the absence of VWF/ristocetin. To confirm involvement of GPIbα, experiments were repeated with BSS-platelets and platelets treated with osge. GPIbα-deficient platelets failed to produce TxA_2. The findings suggest that in the absence of VWF/ristocetin, a change in GPIbα-NTF reflects a conformational change in the receptor that initiates ligand-independent formation of TxA_2.

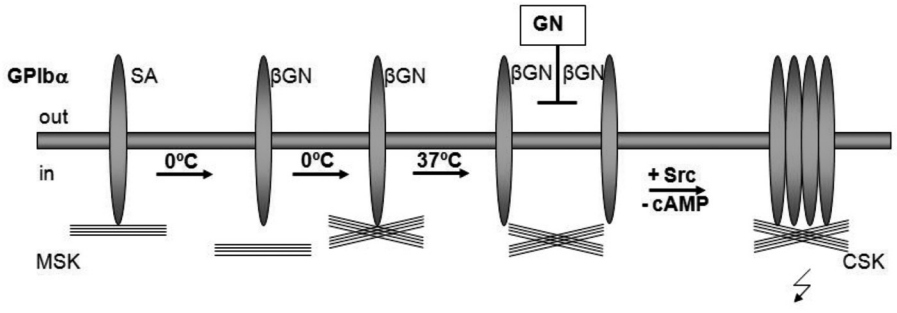
2.5 DISCUSSION

The present results are best explained by assuming two conformations of GPIbα in the plasma membrane: a randomly distributed, dispersed conformation anchored to the membrane skeleton and a clustered conformation anchored to the cytoskeleton. The dispersed conformation is predominant in fresh platelets and distributed in linear arrays on the plasma membrane [9], binds the AN51 antibody with high affinity (this study), is for 70-90 % bound to the membrane skeleton [5] and lacks signalling properties in the absence of an agonist. The conformation predominant in 0/37°C-incubated platelets shows reduced capacity to AN51 binding and strong anchorage to the cytoskeleton. Interestingly, this conformation acquires ligand-independent signalling properties that trigger production of TxA_2.

The different steps between random distribution and concentration in clusters suggest that cold-storage affects GPIbα by two mechanisms. First, there is the extracellular exposure of βGN that tends to interact with βGN on adjacent receptors. Second, there is the intracellular de-attachment from the membrane skeleton which increases lateral receptor mobility. Upon rewarming, released, βGN-exposing receptors cluster while anchoring to the cytoskeleton. At the extracellular side, this process is inhibited by GN, possibly by interfering with sugar-sugar interaction between adjacent receptors. At the intracellular side there is the inhibition by agents that block Src-kinases and raise cAMP, which are treatments that interfere with signalling by ligand-occupied GPIbα ([24, 25] and Figure 7). Earlier Hartwig and Hoffmeister *et al.* [10] described that incubation at 0°C led to GPIbα damage, clusters of βGN-exposing receptors and platelet destruction by macrophages in the absence but not in the presence of added GN. The present findings suggest that GN interferes at an earlier step and prevents the clustering of damaged GPIbα.

Figure 7: Schematic representation of GPIbα changes induced by 0/37° incubation

In fresh platelets, GPIbα is bound to the membrane skeleton (MSK). Incubation at 0°C induces (i) removal of sialic acid (SA) exposing β-*N*-acetyl-*D*-glucosamine (βGN) and, (ii) GPIbα release from the MSK and coupling to the cytoskeleton (CSK). Subsequent incubation at 37°C starts clustering of GPIbα molecules through a mechanisms that depends on Src kinases and a low cAMP level, unless GN interferes with GPIbα - GPIbα interaction.



Inhibitors of actin polymerization are known to release GPIbα from the membrane skeleton [5]. Our data show that they also induce a fall in AN51 binding suggesting that de-attachment alone is sufficient to induce GPIbα clusters. Surprisingly, these clusters are also sensitive to GN although incubation conditions (fresh platelets, 22°C) probably leave GPIbα sugars intact. Cytochalasin D treatment also prevents the formation of a cytoskeleton, and GPIbα molecules will have optimal mobility to form clusters. The fact that GN inhibits this process might indicate that on fresh platelets part of the GPIbα molecules contain exposed βGN. The finding that GN strongly interferes with VWF-ristocetin induced agglutination by fresh platelets is in line with this concept.

Agonist stimulation also appears to trigger GPIbα clusters. Fresh platelets stimulated by VWF-ristocetin, VWF-A1 domain and PAR1 agonist all show a decrease in AN51 binding. An important difference with the 0/37°C-induced GPIbα change is the insensitivity to GN. VWF is a long polymer with multiple bindingsites for GPIbα, a property that might facilitate the concentration of GPIbα molecules in clusters that resists interference by GN. Unexpectedly, also VWF-A1 with a single binding site triggers this change, suggesting that agonist-induced receptor activation induces a change in GPIbα that starts de-attachment from the membrane skeleton and receptor clustering. This reponse is inhibited by Src kinase blockade and therefore dependent on signal transduction by the activated receptor which is associated with Src [24].

The change in antibody binding that might accompany GPIbα clustering is restricted to residues 1-35 of the *N*–terminal flank and binding of antibodies against different epitopes between amino acids 36 - 285 does not change. Residues 1-35 contains the β hairpin which protrudes from the protein surface in models based on crystallographic characterization of GPIbα [13, 28]. GPIbα clustering is known to enhance platelet activation by VWF/ristocetin. In CHO cells, oligomerization of a GPIb-IX (FKBP)2 construct increases VWF/ristocetin binding, Syk phosphorylation and the $\alpha_{IIb}\beta_3$ affinity change that mediates adhesion [29]. Also cytochalasin D treatment induces GPIbα clustering and facilitates VWF/ristocetin binding, adhesion and aggregation [30] as well as mucetin induced-aggregation [31] and snake C-type lectin echicetin-IgM_{κ}-induced agglutination [32]. There is little interference of 0/37°C-induced GPIbα changes by secretion of storage granules. P-selectin expression is low and there is little change when released VWF is neutralized by antibody binding and released ADP cannot contribute to platelet activation by blockade of $P2Y_{1/12}$ receptors.

A comparison between GPIbα conformations and VWF/ristocetin-induced platelet functions learns that aggregation is not affected by GN, whereas agglutination by fresh and particularly by 0/37°C-treated platelets is disturbed by GN. Apparently, changes in GPIbα-NTF contribute to VWF/ristocetin-induced agglutination both in fresh and in cold-rewarmed platelets. Adhesion of fresh platelets to immobilized VWF at 800 and $1600s^{-1}$ was not changed by GN but when cold-rewarmed cells were used, GN increased adhesion by 35%. The better adhesion might relate to the lower spreading caused by severe impairment of stress fibre formation.

The finding that induction of a GPIbα-NTF change by 0/37°C-incubation triggers formation of TxA_2 was unexpected. TxA_2 production was in the range found in fresh platelets stimulated with VWF/ristocetin (without stirring), illustrating a considerable signal induction to

mechanisms that start TxA_2 formation. This TxA_2 formation was inhibited by PP1 and iloprost, which agrees with their blockade of GPIbα clustering. There was also inhibition by $P2Y_{1/12}$ blockade, illustrating the contribution of released ADP, and by bapta, confirming that Ca^{2+} rises contribute to TxA_2 formation. The slight increase in P-selectin positive platelets upon 0/37°C-incubation illustrates that secretion of granule contents does occur. Apart from release of ADP, this would lead to liberation of granule VWF which would initiate TxA_2 formation in experiments without added VWF. The findings that TxA_2 formation persists in platelets with undetectable levels of VWF (VWD type 3 platelets) and is absent in GPIbα deficient platelets (BSS- and osge-treated platelets) are strong indications for VWF-independent signal generation by a change in GPIbα-NTF.

At present, platelet concentrates for transfusion are stored at room temperature for 5-7 days [33]. Potential advantages of a lower storage temperature are suppression of bacterial growth, better preservation of platelet functions and the possibility to extend the storage period to several weeks. So far, the cold-induced platelet damage known as the 'Cold Storage Lesion' has slowed down the search for better storage conditions at low temperature. The present study shows that the cause of cold-induced platelet activation is the re-distribution of GPIbα leading to generation of TxA_2. This insight might pave the way for further studies to interfere with this process during cold-rewarming while preserving its role in haemostasis post-transfusion.

2.5.1 Acknowledgements

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I'm on a wire
my head is on the run
trying to get further
(...)
this time I must go on

(Black Box Revelation)

Chapter 3

Platelet apoptosis by cold-induced glycoprotein Ibα clustering

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3.1 SUMMARY

Background: Cold-storage of platelets followed by rewarming induces changes in Glycoprotein (GP) Ibα-distribution indicative for receptor clustering and initiates thromboxane A_2-formation. GPIbα is associated with 14-3-3 proteins, which contribute to GPIbα-signaling and in nucleated cells take part in apoptosis regulation.
Objectives and methods: We investigated whether GPIbα-clustering induces platelet apoptosis through 14-3-3 proteins during cold (4 hours 0°C)-rewarming (1 hour 37°C).
Results: During cold-rewarming, 14-3-3 proteins associate with GPIbα and dissociate from Bad inducing Bad-dephosphorylation and activation. This initiates pro-apoptotic changes in Bax/Bcl-x_L and Bax-translocation to the mitochondria inducing cytochrome *c* release. The result is activation of caspase-9 which triggers phosphatidylserine exposure and platelet phagocytosis by macrophages. Responses are prevented by *N*-acetyl-*D*-glucosamine (GN), which blocks GPIbα-clustering and by *O*-sialoglycoprotein endopeptidase, which removes extracellular GPIbα.
Conclusions: Cold-rewarming triggers apoptosis through a GN-sensitive GPIbα-change indicative for receptor clustering. Attempts to improve platelet transfusion by cold-storage should focus on prevention of the GPIbα-change.

Keywords: glycoprotein Ibα, cold-storage, platelet, apoptosis, Bad, 14-3-3, thromboxane A_2.

3.2 INTRODUCTION

Glycoprotein (GP) Ibα is associated with GPIbβ, GPV and GPIX in a 2:4:1:2 stoichiometry. GPIbα mediates primary platelet adhesion at high shear by binding von Willebrand factor (VWF) attached to the injured vessel wall. VWF-ligation initiates multiple reactions e.g. phospholipase Cγ-mediated activation of $\alpha_{IIb}\beta_3$ leading to aggregation, GPIbα-release from the membrane skeleton inducing GPIbα-clusters and macrophage recognition [1, 2], and a conformational change in Bax inducing apoptosis [3].

GPIbα is highly glycosylated with *N*-linked carbohydrate chains located at leucine-rich repeats 2 and 4 covered by sialic acid. Incubation at 0-4°C exposes underlying galactose- and β-*N*-acetyl-*D*-glucosamine (βGN)-residues and disrupts anchorage to the membrane cytoskeleton thereby inducing GPIbα-clusters [1, 4]. The clusters then bind to the glycan recognition sites of MAC-1 receptors on macrophages triggering platelet destruction *in vitro* and clearance from the circulation *in vivo* [5]. The damage of GPIbα by low-temperature incubation is the major obstacle for wide-scale application of cold-storage of platelet concentrates prepared for transfusion.

The interference with platelet-macrophage interaction by added *N*-acetyl-*D*-glucosamine (GN) is a strong indication that clusters of damaged GPIbα mediate the destruction of cold-stored platelets [5]. We found earlier that in addition to inhibiting MAC-1 binding to damaged GPIbα, GN might interfere with the formation of GPIbα-clusters before it acts as an inhibitor of platelet-macrophage interaction [1, 6]. Evidence was based on the binding of antibody AN51 directed against amino acids (aa) 1-35 of the *N*-terminus of GPIbα which changes in a reversible manner upon cold-rewarming in the absence but not in the presence of GN [1]. The aa 1-35 region is located in the NH_2-terminal hairpin called β-finger. This domain is about 200 aa's separated from the β-switch (aa 227-241), which is a flexible loop that changes upon GPIbα-binding to the VWF A1-domain [7, 8].

GPIbα-perturbations by VWF/ristocetin (VWF/risto) and cold-storage have in common de-attachment of the membrane skeleton, formation of clusters and generation of thromboxane A_2 (TxA_2). Cold-induced responses primarily show up during subsequent incubation at 37°C, indicating that metabolic processes expose the damage inflicted to GPIbα at low temperature. The finding that VWF-binding to GPIbα affects key intermediates in the apoptotic pathway [3] implies that similar changes might occur during cold-rewarming. If this were true, the possibility of cold-storage of platelets for transfusion would become less attractive.

In the present study we applied incubation at 0°C to cluster GPIbα followed by incubation at 37°C to reveal cold-induced changes in apoptosis regulation. Special focus was on the scaffolding protein 14-3-3ζ, which in addition to contributing to GPIbα-signaling to phosphatidylinositol 3-kinase during VWF-ligation, also controls the de-phosphorylation of Bad, which is an upstream element in the intrinsic apoptotic pathway.

3.3 MATERIALS AND METHODS

We used the following products (with sources): *N*-Acetyl-*D*-Glucosamine (GN, Merck, Darmstadt, Germany), prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI), cantharidin and the InnoCyte Flow cytometric cytochrome *c* release kit (Calbiochem, Darmstadt, Germany), 5,5´,6,6´-Tetrachloro-1,1´,3,3´-tetraethyl-imidacarbocyanine iodide JC-1 (Sigma, St. Louis, MO), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), Cytofix/Cytoperm and Perm/Wash buffer (Becton Dickinson, San Diego, CA), mitochondria isolation kit (Pierce Biotechnology, Rockford, IL), Human VWF was purified as described [7]. The GN- and galactose binding lectin *ricinus communis* agglutinin-1 (RCA-1) was from Vector Laboratories, Burlingame, CA. Sources of other chemicals have been published [1] or are given in the Supplement.

3.3.1 Platelet isolation and incubations

Platelets were isolated [9] while maximally preventing their activation using free-flow blood collection, discarding the first 2 mL of blood and all collections that showed micro-aggregates as determined by particle sizing. Procedures were approved by the Medical Ethical Committee of our hospital. Platelets were resuspended in Hepes-Tyrode's (pH 7.2) to $2x10^{11}$ cells/L. Incubations were without stirring at 0°C for 4 hours in the absence and presence of GN (100 mM) to prevent GPIbα-clustering and *O*-sialoglycoprotein endopeptidase (osge; 80 μg/mL, pre-incubation 30 minutes, 22°C) to cleave the *N*-terminal part of GPIbα [10]. The 0°C-treatment was followed by 1 hour incubation at 37°C to mimic cold-storage and post-transfusion conditions (0/37°, in short). Concurrently, fresh platelets from the same donor were stimulated with VWF/ristocetin (5 minutes, 37°C, without stirring) at suboptimal concentrations (3 μg/mL VWF; 0.3 mg/mL ristocetin) to keep VWF-induced responses in the range induced by 0/37°-incubation [1].

3.3.2 Western blots and immunoprecipitations

Platelet suspensions were added to lysis buffer supplemented with 10 μM cantharidin [9]. Proteins were separated by SDS-PAGE. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 μg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). Quantification was performed with Image-J software (NIH, Bethesda, MD). For immunoprecipitations, 450 μL washed platelets ($5x10^{11}$ platelets/L) was lysed, (15 minutes, 0°C), centrifuged (10 000*g*, 10 minutes, 4°C) to remove cell debris and mixed with 55 μL (10% vol/vol) protein G beads together with antibody (1 μg/mL, 30 minutes, 4°C, rotating). Mitochondria were isolated ($3x10^8$ platelets/sample) using the Mitochondria Isolation Kit, according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL), with extra addition of PMSF (1 μM), leupeptin (1 μg/mL) and cantharidin (10

μM. all f.c.) to reagents A and C. As a control for equal loading, blots were incubated with antibody against cytochrome *c* oxidase IV.

3.3.3 Flow cytometric analysis

For analysis of 0/37°-induced desialylation of GPIbα, platelets were incubated with RCA-1 (0.5 μg/mL, 30 minutes, RT) and binding to exposed GN- and galactose-residues was measured by FACS [11]. FITC-conjugated RCA-1 binding was expressed as ratio of 0° and 0/37°-platelets over fresh platelets. For measurement of Bax and Bcl-x_L, 500 μL platelet suspension was centrifuged (330*g*, no-brake, 10 minutes, RT) in the presence of 10 ng/mL PGI_2. Functionality was recovered by 30 minutes incubation (RT) before the pellet was permeabilized with Cytofix/Cytoperm (30 minutes, 4°C), washed with Perm/Wash buffer, incubated with primary (2 μg/mL, 1 hour, 4°C) and secondary antibodies (2 hours, 4°C). PBS was added and 10 000 events were measured on a FACS Calibur (BD Biosciences, San Jose, CA).
For determination of the mitochondrial membrane potential $\Delta\Psi_m$, 100 μL platelet suspension was incubated with the JC-1 (0.5 μM, 30 minutes, 37°C). In viable cells, the high $\Delta\Psi_m$ promotes a directional uptake of JC-1 into the matrix where JC-1 forms J-aggregates (λ_{ex} 490 nm, λ_{em} 570-610 nm). In apoptotic cells, the low $\Delta\Psi_m$ preserves the monomeric form (λ_{ex} 490 nm, λ_{em} 535 nm) [12, 13]. Changes in $\Delta\Psi_m$ were expressed as the ratio of platelets in lower- over upper-right quartiles [14, 15]. Cytochrome *c* release was measured in 500 μL platelet suspension with the InnoCyte Cytochrome *c* kit, according to the manufacture's protocol. Retained cytochrome *c* in fresh platelets was set at 100%.

3.3.4 Statistics

Data are means ± SEM (n= 3, 4), as indicated. Statistical analysis was performed using GraphPad InStat (San Diego, CA) software. Differences between fresh and treated samples were considered significant at $p<0.05$ (*); between incubations by $\overline{\ \ *\ \ }$.

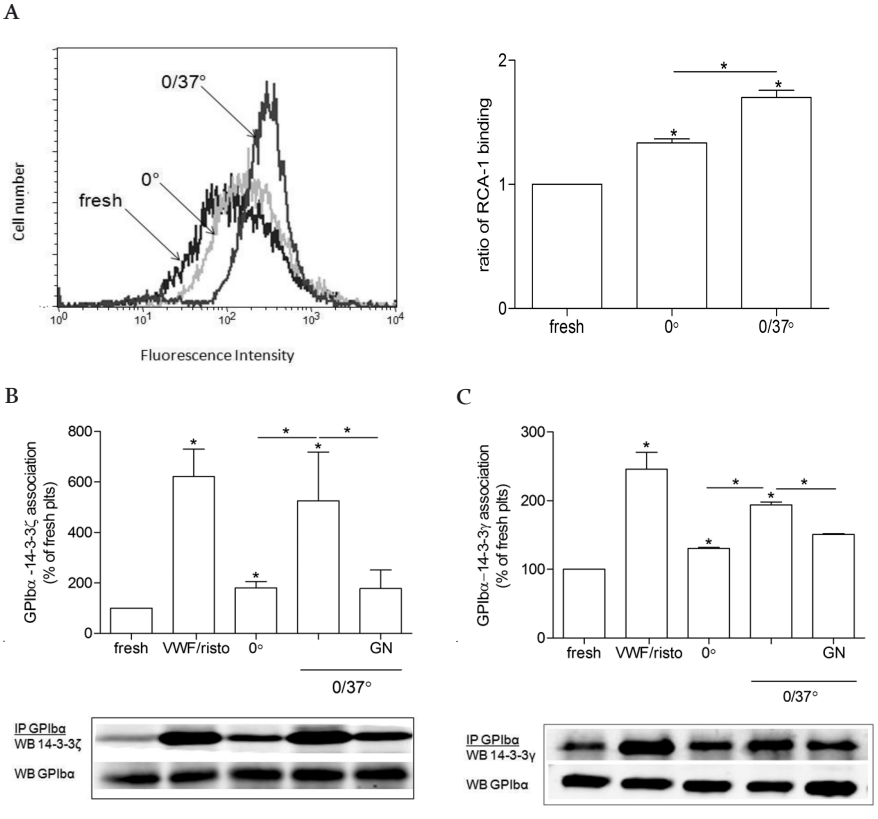
3.4 RESULTS

3.4.1 GPIbα-clustering causes redistribution of 14-3-3 proteins

Earlier work showed that cold-storage of platelets induces GPIbα-clusters that become recognition sites for macrophages [5]. Subsequent incubation at 37°C, starts the production of TxA_2 [1]. The cause of the GPIbα-change is desialylation of GPIbα as illustrated by an increase in RCA-1 binding, confirming earlier observations ([11] and Figure 1A). GPIbα is associated with 14-3-3 proteins which in nucleated cells are known to mediate signaling to cell death [16]. To investigate whether the 0/37°-induced GPIbα-change triggers apoptosis, we measured GPIbα-association with 14-3-3 proteins. Fresh platelets showed a slight association between GPIbα

Figure 1: GPIbα-clustering causes redistribution of 14-3-3 proteins

Fresh platelets and platelets incubated at 0° (4 hours) - and 0/37° (4 hours/1 hour) were studied in the absence and presence of GN (100 mM *N*-acetyl-*D*-glucosamine, added at start of incubations). Stimulation of fresh platelets with VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C, without stirring) served as control for GPIbα activation. A) RCA-1 binding to exposed βGN- and galactose-residues. B) Association between GPIbα and 14-3-3ζ. C) Association between GPIbα and 14-3-3γ. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).



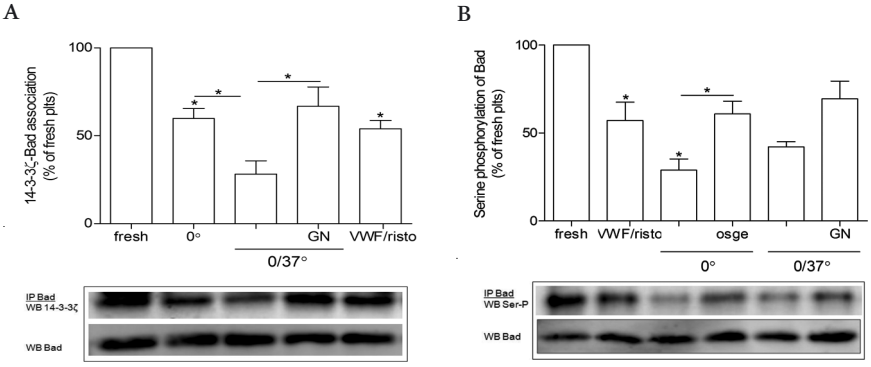
and 14-3-3ζ which increased 6-fold after stimulation with VWF/risto, such in agreement with recent literature [3]. Also incubation at 0°C induced formation of the [GPIbα-14-3-3ζ] complex which increased further during subsequent incubation at 37°C (Figure 1B). Importantly, GN prevented complex formation, illustrating its dependence on GPIbα-clusters. A similar GPIbα-association was observed with 14-3-3β (not shown) and 14-3-3γ (Figure 1C). Much smaller associations were found between 14-3-3ζ and GPIX and GPIbβ (Figure S1).

3.4.2 GPIbα-clustering initiates dephosphorylation of Bad

In nucleated cells, 14-3-3ζ is an upstream regulator of the intrinsic pathway for apoptosis, where it sequesters cytosolic Bad thereby protecting its phosphorylated, dormant state against activation. Since association of 14-3-3ζ with GPIbα might disturb its association with Bad, we investigated immunoprecipitates of total Bad. Cold-incubation induced a 40% fall in 14-3-3ζ-associated Bad, which decreased further during subsequent incubation at 37°C (Figure 2A). GN prevented the decrease, illustrating its dependence on the cold-induced GPIbα-change. VWF/risto induced a similar fall in [14-3-3ζ-Bad] complex as induced by cold-incubation. Analysis of Ser-phosphorylated Bad confirmed that loss of 14-3-3ζ induced dephosphorylation. Again, GN reduced this response and also treatment with osge interfered with the fall in phosphorylated Bad (Figure 2B).

Figure 2: GPIbα-clustering initiates apoptosis through dephosphorylation of Bad

Platelets were treated as indicated in the legend to Figure 1. In addition, extracellular GPIbα was removed by osge-treatment (80 µg/ml, 30 min, 37°C) prior to the 0/37°-incubation. A) Dissociation of the [14-3-3ζ-Bad] complex. B) Dephosphorylation of Ser-phosphorylated Bad. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).

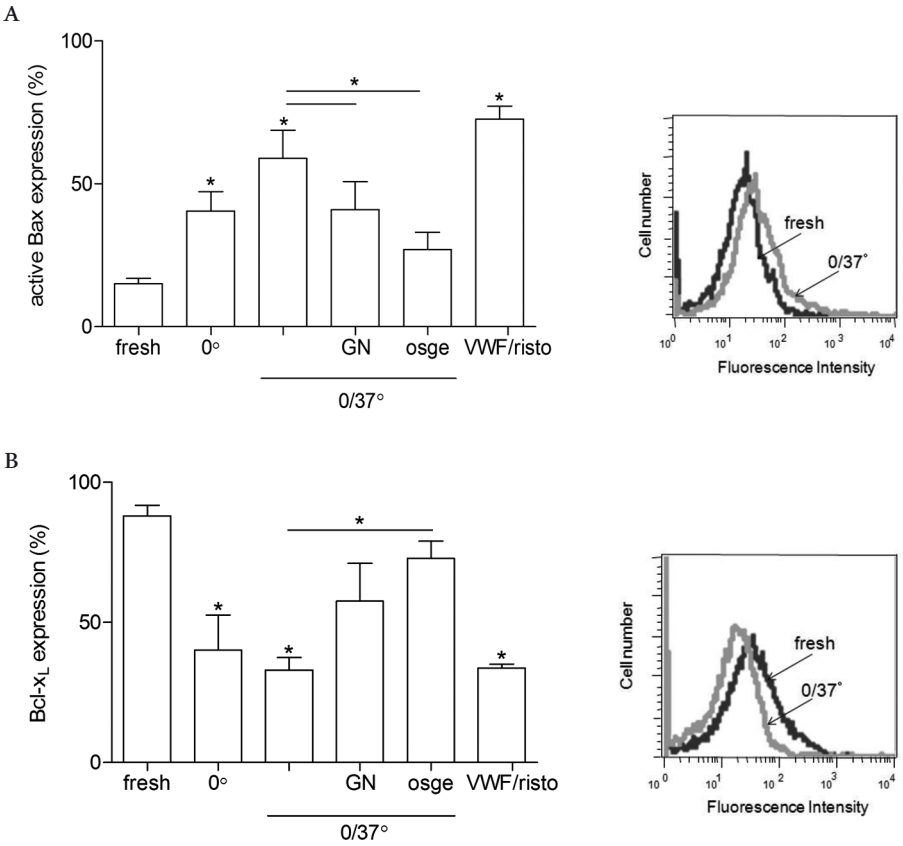


3.4.3 GPIbα-clustering induces changes in Bcl-2 family proteins

To confirm that Bad Ser-dephosphorylation led to its activation, two Bcl-2 family members were investigated known to undergo a conformational change induced by active Bad. FACS-analysis with antibodies against the active epitope of Bax and the active conformation of Bcl-x_L revealed that Bax and Bcl-x_L changed in parallel with the phosphorylation of Bad (Figure 3). Cold-incubation induced a 2-fold increase in active Bax and a similar fall in active Bcl-x_L. Antibody binding to Bax was further increased during subsequent incubation at 37°C, while Bcl-x_L remained unchanged. Prevention of GPIbα-clustering by GN and by GPIbα-removal with osge opposed these changes and again, VWF/risto-stimulation induced similar changes as 0/37°-incubation. Thus, the change in GPIbα-distribution induced by cold-storage is a potent inducer of platelet apoptosis.

Figure 3: GPIbα-clustering induces changes in Bcl-2 family proteins

Platelets were treated as indicated in the legend to Figure 1. Binding of antibodies directed against the Bcl-2 proteins pro-apoptotic Bax (to activation epitope of Bax, A) and pro-survival Bcl-x_L (fall in antibody-binding due to de-activation, B) Data show fluorescence above 1% of background signal. The right panels show representative FACS plots of fresh and 0/37°-treated platelets. Data are means ± SEM (n=4).

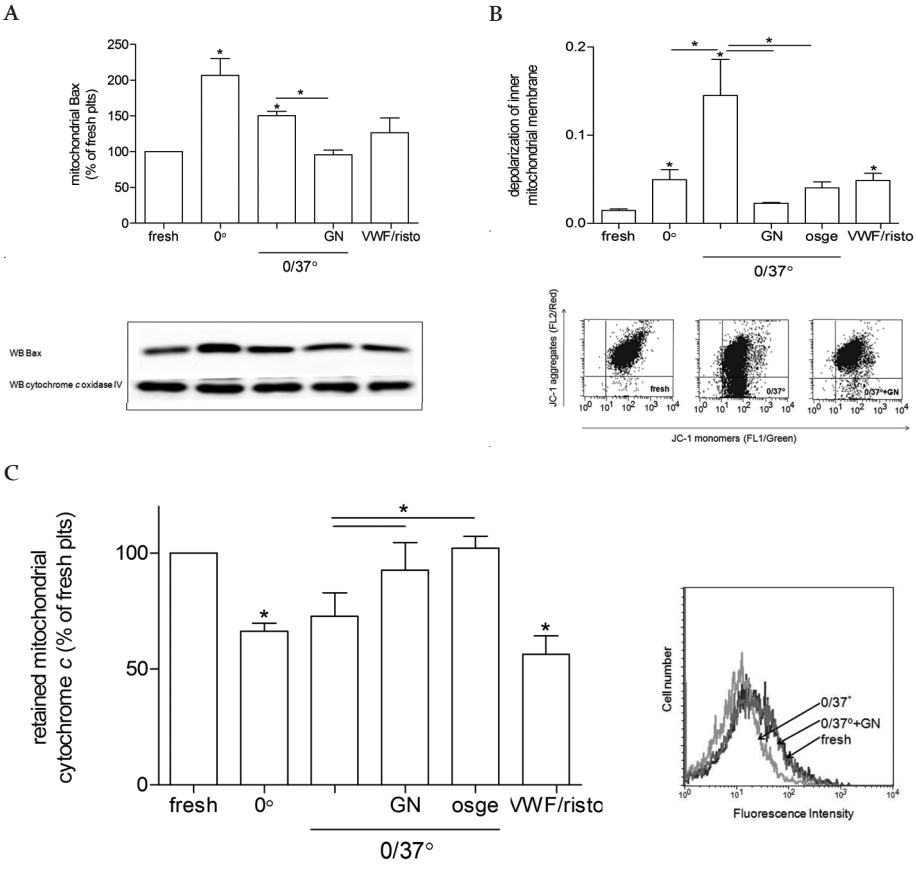


3.4.4 GPIbα-clustering activates apoptosis markers in mitochondria

A next step in apoptosis induction is the translocation of Bax to the mitochondria in a first step in triggering release of cytochrome *c*. To address this issue, mitochondria were isolated and the presence of Bax was measured by immunoblotting. Mitochondria from fresh platelets contained a small amount of Bax and this level increased 2-fold upon cold-incubation and slightly decreased during subsequent 37°C-treatment (Figure 4A). GN completely prevented Bax-translocation to the mitochondria.

Figure 4: GPIbα-clustering activates apoptosis markers in mitochondria

Platelets were treated as indicated in the legend to Figure 1. A) Binding of Bax to mitochondria, as measured by western blotting. Equal protein loading was obtained using adjustments based on the BCA-assay and illustrated by expression of cytochrome *c* oxidase IV. B) $\Delta\psi_m$ measured by JC-1 binding on the FACS (upper panel). Data are expressed as the ratio of fluorescent platelets in the lower-right (green fluorescence) over those in upper-right (green/orange fluorescence) quartiles. C) Cytochrome *c* release was measured by binding of anti-cytochrome *c* antibody. Retained cytochrome *c* in fresh platelets was set at 100%. The left panel shows retained cytochrome *c* at the different incubation conditions. The right panel shows a representative FACS-plot after 0/37°-incubation with an without GN. Data are means ± SEM (n=4).



In contrast to upstream responses, VWF/risto had little effect on the amount of mitochondria-bound Bax. Binding of Bax is known to disrupt the outer mitochondrial membrane, inducing a collapse in electrochemical gradient over the inner mitochondrial membrane. To investigate whether Bax-binding to the mitochondria induced a similar change, platelets were incubated with the JC-1 dye. FACS-analysis showed that cold-treatment induced depolarization of the inner mitochondrial membrane and that subsequent 37°C-incubation greatly augmented this effect (Figure 4B). Importantly, the presence of GN and treatment with osge completely prevented the depolarization induced by 0/37°-treatment. The effect of VWF/risto-stimulation on $\Delta\psi_m$ was much smaller than that induced by cold-rewarming. Thus, the GPIbα-change induced by 0/37°-treatment triggers translocation of Bax to the mitochondria and depolarization of the inner mitochondrial membrane.
Mitochondrial-bound Bax is thought to form oligomers which form transition pores in the outer mitochondrial membrane thereby releasing cytochrome *c* from the intermembrane space [17]. Indeed, the 0/37°-treatment led to a 30% fall in mitochondrial bound cytochrome *c* (Figure 4C). As observed with upstream apoptosis regulators, the presence of GN and treatment with osge prevented the fall in mitochondrial cytochrome *c.* Again, in fresh platelets VWF/risto induced a similar change in cytochrome *c* localization as 0/37°-treatment.

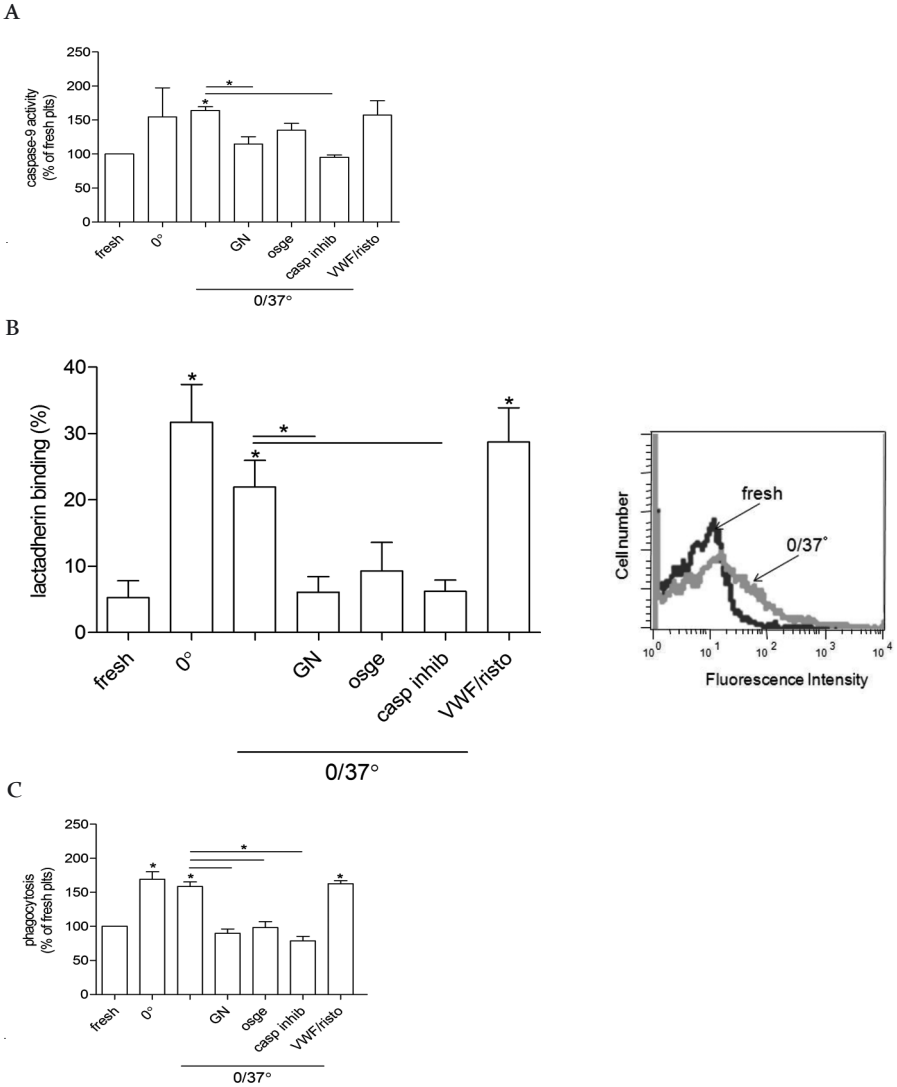
3.4.5 GPIbα-clustering activates caspase-9 and phosphatidylserine surface expression

To investigate whether the changes in mitochondrial apoptosis markers that accompanied GPIbα-clustering affected downstream elements in the apoptotic pathway, caspase-9 was measured, an initiator caspase that controls the effector caspase-3. Cold-incubation led to a 50% increase in active caspase-9 which remained unchanged during rewarming. GN and osge-treatment opposed the activation confirming their dependence on GPIbα-clustering. As expected, the caspase-inhibitor induced full inhibition and VWF/risto induced a similar activation as cold-incubation (Figure 5A). Cold-incubation also induced surface expression of phosphatidylserine (PS), a marker of apoptotic cells. GN- and osge-treatment blocked PS-surface expression completely. Importantly, the caspase-inhibitor induced 100% suppression of cold-induced PS-expression. Stimulation with VWF/risto used as positive control also induced PS-expression in agreement with earlier observations ([3] and Figure 5B and right panel). PS-surface expression is a recognition signal for macrophage binding and platelet phagocytosis [6]. Indeed, increases in surface-PS translated into similar increases in binding (data not shown) and phagocytosis (Figure 5C) of cold-treated platelets by matured THP-1 cells and inhibition of GPIbα-clustering abolished platelet interaction with macrophages.
We demonstrated earlier that 0/37°-incubation triggers the formation of TxA_2 [1]. To clarify whether this pathway contributed to apoptosis regulation, we measured TxA_2 together with the apoptosis markers Bax and $\Delta\psi_m$ and with P-selectin expression as a marker for platelet activation. Cold-incubation alone induced little TxA_2-formation and the markers remained unchanged in the presence of indomethacin (data not shown). Subsequent rewarming started production of TxA_2, a further increase in Bax and $\Delta\psi_m$ and triggered P-selectin expression. These

responses were strongly inhibited by indomethacin, illustrating that rewarming induced a 2nd wave of apoptosis induction through TxA_2, which was accompanied by secretion of α-granules (Supplemental Figure S2).

Figure 5: GPIbα-clustering induces caspase-9 activity, PS-exposure and phagocytosis

Platelets were treated as indicated in the legend to Figure 2 and with Pan-caspase inhibitor Q-VD-Oph (50 μM). A) Caspase-9 activity was measured by cleavage of AFC-conjugated LEHD and expressed as % of fresh platelets. B) PS-exposure was measured by binding of lactadherin, anti- bovine lactadherin and secondary anti-rabbit-FITC antibodies by FACS. The right panel shows a representative FACS-histogram of fresh and 0/37°-treated platelets. Data show fluorescence above 1% of background signal. C) Phagocytosis was measured after incubation of CFMDA-labelled platelets to matured THP-1 cells. Data are means ± SEM (n=4).



3.5 DISCUSSION

The present study shows that 0/37°-treatment of platelets induces apoptosis through a sequence involving (i) a change in GPIbα possibly caused by receptor clustering, (ii) an increase in GPIbα-association with the adapter protein 14-3-3ζ/β/γ, (iii) the release of 14-3-3ζ from Ser-phosphorylated Bad inducing its dephosphorylation and activation, (iv) a conformational change in Bax and Bcl-x_L in favor of apoptosis induction, (v) translocation of Bax to the mitochondria, (vi) depolarization of the inner mitochondrial membrane and release of cytochrome *c*, (vi) activation of caspase-9 inducing PS-exposure and binding to matured THP-1 cells followed by their phagocytosis. These are defined steps in the intrinsic apoptotic pathway of nucleated cells leading to formation of the apoptosome complex, activation of caspases and cell death [18]. Extending the cold-incubation to 48 hours leads to more GPIbα-clustering [6], an increase in $\Delta\Psi_m$-change from 0.14 ± 0.04 (4 hrs 0/37°) to 0.20 ± 0.05 (48 hrs 0/37°) and an increase in exposed PS from 22 ± 4 to 42 ± 4%, illustrating that apoptosis increases at longer periods of cold-incubation.

The change in GPIbα-conformation is caused by extracellular exposure of βGN- and galactose-residues and the intracellular de-attachment from the membrane skeleton [1]. These findings suggest that the glycosylation state of the extracellular domain of GPIbα affects the signalling properties of its intracellular domain. A similar effect is seen with platelet PECAM-1, where a deficiency in α2,6-linked sialic acid leads to decreased tyrosine-phosphorylation of the cytosolic tail [19]. The present data show that GN also blocks GPIbα-induced apoptosis, emphasizing the importance of the GPIbα-change in control of platelet survival. For simplicity, this GPIbα-change has been termed clustering but the molecular mechanisms involved remain to be elucidated. A similar change in GPIbα is seen after prolonged platelet storage at RT and following exposure to a shear of 1600 s^{-1} [1]. If these conditions also initiate apoptosis, this property would seriously affect the survival of RT-stored platelets after transfusion and circulating platelets that roll over VWF bound in a damaged vessel wall. In line with these conclusions are earlier observations showing a $\Delta\psi_m$-change and caspase-3 activation in platelets exposed to high shear stress and together illustrate that GPIbα is extremely sensitive to mechanic stress [14]. Our observation that a poor blood collection alone activates the apoptosis markers identified in this study also supports this reasoning (data not shown).

A next step in GPIbα-induced apoptosis induction is the association with cytosolic 14-3-3-adapter proteins. 14-3-3 denotes a family of ~30 kDa acidic proteins that plays a role in a wide range of cellular functions [20]. The family consists of nine members, but only the β, γ, ε, η, θ and ζ isotypes are present in platelets [21, 22]. All bind to GPIbα at residues 580-590 and 606 [22]. The 0/37°-treatment induces 14-3-3ζ-binding to GPIbα in a similar way as VWF/risto-treatment in fresh platelets and also initiates GPIbα-association with 14-3-3β and 14-3-3γ. Apart from GPIbα, also GPIbβ, GPIX and GPV contain binding sites for 14-3-3ζ [23, 24]. However, 14-3-3ζ binding to GPIbβ and GPIX was low (Figure S1) and absent to GPV (not

shown). The difference might be caused by the high number of 14-3-3ζ-binding sites on GPIbα and the short cytoplasmic domains of GPIbβ and GPIX.

The 14-3-3 family consist of phosphoserine binding proteins and control the activity of pro-apoptotic Bad [25, 26]. This BH3-only protein belongs to the B-cell lymphoma-2 (Bcl-2) superfamily, which contains pro-survival and pro-apoptotic proteins [18]. In pro-survival conditions, Bad is phosphorylated on at least five serine residues [27]. Phosphorylation at Ser-75 and -99 is essential for binding to 14-3-3 proteins [27, 28]. Our data show that association of 14-3-3 proteins with GPIbα is accompanied by dephosphorylation of Bad, which is a crucial step in intrinsic apoptosis induction [28]. Dephosphorylated-Bad replaces Bax on the [Bax-Bcl-x_L] complex liberating active Bax [29]. The ratio of Bak over Bcl-x_L has been proposed as an important determinant of platelet life span [29, 30, 31]. The activation of Bax following 0/37°-treatment is in agreement with findings in RT-stored platelets reported earlier [32, 33].

The increase in antibody-binding to Bax reflects the exposure of the cryptic *N*-terminal after its activation [34]. Its translocation to the mitochondria is accompanied by depolarization of the inner membrane and release of cytochrome *c*. Together these findings reveal a pathway which starts with GPIbα-clustering and signals through 14-3-3 proteins, Bad and Bax to cytochrome *c* release, which is a key step in the induction of later steps in intrinsic apoptosis. In GPIbα-transfected CHO-cells, deletion of the 14-3-3ζ-binding site in GPIbα (aa 551-610) reduced VWF/risto-induced $\Delta\psi_m$-change, caspase-3 activity and PS-exposure, which strongly support the GPIbα-induced apoptosis induction found in the present report [3].

A more detailed analysis of 0/37°-induced apoptosis demonstrates that most activation signals are generated during incubation at 0°C. Prior to rewarming, there is an increase in GPIbα-14-3-3 association, a fall in 14-3-3ζ-Bad association, dephosphorylation of phosphorylated-Bad, an increase in active Bax with concomitant decrease in Bcl-x_L, an increase in mitochondrial-bound Bax and membrane depolarization and a fall in mitochondrial cytochrome *c*. These changes lead to the activation of caspase-9, PS-surface expression ultimately inducing binding of platelets to matured THP-1 cells followed by their phagocytosis. The finding that caspase inhibition aborts PS-expression and platelet-macrophage interaction clearly places these events downstream of caspase activation. Most changes in apoptosis markers remain intact during subsequent rewarming and a few show slight reversibility (mitochondrial Bax, PS-exposure). In contrast, GPIbα-14-3-3 association, 14-3-3ζ-Bad, Bax activation and $\Delta\psi_m$-change increase further during 37°C-incubation. Incubation with indomethacin show that these changes are triggered by TxA_2 which starts when cold-stored platelets enter the 37°C-incubation [1]. Also other platelet activators such as thrombin, VWF/risto [12], epinephrine, U46619 and ADP trigger apoptosis inducing a change in $\Delta\psi_m$ and activation of caspases [35, 36, 37]. The apoptosis induction at 0°C is not accompanied by TxA_2-formation or P-selectin expression. This is a first demonstration of apoptosis induction without concurrent platelet activation in the absence of pharmacological blockade.

In conclusion, cold-storage induces a change in GPIbα that makes the receptor an initiation site for platelet activation [1], apoptosis (this report) and macrophage recognition [5]. Attempts to improve platelet transfusions by cold-storage should focus on the regulation of GPIbα.

3.5.1 Acknowledgements

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Supplementary material

3.7 MATERIALS AND METHODS

We used the following products (with sources): 5-chloromethyl fluorescein diacetate (Cell tracker green\CFMDA, Molecular Probes, Invitrogen, Carlsbad, CA), THP-1 monocytic cells (ATCC/LGC Standards GmBH, Wesel, Germany), phorbal 12-myristate 13-acetate (PMA, MP Biochemicals, Illkirch, France), vitamin D_3, trypan blue and anti- rabbit- FITC-conjugated Fab fragments (Sigma, St. Louis, MO), (platelet derived) human TGF-β1 and Pan-caspase-inhibitor Q-VD-Oph (R&D systems, Minneapolis, MN), anti-human P-selectin antibody (BD Pharmingen, San Diego, CA) and TxA_2 Enzyme Immuno Assay (EIA) kit (Assay Designs, Ann Arbor, MI). Lactadherin (PAS-6/7 or MFG-E8) and anti-bovine lactadherin antibody were from own sources [1]. Antibodies used for western blotting were directed against 14-3-3ζ (C-16), 14-3-3γ (C-16), 14-3-3β (A-15), GPIX, (all Santa Cruz Biotechnology; Santa Cruz, CA), GPIbα (SZ2, Beckman Coulter, Fullerton, CA), GPIbβ, total Bad (Zymed, Invitrogen, Carlsbad, CA), phospho-Ser proteins (Millipore, Bedford, MA), Bax (3/Bax, BD Transduction, San Diego, CA), cytochrome *c* oxidase IV (Abcam, Cambridge, UK) and secondary antibodies Alexa-680 (Molecular Probes, Invitrogen, Carlsbad, CA) and IRDYe 800CW (LI-COR Biosciences, Lincoln, NE). Antibodies for immunoprecipitation (IP) were against GPIbα (AK2), 14-3-3ζ (V-16), (both Santa Cruz Biotechnology), total Bad (Cell signaling Technology, Danvers, MA); for FACS against active Bax (6A7, Sigma, St. Louis, MO) and Bcl-x_L (2H12, BD Pharmingen, San Diego, CA).

3.7.1 Caspase-9 activity

For determination of caspase-9 activity, 1 mL platelet suspension ($2x10^{11}$ cells/L) was centrifuged (330*g*, no-brake, 10 minutes, RT) in the presence of 10 ng/mL PGI2 and the pellet was permeabilized and lysed in 100 µL lysis buffer (10 minutes, 0°C) provided by the caspase-9 fluorimetric activity kit (R&D systems, Minneapolis, MN). 50 µL lysate was used to measure cleavage of the 7-amino-4-trifluoromethylcoumarin (AFC)-conjugated substrate peptide (LEHD). Caspase-9 activity was measured (2 hours, 37°C) on the Spectramax M2 (Molecular Devices, Sunnyvale, CA, λ_{ex} 400 nm, λ_{em} 505 nm) and expressed as % of fresh platelets.

3.7.2 Phosphatidylserine-exposure

Phosphatidylserine (PS)-exposure was measured using the PS-binding protein lactadherin [2]. Bovine lactadherin was isolated from milk. Platelets were incubated with 5.6 µg/mL lactadherin (30 minutes, RT). Rabbit anti-bovine lactadherin antibody (1:100 dilution, 15 minutes, RT) and secondary anti-rabbit-FITC (7 µg/mL, 30 minutes, RT) were added and antibody binding was measured on a FACS Calibur (BD Biosciences, San Jose, CA).

3.7.3 Binding and Phagocytosis

Platelets were resuspended in HEPES-tyrode's, pH 6.5 ($4x10^{11}$ cells/L), labelled with CFMDA (20 μM, 1 hour, RT) and centrifuged (330*g*, no-brake, 10 minutes, RT) in the presence of 10 ng/mL and resuspended in tyrode's, pH 7.2. Functionality was recovered by 30 minutes incubation (RT). For measurement of combined binding and phagocytosis, THP-1 cells were cultured in a 96-wells plate ($5x10^4$ cells/well) and stimulated with 50 nM vitamin D_3 and 1 ng/mL TGF-β1 (12 hours, 37°C) and 250 nM of PMA (2 hours, 37°C). $CaCl_2$ and $MgCl_2$ (1 mM each, f.c.) were added and $2x10^6$ platelets were incubated with the maturated THP-1 cells (90 minutes, 37°C). Unbound platelets were removed and fluorescence was measured on a Fluorstar Galaxy (BMG LABTECH GmbH, Offenburg, Germany). Trypan blue was added, which quenches fluorescence of bound platelets to separate fluorescence from phagocytozed and bound + phagocytozed platelets [2-4]. Phagocytosis of fresh platelets was set at 100%.

3.7.4 P-selectin expression and formation of TxA_2

To measure P-selectin expression, 100 μL platelet suspension ($2x10^{11}$ cells/L) was incubated with anti-human antibody against P-selectin (1:20 diluted, 15 minutes, 37°C) and 10 000 platelets were measured on a FACS Calibur [5].

For measurement of TxA_2-formation, suspensions were incubated without stirring. At the end of incubations, samples were rapidly mixed with indomethacin (30 μM, final concentration) to halt COX-1 activity. Platelets were pelleted (15 000*g*, 30 sec, 22°C) and TxA_2 was measured in the supernatant by EIA. Data were expressed as ng TxA_2 formed/min/10^{11} platelets [5].

3.8 RESULTS

Figure S1: GPIbα-clustering initiates redistribution of 14-3-3 proteins

Fresh platelets and platelets incubated at 0° (4 hours) - and 0/37° (4 hours/1 hour) were incubated in the absence and presence of GN (100 mM *N*-acetyl-*D*-glucosamine, added at start of incubations). Stimulation of fresh platelets with VWF/ristocetin (3 µg/ml VWF, 0.3 mg/ml ristocetin, 5 min, 37°C, without stirring) served as control for GPIbα activation. A) Association of 14-3-3ζ and GPIX (n=2). B) Association of 14-3-3ζ and GPIbβ (n=1). C) Associations of 14-3-3ζ and GPIX or with GPIbβ.

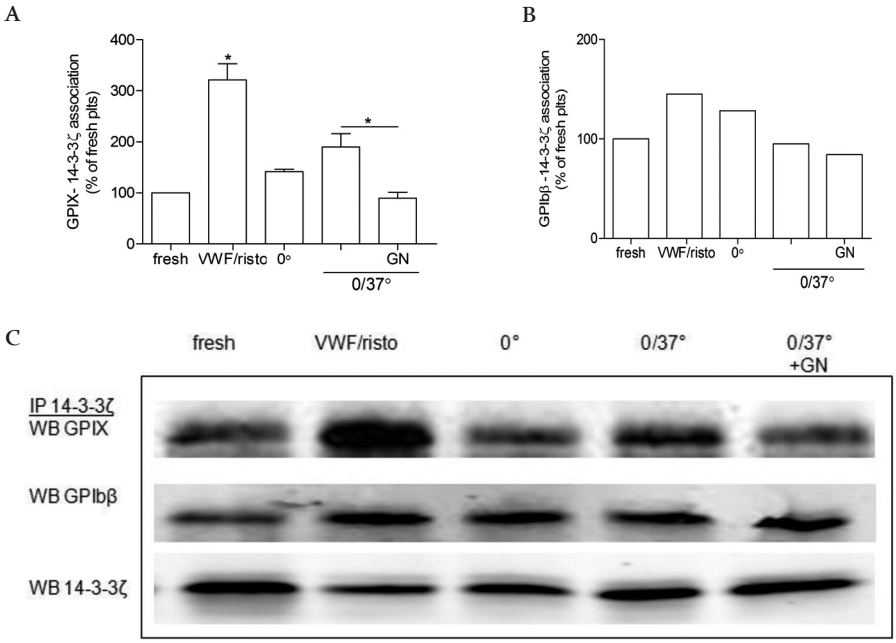
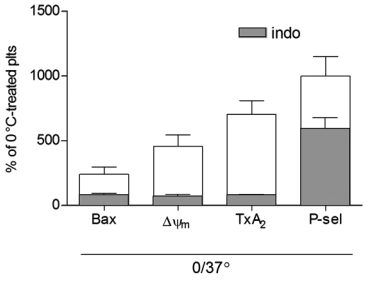


Figure S2: Apoptosis induction and platelet activation during 0/37°-incubation

Platelets were treated as indicated in the legend to Figure S1. Bax, $\Delta\psi_m$, TxA_2-formation [5] and P-selectin (P-sel) expression [5] were measured after 0°C and 0/37°C-incubation. Incubations were without (open bars) and with (grey bars) indomethacin (30 µM). Data found after 0/37°C-incubation were expressed as percentage of those obtained at 0°C. Data are means ± SEM (n=3).



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I'm a scientist, I don't believe in fairy tales

(Avatar)

Chapter 4

Arachidonate-induced platelet apoptosis

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(Submitted)

4.1 SUMMARY

Background: Storage of platelets at low temperature reduces bacterial growth and might better preserve the haemostatic function of platelets than current procedures. However, 0°C-storage triggers a change in Glycoprotein (GP) Ibα, which induces association with the 14-3-3ζ protein, release of phosphoBad from the [14-3-3-phospho-Bad] complex, Bad-activation and apoptosis.

Objectives and methods: We investigated whether during cold-incubation (4 hours, 0°C) arachidonic acid (AA) contributes to GPIbα-induced apoptosis, measured as a change in mitochondrial membrane potential ($\Delta\psi_m$), 14-3-3ζ-release from Bad and COX-1 and platelet survival.

Results: The cold-induced $\Delta\psi_m$-change was reduced by p38MAPK-blockade, increased by COX-1 inhibition and unaffected by thromboxane receptor blockade. Cold-incubation reduced [14-3-3ζ-Bad] and [14-3-3ζ-COX-1] complexes, as did AA-addition. AA-depletion made platelets resistant against cold-induced apoptosis and improved platelet survival. Indomethacin released 14-3-3ζ from the [14-3-3ζ-COX-1] complex and enhanced AA-induced apoptosis.

Conclusions: Cold-incubation triggers the release of AA which becomes a "sink" for the 14-3-3ζ protein, inducing Bad activation and apoptosis. Attempts to improve platelet transfusion by cold-storage should focus on transient AA-depletion.

Keywords: platelets, arachidonic acid, apoptosis, cold-storage, 14-3-3, COX-1

4.2 INTRODUCTION

Current protocols for the storage of platelet concentrates (PCs) recommend a temperature of 22-24°C and a maximum storage of 7 days [1]. A problem of the relative high temperature is that bacteria that occasionally contaminate PCs exponentially increase in number and that platelets rapidly lose their haemostatic functions. Recent improvements are sought in lowering the temperature to 0-4°C, but this approach introduces new problems as it leads to clustering of the receptor for von Willebrand factor (VWF), Glycoprotein (GP) Ibα. Clustering of GPIbα induces platelet apoptosis [2], destruction by macrophages [3] and generation of Thromboxane A_2 (TxA_2) upon rewarming [4].

We described previously, that at 0°C apoptosis is initiated by the cold-induced change in GPIbα, which becomes a "sink" for the scaffolding protein 14-3-3ζ [2]. Formation of a [GPIbα-14-3-3ζ] complex goes in parallel with dissociation of [14-3-3ζ-phosphoBad] complex resulting in Bad-activation, a change in mitochondrial membrane potential ($\Delta\psi_m$) and caspase-9 activation. Subsequent rewarming induces TxA_2-formation and a second wave of apoptosis induction, in agreement with reports that platelet activators trigger apoptosis [5].

Activation of GPIbα by its ligand, VWF is known to activate cytosolic phospholipase A_2 ($cPLA_2$), leading to release of arachidonic acid (AA) from membrane phospholipids initiating TxA_2-formation [6]. $cPLA_2$ is activated through phosphorylation of Ser^{505} by P38-mitogen-activated protein kinase (MAPK) [7, 8]. The major part of released AA is metabolized by cyclooxygenase-1 (COX-1) to endoperoxides and further converted into TxA_2 by thromboxane synthase. Released AA is also a substrate for 12-lipoxygenase which generates hydro (pero)xy-eicosatetraenoic acids [9] and possibly for cytochrome P450 monooxyenase, which catalyzes formation of 14, 15-epoxyeicosatrienoic acids [10]. COX-1 is the target of the non-steroidal anti-inflammatory drug (NSAID) aspirin, which acetylates Ser^{530} blocking access of AA to the active site [11, 12, 13].

The caspase-9 induction followed by surface exposure of phosphatidylserine (PS), and binding/phagocytosis by macrophages initiates major responses to the relative small effect that association with GPIbα will have on 14-3-3 localizations. We therefore searched for other pathways that might contribute to cold-induced apoptosis, acting either in parallel or in synergy with the pathway initiated by GPIbα. A candidate pathway is the release of AA. In U937 phagocytic cells, interference with the reacylation/deacylation cycle of membrane phospholipids triggers accumulation of free AA and starts apoptosis [14]. Neurons stimulated with AA show depolarization of the inner mitochondrial membrane and caspase-3 activation [15, 16]. Overexpression of COX-2 in tumour cells to increase AA-removal blocks apoptosis. The reduction in cell death correlates inversely with the cellular level of AA. Conversely, COX-2 inhibition restored the apoptotic response [15].

In the present report, we show that although TxA_2-formation is absent at low-temperature, upstream steps in its formation contribute significantly to apoptosis induction in cold-stored platelets.

4.3 METHODS

4.3.1 Materials

We used the following products (with sources): prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), 5 , 5 ′, 6, 6 ′ -Tetrachloro-1, 1 ′, 3, 3 ′-tetraethyl-imidacarbocyanine iodide (JC-1), indomethacin, BSA fraction V and fatty acid (FFA)-free BSA (Sigma-Aldrich, St Louis, MO), arachidonic acid (Bio/Data Corporation, Horsham, PA), P38MAPK-inhibitor SB203580 and cytochrome P450 monooxygenase inhibitor SK&F96365 (Alexis Biochemicals/Enzo Lifesciences BVBA, Zandhoven, Belgium), lipoxygenase-inhibitor 5, 8, 11-eicosatriynoic acid (ETI, Cayman chemicals, Ann Arbor, MI) and 5-chloromethyl fluorescein diacetate (Cell tracker green\CFMDA, Molecular Probes, Invitrogen, Carlsbad, CA). Thromboxane receptor (TPα) antagonist SQ30741 was a kind gift from Bristol-Meyers-Squibb (Maarssen, The Netherlands).
Antibodies used for western blotting were directed against COX-1 (Abcam, Cambridge, UK), total Bad (Zymed, Invitrogen, Carlsbad, CA), 14-3-3ζ (C-16, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were: Alexa-680 (Molecular Probes, Invitrogen, Carlsbad, CA) and IRDYe 800CW (LI-COR Biosciences, Lincoln, NE). Antibodies for immunoprecipitation (IP) were against 14-3-3ζ (V-16, Santa Cruz Biotechnology) and total Bad (Cell signaling Technology, Danvers, MA).

4.3.2 Platelet isolation and incubations

Human platelets were isolated [17] while maximally preventing their activation using free-flow blood collection and discarding the first 2 mL of blood and all collections that showed micro-aggregates as determined by particle sizing. Procedures were approved by the Medical Ethical Committee of our hospital; the laboratory is certified for ISO-9001:2008. Platelets were resuspended in Hepes-Tyrode's ($2x10^{11}$ cells/L, pH 7.2) and incubated without stirring for 10 minutes at room temperature (defined as fresh platelets) and 4 hours at 0°C followed by 1 hour at 37°C to mimic cold-storage and post-transfusion conditions, as specified elsewhere [4]. Inhibitors used were for TPα SQ30741 (25 μM), for P38MAPK SB203580 (10 μM), for COX-1 indomethacin (30 μM), for lipoxygenase ETI (25 μM) and for cytochrome P450 monooxygenase SK&F96365 (30 μM), added at the start of the 0°C-incubation. To deplete platelets from AA, fatty acid-free BSA was present (75 g/L in Hepes-Tyrode, pH 7.2) during the 4 hours 0°C-incubation with a concurrent incubation with normal albumin as a control [18]. Then, platelets were washed in the presence of PGI_2 and resuspended to a concentration of $2x10^{11}$ cells/L.
For isolation of murine platelets, we used age-, strain-, and sex-matched C57Bl/6 wild type mice of eight weeks of age, obtained from Harlan (Boxmeer, the Netherlands). The experimental protocols were approved by the local ethics committees for animal experiments.

Mice were anesthetized with Isoflurane and blood was collected in 0.1 volume 130 mM trisodium citrate by cardiac puncture and centrifuged (420*g*, 3 minutes, 22°C, no-brake). The pellet, together with one third of the red blood cell fraction was collected and again centrifuged (960*g*, 1 minute, 22°C, no-brake). Platelets were collected and resuspended in Hepes-Tyrode's (pH 6.5), washed in 0.1 volume ACD and PGI_2 (2700*g*, 2 minutes, 22°C, no-brake) and resuspended in Hepes-Tyrode's (pH 7.2) to a final concentration of $2x10^{11}$ platelets/L.

4.3.3 Western blots and immunoprecipitations

Platelet suspensions were added to lysis buffer [17]. Proteins were separated by SDS-PAGE. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 µg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE). Quantification was performed with Image-J software (NIH, Bethesda, MD). For immunoprecipitations, 450 µL washed platelets ($5x10^{11}$ platelets/L) was lysed, (15 minutes, 0°C), centrifuged (10 000*g*, 10 minutes, 4°C) to remove cell debris and mixed with 55 µL (10% vol/vol) protein G beads together with antibody (1 µg/mL, 30 minutes, 4°C, rotating [2]).

4.3.4 Flow cytometric analysis

For determination of the mitochondrial membrane potential $\Delta\psi_m$, 100 µL platelet suspension was incubated with JC-1 (0.5 µM, 30 minutes, 37°C) and 10 000 platelets were measured on a FACS Calibur (BD Biosciences, San Jose, CA [2]). In viable cells, the high $\Delta\psi_m$ promotes a directional uptake of JC-1 into the matrix where JC-1 forms J-aggregates (λ_{ex} 490 nm, λ_{em} 570-610 nm). In apoptotic cells, the low $\Delta\Psi_m$ preserves the monomeric form (λ_{ex} 490 nm, λ_{em} 535 nm) [19, 20]. Changes in $\Delta\psi_m$ were expressed as the ratio of platelets in lower- over upper-right quartiles [2, 21, 22].

4.3.5 Platelet survival in vivo

Before transfusion, platelets were isolated from donor mice, resuspended in Tyrode's pH 6.5 and labeled with CMFDA (2.5 µM, 1 hour 22°C). After washing in the presence of PGI_2, platelets were resupended in Tyrode's pH 7.2, containing either 75 g/L fatty acid-free BSA or normal BSA and incubated at 0°C for 4 hours. Then, platelets were washed in the presence of PGI_2 and resuspended to a concentration of 10^{12} cells/L. After 30 minutes incubation at 22°C to inactivate PGI_2, $1x10^8$ of CMFDA-labeled platelets were injected into the tail vein of syngeneic recipient mice. For recovery and survival determinations, blood samples were collected at 2 minutes and 2, 24, 48, and 72 hours after injection in small vacuum EDTA tubes by mandibular puncture. A quantity of 1 µL whole blood was diluted in Tyrode's (1/250 by volume), analyzed by FACS and the percentage of CMFDA-positive platelets was determined in a total of 50 000 platelets per sample [23].

4.3.6 Statistics

Data are means ± SEM (n=3), as indicated. Statistical analysis was performed using GraphPad InStat (San Diego, CA) software. Differences between fresh and treated samples were considered significant at $p<0.05$ (*); between incubations by __*__.

4.4 RESULTS

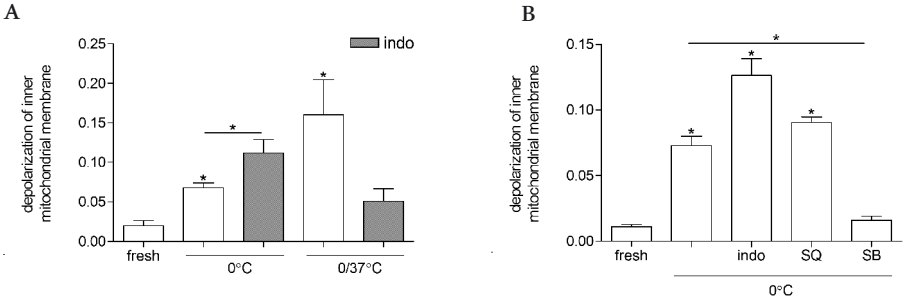
4.4.1 Changes in mitochondrial membrane potential induced by arachidonic acid

We reported previously that platelet incubation at low-temperature triggers apoptosis through activation of the pro-apoptotic Bcl-2 protein Bad, an increase in depolarization of the mitochondrial membrane potential ($\Delta\psi_m$-change), and caspase-9 mediated exposure of surface PS [2]. Figure 1A confirms these observations for the change in $\Delta\psi_m$ and shows a 3-fold change in $\Delta\psi_m$ after 4 hour-incubation at 0°C. COX-1 inhibition by indomethacin did not inhibit the $\Delta\psi_m$-change which agrees with the absent TxA_2-formation seen at 0°C [4]. Instead, there was a small increase in cold-induced $\Delta\psi_m$-change in the presence of the inhibitor. Subsequent rewarming (1 hour, 37°C) induced a further change of $\Delta\psi_m$, as shown earlier [2]. This response was fully blocked by indomethacin, indicating that it was initiated by TxA_2, a known apoptosis inducer in platelets [5]. To clarify the mechanism that starts apoptosis at 0°C, incubations were repeated in the presence of a blocker of the TxA_2-receptor, TPα (SQ30741 [24]) and an inhibitor of P38MAPK (SB203580), which is an upstream regulator of $cPLA_2$ and controls release of AA [8]. TPα-blockade had no effect, but P38MAPK-inhibition strongly reduced the cold-induced $\Delta\psi_m$-change (Figure 1B). Apparently, despite the low-temperature this enzyme contributed to apoptosis induction. Together these findings suggest that cold-incubation initiates a $\Delta\psi_m$-change either by released AA or by a product of AA-metabolism through pathways independent of COX-1 and TPα.

Addition of different doses of AA to platelets induced a dose-dependent increase in $\Delta\psi_m$-change (Figure 2A). Conversely, when platelets were depleted from endogenous AA, the cold-induced

Figure 1: Role of TxA_2-signalling in platelet apoptosis induced by cold-rewarming

Apoptosis induction in platelets incubated at 0°C (4 hours) and 0/37° (4 hours/1 hour) was analyzed by measuring the change in mitochondrial membrane potential ($\Delta\psi_m$) and compared with fresh platelets. A) Platelets in the absence (open bars) and presence of indomethacin (indo; f.c. 30 µM, added at the start of the 0°C-incubation, grey bars), B) Platelets incubated at 0°C (4 hours) without and with indomethacin, the TPα-blocker SQ30741 (SQ; 25 µM, preincubation 15 minutes, 22°C) and the p38MAPK-blocker SB203580 (SB; 10 µM, preincubation 15 minutes, 22°C). Data are means ± SEM (n=3) with significant difference $p < 0.05$ compared with fresh platelets (*) and between treatments (*).



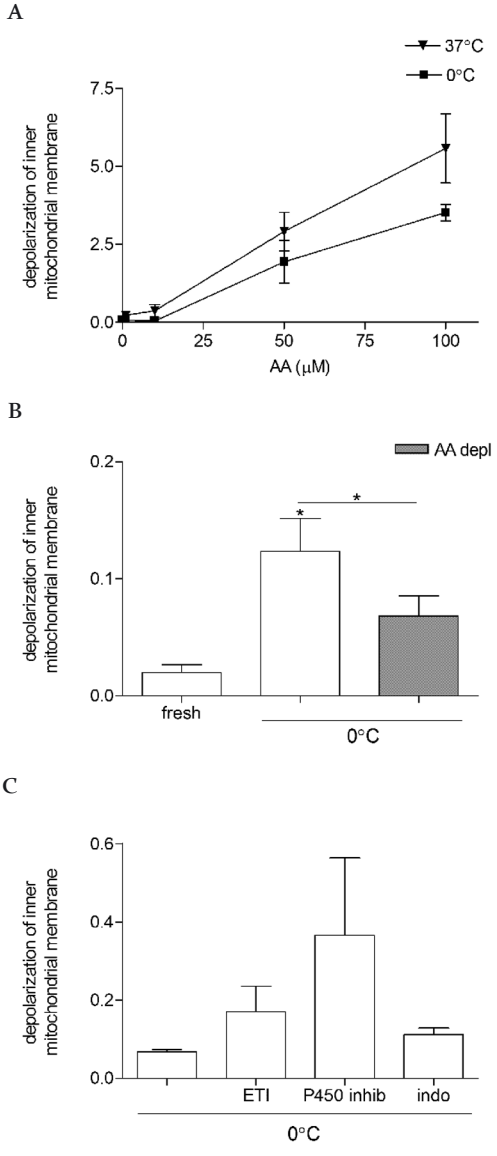
change in $\Delta\psi_m$ was inhibited by about 50% (Figure 2B). Thus, AA has potent apoptotic properties. Apart from being a precursor of TxA_2-formation through COX-1/thromboxane synthase, AA is converted to hydro (pero)xy-eicosatetraenoic acids by 12-lipoxygenase and possibly to 14,15-epoxyeicosatrienoic acids by cytochrome P450 monooxygenase [9, 10].

Addition of the respective inhibitors ETI and SK&F96365 before cold-treatment increased the $\Delta\psi_m$-change by 100% and 300% respectively (Figure 2C). These findings confirm that without being metabolized, AA triggers apoptosis.

At a physiological temperature, AA-release is known to contribute to aggregation and secretion by formation of TxA_2 and extracellular feed-back activation. To investigate whether under these conditions AA preserves apoptotic properties, normal and AA-depleted platelets were stimulated with the Ca^{2+}-ionophore A23187, the PAR-1 activator trap and the TxA_2-mimetic U46619. In all conditions, stimulation led to changes in $\Delta\psi_{m.}$ (Supplementary Figure S1A). In AA-depleted platelets, the change in $\Delta\psi_m$ was much lower (A23187) or virtually absent (trap, U46619) suggesting that apoptosis-induction by activators of platelet aggregation is mediated mainly through AA. Blockade of TPα-mediated signalling reduced the $\Delta\psi_m$-change by ionophore and trap revealing the contribution of TxA_2-induced apoptosis in absence of the blocker. Apoptosis induction by U46619 was virtually absent, confirming complete blockade of the TPα-receptor. AA-depletion further reduced the trap-induced $\Delta\psi_m$-change, but the ionophore response by TPα blocked platelets was not further changed by AA depletion (Supplementary Figure S1B).

Figure 2: Free arachidonic acid triggers platelet apoptosis

The change in mitochondrial membrane potential ($\Delta\psi_m$) was measured in, A) platelets incubated with 100 nM-50 µM arachidonic acid (AA) for 10 minutes at 0°C (squares) and 37°C (triangles), B) platelets depleted from AA with FFA-free BSA (grey bar), using normal BSA as a control (open bar), during incubation at 0°C (4 hours), C) platelets incubated at 0°C (4 hours) with the lipoxygenase-inhibitor ETI (25 µM, preincubation 15 minutes, 22°C), the cytochrome P450-inhibitor SK&F96365 (30 µM, preincubation, 15 minutes, 22°C) and indomethacin (as indicated above). AA-concentrations up to 100 µM did not compromise cell integrity. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).

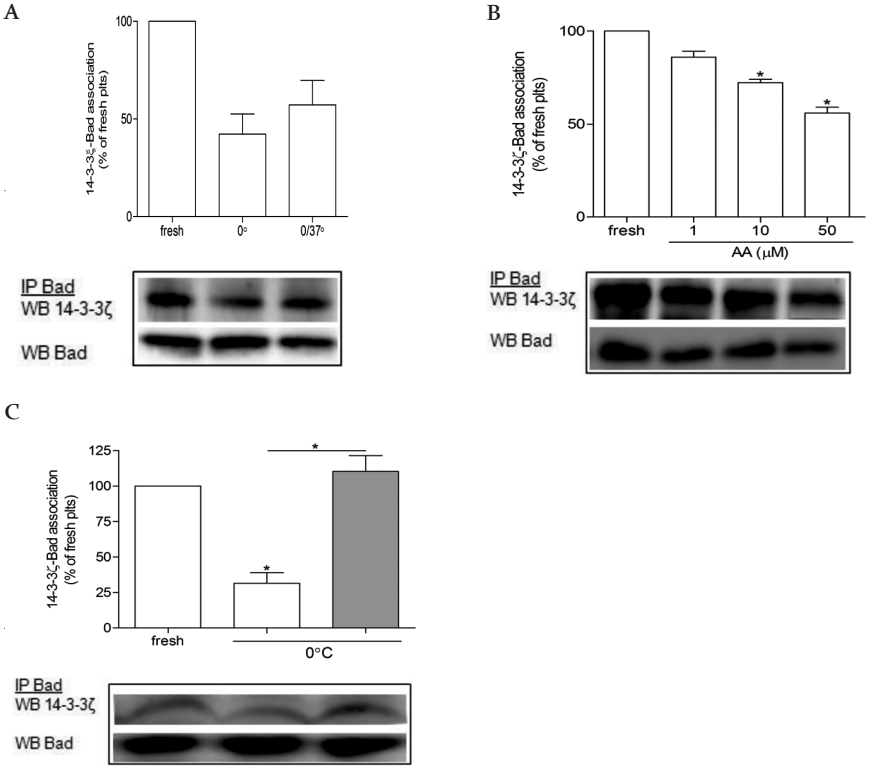


4.4.2 Regulation of [14-3-3ζ-Bad] association by arachidonic acid

A key step in apoptosis induction is the release of 14-3-3 adapter proteins from the [14-3-3-phospho-Bad] complex enabling dephosphorylation of phospho-Bad, Bad activation and further signalling to pro-apoptotic Bax and Bak. We showed earlier that the $\Delta\psi_m$-change in cold-incubated platelets is accompanied by a fall in [14-3-3ζ-Bad], such in line with induction of intrinsic apoptosis in many cell types [2]. To investigate whether AA contributes to this process, platelets were with blocked TPα cold-incubated (4 hours) and subsequently incubated at 37°C (1 hour) and the complex was measured in immunoprecipitates of Bad. Cold and cold/rewarming induced a fall in [14-3-3ζ-Bad] complex (Figure 3A), confirming earlier observations [2]. Also, AA-addition decreased the complex (Figure 3B) and AA-depletion reduced the fall in [14-3-3ζ-Bad] complex found after 0°C-treatment (Figure 3C). Together, these data show that released AA is at least one of the factors that induce dissociation of the [14-3-3ζ-Bad] complex.

Figure 3: Arachidonic acid dissociates the [14-3-3ζ-Bad] complex

Measurement of 14-3-3ζ-Bad association in A) fresh, 0°C- and 0/37°C-treated platelets (see legend Figure 1), B) fresh platelets and platelets incubated with 1, 10 and 50 μM arachidonic acid (AA) for 10 minutes at 0°C, and C) fresh and 0°C-treated platelets incubated with normal BSA (open bar) and FFA-free BSA to deplete platelet arachidonic acid (AA depl, grey bar). (The shape of the spots is disturbed by the high albumin content of the medium). Data are means ± SEM (n=3) with significant difference $p < 0.05$ compared with fresh platelets (*) and between treatments (_*_).

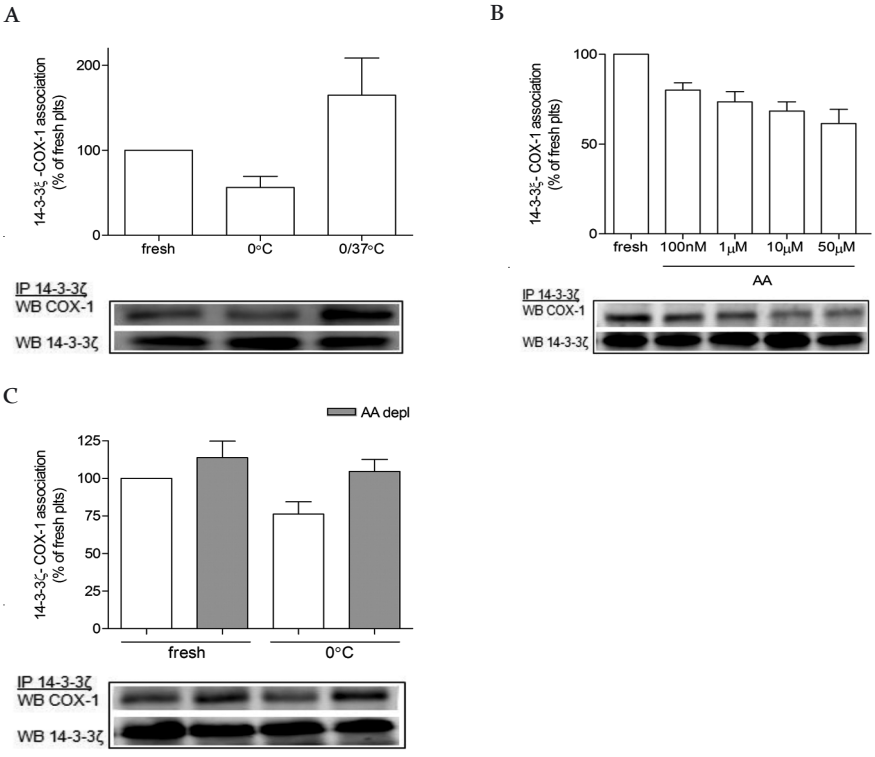


4.4.3 Regulation of [14-3-3ζ-COX-1] association by arachidonic acid

14-3-3 proteins are phosphoserine/phosphothreonine binding proteins through specific interaction with Arg-X-X-X-Ser-X-Pro and Arg-Ser-X-Ser-X-Pro sequences [25]. COX-1 contains two potential 14-3-3ζ binding sites, which are Arg^{60}-Thr-Gly-Tyr-Ser-Gly-Pro^{66} and Arg^{149}-Ile-Leu-Pro-Ser-Val-Pro^{155} (UniProtKB accession number P23219). Immunoprecipitates of 14-3-3ζ protein of fresh platelets with blocked TPα confirmed the association of 14-3-3ζ with COX-1 (Figure 4A). Cold-incubation led to a 40% fall in [14-3-3ζ-COX-1] complex and rewarming induced re-association to levels about 30% above those in fresh platelets. AA-addition induced a dose-dependent fall in the complex (Figure 4B) whereas AA-depletion partially restored the association of 14-3-3ζ to COX-1 in cold-incubated platelets (Figure 4C). Together, these findings show that cold-incubation triggers a fall in [14-3-3ζ-COX-1] complex through accumulation of AA, such in parallel with changes in [14-3-3ζ-Bad] complex. Rewarming restores the complex, probably by AA-removal in the COX-1 mediated conversion to TxA_2.

Figure 4: Arachidonic acid dissociates the [14-3-3ζ-COX-1] complex

Measurement of 14-3-3ζ-COX-1 association in A) fresh, 0°C- and 0/37°C-treated platelets (see legend Figure 1), B) fresh platelets and platelets incubated with 100 nM, and 1, 10 and 50 μM arachidonic acid (AA) for 10 minutes at 0°C, and C) fresh and 0°C-treated platelets incubated with normal BSA (open bar) and FFA-free BSA to deplete platelet arachidonic acid (AA depl, grey bar). Data are means ± SEM (n=3) with significant difference $p < 0.05$ compared with fresh platelets (*) and between treatments (__*__).

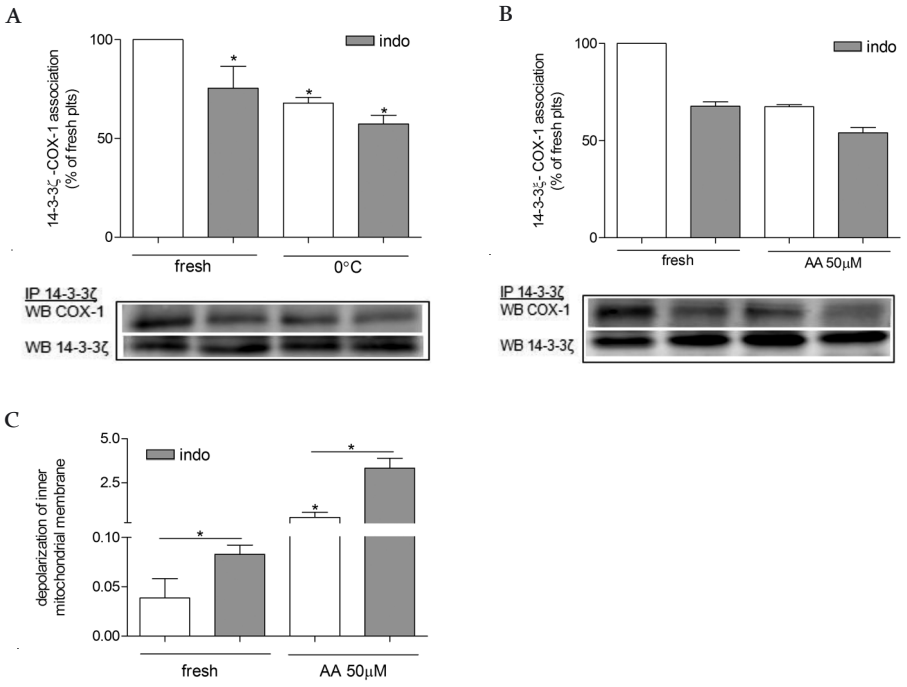


4.4.4 Arachidonic acid is a sink for 14-3-3ζ protein

The fall in [14-3-3ζ-Bad] complex induced by AA and the concurrent change in $\Delta\psi_m$ is in line with apoptosis induction by released Bad observed in many cell types. To address the question whether AA acts merely as a sink for 14-3-3ζ proteins or gains signalling properties upon binding of 14-3-3ζ, platelets with blocked TPα were treated with indomethacin. The 14-3-3 bindings site Arg^{149}-Ile-Leu-Pro-Ser-Val-Pro^{155} of COX-1 is located in the catalytic site of COX-1 (aa 120-385). Indomethacin binds COX-1 at Tyr^{355}, inhibiting the enzyme by preventing binding of AA at Tyr^{385} [26]. Thus, addition of indomethacin to cold-incubated platelets might displace 14-3-3ζ from COX-1, without affecting the pool of free AA since at 0°C, COX-1 is inactive. In fresh platelets, indomethacin induced a 25% fall in [14-3-3ζ-COX-1] complex. In the absence of indomethacin, low-temperature lowered the complex probably as a result of AA-accumulation and again indomethacin induced a further decrease in [14-3-3ζ-COX-1] complex (Figure 5A). AA-addition to fresh platelets also induced a fall in the complex and again indomethacin-

Figure 5: Indomethacin dissociates the [14-3-3ζ-COX-1]

Measurement of 14-3-3ζ-COX-1 association in A) fresh and 0°C-treated platelets in the absence (open bars) and presence of indomethacin (indo; f.c. 30 μM, added at the start of the 0°C-incubation, grey bars), B) fresh platelets and platelets incubated with 50 μM arachidonic acid (AA) for 10 minutes at 0°C. C), 0/37°C-treated platelets (see legend Figure 1), B) fresh platelets and platelets incubated with 50 μM arachidonic acid (AA) for 10 minutes at 0°C. C) The change in mitochondrial membrane potential ($\Delta\psi_m$) in fresh and platelets incubated with 50 μM arachidonic acid (AA) for 10 minutes at 0°C. Data are means ± SEM (n=3) with significant difference $p < 0.05$ compared with fresh platelets (*) and between treatments (*).



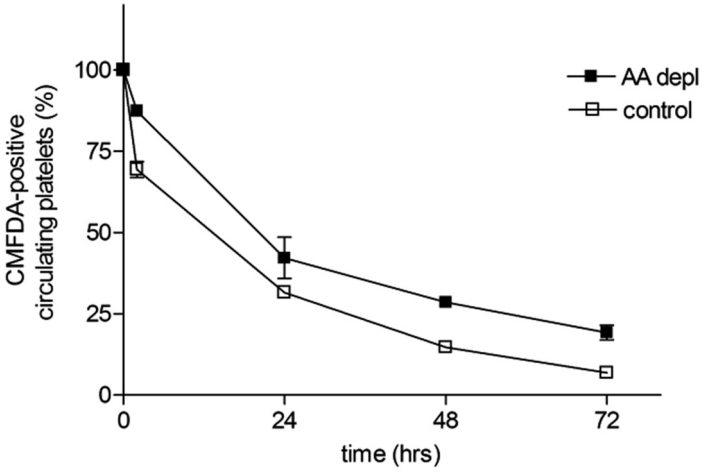
addition led to a further decrease (Figure 5B). Conditions that induced a fall in [14-3-3ζ-COX-1] complex were accompanied by a rise in $\Delta\psi_m$-change (Figure 5C). These results suggest that in addition to its role as a "sink" of 14-3-3ζ proteins, association of 14-3-3ζ with AA leads to further apoptosis induction.

4.4.5 Arachidonic acid-depletion enhances in vivo survival of cold-stored platelets

Apoptosis induction by added AA was also observed in murine platelets and AA-depletion inhibited the cold-induced $\Delta\psi_m$-change (Supplementary Figure 2A, B). These properties made it possible to investigate whether AA-depletion increased the survival of cold-stored platelets. Murine platelets were incubated with FFA-free and normal BSA for 4 hours at 0°C, washed with protection by PGI_2 and injected into recipient mice. AA-depleted platelets survived longer than controls After 72 hours, control platelets were almost completely cleared, while about 20% of the AA-depleted platelets were still in the circulation (Figure 6). These findings suggest that AA-depletion might reduce the induction of clearance signals on cold-stored platelets thereby improving platelet survival.

Figure 6: Arachidonic acid-depletion improves platelet survival of cold-stored platelets

Platelet survival in C57Bl/6 mice. Mice platelets were labelled with CFMDA and incubated with normal BSA (open squares) and FFA-free BSA (closed squares) to deplete platelet arachidonic acid for 4 hours at 0°C, and injected into recipient mice. Blood was collected after 2 minutes, 2, 24, 48 and 72 hours after injection and the percentage recovery of CFMDA-labelled platelets was determined. Data are means ± SEM (n=3).



4.5 DISCUSSION

The main findings of this study are, (i) cold-incubation induces release of AA from membrane phospholipids, which accumulates since COX-1 is inactive at low temperature, (ii) AA induces apoptosis by acting as a "sink" for 14-3-3ζ, thereby releasing Bad from the [14-3-3ζ-Bad] complex, (iii) 14-3-3ζ is also associated with COX-1 and changes in [14-3-3ζ-COX-1] confirm the role of AA, (iv) indomethacin releases 14-3-3ζ from COX-1 and enhances AA-induced apoptosis, (v) AA-depletion during cold-storage increases platelet survival *in vivo*. Cold-storage of platelets triggers desialylation of GPIbα, exposing galactose and β-*N*-acetyl-*D*-glucosamine residues that become recognition sites for the lectin-recognizing moiety of the MAC-1 receptor on macrophages, triggering platelet uptake and destruction [27]. Concurrently, GPIbα is released from the membrane skeleton and undergoes a conformational change that starts signaling to TxA_2-production even in the absence of ligand binding [4]. The GPIbα-change also induces reallocation of 14-3-3ζ-proteins resulting in the release of phosphoBad from the [14-3-3ζ-Bad] complex, Bad-activation and further signaling through the intrinsic apoptotic pathway to caspase-9 activation, PS-exposure and platelet phagocytosis by macrophages [2]. Most inducers of platelet aggregation and secretion have apoptotic properties [5, 19, 28] and activation of Bad appears a logical consequence of the TxA_2 formed during cold/rewarming. However, step-wise analysis of TxA_2 during the incubations showed that TxA_2-formation at 0°C is absent and starts during rewarming [29]. The finding that p38MAPK-inhibition arrests apoptosis-induction at 0°C suggests otherwise. Apparently, cold-incubation activates P38MAPK and $cPLA_2$. The result is release of AA from membrane phospholipids and AA-accumulation. Other examples of cold-induced p38MAPK-activation have been found in the fly *Sarcophaga crassipalpis*, which has a p38MAPK that is highly homologous to the enzyme in mammals [30], and in rat hepatocytes and liver endothelial cells cooled at 4°C [31]. Another enzyme which shows optimal activity at low-temperature is 2, 3-bisphosphoglycerate phosphatase [32] in red blood cells. Also, the strong caspase-9 induction in cold-stored platelets illustrates that many enzymes are active at low-temperature.

p38MAPK is an upstream regulator of $cPLA_2$ and blockade with SB203580 inhibits AA-release and TxA_2-formation in platelets incubated with collagen [7], LPS [33], VWF [34] and low density lipoprotein [35] at physiological temperature. The enzyme activates $cPLA_2$ by phosphorylation of Ser^{505} and Ser^{727}; it is suggested that this induces a conformational change in the catalytic domain bringing the active site close to the membrane, causing tighter binding and penetration into the membrane phospholipids [36, 37]. Together with the increase in cytosolic Ca^{2+} seen in cold-stored platelets [38], $cPLA_2$ is translocated from the cytosol to the membrane, through Ca^{2+} binding to its C2-domain, where it becomes fully active and releases free fatty acids from the *sn*-ester linkage of the second carbon group of the glycerol backbone.

These findings support a concept of cold-induced AA-release by active P38MAPK/$cPLA_2$, which accumulates by apparent lack of COX-1 activity at this low-temperature. A minor metabolism of AA might still take place, as inhibitors of lipoxygenase and cytochrome P450 monooxygenase

slightly raise the change in $\Delta\psi_m$. An earlier study sought the cause of apoptosis in cold-stored platelets in the clustering of GPIbα, which became associated with 14-3-3 proteins thereby releasing Bad for the [14-3-3-phosphoBad] complex. These reactions were inhibited by *N*-acetyl-*D*-glucosamine, which interferes with GPIbα-clustering and by osge, which removes the extracellular part of the receptor. Whether the release of AA by P38MAPK/cPLA$_2$ during cold-incubation is initiated by clustering of GPIbα, remains to be investigated.

The conclusion that AA acts as a "sink" for 14-3-3 proteins is supported by the dose-dependent increase in the $\Delta\psi_m$-change by added AA and the suppression of cold-induced apoptosis induction seen in AA-depleted platelets. The concept was introduced in Cao *et al.* who showed in tumour cells that overexpression of COX-2 inhibited apoptosis and an inverse correlation between AA-levels and reduction of cell death [15]. Upon apoptosis induction in [^{3}H] AA-labelled neurons, immunoprecipitates of 14-3-3ζ became radio-labelled, pointing at the formation of an [14-3-3ζ-AA] complex [39]. This occurred at the expense of the [14-3-3ζ-phosphoBad] complex and is in line with the concept that AA acts as a "sink" for 14-3-3 proteins in the present studies. Release of 14-3-3ζ from phosphoBad leads to activation of pro-apoptotic Bad and Bax and a fall in pro-survival Bcl-x$_L$ resulting in Bax-translocation to the mitochondria, release of cytochrome *c* and further signaling to caspase-activation and platelet destruction by macrophages [2, 40, 41].

At physiological temperature, thrombin, TxA$_2$-analogue U46619 and other inducers of platelet aggregation and secretion are known to start apoptosis including translocation of active Bax to the mitochondria and changes in $\Delta\psi_m$ [5, 19]. Our studies show that also under these conditions free AA might contribute to these processes since the $\Delta\Psi_m$-change is much lower in AA-depleted platelets than in normal cells. Much depends on the rate of AA-release by the agonists and AA-metabolism by COX-1, and COX-1 blockade by indomethacin is likely to further increase AA-mediated apoptosis induction. The Ca^{2+}-ionophore A23187 triggers the entry of extracellular Ca^{2+} and is a potent apoptosis inducer in many cell types while bypassing receptor-mediated cell activation [42, 43]. The A23187-induced $\Delta\psi_m$-change was strongly reduced in AA-depleted platelets illustrating that it acts in part through a similar mechanism. In TPα-blocked platelets, the AA-dependence was lost and revealed a considerable apoptosis induction independent of AA or TxA$_2$-signaling. Apart from binding to phosphoBad and COX-1, 14-3-3ζ proteins couple to many other proteins such as pro-apoptotic Bax [44], members of the GPIb-V-IX complex [2, 45], GTPase-activating protein Rap1GAP2 [46], Phosphoinositide (PI)3 kinase [47], c-Raf-1 and insulin receptor substrate-1 (IRS-1) [48]. 14-3-3ζ depletion by accumulated AA might thereby affect many steps in the regulation of apoptosis and cell functions.

An unexpected finding was the association between 14-3-3ζ and COX-1. Complex-formation was optimal in fresh platelets, fell by about 50% after cold-storage and restored to 30% above pre-treatment values after subsequent incubation at 37°C. These changes are similar as the variations in [14-3-3ζ-Bad] complex, which are coupled to the control of Bad. A direct role of COX-1 in apoptosis regulation is unlikely but its indirect control in 14-3-3ζ translocation might be important, especially since the COX-1 inhibitor indomethacin releases 14-3-3ζ. Phosphorylation of the Ser-residues in 14-3-3ζ binding-proteins is a prerequisite for binding.

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Supplementary material

4.7 METHODS

4.7.1 Materials

We used the following products (with sources): TxA_2-mimetic U46619 (Cayman Chemical, Ann Arbor, MI), calcium ionophore A23187 (Calbiochem, Darmstadt, Germany) and thrombin receptor (PAR1)-activating peptide (TRAP, SFLLRN, Bachem, Switzerland).

4.7.2 Platelet isolation and incubations

For analysis of apoptosis induction by platelet agonists, platelets were resuspended in Hepes-Tyrode's ($2x10^{11}$ cells/L, pH 7.2) in the presence of inhibitors, when indicated, at 22°C. Platelet AA was depleted by incubation with fatty acid-free BSA for 4 hours at 37°C. Then, platelets were washed (330*g*, 10 minutes, 22°C, no-brake, in the presence of 10 ng/mL PGI_2), resuspended in Hepes-Tyrode's pH 7.2 and incubated for 30 minutes at 22°C to inactivate PGI_2 before start of experiments.

4.8 RESULTS

Figure S1: Free arachidonate contributes to agonist-induced platelet apoptosis

In the absence, A), and presence, B), of the TPα-blocker SQ30741, apoptosis induction was analyzed in fresh platelets and in control platelets (4 hours incubation with control BSA at 0°C, open bars) and arachidonic acid depleted platelets (4 hours incubation with FFA-free BSA at 0°C, grey bars) by measuring the change in mitochondrial membrane potential, $\Delta\psi_m$. Platelets were stimulated without stirring with Ca^{2+}-ionophore A23187 (3 µM, with 2 mM extracellular Ca^{2+}), TRAP (20 µM) and the TxA_2-analog U46619 (10 µM). Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).

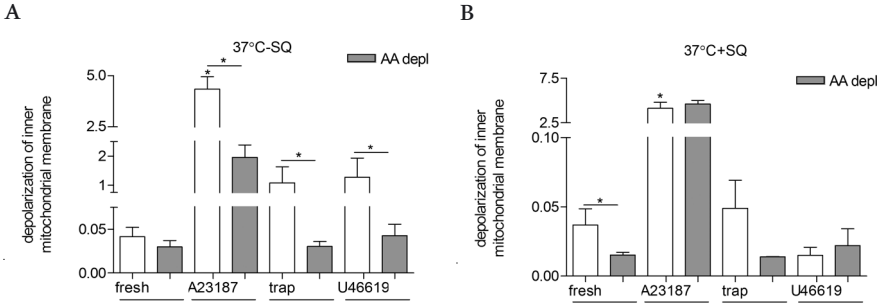
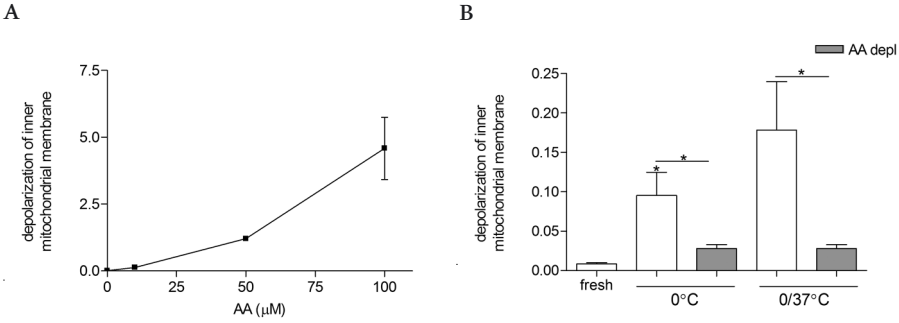


Figure S2: Arachidonate triggers apoptosis in murine platelets

Apoptosis induction in murine platelets incubated at 0°C (4 hours) and 0/37° (4 hours/1 hour) was analyzed by measuring the change in mitochondrial membrane potential, $\Delta\psi_m$ and compared with fresh platelets. A) fresh platelets and platelets incubated with 10, 50 and 100 µM arachidonic acid (AA) for 10 minutes at 0°C, B) fresh and 0/37°C-treated platelets incubated with normal BSA (open bars) and FFA-free BSA to deplete platelet arachidonic acid (AA depl, grey bars). Data are means ± SEM (n=3) with significant difference $p < 0.05$ compared with fresh platelets (*) and between treatments (*).



Won't stop til it's over
Won't stop to surrender

(Temper Trap)

Chapter 5

A patient with enhanced platelet apoptosis

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(In preparation)

5.1 ABSTRACT

We report a 70 year old patient with unexplained thrombocytopenia. The patient had a mild bleeding phenotype with easy bruising and a bleeding time of > 30 minutes. Her platelets aggregated spontaneously during collection in citrate, EDTA and heparin and peripheral blood smears showed micro-aggregates. Blood collection in a citrate/prostacyclin mixture suppressed aggregation. FACS-analysis of patient platelets in the range of single control platelets showed a 5-fold increase in activated GPIIb-IIIa, a 2-fold increase in bound fibrinogen and a 15-fold increase in P-selectin expression compared with controls. Also, the mitochondrial inner membrane potential ($\Delta\psi_m$) and surface-expressed phosphatidylserine were changed (15-fold decrease and 5-fold increase respectively) as was the binding/phagocytosis by matured THP-1 monocytic cells (20-fold increase). In contrast, binding of the anti-Glycoprotein (GP) Ibα antibody AN51 was 30% lower than in controls. Reconstitution of normal platelets in patient plasma induced the same responses, including interference with AN51 binding. These responses were inhibited by prior cleavage of the extracellular part of GPIbα (*O*-sialoglycoprotein endopeptidase) and by interference with GPIbα-GPIbα associations (*N*-Acetyl-*D*-Glucosamine). We conclude that the patient had developed an auto-antibody against GPIbα, which initiates haemostasis and apoptosis responses in platelets, possibly through clustering of GPIbα.

5.2 INTRODUCTION

Acquired thrombocytopenia can be caused by an autoimmune disease, increased platelet consumption, splenomegaly and infectious or drug-mediated bone-marrow suppression [1]. Auto-antibodies against platelets induce platelet responses that contribute to haemostasis and increase their Fc-receptor mediated clearance by the reticuloendothelial system from the circulation. Idiopathic thrombocytopenic purpura (ITP) is characterized by auto-antibodies generated against the platelet Glycoprotein (GP) IIb-IIIa and the GPIb-V-IX complexes with respective contributions of 70-80% and 20-40% of cases. Platelet destruction induced by anti-GPIIb-IIIa antibodies involves activation through the FcγIIa-receptor and patients are generally treated with intravenous administration of immunoglobulin G (IVIG) to neutralize FcγIIa-signalling [2, 3]. The enhanced platelet destruction by anti-GPIIb-IIIa antibodies might be caused by activation of the mechanisms that control apoptosis. These platelets have enhanced platelet caspase-3 activity, which decreases upon treatment with IVIG [4]. In addition, there is a decrease in the number of mature megakaryocytes (MKs). The immature MKs show mitochondrial swelling, an abnormal demarcation membrane system and increased caspase-3 activity, suggesting that an upregulated apoptosis pathway leads to enhanced destruction by bone marrow macrophages [5]. Under *in vitro* conditions, certain anti-GPIIb antibodies decrease the inner mitochondrial membrane potential ($\Delta\psi_m$), activate caspase-3 and expose phosphatidylserine (PS) on the platelet surface, indicating that they trigger apoptosis. IVIG-treatment improves platelet survival in a murine model of ITP [6].

Anti-GPIbα antibodies act independently of FcγIIa-receptor signaling and $F(ab')_2$-fragments of these antibodies induce the same extent of thrombocytopenia as intact IgGs [7]. Consequently, there is no beneficial effect of IVIG-treatment. Instead, the antibodies appear to activate platelets through direct interaction with GPIbα, since administration of a panel of anti-GPIbα antibodies inhibits agglutination of platelets [8].

In attempts to improve the quality of platelets for transfusion, Hoffmeister and colleagues stored platelets at 0°C [9]. Unfortunately, this condition induced loss of sialic acid which normally caps galactose and β-*N*-acetyl-*D*-glucosamine (GN) residues in *N*-linked sugars on GPIbα. Exposure of these sugars led to formation of GPIbα-clusters, which became recognition sites for platelet destruction by macrophages. Addition of GN interfered with GPIbα binding to the lectin-binding MAC-1 receptor on macrophages and blocked platelet phagocytosis [10].

We showed earlier that cold storage (0°C) followed by rewarming (37°C), which mimics conditions when stored platelets are transfused into recipients, initiates the formation of thromboxane A_2 (TxA_2), which is a potent platelet activating agent. These conditions also started apoptosis induction and platelet destruction, which together with the formation of TxA_2 would severely compromise the haemostasis functions of transfused platelets [11, 12]. The responses were inhibited by removal of extracellular GPIbα with *O*-sialoglycoprotein endopeptidase (osge) and interference with GPIbα-GPIbα associations with GN, suggesting that clustering of GPIbα-molecules is the initiation mechanism of these responses.

In the present study, we investigated a patient with thrombocytopenia whose platelets aggregated spontaneously during blood collection. We provide preliminary evidence for the appearance of an antibody against GPIbα in the patient plasma which induces haemostasis and apoptosis functions in patient platelets as well as in normal platelets, possibly by inducing clustering of GPIbα molecules.

5.3 PATIENT, MATERIALS, AND METHODS

5.3.1 Patient characteristics

The patient is a 70 year old Caucasian-female. Her medical history consists of kidney stones in 2004 that caused septicemia in 2005 for which a nefrectomy had to be performed. In 2006 she developed primary hyperparathyroidism due to an adenoma for which a parathyroidectomy was performed. Both surgeries were without bleeding complications. In 2007, routine laboratory investigations revealed thrombocytopenia of $20x10^9$/L (normal 150-$450x10^9$/L). At that time, she had no hemorrhagic diathesis, neither spontaneous nor after surgery. Blood counts showed a hemoglobin level of 12.2 g/L (normal 11.8-15.4 g/L), leucocytes of $6.9x10^9$/L (normal 4.0-$10.0x10^9$/L) with normal differential and platelet counts in citrate and heparin both >$3x10^9$/L. The mean platelet volume was 17.7 fL. Cytomorphology showed multiple platelet aggregates (Figure 1A). Because of iron deficiency, a gastroscopy was performed without abnormalities. Small bowel biopsies were performed; they revealed atrophic mucosa with loss of folds. Antibodies against tissue transglutaminase and endomysium were positive and the diagnosis celiac disease was made. She was given a gluten-free diet. In 2009, she suffered from increased spontaneous skin hematomas and melena. Her medication at that time consisted of bromazepam, paroxetine and enalapril. Laboratory investigations showed a hemoglobin level of 8.5 g/L with platelet counts of >$20x10^9$/L with aggregates. Laboratory investigations are given in Table 1. There was no detectable monoclonal protein and immunoglobulin levels were normal.

Table 1: Laboratory investigations

	Normal values	Patient values
Platelet count (10^9/L) citrate	150-450	3-29
Platelet count (10^9/L) heparin	150-450	3-20
Platelet count (10^9/L) EDTA	150-450	17
Mean platelet volume (fL)	7.0-9.5	14.8-17.7
Factor VIII:C (%)	60-150	163
VWF:Ag (%)	60-130	120-124
VWF:Ristocetin cofactor (%)	40-150	145-149
VWF protease (%)	62-134	97
Erythrocytes (10^{12}/L)	3.7-5.0	4.1-4.5
Haemoglobin (mM)	7.4-9.6	7.5-8.4
Haematocrit (fraction)	0.36-0.46	0.37-0.42
PT (s)	11.5-14.5	13.4
APTT (s)	29-39	36-40
TT (s)	13.9-19.9	15.7
Bleeding time (Surgicut method, min)	2-8.3	>30

She received a blood transfusion with erythrocytes and was treated with IVIG 30mg/d for 5 days with no response. A platelet transfusion showed no increase in platelet counts. Under protection of 2000 U Hemate-P a gastroscopy was performed that revealed a small sliding hernia diafragmatica; biopsies from the small intestine showed normal folds without atrophic mucosa. A subsequent colonoscopy showed mild diverticulosis in the colon ascendens and the sigmoid. A small adenoma was removed without bleeding complications. A clear bleeding focus was not found, but with supportive care using proton pump inhibition, furosemide and tranexamic acid, the patient recovered. She currently has no active bleeding problems, but her platelet counts remain unchanged ($3x10^9$/L).

5.3.2 Chemicals

We used the following products (with sources): *N*-Acetyl-*D*-Glucosamine (GN, Merck, Darmstadt, Germany), prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI), 5,5´,6,6´-Tetrachloro-1,1´,3,3´-tetraethyl-imidacarbocyanine iodide JC-1, phorbol 12-myristate 13-acetate (PMA, MP Biochemicals, Illkirch, France), vitamin D_3 and trypan blue (Sigma, St. Louis, MO), 5-chloromethyl fluorescein diacetate (Cell tracker green\CFMDA, Molecular Probes, Invitrogen, Carlsbad, CA), THP-1 monocytic cells (ATCC/LGC Standards GmBH, Wesel, Germany), (platelet derived) human TGF-β1 (R&D systems, Minneapolis, MN), thrombin receptor (PAR1)-activating peptide (TRAP, SFLLRN, Bachem, Switzerland), *O*-sialoglycoprotein endopeptidase (osge, Cederlane Laboratories, Hornby, Ontario, Canada), Retic-Count (Thiazole Orange), Fibrinogen (Fg, Enzyme Research Laboratories, South Bend, IN), Annexin-V, P-selectin and PAC-1 FITC-conjugated antibodies (BD, San Diego, CA) and FITC-conjugated anti-Fibrinogen antibody (Emfret Analytics, Würzburg, Germany). We used the following GPIbα-antibodies: AN51 directed against aa 1-35 (DakoCytomation, Glusdorp, Denmark), HIP-1 directed against aa 59-81 (BD, San Diego, CA) and SZ2 directed against aa 268-282 (Immunotech (Marseille, France).

5.3.3 Blood collection and platelet isolation

Procedures were approved by the Medical Ethical Committee of our hospital; the laboratory is certified for ISO 9001:2008. Platelets from healthy, medication-free volunteers were isolated [13] and resuspended in Hepes-Tyrode's (pH 7.2). Whole blood and isolated platelets were analyzed on haematology analyzers CELL-DYN Sapphire and CELL-DYN 1800 (Abbott Diagnostics, Santa Clara, CA) respectively. The patient platelets aggregated spontaneously during blood collection in standard vacuum tubes containing citrate, coated EDTA-K2 (BD, San Diego, CA) or sodium heparin (Greiner bio-one, Frickenhausen, Germany), preventing standard laboratory investigations. To reduce aggregation to a minimum, patient blood was collected by free-flow, the first 2 mL was discarded and the remainder collected in 13 mM citrate (f.c.) supplemented with 10 ng/mL PGI_2 to induce transient platelet inhibition. Plasma was isolated from patient blood by high-speed centrifugation (10 minutes, 2000*g*, 22°C).

5.3.4 Platelet incubations

After platelet isolation and subsequent incubation at 22°C for 30 minutes to inactivate PGI_2, platelets were stimulated (5 minutes; 37°C, without stirring) with TRAP (20 μM) in the presence of fibrinogen (100 μg/mL). For reconstitution experiments, normal platelet-rich plasma was mixed with 0.1 volume of ACD (2.5% trisodium citrate, 1.5% citric acid and 2% *D*-Glucose) to prevent activation, centrifuged (15 minutes, 330*g*, no-brake, 22°C) and resuspended in autologous or patient plasma (2 hours, RT). Platelets were washed (15 minutes, 330*g*, no-brake, 22°C, in presence of 10 ng/mL PGI_2) and resuspended to a platelet concentration of $2x10^{11}$ cells/L in Tyrode's pH 7.2 and incubated for 30 minutes at RT to inactivate PGI_2. The contribution of GPIbα was investigated by removal of the extracellular of GPIbα with osge (80 μg/mL) during incubation with plasma and by addition of GN (100 mM), as described [12].

5.3.5 Flow cytometric analysis

Characterization of patient platelets by FACS was restricted to the range typical for single platelets from healthy controls based on FSC and SSC-scatter (FACS Calibur; BD Biosciences, San Jose, CA). Appropriate antibodies were added in 1/20 (vol/vol) dilutions and suspensions (100 μL) were incubated (15 minutes, 37°C); 10 000 platelets were measured. We measured activated GPIIb-IIIa (PAC-1) and surface-expressed fibrinogen and P-selectin. For determination of the mitochondrial membrane potential $\Delta\psi_m$, 100 μL platelet suspension was incubated with the JC-1 (0.5 μM, 30 minutes, 37°C) [14]. In viable cells, the high $\Delta\psi_m$ promotes a directional uptake of JC-1 into the matrix where JC-1 forms J-aggregates (λ_{ex} 490 nm, λ_{em} 570-610 nm). In apoptosis cells, the low $\Delta\psi_m$ preserves the monomeric form (λ_{ex} 490 nm, λ_{em} 535 nm) [15, 16]. Changes in $\Delta\psi_m$ were expressed as the ratio of platelets in lower- over upper-right quartiles [17, 18]. Surface-exposed PS was deduced from the binding of FITC-conjugated Annexin V, measured in the presence of 2.5 mM $CaCl_2$. The number of reticulated (young) platelets was measured by incubation with thiazole orange (1/10 dilution, 1 hour, RT) and fluorescent platelets were detected by FACS.

5.3.6 Platelet binding and phagocytosis

Platelets were resuspended in HEPES-tyrode's, pH 6.5 ($4x10^{11}$ cells/L), labelled with CFMDA (20 μM, 1 hour, RT), centrifuged (330*g*, no-brake, 10 minutes, RT) in the presence of 10 ng/mL PGI_2 and resuspended in tyrode's, pH 7.2. Functionality was recovered by 30 minutes incubation at RT. For measurement of combined binding and phagocytosis, monocytic THP-1 cells were cultured in a 96-wells plate ($5x10^4$ cells/well) and stimulated with 50 nM vitamin D_3 and 1 ng/mL TGF-β1 (12 hours, 37°C) and 250 nM of PMA (2 hours, 37°C). $CaCl_2$ and $MgCl_2$ (1 mM each, f.c.) were added and $5x10^5$ platelets were incubated with the maturated THP-1 cells (90 minutes, 37°C). Unbound platelets were removed and trypan blue was added. Trypan Blue quenches fluorescence of bound, but not phagocytosed CFMDA-labelled platelets and the fluorescence of phagocytozed and bound plus phagocytozed platelets was measured, as

described [14]. Phagocytosis was measured on a Fluorstar Galaxy (BMG LABTECH GmbH, Offenburg, Germany). Binding and phagocytosis of fresh control platelets were set at 100%.

5.3.7 Statistics

Data are means ± SEM (n=3), as indicated. Statistical analysis was performed using GraphPad InStat (San Diego, CA) software. Differences between fresh and treated samples were considered significant at $p<0.05$, as indicated (*).

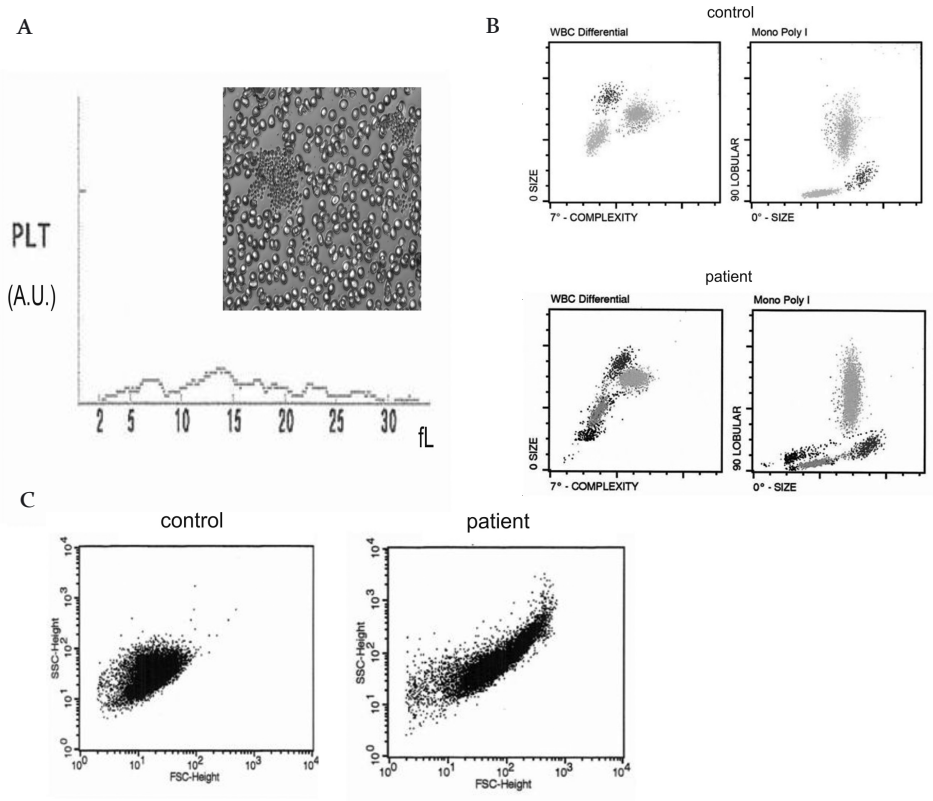
5.4 RESULTS

5.4.1 Spontaneous platelet aggregation during blood collection

Particle seizing of patient blood collected in citrate revealed the presence of small aggregates ranging from 2 to 30 fL in size (Figure 1A). A blood smear showed clumps of normal-sized platelets between red blood cells (Figure 1A, insert). Also, whole blood analysis by the Haematology Analyzer CELL-DYN Sapphire showed clumping of platelets (Figure 1B, lower panel), compared with control (Figure 1B, upper panel). When patient blood was collected by free flow in citrate supplemented with PGI_2, the number of aggregates decreased but a small

Figure 1: Morphology of patient platelets

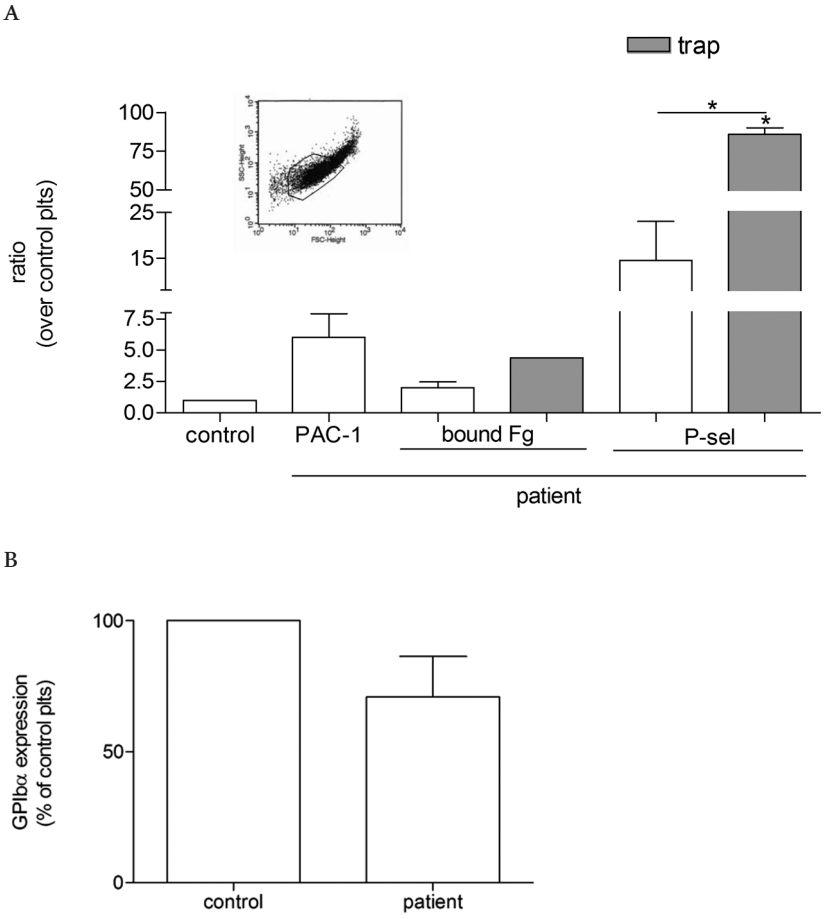
A) Patient platelets were analyzed on the hematology analyzer CELL-DYN 1800. A peripheral blood smear after blood collection without PGI_2 was made and stained with May-Grünwald-Giemsa (insert), B) In whole blood from control subjects (upper panel) or the patient (lower panel), platelet count and MPV were measured by CELL-DYN Sapphire (orange, blue, purple, green and black populations are neutrophils, lymphocytes, monocytes, eosinophils and clumped platelets respectively), C) whole blood was collected in citrate supplemented with PGI_2 (10 ng/mL) and FACS-analysis of isolated control platelets (left panel) or patient platelets (right panel) was performed by FFC/SSC scatter.



population of aggregated platelets remained detectable. A FFC/SSC-scatterplot confirmed that the distribution of patient platelets extended beyond the range of single platelets from healthy individuals indicative for the presence of small aggregates (Figure 1C). Further FACS-analyses were therefore restricted to patient platelets in the range of single platelets obtained from healthy volunteers (Figure 2A, insert).

Figure 2: Increased levels of haemostasis markers

A) Fresh control and patient platelets without and with stimulation with trap (20 μM, 5 minutes, 37°C), supplemented with fibrinogen (100 μg/mL), as indicated. FACS-analysis of activated GPIIbIIIa (PAC-1), bound fibrinogen (bound Fg) and surface P-selectin (P-sel). Gating of single platelets was used for analysis of the FACS results (A, insert), B) Binding of anti-GPIbα antibody AN51. Results are expressed as ratio of patient platelets/ control platelets. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*) and between treatments (*).



5.4.2 Platelets are activated during blood collection

Use of citrate supplemented with Iloprost or PGI_2 kept platelet clumping to a minimum. After 30 minutes incubation at RT to let platelets recover from the inhibition by PGI_2, FACS-analysis revealed a strongly increased binding of PAC-1 antibody and surface-expressed fibrinogen and P-selectin (Figure 2A). Stimulation with 20 µM TRAP (in the presence of 100 µg/mL fibrinogen) almost doubled the amount of bound fibrinogen and induced a 5-fold increase in P-selectin expression (Figure 2A). These data indicate that patient platelets have been activated during blood collection but not to a maximal extent. ITP-patients can be divided in those with antibodies against GPIIb-IIIa and respond to IVIG-administration and those with antibodies against GPIb-V-IX and do not respond to IVIG. Our patient failed to benefit from IVIG administration and may therefore suffer from an anti-GPIb-V-IX antibody. Indeed, FACS-analysis revealed a 30% lower binding of the anti-GPIbα antibody AN51 than controls suggesting that part of the patient GPIbα is covered by an endogenous anti-GPIbα antibody (Figure 2B).

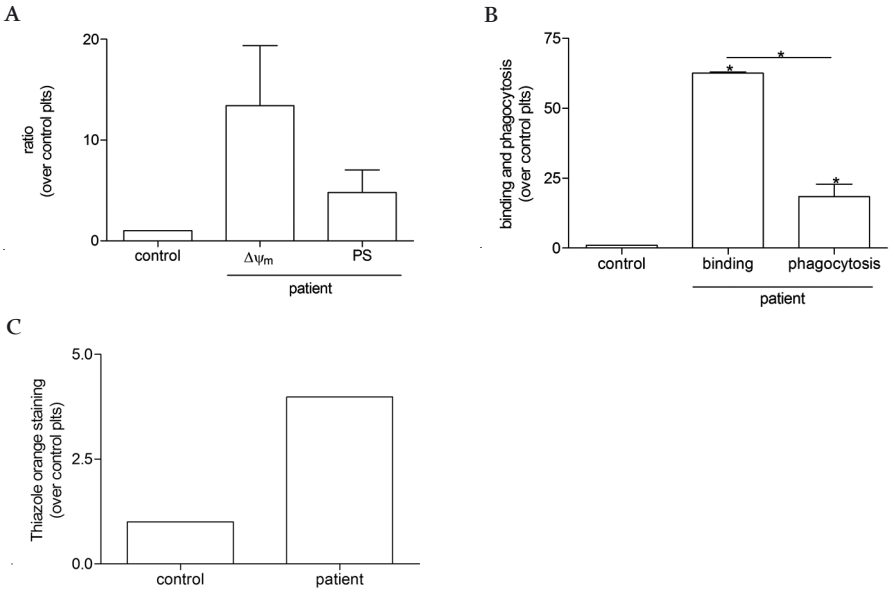
5.4.3 Patient platelets show enhanced apoptosis and destruction by macrophages

Platelet functions which are important for haemostasis are accompanied by induction of apoptosis and surface expression of 'eat me" signals for phagocytosis by liver macrophages and hepatocytes. To address the possibility of increased apoptosis induction, the membrane potential over the inner mitochondrial membrane ($\Delta\psi_m$) was inferred from the binding to the JC-1 dye. Patient platelet showed a 15-fold increased $\Delta\psi_m$-change, reflecting a strongly upregulated apoptosis (Figure 3A). Also, surface-expressed PS, a marker for apoptosis and an important "eat me" signal was increased. Thus, the activated state of patient platelets was accompanied by a strong upregulation of the apoptosis pathway.

Surface-expression of P-selectin, PS and GPIbα-clusters are potent "eat-me" signals for macrophage-mediated platelet destruction. Earlier work on platelets confirmed that these markers mediate platelet binding (P-selectin) and phagocytosis (PS, GPIbα-clusters) by macrophages [19]. Indeed, matured monocytic THP-1 cells responded to patient platelets with a 60-fold increase in platelet binding and a 20-fold enhanced phagocytosis by macrophages (Figure 3B). Possibly, these *in vitro* findings indicate that *in vivo* patient platelets are cleared much faster than normal platelets. The low platelet count (approximately 4-27x10^9/L) and the increase in reticulated platelets appear to support this conclusion (Figure 3C).

Figure 3: Increased levels of markers of apoptosis, destruction and platelet production

A) Apoptosis induction in platelets was analyzed by measuring the change in mitochondrial membrane potential, $\Delta\psi_m$, measured by JC-1 binding and compared with control platelets. PS-exposure was measured by binding to Annexin V, B) CFMDA-labelled platelets were incubated with matured monocytic THP-1 cells and binding/ phagocytosis were determined. C) Presence of reticulated platelets in control and patient platelets. Results are expressed as ratio of patient platelets/control platelets. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).



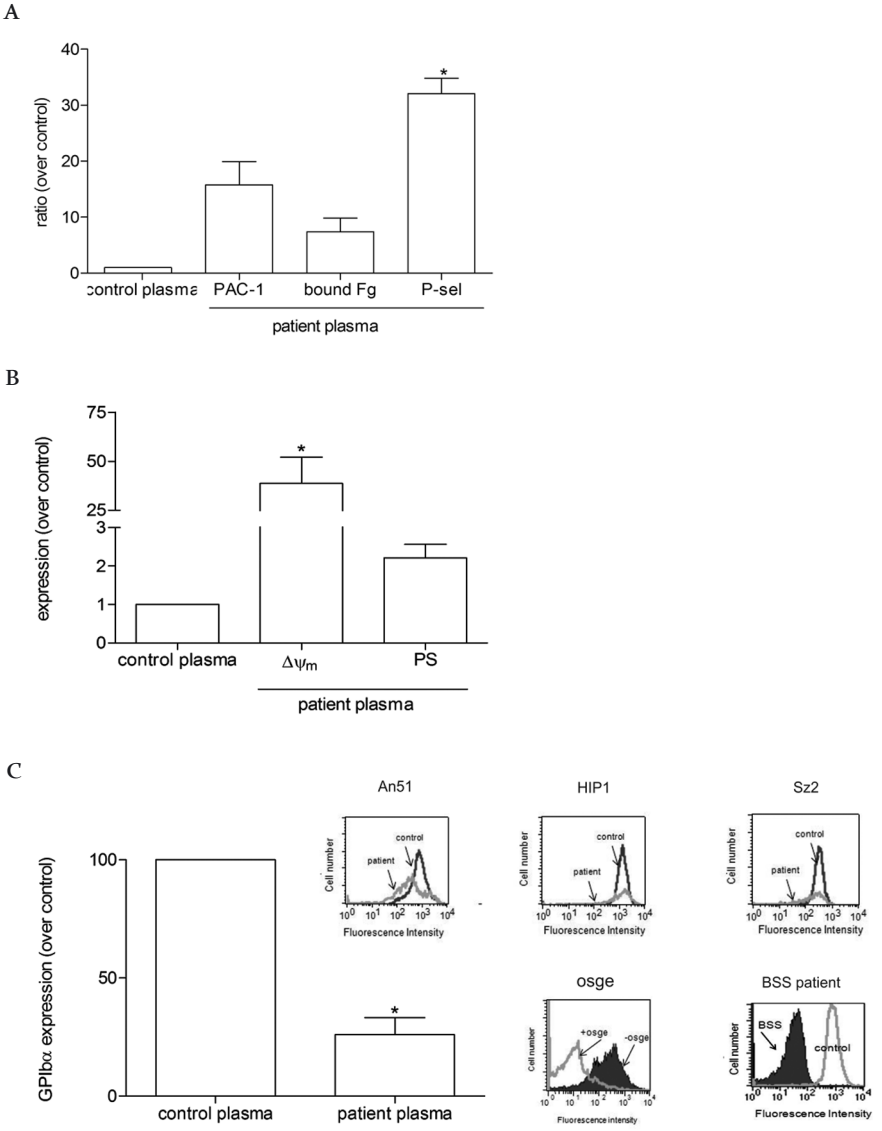
5.4.4 Patient plasma starts haemostasis and apoptosis responses in normal platelets

To address the question whether the increased activation phenotype of patient platelets reflects an intrinsic property or is the results of extracellular factors, platelets from control individuals were resuspended in patient plasma. Again, FACS-analysis revealed a strongly increased binding of PAC-1 antibody and surface-expressed fibrinogen and P-selectin compared with the same platelets in autologous plasma (Figure 4A). Concurrently, patient plasma induced a strong increase in $\Delta\psi_m$-change and PS-expression (Figure 4B). Thus, patient plasma induced haemostasis and apoptosis responses in normal platelets.

Incubation with patient plasma also reduced the binding of the anti-GPIbα antibody AN51 to about 40% of controls (Figure 4C, left panel and insert). A decrease in binding was also seen with anti-GPIbα antibodies HIP1 directed against aa 59-81 and SZ2 against aa 268-282 (Figure 4C, upper-right panel). The FACS patterns for antibody binding differed from those following GPIbα removal by osge and observed with GPIbα-deficient Bernard Soulier Syndrome (BSS)-patients (Figure 4C, lower-right panel), suggesting that they reflect competition with antibody binding by the patient plasma.

Figure 4: Patient plasma activates normal platelets

Control platelets were incubated with autologous or patient plasma (2 hours, 22°C), washed (in the presence of 10 ng/mL PGI_2) and resuspended in Tyrode's, pH 7.2. A) Haemostasis markers measured by FACS, B) Apoptosis markers indicating the change in mitochondrial membrane potential, $\Delta\psi_m$ and PS-exposure. C) Binding of the anti-GPIbα antibodies AN51 (FACS-histogram is shown as insert, left panel), HIP1 and SZ2 (upper-right panels). Also shown are results of platelets treated with osge to cleave GPIbα (lower-right, left panel) and platelets from a GPIbα-deficient patient (lower-right, right panel). Results are expressed as ratio of control platelets in patient plasma/control platelets in normal plasma. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).

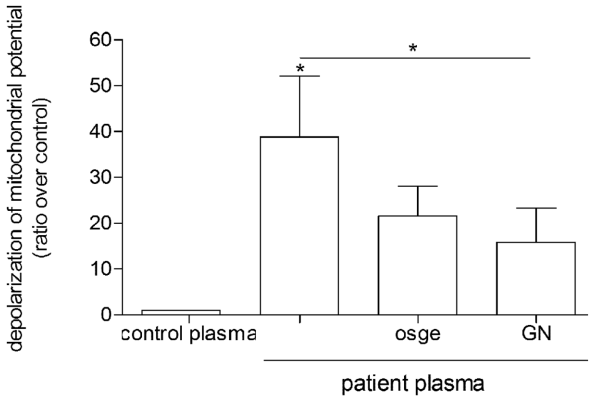


5.4.5 Activation of patient platelets through clustering of GPIbα?

To understand the role of GPIbα in the induction of haemostasis and apoptosis functions in more detail, normal platelets were incubated with osge to remove the extracellular part of the receptor and incubated with patient plasma. Compared with untreated platelets, osge-treatment caused a 40% reduction in the $\Delta\psi_m$-change, indicating that it strongly depended on an intact GPIbα (Figure 5). Also without osge-treatment a role for GPIbα became evident as interference with GPIbα-clustering by addition of GN strongly reduced the change in $\Delta\psi_m$. Together, these data provide preliminary evidence that patient plasma contains an antibody that starts haemostasis and apoptosis responses in normal platelets by inducing clustering of GPIbα.

Figure 5: Interferences with GPIbα reduces apoptosis induction by patient plasma

Normal platelets were incubated in patient plasma, as described in the legend to Figure 4, without and with treatment with osge (80 μg/mL) or the presence of GN (100 mM). The change in mitochondrial membrane potential, $\Delta\psi_m$ was measured. Results are expressed as ratio of normal platelets in patient plasma / normal platelets in control plasma. Data are means ± SEM (n=3) with significant difference $p < 0.05$ (*).



5.5 DISCUSSION

We describe a patient with hyper-reactive platelets which aggregated spontaneously during blood collection in citrate, EDTA or sodium heparin. A combination of citrate and PGI_2 reduced aggregation to a considerable extent and made further studies possible on the FACS by selecting ranges typical for single platelets. PGI_2 raises the intracellular platelet inhibitor cAMP, which through cAMP-dependent protein kinase A, interferes with many activating pathways and prevents further activation of the fibrinogen receptor GPIIb-IIIa. Its short half-life makes its inhibition transient and thirty minutes incubation at room temperature is normally sufficient to restore platelet responsiveness. Compared with platelets from healthy individuals, patient platelets have a number of abnormal characteristics as they express increased levels of activated GPIIb-IIIa, bound fibrinogen, P-selectin, $\Delta\psi_m$-change, PS and show increased binding/phagocytosis by macrophages. Important is the decreased binding of the anti-GPIbα antibody AN51.

Spontaneous platelet aggregation is an extremely rare abnormality. The few congenital defects described so far include the Von Willebrand Factor (VWF)-related syndromes Von Willebrand Disease type IIB Tampa and the Montreal platelet syndrome [20, 21, 22]. The patient presented here has no family history of increased bleeding and VWF-antigen levels, ristocetin-cofactor activity, protease activity and VWF-multimers were in the normal range, suggesting that she does not suffer from one of these VWF-related diseases. A syndrome called sticky platelet syndrome has described showing hyper-reactive platelets but the causes of these abnormalities remained uncertain [23].

Reconstitution experiments show that the cause of the platelet abnormalities is present in the patient´s plasma. Platelets from healthy individuals suspended in patient plasma developed the same abnormalities with signs of increased haemostasis and apoptosis properties without being stimulated with a platelet activating agent. The cause of the activation is probably an autoantibody generated against GPIbα, since patient plasma strongly interfered with the binding of three antibodies directed against different epitopes on this receptor. Apparently, GPIbα occupancy by the antibody starts signaling through the receptor inducing activation of the fibrinogen receptor GPIIb-IIIa, fibrinogen-binding and secretion of α-granules leading to surface-exposed P-selectin. The activation pathways may be similar to those initiated by VWF, which also triggers these functions. Platelet activation by VWF is facilitated by cytochalasin D treatment which releases GPIbα from cytoskeletal restrains and facilitates GPIbα clustering upon ligation. We demonstrated a similar GPIbα activation during cold-incubation leading to P-selectin expression and TxA_2-formation upon rewarming. These responses were inhibited by removal of extracellular GPIbα (osge) and prevention of GPIbα-clustering (GN). Exactly the same treatments interfered with the platelet activation by patient plasma, suggesting that its property to induce haemostasis and apoptosis properties resides in its capacity to cluster GPIbα. The question remains as to whether the patient's antibody is directed exclusively to GPIbα or also against other platelet receptors such as GPIIb-IIIa.

5.5.1 Acknowledgements

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Jigsaw falling into place

(Radiohead)

Chapter 6

General Discussion

6.1 CLUSTERING OF MEMBRANE RECEPTORS

Current attempts to improve the quality of platelet concentrates for transfusion focus on low temperature storage since this might better preserve the haemostasis functions and will suppress the growth of bacteria that occasionally contaminate platelet concentrates (Chapter 1). This thesis describes that cold storage induces clustering of the receptor for VWF, the GPIb-V-IX-complex, inducing apoptosis and upon rewarming formation of the platelet activator Thromboxane A_2 (TxA_2). Both properties are severe drawbacks in the search for better preservation methods since the combination of activated platelets with an accelerated death pathway and surface-expressed 'eat me'-signals for macrophages is not an attractive basis to improve the haemostasis properties post transfusion. The GPIbα-clustering upon cold/rewarming is not a unique property since GPIbα can also form clusters after stimulation by thrombin [1]. Many platelet receptors form homo- or heterodimeric complexes that start signaling. Examples include the fibrinogen receptor GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$), which forms clusters upon ligand-occupancy [2, 3], the thrombopoietin (TPO)-receptor cMpl, which forms homodimers after ligand binding [4], GPVI which oligomerizes to interact with collagen [5] and the *C*-type lectin receptor (CLEC-2) which forms large complexes upon activation [6]. Examples of receptors which form hetero-dimers are GPVI, which forms an oligomer with FcRγ [5], PECAM-1, which interacts and inactivates the GPVI-FcRγ-complex [7] and insulin-like growth factor-1 (IGF-1)-receptor, which heterodimerizes and thereby decreasing the binding to insulin [8]. GPIbα can also cluster with Fc-receptors FcγIIA and FcRγ [9].

6.2 GPIBα-CLUSTERING

6.2.1 Definition of GPIbα-clustering

It is still difficult to define the "clustering" of GPIbα. The question arises: what exactly is "clustering"? In the dictionary clustering is defined as the formation of groups. According to this definition, different GPIb-V-IX-complexes may associate or different GPIbα-molecules within one complex may interact with each other. Cold-incubation triggers the clustering of GPIbα-receptor, but the mechanism is not understood [10]. First, we demonstrated that after 0°C-incubation, GPIbα translocates from the membrane skeleton to the cytoskeleton. During this transition, GPIbα-molecules are released from their actin-anchors in the membrane skeleton increasing their lateral mobility and favoring conditions that lead to cluster formation. This is in line with the effect of the actin-inhibitor cytochalasin D, which increases the mobility of the receptor by release from the membrane skeleton thereby strongly enhancing the VWF-binding capacity of GPIbα [11, 12]. Second, we used the binding properties of the anti-GPIbα antibody AN51 directed against the *N*-terminal flank as an indicator of GPIbα-clustering. AN51 showed a lower binding affinity after cold-storage and subsequent rewarming at 37°C suggesting that GPIbα had undergone a conformational change (Chapter 2). Third, we demonstrated that cold-storage led to exposure of galactose-residues, as shown by enhanced binding to the sugar-binding lectin RCA-1, illustrating deglycosylation of GPIbα (Chapter 3). Together, these observations suggest that after cold-incubation GPIbα has shifted from a random distribution in the plasma membrane to clusters with enhanced VWF binding capacity. How these properties interact remains obscure. Hartwig/Hoffmeister measured the distribution of GPIbα-clustering on platelets by Transmission Electron Microscopy (TEM) with immunogold-labelled-GPIbα. After cold-storage, a shift was observed from linear arrays to a group-like appearance [10]. These authors indicated that in fresh platelets, GPIbα is connected to underlying F-actin filaments by Filamin A. Cold-storage leads to a re-arrangement of actin-filaments which drives the formation of GPIbα-clusters in the membrane. The clusters of deglycosylated GPIbα are recognized by MAC-1 receptors on macrophages, which bind exposed sugar-residues and start phagocytosis of cold-incubated platelets. Addition of β-*N*-Acetyl-*D*-Glucosamine (βGN) and UDP-Galactose reduced phagocytosis by macrophages *in vitro*. Unfortunately, the question whether these additions also preserved the linear arrays of GPIbα-molecules during cold-incubation was not addressed in these TEM studies.

6.2.2 Measurement of GPIbα-clustering

In the present studies we used another approach to detect changes in GPIbα conformation induced by cold-storage and rewarming. AN51 is an antibody against the *N*-terminal flank of GPIbα. We showed that cold-storage and subsequent rewarming at 37°C induced a shift in the affinity of AN51-binding to GPIbα (Chapter 2). The shift was inhibited when the monosaccharide

N-acetyl-*D*-Glucosamine (GN) was added, suggesting that the affinity-shift was a reflection of GPIbα-clustering. Cold alone did not result in affinity changes for AN51, and the major part of the shift was induced during rewarming suggesting that metabolic processes might be involved. Alternatively, the AN51-appraoch might be too insensitive to detect minor changes in GPIbα that precede the larger shift induced at 37°C. Direct analyses of changes in GPIbα-conformation and/or distribution can be obtained by applying the Resonance Energy Transfer (FRET) technique. This technique allows detection of clustering of two different proteins or within one complex. Preliminary data by our laboratory show strong correlations between changes in AN51-binding affinity and FRET-measurements including the inhibition by GN [Gitz E *et al,* NVTH symposium 2010].

6.2.3 Intracellular interference with GPIbα-clustering

Apart from the extracellular deglycosylation of GPIbα that induce clusters, intracellular mechanism may contribute. We used different metabolic inhibitors to understand the affinity changes of AN51 (Chapter 2). An inhibitor of Src-family kinases (PP1) decreased the changes in AN51-binding by cold-rewarming. After stimulation with VWF/ristocetin, Src binds to GPIbα and is co-translocated to the cytoskeleton triggering further signaling [13]. Possibly, Src is involved in the clustering of GPIbα. Also, the cAMP-raising agent Iloprost inhibited the changes in AN51-binding, again indicating that GPIbα-clustering is under control of metabolic processes. The important role of cAMP is illustrated in a report by Englund *et al.* After stimulation with VWF/ristocetin, GPIbα translocated from the membrane skeleton to the cytoskeleton. The actin-inhibitor cytochalasin D induced the same effect and enhanced VWF-binding. Importantly, the cAMP-raising agent PGE_1 inhibited VWF-binding and translocation of GPIbα to the cytoskeleton. CD-treatment interfered with this cAMP-dependent translocation of GPIbα to the cytoskeleton, showing that cAMP is important in mobility of GPIbα [11].
After VWF-stimulation, GPIbα together with the Src kinase family member Lyn, becomes associated with lipid rafts anchored to the cytoskeleton [14]. To investigate whether rafts play a role in cold-induced GPIbα-reallocation, we used the cholesterol-depleting agent methyl β-cyclodextrin. When we treated platelets with methyl β-cyclodextrin before cold-storage, there was no change in AN51-binding, suggesting that the cold-induced clustering of GPIbα does not depend on intact lipid-rich clusters (data not shown).
In addition to cold-treatment, we observed a fall in AN51-binding after other treatments including stimulation with the major ligand for GPIbα, VWF, platelet storage at Room Temperature (RT) for 24 hours and shear stress at 1600 s^{-1}; all these treatments activate GPIbα, triggering clustering. Actin-inhibitors cytochalasin D (CD) or Latrinculin A also induced AN51-changes. These findings demonstrate that clustering is induced when mobility of GPIbα is increased. GN inhibited the shift in all these treatments, except after stimulation with VWF. Because of the multimeric character of VWF, VWF might combine different GPIbα-molecules, leading to irreversible clustering.

By using an anti-VWF antibody, we investigated the contribution of VWF-secretion from platelet granules after cold-storage and rewarming. Secreted VWF could further enhance GPIbα-clustering. However, an anti-VWF antibody inhibited AN51-shift only for 30%, meaning that secretion of VWF plays only a minor role.
Importantly, we found that clustering of GPIbα is the main trigger of TxA_2-formation after cold-storage and subsequent rewarming. Studies with VWF-deficient platelets and show for the first time that upon clustering; GPIbα induces TxA_2-formation even without binding of a ligand.

6.2.4 Extracellular interference with GPIbα-clustering

Addition of GN interfered with GPIbα-clustering. GN inhibited many important platelet functions like agglutination, stress fiber-formation and spreading and TxA_2-formation (Chapter 2). This suggests that after VWF-induced signaling, there are changes in glycosylation status of GPIbα making the receptor sensitive to GN. Alternatively, this finding may indicate that GN inhibited clustering of GPIbα. Thus, there is a strong association between the glycosylation-status of extracellular GPIbα and intracellular signaling in platelets.

6.2.5 GPIbα as mechanic stress receptor

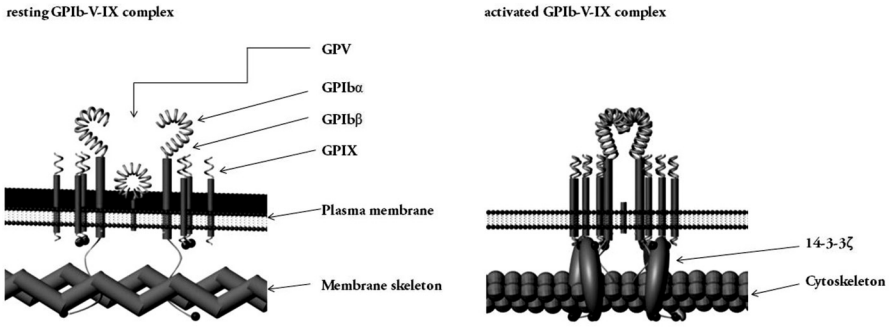
Mechanic stress receptors are regulated when physical forces are applied, like mechanical loading or shear stress. GPIbα and the fibrinogen receptor or integrin $\alpha_{IIb}\beta_3$ have been referred to as a mechanic stress receptors, sine they respond to increased shear with enhanced adhesive and signalling functions [15, 16]. When a magnetic drag force was used, the tyrosine phosphorylation of $\alpha_{IIb}\beta_3$ was enhanced and induced anchorage to the cytoskeleton in a reaction mediated by the MAPK ERK [15]. Arterial blood flow conditions enhance GPIbα-binding to its ligand VWF, initiating activation of GPIbα and platelet adhesion [18].
A related phenomenon was observed in our laboratory. Part of the blood collections and platelet isolations showed platelets with a lower basal affinity for AN51, suggesting that clustering of GPIbα had occurred before the start of the incubations. Further stimulation of these platelets did not result in good responses, because of this preactivation. These findings may imply that AN51-binding can be used as a sensitive marker for preactivated platelets in quality control regimen in blood banks.

6.2.6 Mobility of GPIbα in the plasma membrane

GPIbα is anchored in the membrane skeleton by filamin A. We and others found that after VWF-stimulation or CD-treatment, GPIbα was released from the membrane skeleton and translocated to the cytoskeleton (Figure 1 and [11]).
CD-addition is thought to increase the mobility of GPIbα, enhancing VWF-binding and VWF-induced agglutination. This mechanism was elegantly shown by Kasirer-Friede *et al.,* who constructed a chimeric GPIX-subunit which contained two tandem FKBP-repeats fused to the *C*-terminus of the cytoplasmic tail. GPIbα and GPIbβ were co-expression in CHO-cells; the

Figure 1: Translocation of GPIbα from the membrane skeleton (resting platelet) to the cytoskeleton (stimulated platelet)

(Courtesy of Arjan Barendrecht)



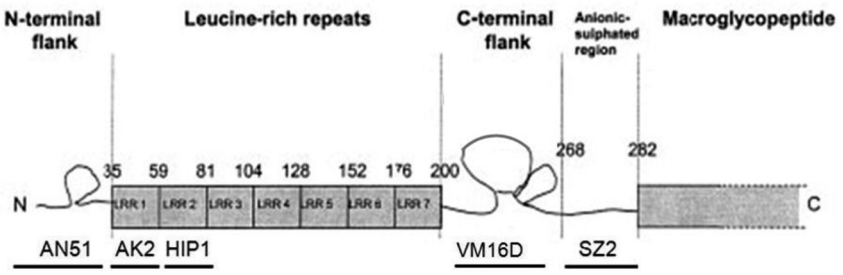
receptor complex was clustered by the addition of a FKBP-ligand. When GPIb-IX functions were investigated under static and flow conditions, these authors established that oligomerization of GPIb-IX increased cell adhesion, indicating that clustering of GPIbα is required for platelet adhesion [12]. We found that cold-storage, like CD-treatment, triggers translocation of GPIbα to the cytoskeleton. Addition of CD also induced AN51-changes, indicating that clustering of GPIbα is a prerequisite for GPIbα-signaling to the cytoskeleton (Chapter 2). GN inhibited the translocation of GPIbα to the cytoskeleton, illustrating that extracellular interference has a great effect on these intracellular signaling pathways.

6.2.7 Conformational change of GPIbα

AN51 is an antibody against aa 1-35 of the *N*-terminal flank of GPIbα. Antibodies against other epitopes of GPIbα (Figure 2), including aa 35-59 (AK2), aa 59-81 (HIP1), aa 200-268 (VM16D) and aa 276-282 (SZ2) did not show the cold-induced changes in affinity as observed with AN51. This suggests that GPIbα undergoes a conformational change that is restricted to aa 1-35.
The the crystal structure of GPIbα published by Huizinga *et al.* predicts that only the *C*-terminal flank of GPIbα is flexible, while the remainder of the molecule is rigid [20]. In contrast, Deckmyn *et al.* described explained the conformational changes in GPIbα with the 'horse-shoe'-model. Using antibodies for different epitopes of GPIbα, they found that after binding to VWF, the *N*-terminal flank region aa 1-81 was topographically associated with aa 201-268 of the *C*-terminal region [19]. These observations are more in agreement with our results and may indicate that the *N*-terminal flank of GPIbα can undergo a conformational change which is important for the function of GPIbα.

Figure 2: Epitopes for anti GPIbα antibodies

adapted from [19].



6.2.8 GPIbα-clustering: new insights and questions

An alternative explanation for the fall in anti-GPIbα antibody binding is that the receptor becomes inaccessible by internalization. Platelet activation by the PAR-1 agonist TRAP or by thrombin triggers a redistribution of GPIbα which is accompanied by P-selectin expression and an increase of GPIbα within the open canalicular system (OCS). This redistribution of GPIbα was found to be reversible [21]. Possibly, cold-induced GPIbα-clustering might induce a similar redistribution to the OCS, leading to lower affinity to AN51-antibody. The fact that antibodies directed against more proximal membrane regions of GPIbα do not show this decrease in binding, argues against this possibility. This issue should be addressed in later TEM-studies.

Because GPIbα is associated with GPIbβ, GPV and GPIX, one has suggested that shedding of GPV is required for GPIbα-clustering within this complex [1, 22]. The release of GPV would provide the space for GPIbα-reassociations that lead to cluster-formation. Shedding of GPV is seen after 60 minutes incubation with thrombin. Whether a similar process occurs during our incubations, remains to be established in the future.

It has recently been demonstrated that a 16 hours incubation at RT or 37°C leads to shedding of GPIbα from the platelet surface through a mechanism mediated by P38MAPK and ADAM17 [23]. The shorter incubation times and lower temperature applied in our incubations make it unlikely that this process interferes with our experiments. When we repeated our experiments in the presence of the ADAM17-blocker Tapi-2, the same results were found as in the absence of the inhibitor. It is possible, however, that at longer incubation times this P38MAPK induced GPIbα-release occurs even at low-temperature especially in view of the strong inhibition of the cold-induced arachidonic acid (AA)-accumulation by a P38MAPK-inhibitor (Chapter 4).

6.3 DEGLYCOSYLATION AND SECRETION AND AGGREGATION

6.3.1 Deglycosylation of GPIbα

According to the Hartwig/Hoffmeister group, GPIbα-clustering is related to the extracellular glycosylation status of GPIbα [10]. Already back in the 80's publications appeared showing that platelet stimulation by ADP, collagen or thrombin leads to more susceptibility to treatment with neuraminidase (NA), the enzyme responsible for the cleavage of sialic acid. Wu *et al.* claimed that this was caused by reorganization of Glycoproteins on the platelet membrane [24]. NA-treatment before platelet stimulation did not influence agglutination, whereas removal of *O*-linked glycans by *O*-glycosidase reduced responses by 50% [25]. NA is present in platelets at a low, but detectable level and can therefore play a role in GPIbα-signalling [26]. The enzyme peptide-*N*-glycosidase F (PGNase F) cleaves between the innermost GN and asparagines-residues cleaving all complex *N*-glycans. A study demonstrated that PGNase F impaired VWF-binding and platelet agglutination, while leaving surface-expression of GPIbα intact. An inhibitor of *N*-linked oligosaccharide synthesis in the Golgi, tunicamycin, decreased VWF-dependent aggregation of GPIb-IX expressing CHO-cells by 3-fold, again demonstrating the importance of glycans for the function of GPIbα [27].
The sugars βGN and UDP-Galactose inhibited platelet phagocytosis by restoring the glycosylation of GPIbα, measured by a panel of sugar-binding lectins. The enzyme β-1, 4-galactosyltransferase (β4GalT1) catalyzes the linkage Galβ-1GNβ1-R, restoring sugar-moieties on GPIbα after deglycosylation. It was demonstrated that there was no requirement for addition of β4GalT1 since UDP-Galactose alone was sufficient to inhibit phagocytosis of platelets [28]. The presence of β4GalT1 was demonstrated in the supernatant of washed platelets and it was suggested that it is possibly associated with the platelet membrane [28].
Deglycosylation is also an important trigger in platelet clearance in different diseases. Enhanced removal of desialylated platelets is observed in sepsis and caused by NA-activity of *Streptococcus pneumonia*. The parasite *Trypansoma cruzi* causes Chagas' Disease. It contains high levels of NA, leading to desialylation of platelets and enhanced clearance, which may explain the thrombocytopenia observed in infected patients [29].

6.3.2 Deglycosylation and apoptosis induction

We demonstrated in chapter 3, that the cold-induced change in GPIbα triggers platelet apoptosis. The finding that addition of GN inhibited all apoptosis responses suggests that exposure of sugars plays an important role in GPIbα-mediated apoptosis. It is interesting to note that extracellular changes in glycosylation of a receptor lead to intracellular signaling and apoptosis. A recent publication demonstrated that absence of the β-galactoside sialyltransferase (ST6Gal I) caused a functional abnormality of platelet endothelial cell adhesion molecule (PECAM-1). In ST6Gal I-deficient mice, the level of tyrosine-phosphorylation was significantly decreased. Because

PECAM-1 is an inhibitor of apoptosis, PECAM-deficient endothelial cells were cleared faster than WT. PECAM-1 functions by homophilic interactions and in ST6Gal I-deficient mice, these reactions were abolished. Again, this finding shows that the extracellular glycosylation status of GPIbα is important in signal induction [30]. Another report demonstrated that sialylation by *Trypanosoma cruzi* triggered apoptosis in thymocytes. Again, this shows a link between deglycosylation and signaling to apoptosis [31].

6.4 PLATELET APOPTOSIS

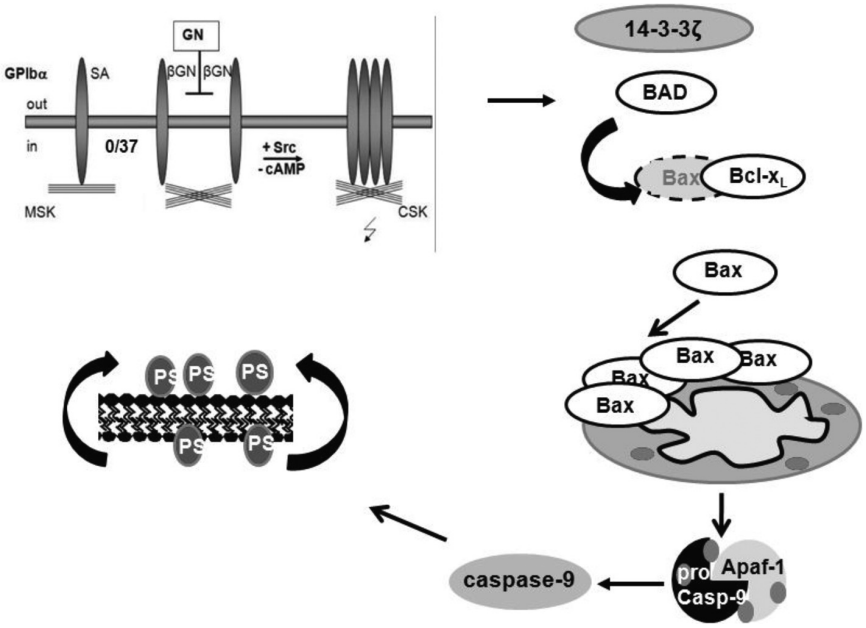
6.4.1 Role of GPIbα

In chapter 2, we demonstrate that clustering of GPIbα by cold/rewarming triggers apoptosis (Figure 3). Also, platelet stimulation by VWF, lead to an increase in apoptosis markers such as a change in $\Delta\psi_m$ [our data and 32, 33, 34].

An interesting disease for further studies of the role of GPIbα in apoptosis is the Bernard-Soulier Syndrome (BSS). BSS-patients lack the GPIb-V-IX-complex or possess mutations leading to truncated receptors in the complex. One would expect that platelets deficient in GPIbα have become resistant against apoptosis and would have a higher platelet count than controls. The opposite is found: BSS-patients are thrombocytopenic and display enhanced apoptosis characteristics [35]. This discrepancy might be explained by the fact that BSS-patients have aberrant apoptosis in the MKs, resulting in impaired proplatelet formation and release of giant platelets [36].

Akt/PKB, a serine/threonine kinase activated by Phosphoinositide 3-kinase (PI3K), triggers cell survival and prevents apoptosis. Phosphorylation by Akt/PKB affects many different cell death players, like the BH3-only protein Bad promoting its association with 14-3-3 proteins or the

Figure 3: GPIbα-clustering leads to platelet death via the intrinsic apoptosis pathway



pro-apoptosis Bax inducing interaction with pro-survival Bcl-x_L [37]. The PI3K/Akt-pathway is also important in platelet-signaling [38]. After stimulation with VWF, PI3K binds GPIbα and together with 14-3-3ζ translocates to the cytoskeleton. Interestingly, in GPIbα-deficient mice, an increase in TPO-mediated Akt/PKB-phosphorylation was found leading to a higher percentage of high ploidy MKs [36]. This suggests that in MKs, GPIbα normally contributes to regulation of megakaryopoiesis by inhibiting the Akt/PKB-pathway.

6.4.2 Role of P38MAPK and cPLA$_2$

We found that cold-storage triggers a first wave of apoptosis (Chapter 3). Subsequent rewarming initiated a second wave of GPIbα-dependent apoptosis by the formation of TxA_2. In chapter 4, we show that cold-storage also leads to the release of AA from the membrane, by activation of P38MAPK/$cPLA_2$.

After VWF-stimulation at 37°C, GPIbα is known to release of AA from membrane phospholipds leading to formation of TxA_2 [39]. Platelets contain four isoforms of P38MAPK (α, β, γ and δ), the most abundant forms being the α- and β-isoforms [40, 41]. P38MAPK-blocker SB203580 abolished cold-induced $\Delta\psi_m$-changes completely, indicating that P38MAPK is active (Chapter 3). SB203580 did not affect AN51 binding to GPIbα, indicating that P38MAPK is downstream of GPIbα-clustering. We found that after blockade of P38MAPK by SB203580, the GPIbα-induced TxA_2-formation after 0/37°-incubation was strongly inhibited (Chapter 2). SB203580 is not a very specific inhibitor and also inhibits COX-1 and thereby the formation of TxA_2 [42]. We showed that during cold-storage, the COX-1 inhibitor indomethacin rather increased than decreased the change in $\Delta\psi_m$ indicating that the effect of SB203580 is not at the site of COX-1 activity.

As stated earlier, prolonged storage of platelets at blood bank conditions triggers ADAM17-mediated shedding of GPIbα, leading to enhanced platelet clearance [23]. After storage for 7 days, about 45% of GPIbα was shed. Refrigeration of murine platelets for 48 hours and subsequent rewarming also led to a partial fall of platelet-bound GPIbα. Pharmacological inhibition of P38MAPK and ADAM17 prevented GPIbα shedding [43]. Interestingly, shedding could also be induced by the uncoupler of the mitochondrial potential carbonyl cyanide m-chlorophenylhydrazone (CCCP). This raises the possibility that ADAM17-activity is downstream of mitochondrial damage.

6.4.3 Bcl-2 proteins

Although platelets have no nucleus, they are capable of synthesis of different proteins such as Tissue factor [44], Bcl-3 [45] and COX-1 [46]. Today, it is unclear whether the increase in expression of pro-apoptosis proteins Bax or Bak after apoptosis-induction in platelets is due to protein synthesis or reflects a conformational change in these proteins. Bax contains a cryptic *N*-terminal domain, which is only exposed after activation. We used an anti-Bax antibody that specifically recognizes this exposed residue. Only activated-Bax can insert into the mitochondrial membrane. The Bak-protein already resides in the mitochondria and it is

therefore not necessary to be translocated. Cold-storage not only showed enhanced binding to the Bax-antibody, but also a translocation of Bax to the mitochondrial fraction. This suggests Bax is in an activated conformation initiating apoptosis (Chapter 3). These findings suggest that the increase in Bax-expression reflects its activation rather than *de novo* synthesis.

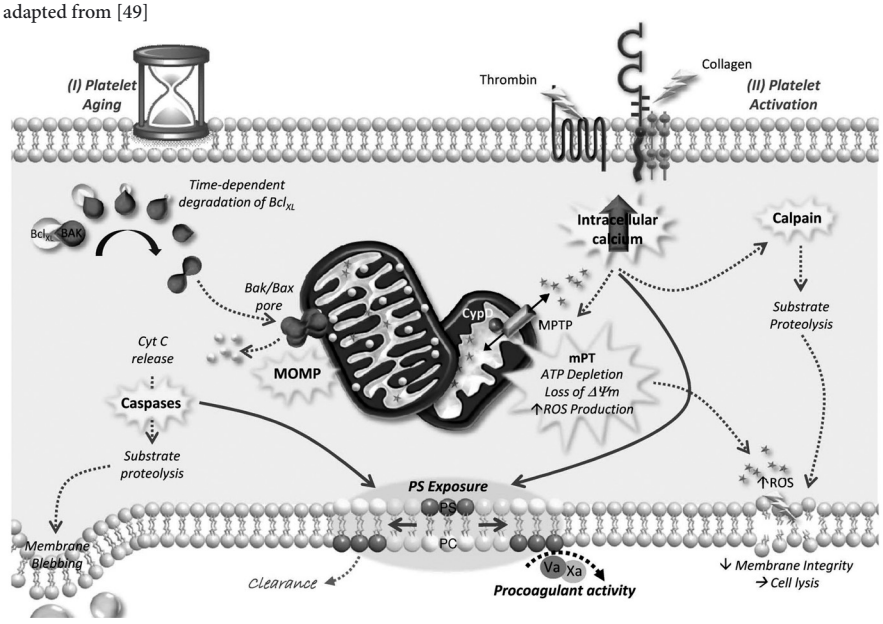
Genetic ablation of Bcl-2 family member proteins provided clues which protein(s) are crucial for the inhibition or initiation of platelet apoptosis. Platelets deficient in the prosurvival protein Bcl-x_L are more prone to apoptosis and clearance leading to thrombocytopenia [47]. Also, when Bcl-x_L is pharmacologically inactivated by the BH3-mimetic ABT-737, thrombocytopenia is induced. These results indicate the importance of this protein in platelet survival. Bcl-x_L constrains the apoptosis proteins Bax/Bak. Surprisingly, only lack of Bak or Bax/Bak, but not Bax alone, protected platelets against apoptosis induction by ABT-737 [47]. This suggests that Bak plays a crucial role in platelet apoptosis. In chapter 3, we found a major role for pro-apoptosis Bad protein in platelet survival. In fresh platelets, phosphoBad is bound to 14-3-3ζ protein, which inhibits its pro-apoptosis function. However, after cold-storage of platelets, Bad is released from this complex and can trigger apoptosis. Earlier findings already showed that Bad-knockout mice are characterized by elevated platelet numbers caused by a prolonged life span, again demonstrating that Bad is a key player in platelet apoptosis [48].

6.4.4 Platelet secretion and aggregation vs apoptosis

According to Schoenwaelder *et al.*, in platelets two important signaling pathways can be distinguished (Figure 4). First, apoptosis can be induced after stimulation with platelet agonists,

Figure 4: Platelet aging leading to apoptosis

adapted from [49]

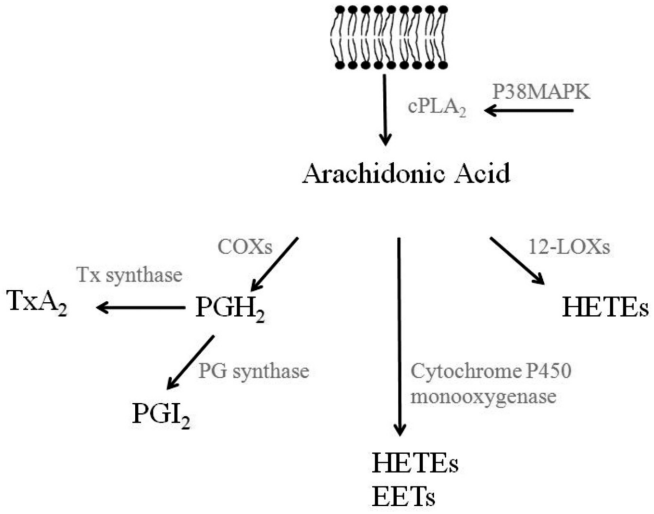


like thrombin or collagen. Also, these agonists trigger the procoagulant function of platelets. This apoptosis induction was inhibited by metabolic platelet inhibitors [49].
It was suggested that this process was independent of caspases and Bax/Bak proteins. Second, use of the Bcl-x_L inhibitor ABT-737 also induced platelet apoptosis but no thrombin-generation was observed. Also, this apoptosis induction was insensitive for metabolic platelet inhibitors. In contrast to these findings, we showed a link between platelet activation (aggregation and secretion) via GPIbα and the induction of platelet apoptosis. GPIbα-changes after cold-storage were the result of signaling of GPIbα, shown by the interference with metabolic inhibitors such as the Src-kinase inhibitor PP1 and the cAMP-raising agent Iloprost (Chapter 2). Clustering of GPIbα leads to Bax-activation and translocation to the mitochondria, depolarization of the inner mitochondrial membrane, caspase-9 activation and PS-exposure (Figure 3 and Chapter 3). These findings clearly demonstrate that the induction of apoptosis by GPIbα is dependent on platelet signaling.
When we stimulated platelets by the non-physiological ABT-737, a dose-response in aggregation was found which was blocked by COX-1 inhibitor indomethacin, also indicating platelet activation via TXA_2-formation (data not shown).

6.5 ENZYME ACTIVITY AT LOW TEMPERATURE

We found evidence for enzymatic activity of P39MAP/cPLA$_2$ involved in AA-metabolism at 0°C incubation (Figure 5).

Figure 5: AA-metabolism in platelets



There are few reports of enzyme activities at low-temperature. RBCs are generally stored at 1-6°C and under these conditions 2, 3-bisphosphoglycerate phosphatase is active and contributes to the storage lesion [50]. Moreover, in the more northerly latitudes, certain organisms have the ability to adapt to low-temperatures, including certain fishes, insects and hibernating mammals. Glyceraldehyde-phosphate dehydrogenase (GAPDH) retains complete activity at 0°C in an arctic fish (*Dissostichus mawsonini)*, the carpenter ant (*Camponotus pennsylvanicus)* and clam (*Mya arenaria)*. In general, the more primitive invertebrate animals contain GAPDH capable of withstanding cold-stress more effectively than more developed animals [51]. During hibernation, squirrels maintain a body temperature of 3-4°C and despite this, the enzyme involved in serotonin metabolism, monoamine oxidase remains active [52].

We describe that after storage at low-temperature, platelet apoptosis was induced by the release of AA (Chapter 4).

First, we demonstrated indirect evidence for active cPLA$_2$ leading to AA-mediated changes in $\Delta\psi_m$. Second, the enzyme P38MAPK was active, as demonstrated by the inhibitor SB203580 which abolished all apoptosis responses. Third, lipoxygenase and cytochrome P450 monooxygenase were active since inhibition of these enzymes enhanced platelet apoptosis. To address whether P38MAPK is indeed active at low-temperature, future experiments focusing on the phosphorylation of this enzyme at 0°C may provide more insight.

6.6 GPIBα-INDUCED THROMBOCYTOPENIA

In this thesis, we describe a patient with spontaneous aggregation, caused by anti-platelet antibodies, probably directed against GPIbα (Chapter 5). We showed that the patient platelets had enhanced markers for haemostasis functions and apoptosis functions and were faster phagocytosed by macrophages. We found enhanced P-selectin expression, PAC-1 binding, bound fibrinogen, PS-exposure, $\Delta\psi_m$-changes, binding and phagocytosis compared with controls. Patient platelets had reduced binding of three different GPIbα-antibodies. Importantly, patient plasma induced secretion, aggregation and apoptosis in platelets from healthy individuals, responses that were strongly impaired after removal of extracellular GPIbα by osge or inhibition of GPIbα-clustering by GN. These results suggest the presence of a plasma factor specifically affecting GPIbα-signaling. Whether the patient antibodies are exclusively directed against GPIbα or also bind other receptors such as $\alpha_{IIb}\beta_3$ has to be determined in future experiments.

6.7 CONSEQUENCES FOR THE STORAGE OF PLATELET CONCENTRATES

Although the application of low temperature for the storage of platelets for transfusion has deepened our insight in induction of haemostasis and apoptosis signaling, attempt to apply this technique in the clinic have met little success. The new approaches reduced platelet phagocytosis by macrophages *in vitro*. The use of UDP-Galactose for galactosylation of platelet GPIbα was only successful after short-term storage at low-temperature (< 48 hours). When galactosylated platelets were administrated into human subjects after long-term storage, it resulted in poor survival [53]. Whether the use of GN to prevent GPIbα changes after long-term storage leads to better survival has also still to be determined. A complicated factor is the recent observation that long-term storage of platelets at low-temperature leads to clearance by hepatocytes rather than by macrophages [54].

Changes in AN51-binding were shown to be reversible upon rewarming at 37°C, suggesting GPIbα-clustering is reversible in nature (Chapter 2). This can provide opportunities for future experiments *in vivo*. Cold-storage can still be useful to preserve platelet function, compared to the current storage at RT in blood banks. The reduction of bacterial growth in cold-storage is also an important advantage preventing sepsis after transfusion in patients.

Alternatively, focus could be on prevention of the intracellular changes caused by GPIbα-clustering rather than on interference with the clustering itself. New approaches can be sought in the depletion of AA (Chapter 4). After 4 hours of storage at 0°C, apoptosis and *in vivo* clearance were much reduced. These positive effects on apoptosis induction remain present after long-term storage of 72 hours at 0°C (data not shown). Future experiments should investigate whether these platelets regain their haemostasis functions by reuptake of AA during circulation.

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Nederlandse Samenvatting

INTRODUCTIE

Bloedplaatjes zijn kleine schijfvormige cellen in het bloed. Na verwonding aan de beschadigde bloedvatwand kleven de bloedplaatjes ter plekke aan elkaar en aan de bloedvatwand. Hierdoor wordt de bloeding snel gestopt.
Het toedienen van bloedplaatjes (transfusie), kan levensreddend zijn bij patiënten met kanker of patiënten met een bloedingsneiging die een chirurgische ingreep moeten ondergaan.
Het bewaren van bloedplaatjesconcentraten (PCs) is al jaren een struikelblok in de bloedbanken. De huidige bewaarcondities zijn maximaal 5-7 dagen bij een temperatuur van 22°C. Bewaren onder deze condities leidt tot een vermindering van de hemostatische functies van de bloedplaatjes. Ook is er een kans op bacteriegroei en daardoor een kans op bacteriële infecties zoals sepsis, als deze PCs toegediend worden aan patienten. Een nieuwe methode om PC te bewaren is bij lagere temperaturen (0-4°C). Echter het gekoeld bewaren leidt tot een snellere afbraak van de getransfundeerde bloedplaatjes door de levermacrofagen. Dit wordt veroorzaakt door het bijelkaar komen (clustering) van de Glycoproteine Ib (GPIb) receptoren op de bloedplaatjes. Deze clusters kunnen worden herkend door de MAC-1 receptor op macrofagen en op deze manier opgeruimd worden. De GPIb-receptor is een membraanreceptor en bevat veel suikerstaarten aan de buitenkant. Het is aangetoond dat er een verband is tussen clusteren van GPIb-receptor en de veranderingen van die suikers aan de buitenkant. Deze suikers laten los van GPIb bij lage temperaturen en dit zou clustering veroorzaken met de nadelige gevolgen vandien. De bloedplaatje-bloedplaatje interactie na een beschadiging van de bloedvatwand wordt gestart door binding van GPIb aan het klevingseiwit von Willebrand factor (VWF). GPIb wordt geaktiveerd en daardoor geclusterd door toevoeging van VWF.

GPIB-CLUSTERING

De afgelopen jaren hebben wij onderzoek gedaan naar de effecten van koud bewaren op de GPIb-receptor van bloedplaatjes. Eerst, hebben we de bloedplaatjes geïsoleerd uit bloed van vrijwillige donoren uit het UMC en vervolgens bewaard voor 4 uur op ijs (0°C). Hierna hebben we ze 1 uur bij lichaamstemperatuur (37°C) gezet om de post-transfusie condities na te bootsen. We hebben gevonden dat het toevoegen van een suiker, GN, het clusteren van de receptor tegengaat. Wanneer GN gebruikt wordt, is ook de afbraak in het lichaam van bloedplaatjes nadat ze koud bewaard zijn, verminderd. Op deze manier blijven bloedplaatjes langer in het lichaam circuleren en kunnen ze hun werk blijven doen.
In Hoofdstuk 2 is een methode beschreven om de clustering van GPIb te meten. We hebben een GPIb-bindende antistof (AN51) gebruikt en we zagen na koud bewaren van bloedplaatjes dat er verminderde binding is aan deze antistof. Dit betekent dat de GPIb-receptor zo veranderd is dat hij minder herkend/gebonden wordt door AN51. De suiker GN remde de verandering in AN51-binding na koud bewaren, zodat kon worden vastgesteld dat AN51 geclusterde GPIb kan meten.

De AN51-veranderingen waren gevoelig voor verschillende metabole bloedplaatjes remmers, dit duidt op signalering van GPIb. Het toevoegen van GN heeft een remmende invloed op belangrijke bloedplaatjesfuncties, zoals binding aan VWF, bloedplaatje-bloedplaatje interacties, verklontering van bloedplaatjes en vorming van thromboxaan. Hierdoor was duidelijk dat de GPIb-receptor essentieel is in al deze bloedplaatjesfuncties.
Ook is beschreven dat het koud bewaren van bloedplaatjes leidt tot het vrijkomen van de bloedplaatjes stimulator thromboxaan. Normaal gesproken komt thromboxaan alleen vrij als bloedplaatjes geaktiveerd worden door toevoeging van een stimulator, zoals VWF. In bloedplaatjes zelf is ook VWF aanwezig en dit komt vrij na aktivatie, dit zou ook bloedplaatjes verder kunnen aktiveren en zodoende GPIb kunnen clusteren. Patienten met von Willebrand ziekte hebben geen VWF en daardoor een grote bloedingsneiging. Het koud bewaren van deze bloedplaatjes leidde ook tot vorming van thromboxaan en dus is het uit te sluiten dat er veel VWF vrijkomt uit bloedplaatjes na koud bewaren. Patienten met het Bernard Soulier Syndrome hebben geen GPIb en ook een grote bloedingsneiging daardoor; na koud bewaren van bloedplaatjes van zo'n patient was er geen thromboxaan gevormd, zodat we konden concluderen dat de thromboxaan-vorming door GPIb wordt gestart.

DOOD VAN EEN BLOEDPLAATJE

Het is beschreven dat bloedplaatjes maximaal 10 dagen leven in het lichaam. Hierna worden ze door het lichaam opgeruimd door macrofagen uit de lever, milt of longen. Als bloedplaatjes geaktiveerd worden door aktivatoren, krijgen ze verschijnselen van gereguleerde celdood (apoptose). Dit vindt ook plaats als bloedplaatjes lang bewaard worden bij bloedbank condities. In Hoofdstuk 3 hebben we onderzocht of er een link was tussen het clusteren van GPIb en deze gereguleerde celdood. We hebben gevonden dat koud bewaren van bloedplaatjes leidt tot celdood, gestart door GPIb-clustering. Er zijn verschillende celdood markers en eiwitten. Nadat celdood gestart wordt, komt een hele signaalcascade op gang: verschillende eiwitten worden actief; de zg. Bcl-2 eiwitfamilie speelt hierin een cruciale rol. De Bcl-2 eiwitfamilie bestaat uit zowel celdoodeiwitten als overlevingseiwitten. In een gezonde cel zit celdoodeiwit Bax vast aan overlevingseiwit Bcl-x_L, wanneer een cel verouderd/doodgaat gaat Bax los en wordt actief. Bax kan gaten maken in de membraan waardoor de cel celdoodeiwitten gaat lekken, met celdood tot gevolg.
Na koud bewaren van bloedplaatjes vinden de volgende processen plaats: GPIb bindt het eiwit 14-3-3, zodat 14-3-3 loskomt van een celdoodeiwit genaamd Bad. Bad kan vervolgens binden aan overlevingseiwit Bcl-x_L waardoor celdoodeiwit Bax wordt geaktiveerd. Uiteindelijk leidt dit tot veranderingen in de energiecentrale van de cel (mitochondrium), waardoor cytochrome *c* vrijkomt en de caspase enzym geaktiveerd wordt. De laatste stap is het op de oppervlakte komen van phosphatidylserine (PS) wat ook herkend wordt door de macrofagen en op die manier de bloedplaatjes opgeruimd kunnen worden. Het gebruik van GN om de clustering van GPIb tegen te gaan, remde ook de celdood routes.

In Hoofdstuk 4 zijn we verder gegaan met het onderzoeken van de celdoodroutes in bloedplaatjes na koud bewaren. We vonden dat koud bewaren leidde tot het vrijkomen van vetzuur AA uit de celmembraan. Normaal gesproken vindt dit plaats bij stimulatie van de bloedplaatjes en wordt van AA uiteindelijk thromboxaan gemaakt. Het koud bewaren van bloedplaatjes leidde tot het starten van gereguleerde celdood. Wanneer we een remmer gebruikten voor een enzym (P38MAPK) die normaal verantwoordelijk is voor het aktiveren van het enzym wat AA vrijmaakt uit de membraan, zagen we een sterke daling in celdood. Ook zagen we dat dit enzym aktief is bij lage temperatuur. Het toevoegen van AA aktiveerde ook de gereguleerde celdood van de bloedplaatjes, gemeten door veranderingen in het mitochondrium en in verschillende eiwit-eiwit complexen, van bijvoorbeeld 14-3-3 met celdoodeiwit Bad of 14-3-3 met het enzym verantwoordelijk voor het maken van thromboxaan (COX-1). AA-toevoeging aan bloedplaatjes leidde tot dezelfde veranderingen in eiwit-eiwit complexen. Na het koud bewaren van bloedplaatjes bleken een aantal enzymen verantwoordelijk voor de omzetting van AA tot andere stoffen, zoals LOX, actief te worden. Normaal is dit alleen bij lichaamstemperatuur van 37°C. Wanneer we remmers voor deze enzymen gebruikten, werd celdood gestimuleerd. Het lijkt erop dat deze enzymen en de stoffen die ze maken, normaal een beschermende functie hebben in bloedplaatjes. Door het wegvangen van AA uit de bloedplaatjes met vetzuur-vrije eiwit albumine, werd de celdood geremd. Als voorstadium voor transfusie van de bloedplaatjes in mensen, hebben we een transfusie gedaan in muizen.Wanneer we de afbraak van deze bloedplaatjes bekeken in muizen, zagen we dat bloedplaatjes zonder AA langer overleefden dan bloedplaatjes met AA. In mensen zou dit ook het geval kunnen zijn. Op deze manier kunnen bloedplaatjes toch koud bewaard worden zonder dat ze doodgaan tijdens het bewaren of worden opgeruimd in het lichaam.

PATIENT MET HYPERACTIEVE BLOEDPLAATJES

In Hoofdstuk 5, hebben we onderzocht hoe het kan dat een vrouwelijke patient van 70 jaar hyperactieve bloedplaatjes heeft. De bloedplaatjes in het bloed verklonteren spontaan na bloedafname. De patient had geen familie geschiedenis van bloedingen en had zelf ook geen last van bloedingen, alleen milde klachten. We hebben gevonden dat de bloedplaatjes van de patient sterk verhoogde aktivatie- en ook celdoodmarkers hebben. Ook zagen we minder GPIb in de bloedplaatjes. Wanneer we bloedplaatjes van gezonde donoren namen en we plasma van de patient toevoegden, zagen we dezelfde activatie. Deze activatie was te remmen wanneer GPIb verwijderd werd met een enzym of met de suiker GN. Dit duidt op activatie via GPIb. Ook zagen we minder binding van antistoffen tegen GPIb. We hebben geconcludeerd dat de patient afweerstoffen maakt tegen GPIb waardoor deze gaat clusteren en aktief wordt. Hierdoor worden de bloedplaatjes extreem geactiveerd, gaan waarschijnlijk sneller dood en worden daardoor sneller opgeruimd in het lichaam.

CONCLUSIES EN TOEKOMSTPLANNEN

De resultaten van dit proefschrift kunnen gebruikt worden om de houdbaarheid van bloedplaatjes te vergroten. Ook is het belangrijk dat de bloedplaatjes nog goed kunnen functioneren in het lichaam na het bewaren en na een transfusie. Het bewaren bij lage temperaturen zou nog steeds een geschikte methode zijn. Wel moet er goed gekeken worden op welke manier clustering van GPIb of de consequenties van deze clustering kunnen worden tegengegaan.

Curriculum Vitae

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 12 December, 1978 in Delfzijl. Ze begon met de opleiding Hoger Laboratorium Onderwijs (HLO), richting Biotechnologie aan de Hanzehogeschool in Groningen in 1996. Nadat ze haar diploma had behaald in 2000, werkte ze een aantal jaren als research analist voor ze begon als research analist voor twee jaar bij de afdeling Anatomie en Fysiologie van de Faculteit Diergeneeskunde in Utrecht, onder begeleiding van Dr. K. Eizema. In 2006 begon ze als AIO aan een promotieonderzoek op de afdeling Laboratorium voor Klinische Chemie en Hematologie (LKCH) in het Universitair Medisch Centrum in Utrecht, onder begeleiding van Prof. Dr. J.W.N. Akkerman; de resultaten hiervan staan beschreven in dit proefschrift. In de toekomst, zal ze haar wetenschappelijke carrière voortzetten.

The author of this thesis was born on the 12th of December, 1978 in Delfzijl, the Netherlands. She started her Bachelor study Biology and Medical Research at the Hanzehogeschool in Groningen in 1996. After graduation in 2000, she worked for several years as a research technician, before she started as a research technician for two years at the Department of Anatomy and Physiology at the Faculty of Animal Sciences in Utrecht, under the supervision of Dr. K. Eizema. In 2006 she started as a Ph.D-student, at the Department of Laboratory of Clinical Chemistry and Haematology at the University Medical Center in Utrecht, under the supervision of Prof. Dr. J.W.N. Akkerman; the results are described in this thesis. In the future, she will be continuing her scientific career.

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Martin: grote labopperhoofd met al die perfusiekennis, bedankt. Eindelijk nu lekker zweefvliegen en sporten wanneer je maar wil.

Michael: Nog even doorzetten, het komt goed uiteindelijk! Goed om af en toe de laatste-jaars stress te delen.

Milan: Lieve Milan, een zeer onterechte plaats zo in het midden van dit ABC... Maar, wat ben ik trots met iemand aan mijn zij, zoals jij! Woorden schieten tekort in hoe ik jou moet bedanken voor je liefde, geloof in mij en het aanhoren van mijn saaie werkverhalen. (en het voorkomen dat het huis een puinhoop werd!) Je hebt nooit geklaagd, zelfs niet als ik weer eens laat thuis kwam of bekaf was. En wat mag ik gelukkig zijn dat je met me mee gaat, waar dan ook heen! Ik weet zeker dat 'onze' tijd nu echt begint, ik verheug me erop!

Mirjam: Wat ben jij een chaoot, maar alles lukt toch altijd weer. Ook al was je er maar 1x per week (altijd bij de borrels!), het was altijd meteen weer gezellig. Heel erg bedankt voor je hulp bij promotiedingetjes en sollicitatiebrieven. Veel geluk in Caïro met de kleine Roos, ik kom langs hoor!

Margo: Een goed voorbeeld van collega's die vrienden zijn geworden. Ook al zijn onze levens enorm verschillend, we blijven nooit uitgepraat! Bedankt voor je begrip, altijd.

Ouders: de allerliefste ouders zijn jullie. Wat goed dat ik mijn verhaal altijd kan doen, ook al zijn jullie ver weg. Superbedankt voor jullie steun, altijd!

Robbert: Op borrels veel gezellig geouwehoerd, succes in de States.

Roger: bedankt voor je goede samenwerking met die bijzondere patienten.

Rolf: 'de oude wijze man' van het lab, is er iets wat jij niet weet? Bedankt voor het meedenken, je luisterend oor en je goede muzieksmaak. Ben blij dat je sociale kant weer helemaal terug is!

Ronan: You left way too early; I miss your dancing moves and fun. Sorry for not coming to your lab…

Rutger: blij om mijn ei kwijt te kunnen in avondjes in 'de Zaak', ook al ben je toch een alphaman, het was altijd weer leuk om te ouwehoeren over vanalles en nog wat. Kom langs ok?!

Ruth: Nu al ben ik blij dat ik jou heb 'ingehuurd', bedankt alvast voor dit mooie boekje!

Sabina: Lieve zus, jij weet hoe je door moet zetten en je gaat het zeker halen! Veel succes met het redden van mensenlevens, daar heb ik veel respect voor. Ik ben erg trots op je meid!

Sabine: Bedankt voor je goede samenwerking bij het GDL, ook al was mijn planning soms nogal op het laatste moment... Als ik jou niet had!

Sandra: Waar moet ik beginnen?! Wat heerlijk om een collega te hebben die ook lekker grof en lomp kan zijn, soms nog erger dan ikzelf! Je bent zowel de jongere als een oudere versie van mezelf. Bedankt voor al je uitleg toen ik net begon, je grote gezellige aanwezigheid in labIII (het

keihard draaien van foute liedjes en het theeleuten over 'vrouwenzaken') en voor de enorme hoeveelheid werk wat je hebt verricht (10 blotjes tegelijk)! Ik heb je erg gemist op het lab en doe dat eigenlijk nog steeds...

Suzanne Kroon: Nog nooit zo'n gezellige buuf gehad op een lab. Wat was het opeens saai toen je weg was!

Suzanne Korporaal: Je bent mijn grote voorbeeld! Bedankt voor het opleuken van labIII, je tips en hulp bij experimenten en sollicitaties.

Steve Watson: Thank you so much for all your help in finding a Postdoctoral position. Hope I will see you again soon.

Sylvie: Dank voor je hulp bij de perfusies, je gezelligheid en goede muzieksmaak. Ben blij dat er mensen zijn die over sommige dingen hetzelfde denken!

Teun: collega van de borrelcommissie, een goede aanwinst. Was toch altijd weer leuk om met je over studenten te klagen.

Thijs: Blij om weer eens goede muziek live te zien, wat een goed rockshow was dat in de ACU! Thanks voor je gezelligheid als ik weer eens tegen iemand wilde aanpraten in de AIO-kamer. Jij ook nog succes natuurlijk en niet teveel redbulletjes hè?!.

Tineke: door jou is mijn meest gehate apparaat, de FACS, de meest geliefde geworden. Het was altijd weer gezellig als je er was!

Tuna: bedankt voor het gezellige geklaag op de researchmeetings en de borrels.

Valentina: the little hurricane. I love your unstoppable energy. Good luck with all the perfusions of your patients and don't be too stressed out afterwards, ok?

Vivian: You helped me so much with your fabulous blotting skills, many thanks! Also for your scientific input and chats in the lab. Good luck with your new project.

Vivianne: Bedankt voor al je hulp met de DECs en de experimenten!

Yaping: Thank you for your help in making nice EM pictures. Enjoy your life after work.

'Je moet een keer stoppen', bij deze dus.

The End

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van der Wal DE, Verhoef S, Wu Y, Lenting PJ, Akkerman JWN. *Role of glycoprotein Ibα mobility in platelet function and destruction.* Poster presentation. XXIst Congress of the International Society on Thrombosis and Haemostasis (ISTH), Geneva, Switzerland, July 6-12, 2007.

als je luistert naar de wolken,
als je luistert naar de wind,
als je luistert naar de wolken,
als je luistert naar de wind...

(Spinvis)

Abbreviations

ABBREVIATIONS

AA	arachidonic acid
ABT-737	4-[4-[[2-(4-chlorophenyl)phenyl] methyl] piperazin-1-yl]-N-[4-[[(2R)-4-(dimethylamino) -1-phenylsulfanylbutan-2-yl] amino]-3-nitro phenyl] sulfonylbenzamide
ACD	citric acid, trisodium citrate and dextrose
ADAM17	A Disintegrin And Metalloproteinase-17
ADP	adenosine di-phosphate
AFGPs	antifreeze glycoproteins
APAF-1	apoptotic protease-activating factor-1
cAMP	cyclisch adenosine mono-phosphate
cGMP	cyclic guanosine monophosphate
BAPTA-AM	Bis (*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester)
BSA	bovine serum albumin
β_2GPI	β_2-glycoprotein I
cAMP	3',5',-cyclic AMP
CARD	caspase recruitment domain
CCI	corrected count increment
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CD	cytochalasin D
CFMDA	5-chloromethylfluorescein diacetate

CHO	Chinese Hamster ovary
CLEC-2	*C*-type lectin receptor-2
COX-1	cyclooxygenase-1
CSK	cytoskeleton
$cPLA_2$	cytosolic phospholipase A_2
C1q	collectin-related first component of the classical complement cascase
DANA	2, 3-dehydro-2-deoxy-*N*-acetylneuraminic acid
DED	death effector domain
EET	14, 15-epoxyeicosatrienoic acid
ER	Endoplasmatic Reticulum
ERK1/2	extracellular kinase 1/2
ETI	5,8,11-eicosatriynoic acid
FACS	Fluorescence Activated Cell Sorter
Fg	Fibrinogen
FITC	fluorescein thioisocyanate
FRET	fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-phosphate dehydrogenase
Gas-6	growth arrest specific gene 6
GN	*N*-acetyl-*D*-glucosamine
GPIbα	glycoprotein Ibα
GTP	Guanosine-5'-triphosphate
12-HETE	12-hydroxyeicosatetraenoic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

IGF-1	insulin-like growth factor-1
IVIG	intravenous immunoglobulin G
IgG	immunoglobulin G
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl carbocyanine iodide
LB	ligand binding
LDL	low density lipoprotein
12-LOX	12-lipoxygenase
LPC	lysophosphatidylcholine
LRR	Leucine-rich repeats
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
MFG-E8	Milk fat globule-EGF factor 8 protein/lactadherin
MKs	megakaryocyte
MPTP	mitochondrial permeability transition pore
MPV	mean platelet volume
MSK	membrane skeleton
NA	neuraminidase
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
NTF	*N*-terminal flank
OCS	open canalicular system
OSGE	*O*-Sialoglycoprotein Endopeptidase
PAS	platelet additive solution
PBS	phosphate-buffered saline

PCs	platelet concentrates
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PECAM-1	platelet endothelial cell adhesion molecule-1
PGI_2	prostaglandin I_2 (prostacyclin)
PI3-K	phosphoinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phosholipase A_2
PLCγ2	phosholipase Cγ2
PLADO-study	prophylactic platelet dose-study
PMA	phorbol 12-myristate 13-acetate
PGNAse F	Peptide N-Glycosidase F
PP1	pyrazolopyrimidine, 4-amino -5-(4-methylphenyl) -7-(t-butyl)pyrazolo[3,4-d] pyrimidine
PRP	platelet rich plasma
PS	phosphatidylserine
PSGL-1	P-selectin protein ligand-1
PSL	platelet storage lesion
RBCs	red blood cells
RCA-1	*Ricinus communis* agglutinin-1
RGDS	Arginine-Glycine-Aspartic acid-Serine
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
Ser	Serine
SIRPα	signal-regulatory-proteinα

SK&F96365	1-[β-[3-(4-Methoxyphenyl) propoxy]-4- methoxyphenethyl]-1H-imidazole·HCl
SP-A/D	surfactant protein A/D
SR	scavenger receptor
STOP-study	strategies for the transfusion of platelets study
sWGA	succininylated wheat germ agglutinin
TAPI-2	*N*-(R)-(2-(Hydroxyaminocarbonyl) methyl)-4-methylpentanoyl-L-*t*-butyl-glycine-L-alanine 2-aminoethyl amide
TF	Tissue Factor
TNF	tumor necrosis factor
TNFR-1	TNF receptor-1
TOPPs-study	trial of prophylactic platelets study
TPα	Thromboxane A_2 receptor
TPO	thrombopoietin
TRAIL	TNF-related apoptosis-inducing ligand
TRALI	transfusion related acute lung injury
TRAP	thrombin receptor-activating peptide
TSP-1	thrombospondin-1
TxA_2	thromboxane A_2
UDP-Gal	uridine 5'-diphosphogalactose
VWD	von Willebrand disease
VWF	von Willebrand factor
WT	wild-type