From the Department of Medicine Solna,
Clinical Pharmacology Unit, Karolinska Institutet at
Karolinska University Hospital (Solna), Stockholm, Sweden

PLATELET DYSFUNCTION IN DIABETES:

IMPACT OF HYPERGLYCEMIA AND GPIIb/IIIa INHIBITION

Masoud Razmara

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پرواز را به خاطر بسپار،
پرنده مردنی است.
Keep the flight in mind,
The bird is mortal.
Forough Farrokhzad

To my families, especially my Father, who left us too soon

TABLE OF CONTENTS

ABSTRACT

Diabetes mellitus (DM) is associated with increased cardiovascular morbidity and mortality, due to diabetic angiopathy. Hyperglycemia is one of the factors that may cause platelet dysfunction in diabetic patients. This work investigated mechanisms underlying hyperglycemia-induced platelet dysfunction, and its impact in DM patients. Antiplatelet treatment by glycoprotein (GP) IIb/IIIa blockade provides more efficient thrombotic protection in diabetic than in non-diabetic subjects. We therefore also examined the effect(s) of GPIIb/IIIa blockade on platelet procoagulant activity *in vitro*, and their possible impact in type 2 DM (T2DM) patients.

High glucose dose-dependently enhanced agonist-induced platelet activation in vitro through increased osmolality, as shown by experiments with different sugars. High glucose enhanced platelet P-selectin expression but not fibrinogen binding in ADP-stimulated samples, and enhanced both platelet P-selectin expression and fibrinogen binding in thrombin receptor activating peptide (TRAP)-stimulated whole blood. Protein kinase C (PKC) blockade did not counteract the enhancement of platelet P-selectin expression by high glucose, but abolished the enhancement of TRAP-induced platelet fibrinogen binding. Superoxide anion scavenging by superoxide dismutase attenuated the enhancement by high glucose of ADP- and TRAP-induced platelet P-selectin expression, but did not influence platelet fibrinogen binding.

The influence of a standardized carbohydrate-rich meal on platelet reactivity was assessed in T2DM patients and matched healthy controls; the patients also received oral antidiabetic treatment with repaglinide and glibenclamide in a cross-over study. In T2DM patients food intake caused postprandial hyperglycemia and markedly augmented platelet P-selectin expression and platelet-leukocyte aggregation induced by the thromboxane A_2 (TxA_2) analogue U46619, and slightly enhanced ADP-induced platelet P-selectin expression. The meal caused no postprandial hyperglycemia or enhancement of platelet reactivity in the healthy controls. Both repaglinide and glibenclamide treatment only mildly reduced the postprandial hyperglycemia, and did not attenuate the meal-induced enhancement of platelet reactivity in the T2DM patients.

Platelet procoagulant activity was assessed by annexin V binding, reflecting phosphatidylserine appearance on the platelet surface membrane (PS exposure), and effects of GPIIb/IIIa blockade were examined in washed platelets from healthy volunteers. Thrombin-induced PS exposure was cell-cell contact dependent. GPIIb/IIIa blockade inhibited this by enhancing translocase activity and inhibiting scramblase activity. Thrombin-induced platelet derived microparticle (PDMP) formation was not influenced by GPIIb/IIIa blockade.

Platelet procoagulant activity, i.e, PS and factor Va (FVa) expression, and PDMP generation, was compared in T2DM patients and healthy subjects. The unstimulated platelet procoagulant activity was only slightly higher in patients compared to controls. TRAP enhanced the PS exposure and FVa expression of platelets, and PDMP generation more markedly among T2DM patients. These hyperprocoagulant alterations in T2DM patients were associated with elevated thrombin generation and a shortened plasma clotting time. GPIIb/IIIa blockade with c7E3 or SR121566 reduced the platelet PS exposure and FVa expression, and also reduced the procoagulant activity seen in T2DM patients.

In conclusion, high glucose levels enhance the platelet reactivity to agonist stimulation through elevated osmolality. This occurs via superoxide anion production that enhances platelet P-selectin expression (secretion), and PKC signalling that enhances TRAP-induced fibrinogen binding (aggregablity). Food intake markedly enhances TxA_2-induced platelet activation in type 2 diabetic patients but not in healthy subjects, presumably due to postprandial hyperglycemia. The procoagulant PS exposure is cell-cell contact dependent and it is inhibited by GPIIb/IIIa blockade. The inhibition of PS exposure by GPIIb/IIIa blockade occurs via increased translocase and reduced scramblase activity. Moreover, GPIIb/IIIa blockade attenuates the platelet hyperprocoagulant activity which is seen in T2DM patients. This thesis provides additional information on mechanisms that probably contribute to the clinical benefits of glycemic control, and of GPIIb/IIIa blocker treatment, which might be useful in shaping more efficient antithrombotic therapy for T2DM patients.

ORIGINAL PAPERS

The present thesis is based on the following papers:

I. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N. High glucose levels enhance platelet activation: Involvement of multiple mechanisms. *Br J Haematol* 2006; 133: 315-322.

II. Razmara M, Hjemdahl P, Yngen M, Östenson CG, Wallén NH, Li N. Food intake enhances thromboxane receptor-mediated platelet activation in type 2 diabetic patients but not in healthy subjects. *Diabetes Care* 2007; 30: 138-140.

III. Razmara M, HU H, Masquelier M, Li N. Glycoprotein IIb/IIIa blockade inhibits platelet aminophospholipid exposure by potentiating translocase and attenuating scramblase activity. *Cell Mol Life Sci* 2007; 64: 999-1008.

IV. Razmara M, Hjemdahl P, Östenson CG, Li N. Platelet hyperprocoagulant activity in type 2 diabetes mellitus: attenuation by glycoprotein IIb/IIIa inhibition. *J Thromb Haemost* 2008; 6: 2186-2192.

These papers are referred to by their Roman numerals (I-IV) in the text. The published papers are reproduced with permissions from the publishers.

ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
BMI	Body mass index
DM	Diabetes mellitus
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FVa	Factor Va
GP	Glycoprotein
Hepes	N-2-hydroxy-ethyl-piperazine-N-2-ethanesulfonic acid
MAb	Monoclonal antibody
MFI	Mean fluorescence intensity
NO	Nitric oxide
PAR	Protease activated receptor
PCI	Percutaneous coronary intervention
PDMP	Platelet-derived microparticle
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLA	Platelet-leukocyte aggregate
P-Lym	Platelet-lymphocyte aggregate
P-Mon	Platelet-monocyte aggregate
P-Neu	Platelet-neutrophil aggregate
PI_3	Phosphotidylinositol-3
PS	Phosphatidylserine
sP-selectin	Soluble P-selectin
SOD	superoxide dismutase
TRAP	Thrombin receptor activating peptide
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
T2DM	Type 2 diabetes mellitus
vWf	von Willebrand factor

INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disease in humans, with a prevalence of approximately 2% worldwide, and 3-5% in developed countries [1]. DM is characterized by disturbed glucose metabolism and chronically elevated blood glucose levels (hyperglycemia) [2]. According to the underlying pathophysiological mechanisms, DM is classified as type 1 (T1DM), which is due to reduced insulin production, and type 2 (T2DM), which is due to insulin resistance and/or relative insulin deficiency. T1DM and T2DM account for approximately 10% and 90% of diabetic cases, respectively [3].

DM is associated with an elevated cardiovascular morbidity and mortality, which is due to diabetic angiopathy, and involves thrombosis, inflammation and atherosclerosis [4-6]. The pathological process of diabetic angiopathy is closely linked to platelet dysfunction, endothelial dysfunction, enhanced coagulation and vascular inflammatory activity, as well as impaired fibrinolysis [7].

Platelet Physiology

Platelets, which are anucleated cytoplasmic fragments shed from megakaryocytes in the bone marrow, are the smallest blood cells. Resting platelets have a discoid shape with an average diameter of 3±0.5 μm and a mean platelet volume of 7.0±4.8 fl. Of the total population of platelets, 70% are present in the circulation with a concentration of 150 to 450×10^9 cells/L, while the rest are stored in the spleen. Platelets remain in the circulation for an average period of 10 days, and old/activated platelets are removed by macrophages in the spleen and the liver.

Apart from lysosomes, platelets contain two kinds of secretory granules: α-granules and dense granules, which store high molecular weight proteins (e.g., fibrinogen, factor V, von Willebrand factor and GPIIb/IIIa) and small molecules (e.g. ADP and calcium; Ca^{2+}), respectively. Platelets are equipped with two tubular systems. The open canalicular system (OCS) provides a route for the release of platelet granule contents. Its membrane is an intracellular storage site for membrane receptors. The dense tubular system is the site for Ca^{2+} storage and prostaglandin synthesis.

The platelet membrane is composed of bilayered, polarized phospholipids enriched with arachidonic acid (AA), and a number of glycoproteins (GP's; table I). The GP's are receptors for various ligands and play key roles in platelet function. GPIIb/IIIa, the principal receptor for fibrinogen, is the most abundant platelet membrane receptor with approximately 80.000 surface copies per platelet.

Table I: Major platelet membrane adhesion molecules, their ligands, and their functions.

Glycoprotein	CD nomenclature	Ligand(s)	Biological functions
GPIa/IIa	CD49b/CD29	Collagen	Adhesion
GPIb/IX	CD42b/CD42a	von Willebrand Factor (vWF)	Adhesion to subendothelium
GPIc/IIa	CD49e/CD29	Fibronectin and laminin	Adhesion
GPIIb/IIIa	CD41/CD61	Fibrinogen, VWF	Aggregation and adhesion
GPIV	CD36	Collagen, thrombospondin	Adhesion
GPVI	-	Collagen	Adhesion
P-selectin	CD62P	PSGL-1, GPIb	Adhesion and stabilization of aggregation

Platelet activation

The main physiological function of platelets is to seal and repair vascular injuries in order to prevent hemorrhage. Platelets can be activated by a number of agonists (e.g., ADP, thrombin, thromboxane A_2, platelet activating factor, and collagen). These agonists exert activating effects via binding to or cleavage of specific receptors on the plasma membrane (fig 1). This process initiates intracellular signal transduction leading to complex biochemical and morphological changes, such as cytoskeletal reorganization, ion fluxes, granule secretion, and fibrinogen receptor exposure. Platelet activation results in platelet shape change, adhesion, secretion, aggregation, and, upon intense stimulation, vesiculation (microparticle generation).

Phospholipid metabolism in the platelet plasma membrane plays a central role during platelet activation. Taking thrombin-induced platelet activation as an example, thrombin recognizes and cleaves the amino-terminal exodomain of G protein-coupled thrombin receptors (protease-

activated receptors 1 and 4, PAR-1/4). This cleavage creates a new amino-terminus, SFLLRN/GYPGQV, that docks intramolecularly with the body of the receptor to initiate transmembrane signalling [8]. The latter transforms G-protein into an activated form that allows it to interact with and activate phospholipase C (PLC) and phospholipase A_2 (PLA_2).

PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 mobilizes ionized calcium stored in the dense tubular system and thus increases cytosolic Ca^{2+}, which serves as a second messenger and initiates downstream events, such as cytoskeletal rearrangement and granule secretion. In the presence of elevated cytosolic Ca^{++}, DAG activates protein kinase C (PKC) that phosphorylates a number of signalling proteins and triggers multiple downstream signalling pathways, such as the mitogen activated protein (MAP) kinase pathway [9,10] (fig 1).

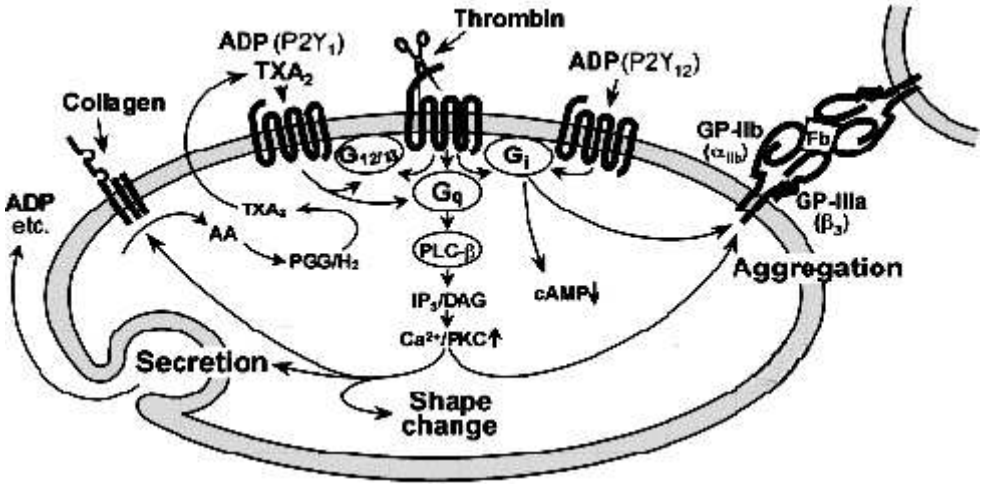

Figure 1. Signal transduction pathways in platelet activation. The figure is adapted and modified from Offermans et al [17].

Activated phospholipase A_2 (PLA_2) cleaves membrane phospholipids and releases AA and lyso-glycerol phosphocholine (lyso-PAF). AA is then oxygenated by cyclooxygenase (COX) to form cyclic endoperoxides, which are subsequently converted to thromboxane A_2 (TxA_2) by thromboxane synthase or to prostacyclin (PGI_2) by prostacyclin synthase. Lyso-PAF is converted to PAF by acetyltransferase. Importantly, TxA_2 synthesis and release is an example of positive feedback during platelet activation. Thus, TxA_2, together with other substances released from activated platelets (e.g., ADP and serotonin), stimulates adjacent platelets to amplify platelet activation.

A synthetic peptide mimicking the PAR-1 ligand sequence, thrombin receptor-activating peptide (TRAP), is capable of activating the receptor independently of the cleavage of the peptide bond. The PAR-1 receptor is connected to a significant number of intracellular signalling pathways causing rapid platelet shape change, secretion, aggregation, and calcium mobilization. Physiological differences between PAR-1 and PAR-4 receptors on human platelets, suggest that PAR-1 is the high affinity receptor for thrombin. It is exposed upon platelet activation from an additional pool located in OCS [11], and contains a leech anticoagulant, hirudin-like sequence in the exodomain of the receptor, which is important for the receptor cleavage at low concentrations of thrombin. The PAR-4 receptor, which lacks the hirudin-like site, is functional only at high thrombin concentrations [12,13]. Activation of the PAR-1 receptor induces procoagulant responses, whereas agonists to PAR-4 do not stimulate procoagulant activities [14]. ADP is another important platelet agonist with two distinct G protein-coupled receptors; the P2Y1 receptor is coupled to PLC, and the P2Y12 receptor to inhibition of adenylyl cyclase [15,16]. Stimulation of these ADP receptors activates the fibrinogen receptor, causing platelets to bind fibrinogen and aggregate. For this reason, pharmacological blockade of P2Y12 is an effective therapeutic tool in the prevention of thrombosis.

Glycoprotein IIb/IIIa

GPIIb/IIIa is a member of the supergene family of adhesive protein receptors called integrins [18,19], and is exclusively expressed on platelets and megakaryocytes. The GPIIb/IIIa receptor is a heterodimer of αIIb and β3 subunits. In its activated form it recognizes several Arg-Gly-Asp (RGD) containing ligands, such as fibrinogen and vWF. The interaction between GPIIb/IIIa and its principal ligand fibrinogen is crucial for platelet aggregation, and is the key event for the normal formation of a hemostatic plug as well as for pathologic thrombus formation [20]. Therefore GPIIb/IIIa has become a target to control platelet function in athero-thrombotic diseases [21].

The affinity of the GPIIb/IIIa receptor for its ligands is dynamically regulated during thrombogenesis and hemostasis [22,23]. Activation of GPIIb/IIIa receptors may be elicited by a wide variety of agonists such as thrombin, TXA_2 and ADP, each of which binds to a distinct receptor on the platelet surface. The receptors for these agonists act through G-proteins, which initiate interactions of intracellular signalling molecules, including talin, with the cytoplasmic

tail of the GPIIb/IIIa receptor. This causes dissociation of the αIIb and β3 subunits, leading to conformational changes in the extracellular domain of the receptor from a low-affinity to a high-affinity state [23,24]. This, in turn, allows the activated GPIIb/IIIa receptor to bind its principal ligand, fibrinogen (fig 2). Activation of GPIIb/IIIa receptors by 'inside-out' signalling represents a final common pathway for both soluble and subendothelial agonists. Elevation of cytosolic Ca^{2+} levels and PKC activation are two major processes involved in the inside-out signalling [23,25].

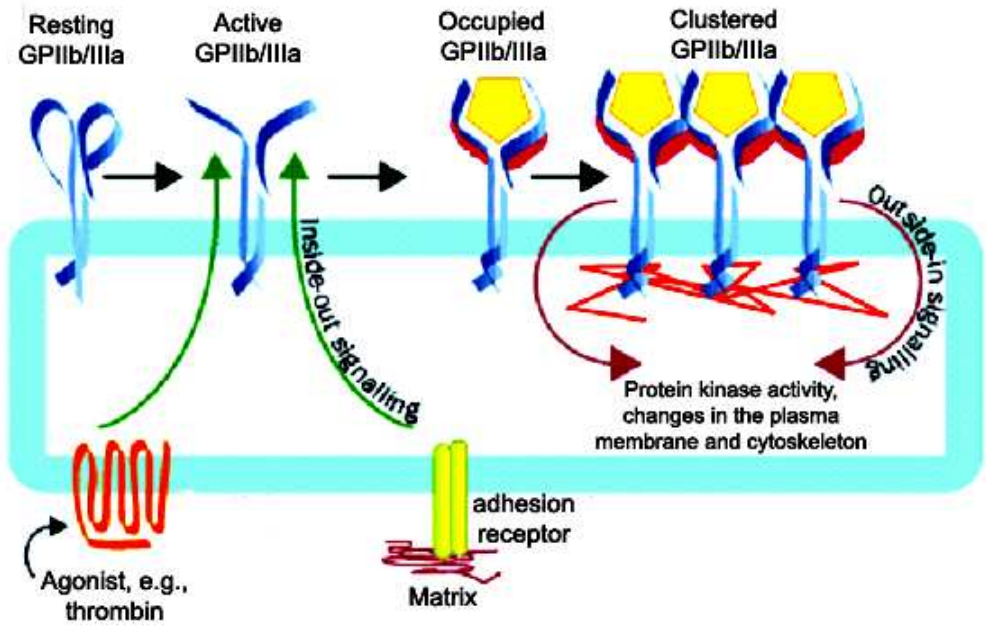

Figure 2. Signal transduction through platelet receptor glycoprotein (GP) IIb/IIIa. The image is modified from Topol et al [26].

The initial ligand binding to the GPIIb/IIIa receptor is reversible, but it progressively becomes irreversible. After ligand binding to, and occupancy of the GPIIb/IIIa receptor, clustering leads to the generation of 'outside-in' signalling which initiates post-ligand events by direct or indirect interactions of different intracellular signalling proteins with either or both of the cytoplasmic tails of the αIIb and β3 subunits. Further modifications of the cytoplasmic domains of the receptor result in a series of intraplatelet responses, such as cytoskeletal and biochemical changes including tyrosine phosphorylation of intracellular proteins (fig 2). Outside-in signalling can promote the presentation of a procoagulant surface, which generates an efficient setting for the assembly of activated coagulation factors, and leads to enhanced thrombin formation [27].

Platelet procoagulant activities

The two leaflets of the platelet plasma membrane differ markedly in phospholipid composition, with the choline phospholipids, phosphatidylcholine (PC) and sphingomyelin (Sph) concentrated in the external leaflet, and the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), concentrated in the inner leaflet (fig 3). This asymmetrical distribution of the phospholipids is controlled by the functional balance between two enzymes: the aminophospholipid translocase that selectively translocates aminophospholipids, mainly PS, from the outer to the inner leaflet, and scramblase that non-selectively transfers all phospholipids from one leaflet to the other. In the resting state, at physiologic Ca^{2+} concentrations, PS asymmetry is maintained due to an active translocase but an inactive scramblase [28,29]. Upon

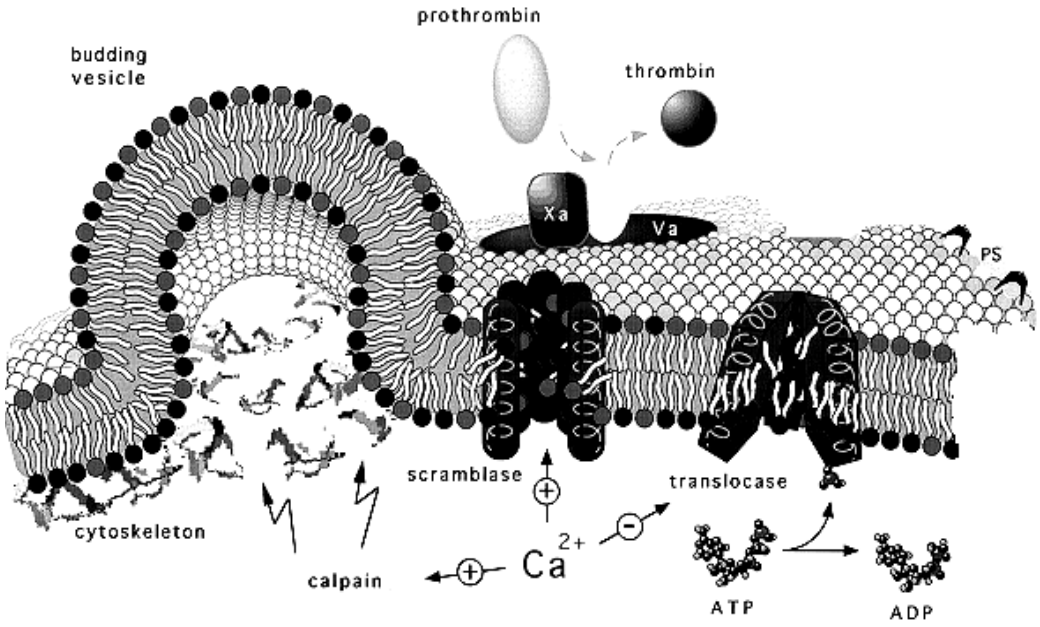

Figure 3. Mechanisms regulating phospholipid asymmetry and vesiculation (i.e., PDMP generation) upon platelet activation. Platelet agonist stimulation leads to intracellular calcium mobilization and calcium influx. Elevated cytosolic calcium inhibits aminophospholipid translocase, which selectively and rapidly transports the negatively charged aminophospholipids (with grey heads), PS and PE, from the outer leaflet to the inner leaflet, but enhances scramblase activity, which randomly and nonselectively moves phospholipids between the two leaflets. The altered balance between these two enzyme activities results in aminophospholipid, mainly PS, exposure that provides a catalytic surface for the assembly of the prothrombinase and tenase complex, and enhances coagulation and thrombosis. Elevated cytosolic calcium also activates calpain, which facilitates membrane vesiculation or microparticle generation. The figure is modified from Zwaal et al [31].

platelet activation, elevated intracellular Ca^{2+} enhances scramblase activity and inhibits translocase activity via intracellular signalling mechanisms not yet defined. This alteration results in the loss of phospholipid asymmetry and PS exposure on the cell surface (fig 3). The content of scramblase in human platelets is greater than that of the erythrocytes, which is important for the rapid exposure of PS and the prompt formation of the platelet procoagulant surface for thrombin generation [30].

Platelet factor V (FV) is crucial for the maintenance of physiological hemostasis. Approximately 20% of FV in whole blood is found in platelet alpha granules [32]. FV is rapidly released upon platelet activation and may elevate the local concentrations of FV up to 600-fold [33]. Subsequently, activated FX and FV assemble on negatively charged aminophospholipids exposed on activated platelets in the presence of calcium ions. These four components form an efficient prothrombinase complex to catalyze the conversion of prothrombin to thrombin, which is a critical reaction in the coagulation cascade. Thrombin can, in turn, activate upstream FVII and FXI, especially when the latter is bound to the platelet GPIb/IX/V complex [34]. This positive feedback enhances the assembly of the tenase complex FIXa/FVIIIa and the prothrobinase complex FXa/FVa on the activated platelet surface, and causes further thrombin formation [35,36]. Thus, a burst of thrombin generation may take place on the platelet membrane surface when platelets are activated [34] (fig 4).

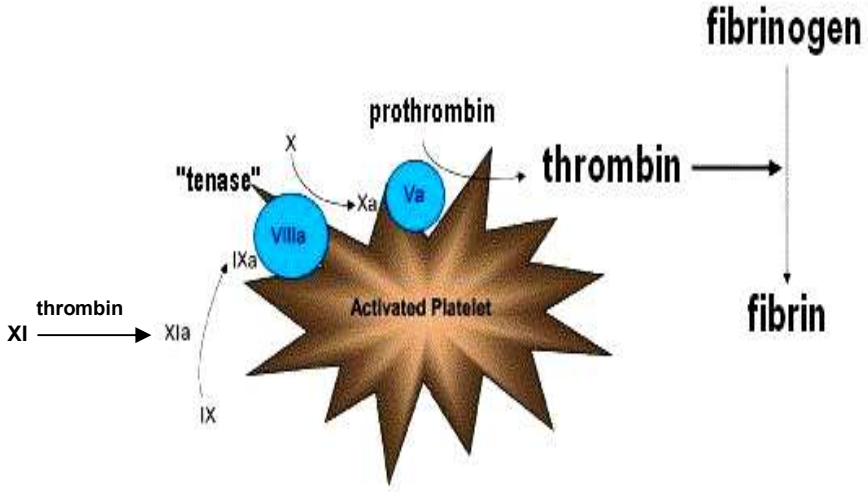

Figure 4. Mechanisms behind thrombin formation on activated platelets. The figure is modified from Wolberg et al [37].

Platelet-derived microparticles

Microparticles are plasma membrane fragments (~0.1–1 μm) released from a variety of cells upon activation [38,39]. Microparticles display membrane antigen that are specific for the parent cell of their origin. Platelet activation by various agonists induces the formation of platelet-derived microparticles (PDMPs). PDMPs are the most abundant microparticles in human blood, and account for more than 90% of the plasma microparticles in healthy individuals [38].

PDMP generation involves complex intracellular signalling and biochemical changes in the platelets. As demonstrated in fig 3, elevation of the cytosolic Ca^{2+} level acts as an intracellular second messenger and activates calpain, a key enzyme associated with PDMP formation upon platelet activation [31]. Calpains comprise a family of calcium-dependent cysteine proteases with two tissue-specific isoforms, μ- and m-calpain [40-42]. Platelets contain both isoforms, but μ-calpain represents ~90% of the total calpain activity [43,44]. The calpains differ in their responses to Ca^{2+}; μ-calpain has a high affinity for Ca^{2+}, requiring only micromolar concentrations for activation, while m-calpain activation requires millimolar concentrations of Ca^{2+} [41]. Calpain cleaves a number of focal adhesion proteins (such as talin, spectrin and β3 integrin) [45-47] and several signalling enzymes (PLCγ and PKC) [48,49].

PDMPs express negatively charged aminophospholipids, and provide additional procoagulant surfaces for the assembly of prothrombin/tenase complex of the coagulation cascade, thereby promoting thrombin generation and further platelet stimulation. Hence, therapeutic interventions resulting in reduced PDMP generation would be attractive for patients in need of antithrombotic treatment. It has been claimed that GPIIb/IIIa inhibition reduces the PDMP generation from stimulated platelets [50,51], but there are also divergent findings [52]. Therefore, it is of interest to clarify if and how GPIIb/IIIa inhibition affects PDMP generation.

Platelet dysfunction and procoagulant activity in diabetes mellitus

Patients with diabetes, especially T2DM, have a higher cardiovascular risk than those without diabetes [53-56]. This increased risk is at least partially due to platelet dysfunction and platelet-related and other procoagulation alterations in DM [57-59], and antithrombotic therapy is a cornerstone in the management of high risk diabetic patients [53,54]. The development of atherosclerotic vascular disease in T2DM appears to be related to impaired glycemic control

[53,54,60,61], but recent trials have cast some doubt on the paradigm that intense glycemic control is better than moderate control [62-64].

Both T1DM and T2DM have been shown to be associated with platelet hyperactivity [79,91,128]. Diabetic platelet dysfunction is evidenced by increased circulating activated platelets [69] and platelet-leukocyte aggregates [148], elevated plasma levels of platelet secretory substances (such as beta-thromboglobulin and soluble P-selectin) [58,68,69], and by platelet hyperreactivity to *in vitro* stimuli [72,91]. Furthermore, enhanced AA metabolism and TxA_2 synthesis [73], decreased production of platelet inhibiting nitric oxide and prostacyclin [74,75], as well as altered Ca^{2+} and Mg^{2+} homeostasis [149,150] have been described in patients with diabetes.

The mechanisms underlying diabetic platelet dysfunction are multiple and complex. Hyperglycemia appears to be one of the most important factors causing platelet hyperactivity [2,76]. Elevated glucose may facilitate protein glycation, due to nonenzymatic reactions with primary amines of proteins which form glycated compounds [146]. Increased nonenzymatic glycosylation of platelet proteins has been suggested as a major factor in the platelet hypersensitivity [77-79]. For instance, glycation of calmodulin reduces its regulatory efficacy during platelet activation [147]. Hyperglycemia reduces nitric oxide synthase (NOS) activity and thus nitric oxide production in platelets, and also reduces the concentrations of antioxidant compounds such as glutathione [80]. High glucose levels decrease the fluidity of the platelet membrane, but increase the exposure of GP receptors such as GPIIb/IIIa on the platelet surface. It has also been shown that high glucose increases phosphoinositide turnover leading to increased protein phosphorylation, enhanced inositol trisphosphate (IP3) production, and subsequently accelerated Ca^{2+} mobilization [79]. Hyperglycemia also enhances platelet activation *in vivo* via PKC-mediated intracellular signalling [81]. Furthermore, hyperglycemia is associated with enhanced platelet-dependent TxA_2 formation in vivo [73,82] and increased superoxide anion production [80].

There is accumulating evidence indicating that procoagulant activity is also enhanced in diabetic patients [83,84]. Platelet-dependent thrombin generation has been found to be elevated in patients with T2DM [85,86], and these patients have elevated levels of fibrinogen, vWF, coagulation factors X and VII [7], as well as significantly shortened prothrombin time [87]. More recently, it has been shown that there is a tight association between high blood glucose

levels *in vitro*/*in vivo* and elevated procoagulant activity [88,89]. Thus, hyperglycemia probably contributes to the rise of procoagulant proteins and to the prothrombotic state in diabetic patients, In addition, elevated levels of plasminogen activator inhibitor-1 (PAI-1) indicates decreased fibrinolysis in diabetic patients [90]. These alterations may, together with alterations of the procoagulant properties of diabetic platelets, contribute to development of vascular complications in DM patients [7].

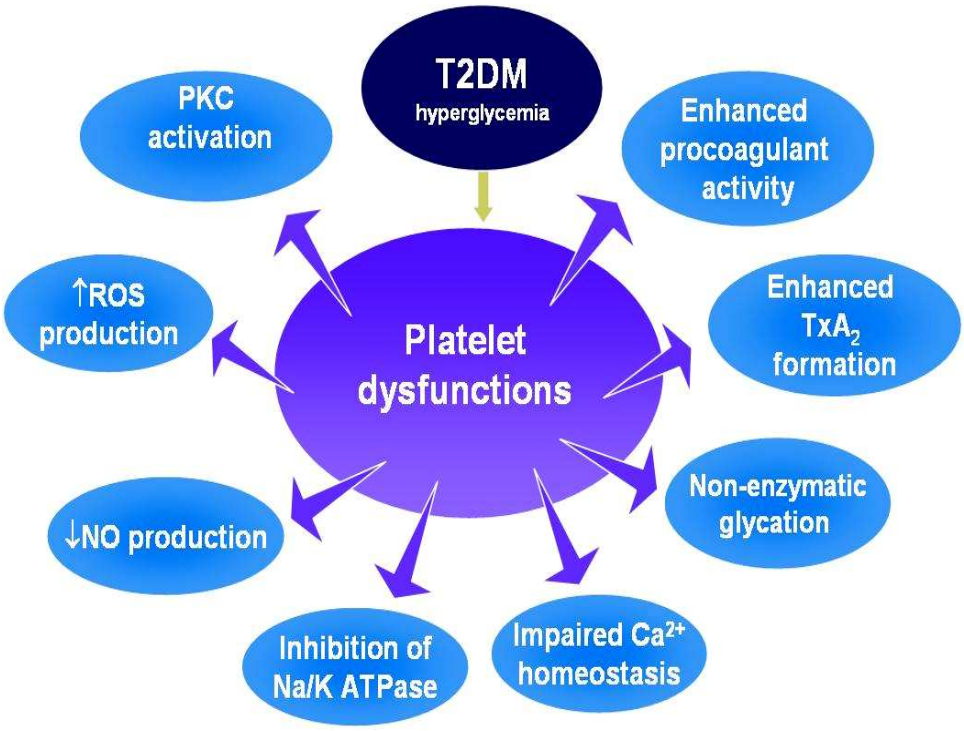

Figure 5. Pathogenetic mechanisms that may be influenced by hyperglycemia-induced platelet dysfunction in T2DM. The figure is modified from Ferroni et al [91].

Increased circulating PDMPs may be one of the procoagulant determinants in T2DM patients [92,93], and may be directly involved in the thrombotic complications in diabetes mellitus [39]. The role of PDMPs is not limited to enhanced procoagulant activity, but extends to inflammation and vascular function [94,95]. Thus, the pro-inflammatory and procoagulatory functions of PDMPs may be important in the establishment and/or progression of vascular disease. Numerous studies have shown that PDMPs may enhance thrombin generation [96], increase the expression of adhesion molecules on endothelial cells and monocytes [97], alter vascular reactivity by increasing TxA_2 production [98], and induce angiogenesis [99].

Antiplatelet treatment in T2DM

A growing body of evidence indicates important contributions of diabetic platelet dysfunction to cardiovascular complications in DM patients. Thus, anti-platelet treatment is an important component of the management of high-risk patients with DM. Three categories of antiplatelet agents are currently in clinical use: the COX-1 inhibitor aspirin, the ADP (P2Y12) receptor antagonist clopidogrel, and GPIIb/IIIa inhibitors such as abciximab.

Aspirin is the most commonly used antiplatelet agent. It irreversibly inhibits platelet cyclooxygenase, and thus blocks TxA_2 formation. Low dose aspirin is recommended as a preventive strategy in high-risk DM patients and for secondary prevention in DM patients who have evidence of large vessel disease by the American Diabetes Association (ADA) [54,100]. However, recent clinical trials show that DM is associated with a poor primary preventive effect of aspirin therapy [101,102]. The reasons underlying the reduced aspirin efficacy in DM patients have not been defined, but they are most likely multiple. For instance, hyperglycemia may lead to by-passing of the COX step, mediated by a free radical-catalyzed mechanism causing nonenzymatic peroxidation of AA and production of TxA_2 [103,104]. Hyperglycemia may also interfere with the irreversible acetylation of platelet COX-1 [105]. Furthermore, an increased platelet turnover may result in less complete platelet COX inhibition at the end of a dosing interval [106].

Another class of frequently used antiplatelet drugs is thienopyridine ADP-receptor antagonists, such as clopidogrel. Clopidogrel inhibits ADP-induced platelet activation by irreversible blockade of P2Y12 receptor by an active metabolite of the drug. Clinical trials have shown that clopidogrel alone or in combination with aspirin provides protection against cardiovascular events. Clopidogrel is used as add-on therapy to aspirin during limited periods of time in high risk patients with or without DM, or as an alternative to aspirin in patients who are in need of antiplatelet therapy but cannot take aspirin due to allergic/supersensitive reactions [53,107].

GPIIb/IIIa antagonists block the final common pathway of platelet activation, fibrinogen binding to GPIIb/IIIa, and thus protect against platelet-dependent thrombus formation by blocking platelet aggregation. Mechanisms other than blockade of fibrinogen-GPIIb/IIIa ligation may also contribute to the clinical benefits of GPIIb/IIIa antagonist treatment. Thus, GPIIb/IIIa inhibition may attenuate thrombin generation [67,70], which may be related to their abilities to reduce

platelet surface FV/Va binding [71], phosphatidylserine expression [71], and microparticle generation [50]. The GPIIb/IIIa inhibitors have also been shown to reduce leukocyte-platelet cross-talk [108,109], and platelet thrombus clot stability [110] and to enhance thrombolysis and the dissolution of platelet-rich clots [111-114]. The GPIIb/IIIa antagonists in clinical use are the chimeric monoclonal antibody fragment Abciximab (c7E3, ReoPro®), the snake venom disintegrin-mimicking cyclic peptide eptifibatide (Integrilin®), and the non-peptide RGD analogue tirofiban (Aggrastat®). They are used as add-on drugs for short term therapy in patients with acute coronary syndromes (ACS) and high risk patients undergoing percutaneous coronary interventions (PCIs) [65,66]. Several trials of GPIIb/IIIa antagonists in patients with acute coronary syndromes have indicated that such treatment is more efficient in decreasing cardiovascular events and mortality in diabetic individuals compared to non-diabetic individuals [115]. Thus, GPIIb/IIIa blockade reduces post-PCI restenosis in diabetic patients receiving bare metal stents but not in non-diabetic patients [115]. GPIIb/IIIa blockade also reduces 30-day and one-year mortality in diabetic but not in non-diabetic patients [115,116]. Therefore, it is of interest to elucidate the mechanisms underlying the extra therapeutic benefits of GPIIb/IIIa inhibition seen in diabetic patients.

AIMS OF THE STUDY

The aims of the present study were to investigate:

- Mechanisms underlying hyperglycemia-induced platelet hyperactivity in vitro
- How food intake and postprandial hyperglycemia influences platelet function in T2DM
- Mechanisms underlying the exposure of platelet phosphatidylserine (PS), and the influence of GPIIb/IIIa blockade
- Whether T2DM patients have increased platelet procoagulant activity, and the possible impact of GPIIb/IIIa blockade on the procoagulant activity of platelets

MATERIALS AND METHODS

Study subjects

Fourty-five healthy volunteers (25 females and 20 males; aged 19-63 years) were recruited for the mechanistic studies of papers I and III. Twenty-seven T2DM patients were enrolled from the out-patient clinic of the Endocrinology and Diabetes Unit, Karolinska University Hospital (Solna) for the clinical studies of papers II and IV; their healthy controls were recruited among hospital staff and were matched for gender, age, and body mass index (BMI).

All subjects denied taking aspirin or any other platelet-active medication during at least 14 days preceding the experiments. The subjects in the hyperglycemic studies donated blood after an overnight fast and were instructed to refrain from caffeine and tobacco on the day of sampling.

Informed consent was obtained from all participants, and the studies had been approved by the regional Ethics Committee of the Karolinska Institute.

Blood collection and handling

Blood was collected from an antecubetal vein after ≥15 min rest. Venepuncture was performed without stasis using siliconized vacutainers containing recombinant hirudin (final concentration 20 μg/ml) or trisodium citrate (0.38%). Platelet rich plasma (PRP) was prepared by centrifugation at 190 × g for 10 min at 22°C, and plasma samples were prepared by centrifugation at 1400 × g, 10 min, at 4°C.

Flow cytometry

Flow cytometric analyses were performed with a Beckman-Coulter EPICS XL-MCL flow cytometer. Platelets and PDMPs were identified by their chrematistic light scattering signals and their platelet specific staining with a fluorescein isothiocyanate (FITC) conjugated anti-CD42a (GPIX) MAb Beb1 (Becton Dickinson, San Jose, CA, USA). Platelet P-selectin expression and fibrinogen binding were determined by an R-phycoerythrin (RPE)-conjugated MAb AC1.2 (Becton Dickinson) and by FITC-conjugated polyclonal rabbit anti-human fibrinogen antibodies (DAKO, Glostrup, Danmark), respectively. Leukocytes were identified with RPE conjugated anti-CD45 MAb J33 (Immunotech, Marseille, France). Leukocyte CD11b expression was

determined by a FITC-conjugated MAb BEAR 1 (Immunotech). PS exposure and FVa expression were identified by RPE/FITC-Annexin V (Becton Dickinson) and the FITC-FV MAb V237 (American Diagnostica, Greenwich, CT, USA) respectively. The fluorescent lipid probe NBD-PS (Avanti Polar Lipids, Alabaster, AL, USA) was used as aminophospholipid tracer across the membrane. FITC- and RPE-conjugated isotypic MAb's were used as negative controls.

Aliquots of whole blood or washed platelets were added to Hepes-buffered saline or binding buffer containing appropriately diluted fluorescent MAbs and incubated at room temperature for 20 min. Thereafter, the samples were mildly fixed with 0.5% (v/v) formaldehyde saline or diluted with binding buffer before flow cytometric. Platelet activation is reported as the percentages of P-selectin- or fibrinogen-positive cells in the total platelet population [117]. Leukocyte activation was monitored by the mean fluorescence intensity (MFI) of CD11b expression of the total leukocytes and leukocyte subpopulations [118]. Platelet-leukocyte aggregates (PLA) are presented as percentages of platelet-conjucated leukocytes in the total leukocyte population and among lymphocytes (P-Lym), monocytes (P-Mon) and neutrophils (P-Neu) [119]. PDMPs were gated after their characteristic light scattering signals in reference to A23187-stimulated platelet samples. Annexin V-positive and NBD-PS-positive platelets and PDMPs were recorded as both percentages and MFIs. Fluorescent beads (Rainbow particles 3.0–3.4 mm; Becton Dickinson) were used to determine platelet and PDMP counts.

Immunoblotting

Platelet suspensions were incubated with vehicle or the GPIIb/IIIa inhibitors for 10 min without stirring. Vehicle or platelet agonists (thrombin and A23187) were subsequently added with further incubation for 20 min. The reactions were terminated by adding 5× ice-cold lysis buffer containing a protease inhibitor cocktail. The lysates were then aliquoted and stored at -80°C before immunoblotting.

Platelet lysates containing equal amounts of protein were subjected to immunoblotting. Proteins were separated by 4-12% SDS-PAGE gel, and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were probed with the anti-calpain MAbB27D8 (1:5000) and then with polyclonal goat anti-mouse secondary antibodies. The immunoreactive bands were detected by chemiluminescence.

Caspase activity assay

Caspase-3 like activity was measured using a modified fluorometric assay [120]. Washed platelets were incubated with vehicle or platelet agonists (thrombin and A23187) for 20 min in the absence or presence of GPIIb/IIIa inhibitor. The reaction was terminated by adding a cell lysis buffer. The lysate aliquots were added to a microplate and the caspase activity assay was initiated by adding the 7-amino-methylcoumarin-derived substrate DEVD-AMC, and reaction buffer (100 mM HEPES, 10% sucrose, 5 mM, dithiothreitol (DTT), 0.0001%NP40, and 0.1%CHAPS, pH 7.25). The fluorescence was measured at 37°C during 120 min using a FL600 microplate fluorescence reader (Labsystem, Stockholm, Sweden). The data are presented as caspase-3 activity units/10^6 platelets.

Measurements of translocase and scramblase activity

Translocase selectively transfers aminophospholipids from the outer to the inner leaflet, while scramblase bidirectionally moves all phospholipids. Platelet translocase and scramblase activities were assessed by the distribution of the fluorescent lipid probe NBD-PS over the two leaflets of the plasma membrane using a modified BSA-back-exchange protocol [121] and flow cytometry [96]. To measure translocase activity, platelets were preincubated with vehicle or GPIIb/IIIa inhibitors for 5 min at 37°C, and then incubated for 5 min at 37°C without or with thrombin. NBD-PS amounting to approximately 2% of the endogenous phospholipids content was added, and sample aliquots were taken at different time points, mixed with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose; pH 7.4) in the presence or absence of BSA (final concentration 1%) to monitor the amount of NBD-PS present in the inner leaflet and the total amount present in both leaflets, respectively. After an additional 5-min incubation to allow BSA-back extraction of NBD-PS on the outer leaflet, the samples were labeled with RPE-Annexin V to detect surface exposed PS by flow cytometry.

To measure scramblase activity, platelets were preloaded at 37°C for 30 min with NBD-PS amounting to approximately 1% of the endogenous phospholipid concentration, and the GPIIb/IIIa inhibitors were added during the last 5 min. The NBD-PS loaded platelets were further incubated in the presence or abcence of platelet agonists (thrombin and A23187) at 37°C for 5 min. Thereafter, sample aliquots were transferred to HEPES buffer without or with BSA and further processed according to the procedures of the translocase assay.

Thrombin generation and plasma clotting-time

PRP adjusted to $300x10^9$/L and PPP were incubated with or without a GPIIb/IIIa inhibitor. Thrombin generation and clotting were initiated by adding 1/10 volume of 250 mM $CaCl_2$ to the plasma. The clotting status of the samples was observed and recorded, and sample aliquots were collected at different time points during 45 minutes. The collected aliquots were mixed with 1/10 volume of ice-cold anticoagulation cocktail (250 mM EDTA and 100U/ml heparin) and centrifuged at 2500 g for 10 min at 4 °C. The supernatants were stored at -80 °C before analyses of prothrombin fragment F1.2 (F1+2) using an EIA kit (Enzygnost, Behringwerke AG, Marburg, Germany).

Data presentation and statistics

Data are presented as mean±SEM. Differences between treatments were analyzed by paired *t*-tests and repeated measures ANOVA's using StatView 4.5 and SuperANOVA (Abacus Concepts, Barkeley, CA, USA). $P<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Study I

High glucose levels enhance platelet activation: Involvement of multiple mechanisms.

The study was designed to investigate how high blood glucose levels influence platelet function under physiological conditions in vitro. Glucose dose-dependently enhanced agonist induced platelet activation *in vitro*. Glucose (30 mM) increased 1 μM ADP-induced platelet P-selectin expression from 50.6±6.0% to 61.8±6.2% ($P<0.05$), but did not enhance fibrinogen binding. In contrast, high glucose enhanced both platelet P-selectin expression and fibrinogen binding induced by TRAP. The agonist dependence of the enhancement of platelet fibrinogen binding suggested that hyperglycemia influenced specific intracellular signalling pathways activated by TRAP, but not ADP. The enhancement of platelet activation was not specific for D-glucose, as 30 mM L-glucose, sucrose, or galactose enhanced platelet responses to ADP and TRAP similarly. For example, TRAP-induced platelet fibrinogen binding was increased from 66.9±12.3% at 5 mM glucose to 87.6±2.5% with 30 mM D-glucose, to 90.9±1.5% with 30 mM L-glucose, to 88.4±3.2% with 30 mM sucrose, and to 88.2±3.2% with 30 mM galactose.

The PKC antagonist BIM-I attenuated ADP-induced platelet P-selectin expression both at 5 mM and 30 mM glucose; the enhancement of ADP-induced platelet P-selectin expression by high glucose tended (*P<0,07*) to be reduced by BIM-I (fig 6A). However, PKC blockade did not influence ADP-induced platelet fibrinogen binding (fig 6B). These data indicate that PKC signalling is involved in ADP-induced platelet secretion and its enhancement by high glucose, but not in ADP-induced platelet aggregability. PKC blockade by BIM-I attenuated both platelet P-selectin expression and fibrinogen binding induced by TRAP, suggesting that PKC signalling is involved in thrombin-induced platelet secretion and aggregability at normal glucose levels. Interestingly, PKC blockade abolished the effect of high glucose on TRAP-induced platelet fibrinogen binding, but did not influence the enhancement of TRAP-induced P-selectin expression (fig 6C and D). This finding implies that the enhancement of TRAP-induced platelet aggregability by hyperglycemia is entirely dependent on PKC signalling, and that the

enhancement of TRAP-induced platelet secretion is achieved via mechanisms other than PKC signalling.

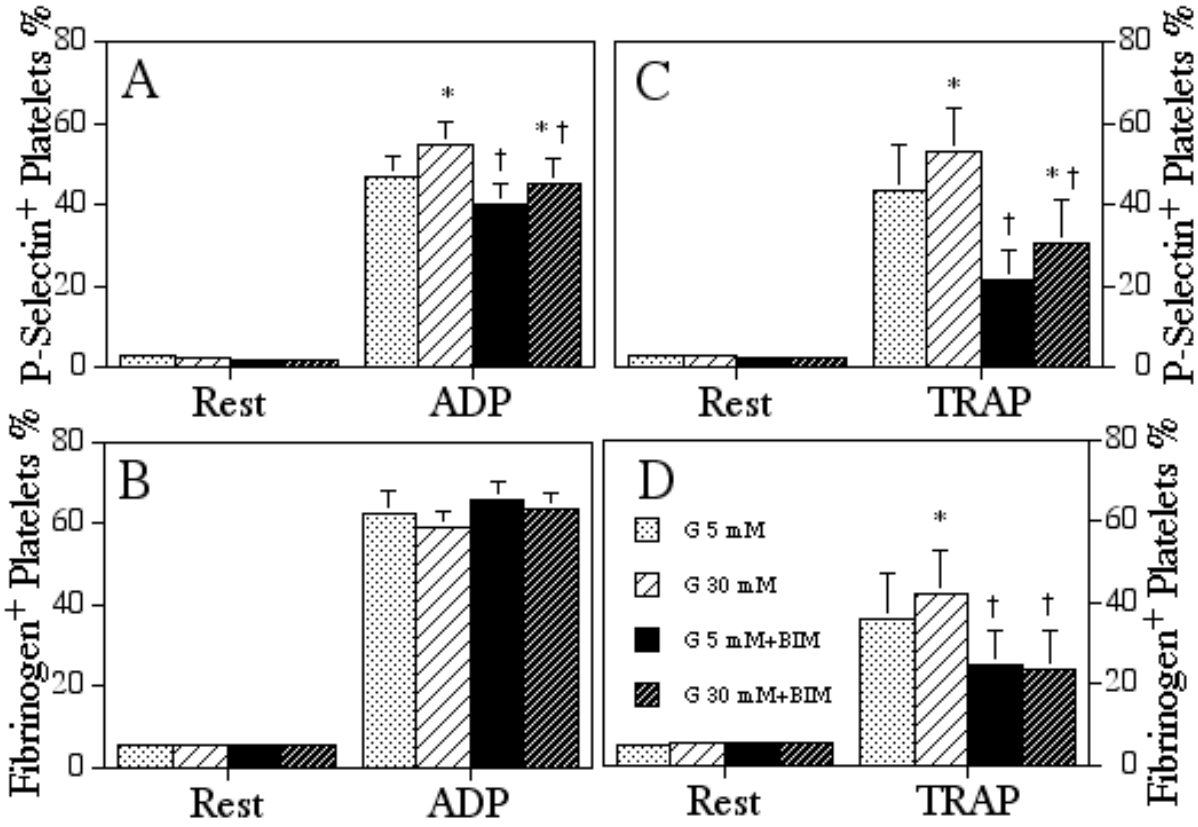

Figure 6. Influence of PKC blockade on high glucose-enhanced platelet P-selectin expression. Whole blood was pre-incubated without or with the PKC inhibitor BIM-I (10 µM) for 5 min at 37°C for 5 min. Afterwards, 5 µl blood aliquots were labelled for flow cytometric analysis without or with ADP (1 µM) or TRAP (4 µM) in the presence of 5 mM or 30 mM glucose. Mean ± SEM; * $P<0.05$, 30 mM glucose vs 5 mM glucose; † $P<0.05$ compared to the corresponding samples incubated without BIM-I; n=9.

Superoxide anion scavenging by superoxide dismutase (SOD) influenced ADP- or TRAP-induced platelet activation little at normal glucose levels (fig 7). SOD reduced the hyperglycemic enhancement of both ADP- and TRAP-induced P-selectin expression (fig 7) but did not attenuate the enhancement of TRAP-induced fibrinogen binding by high glucose, suggesting that superoxide anion production is involved in the hyperglycemic enhancement of platelet secretion but not aggregability.

Hyperglycemia enhanced platelet-leukocyte aggregation in TRAP-stimulated, but not ADP-stimulated samples. This is probably because hyperglycemia only increased ADP-induced platelet P-selectin expression, whilst it enhanced both platelet P-selectin expression and

fibrinogen binding in TRAP-stimulated samples, and supports the contention that both bridging molecules are involved in this heterotypic cellular conjugation [122].

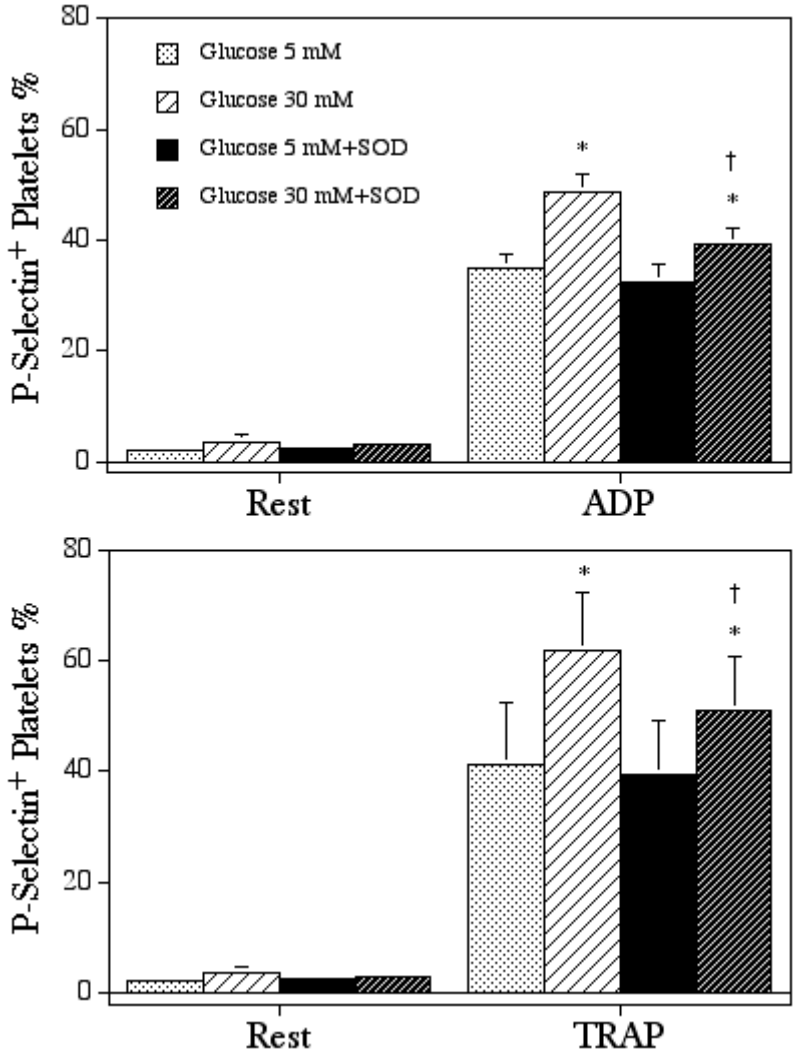

Figure 7. Influence of superoxide anion scavenging on high glucose-enhanced platelet P-selectin expression. Whole blood was pre-incubated (37°C; 5 min) without or with the superoxide anion scavenger superoxide dismutase (SOD; 100 µM) in the presence of 5 mM or 30 mM glucose. Afterwards, 5 µl blood aliquots were labelled for flow cytometric analysis of platelet P-selectin expression without or with ADP (1 µM) or TRAP (4 µM) in the presence of 5 mM or 30 mM glucose. Mean ± SEM; * $P<0.05$ compared to the corresponding samples incubated with 5 mM glucose, † $P<0.05$ compared to samples incubated at 30 mM glucose without SOD; n=7.

Taken together, this *in vitro* investigation shows that acute hyperglycemia induces platelet hyperreactivity through an elevation of osmolarity that affects platelet intracellular signalling. Hyperglycemia enhances platelet secretion via mechanisms involving both oxidative stress and PKC signalling. The hyperglycemia enhancement of platelet fibrinogen binding appears to be stimulus specific and mediated by PKC signalling.

Study II

Food intake enhances thromboxane receptor-mediated platelet activation in type 2 diabetic patients but not in healthy subjects.

Food intake markedly elevated the blood glucose concentration 90 min after the meal, from 10.5±2.7 to 13.6±3.6 mM ($P<0.05$) in T2DM patients when on diet only. During randomized antidiabetic treatment with repaglinide or glibenclamide, the meal elevated blood glucose concentrations from 9.7±2.5 to 12.3±3.2 mM ($P<0.05$) and 9.2±2.0 to 11.2±2.9 mM ($P<0.05$), respectively. Thus, the postprandial glucose levels were only slightly but significantly lower than the levels on diet only ($P<0.05$). The same meal did not influence postprandial blood glucose levels in healthy controls who were matched for age, gender, and body mass index.

In these fairly well controlled T2DM patients (as evidenced by HbA1c of 6.8±1.7%), platelet P-selectin expression at rest and upon stimulation by the TxA2 analogue U46619 (fig 8A) or ADP was similar to that seen in healthy controls. After the carbohydrate-rich meal, platelet P-selectin expression induced by U46619 was markedly enhanced (fig 8A) and that elicited by ADP moderately enhanced [123] in the T2DM patients, but the meal did not influence platelet reactivity in the healthy subjects. The difference in meal effects between patients and controls is most likely due to the postprandial hyperglycemia, which was seen among diabetic patients only. Antidiabetic treatment with repaglinide or glibenclamide did not affect the enhancement of platelet reactivity (fig 8A), probably because both oral hypoglycaemic agents only slightly reduced the postprandial elevation of blood glucose.

Consistent with the marked enhancement of platelet P-selectin expression, food intake also enhanced U46619-induced platelet-leukocyte aggregate formation in diabetic patients but not in healthy controls (fig 8B). Similar to the findings regarding platelet P-selectin expression, the postprandial enhancement of platelet-leukocyte aggregation was not influenced by repaglinide or glibenclamide treatment (fig 8B).

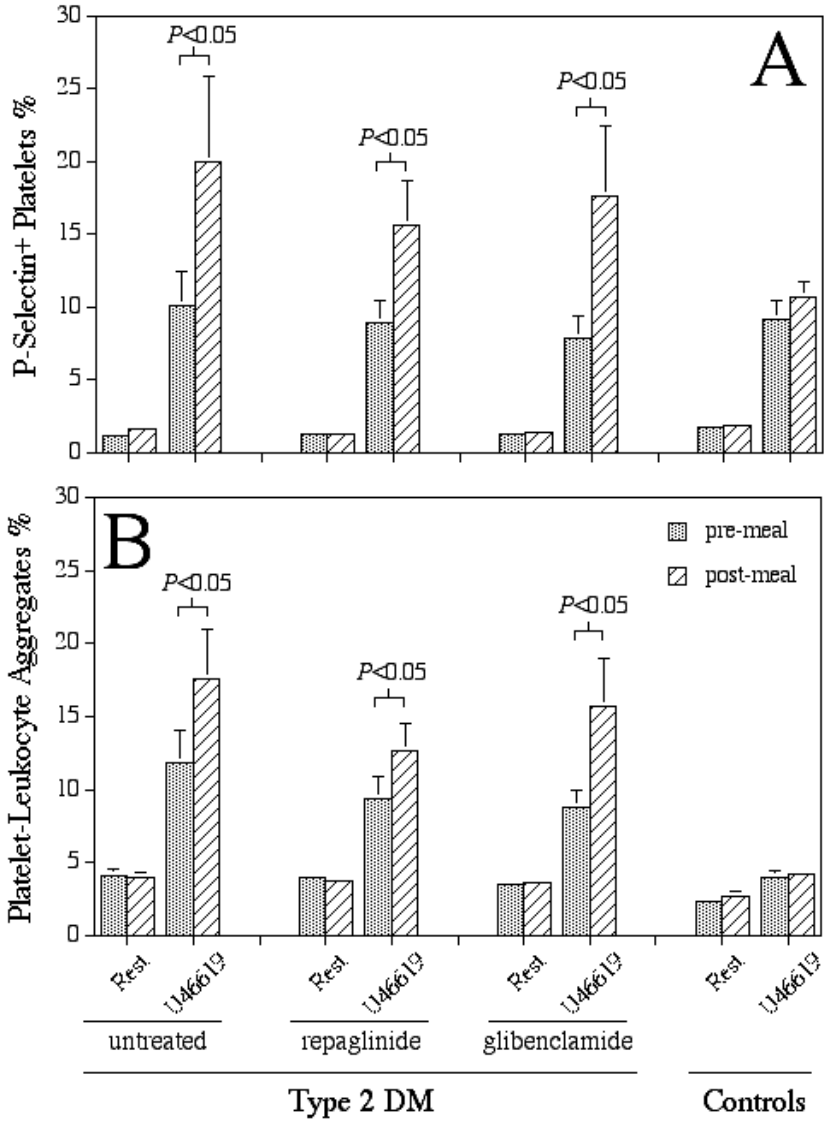

Figure 8. Effects of food intake on platelet reactivity and platelet-leukocyte aggregation upon TxA_2 receptor stimulation by U46619. Venous blood was obtained before (dotted bars) and 90-min after a standardized carbohydrate-rich meal (stippled bars). Blood samples were incubated without or with U46619 in the presence of fluorescent antibodies. Platelet P-selectin expression (panel A) and platelet-leukocyte aggregates (B) were measured using whole blood flow cytometry. U46619-induced PLA formation was less pronounced among controls compared to patients ($p<0.05$) before the meal. Data plotted are mean ± SEM from 15 DM patients and 10 controls.

Study III

Glycoprotein IIb/IIIa blockade inhibits platelet aminophospholipid exposure by potentiating translocase and attenuating scramblase activity

This study investigated the influence of GPIIb/IIIa blockade on platelet aminophospholipid (PS) exposure and PDMP generation. Platelet and PDMP PS exposure was monitored by Annexin V binding. Thrombin (1 U/ml) stimulation markedly increased both the percentage of platelets expressing PS (5×10^8 platelets/ml; from 3.5±0.9% to 53.2±3.6%; $P<0.05$; fig 9A) and the MFIs for PS expression of positive platelets (from 17.2±2.4 to 28.2±5.4; $P<0.05$; fig 9B). When the

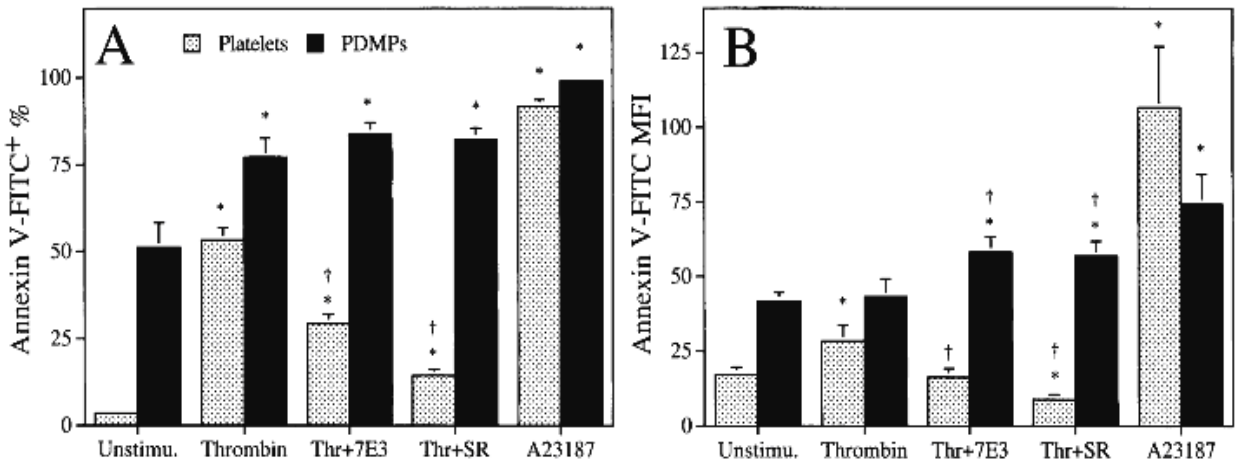

Figure 9. Effect of glycoprotein (GP) IIb/IIIa blockade on platelet phosphatidylserine (PS) exposure. Washed platelets were preincubated without or with the GPIIb/IIIa inhibitors c7E3 or SR121566 in the absence or presence of thrombin or A23187 without stirring. Annexin V binding of platelets and platelet-derived microparticles (PDMPs) were measured by flow cytometry. Mean ± SEM for Annexin V positive percentages (a) and their mean fluorescence intensity (MFI) (b) are plotted. * $p<0.05$ compared to unstimulated samples; † $p<0.05$ compared to corresponding thrombin-stimulated samples.

time course for platelet PS exposure was studied at a lower platelet concentration (0.5×10^8/ml), stimulation with the calcium ionophore A23187 (10 μM) induced a rapid burst of platelet PS expression, whilst thrombin stimulation did not alter platelet PS expression during 10 min of continuous monitoring (fig 10A). This suggests that the mechanisms underlying thrombin- and A23187-induced platelet PS exposure are different, and that a low platelet concentration (i.e.,

little direct cell-cell contact) may be the factor limiting PS exposure in thrombin-stimulated samples. Further time course studies showed that thrombin (1 U/ml) stimulated platelet PS expression reached a plateau after approximately 15 min at the higher cell concentration 5×10^8/ml (fig 10B). To elucidate the dependency on cell-cell contacts further, we showed that thrombin-enhanced platelet PS exposure was positively correlated to the cell concentrations of the platelet suspensions (fig 10C), and that stirring of the sample enhanced thrombin-induced platelet PS expression (fig 10D). These data support the concept that thrombin-induced platelet PS exposure is cell-cell contact dependent. Furthermore, the thrombin-induced platelet PS expression was reduced by GPIIb/IIIa inhibition by c7E3 (20 μg/ml) or SR121566 (50 μM) (fig 9 and 10). The reduction was seen for both the positive percentage (fig 9A) and the intensity (fig 9B) of PS expression of the platelets.

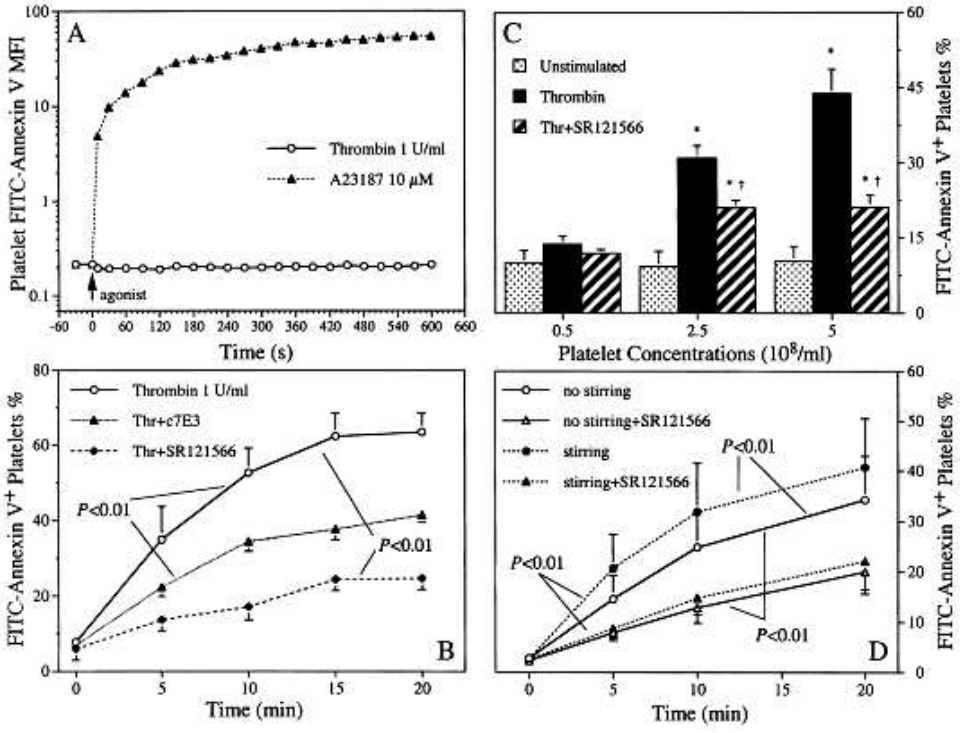

Figure 10. Influence of cell-cell contact and GPIIb/IIIa inhibition on platelet PS exposure. (a) Annexin V binding of washed platelets (10^6/ml) was continuously monitored by FITC-AnnexinV before and after thrombin or A23187 stimulation. (b) Washed platelets (5 ± 10^8/ml) AnnexinV binding measured without or with GPIIb/IIIa inhibitor stimulated with thrombin. (c) Annexin V binding measured in different platelet concentrations without or with SR121566, in the absence or presence of thrombin. (d) Annexin V binding in washed platelets (2.5 ± 10^8/ml) without or with SR121566, stimulated by thrombin without or with stirring. * $p<0.05$ compared with unstimulated samples; † $p<0.05$ compared with corresponding thrombin-stimulated samples without SR121566.

The asymmetric distribution of negatively charged phospholipids is maintained by predominance of aminophospholipid translocase activity over scramblase activity in platelets [31]. The fluorescent lipid probe, 1-Oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-sn-glycero-3-phospho-L-serine (NBD-PS), was rapidly transferred to the inner leaflet of the plasma membrane upon addition to unstimulated platelets (fig 11A), suggesting high translocase activity in resting platelets. Thrombin stimulation markedly reduced the velocity and extent of NBD-PS internalization, and the reduction was attenuated in the presence of GPIIb/IIIa inhibition by c7E3 or SR121566 (fig 11A). These data indicate that thrombin stimulation reduces the translocase activity. Furthermore, the reduction of translocase activity was more marked in PS-expressing platelets (fig 11C) than in platelets that did not expose PS, i.e. bind Annexin V (fig 11B). Fig 11D shows that BSA back extraction only slightly reduced the percentage of NBD-PS positive cells in NBD-PS-loaded unstimulated platelets, indicating limited scramblase activity in resting platelets. Fig 11D also shows that NBD-PS positive platelets were reduced by thrombin stimulation, and further reduced in the presence of BSA, suggesting that thrombin stimulation enhances scramblase activity. The enhancement of scramblase activity was attenuated by GPIIb/IIIa blockade. Furthermore, thrombin-enhanced scramblase activity was more marked in PS-expressing platelets (fig 11F) than in non-PS-expressing platelets (fig 11E). Our observations are in agreement with a recent report [96] stating that translocase activity was limited in PS-expressing activated platelets and that scramblase activity is mainly seen in PS-expressing platelets. Our results also suggest that GPIIb/IIIa inhibitors reduce thrombin-enhanced platelet PS exposure by enhancing translocase and attenuating scramblase activity. This property of GPIIb/IIIa inhibition may contribute to the reduction of thrombin generation and platelet thrombus formation by GPIIb/IIIa blockade [67,113].

Stimulation by the calcium ionophore A23187 induced extensive PDMP generation, while thrombin stimulation induced only mild PDMP generation. Thrombin-induced PDMP generation was slightly increased by GPIIb/IIIa inhibition, presumably due to reduced PDMP adhesion to platelets or platelet aggregates. Moreover, the GPIIb/IIIa inhibitors influenced calpain and caspase activation, which are known to be important in platelet vesiculation [31], little. Thus, our results fail to support earlier claims that GPIIb/IIIa blockade inhibits agonist-induced PDMP generation [50,51].

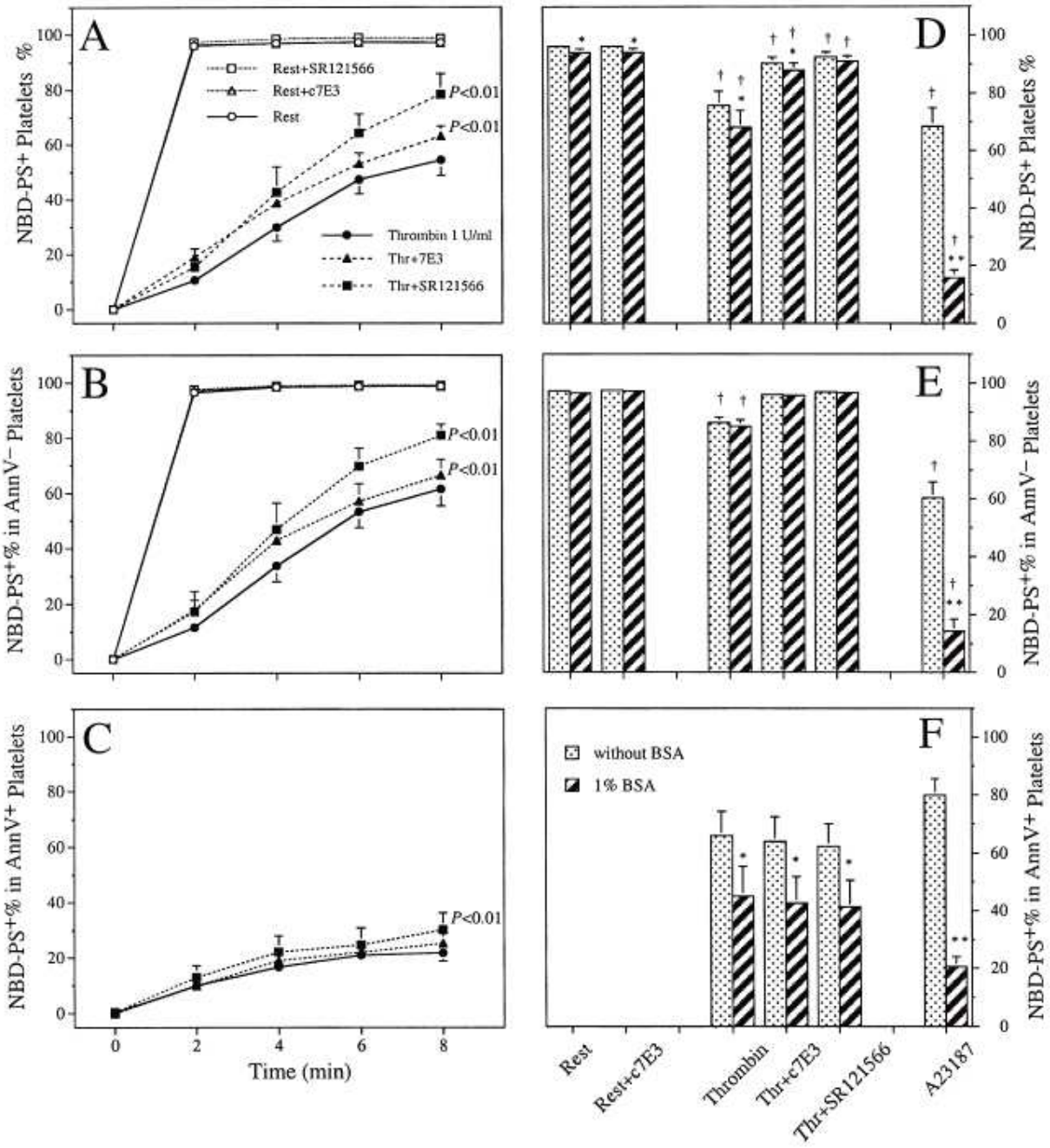

Figure 11. Effect of GPIIb/IIIa blockade on platelet aminophospholipid translocase and phospholipid scramblase activity. Inward transport of NBD-PS by translocase was monitored after BSA-back extraction and is shown in total platelets (a), non-PS-expressing (b), and PS-expressing (c; the events in unstimulated samples were rare, and the data were thus not shown) platelets. Scramblase activity was monitored by the difference of NBP-PS-positive platelet percentages between the samples without and with BSA in the buffer. Mean±SEM of the percentages of NBD-PS-positive cells in all platelets (d), non-PS expressing (e), and PS expressing (f; similar to c, the data were only from stimulated samples) platelets are shown. * $p<0.05$, ** $p<0.01$ compared to corresponding samples without BSA, † $p<0.01$ compared to unstimulated samples without BSA; n=5.

Study IV

Platelet hyperprocoagulant activity in type 2 diabetes mellitus: attenuation by glycoprotein IIb/IIIa inhibition

Twelve T2DM patients and 12 matched controls were examined regarding platelet procoagulant activity, and how GPIIb/IIa blockade influenced the platelet procoagulant activity. Multiple aspects of platelet procoagulant activity were assessed, including platelet phosphotidylserine (PS) exposure and FVa binding, thrombin generation in recalcified plasma, as well as plasma clotting time. We found that the basal levels of platelet PS exposure and FVa binding (fig 12), and plasma F1+2 levels were similar between T2DM patients and the matched healthy controls (paper IV, table 2). For example, platelet FVa binding in unstimulated samples was 1.6 ± 0.25% in T2DM patients and 1.1 ± 0.24% in the controls (P=0.33). A significant difference was, however, seen in stimulated samples, as 10 μM TRAP increased platelet FVa binding to 29.8±0.3% in the patients but only to 24.7±2.2% in the matched controls (fig 12B; $p<0.05$ for difference). To generate an overall index of platelet procoagulant activities, a platelet procoagulant index (PPI) was calculated, which takes into consideration both the percentages of Annexin V binding positive cells and FVa expression, and the intensities of these procoagulant parameters: PPI=[(Annexin V %×MFI)+(FVa %×MFI)]×100. The basal PPI tended to be higher among the patients, and the PPI evoked by TRAP stimulation was significantly higher in the patients than in the controls (fig 12C). In line with the above findings, thrombin generation in recalcified PRP and PPP, as reflected by accumulation of F1+2, was faster and greater in the T2DM patients than in the controls (paper IV, table 2). The clotting times of recalcified PRP and PPP were shorter in the patients than in the controls (fig 13). Hence, our findings support the notion that the platelets and PDMP´s contribute to a hyperprocoagulant status in T2DM.

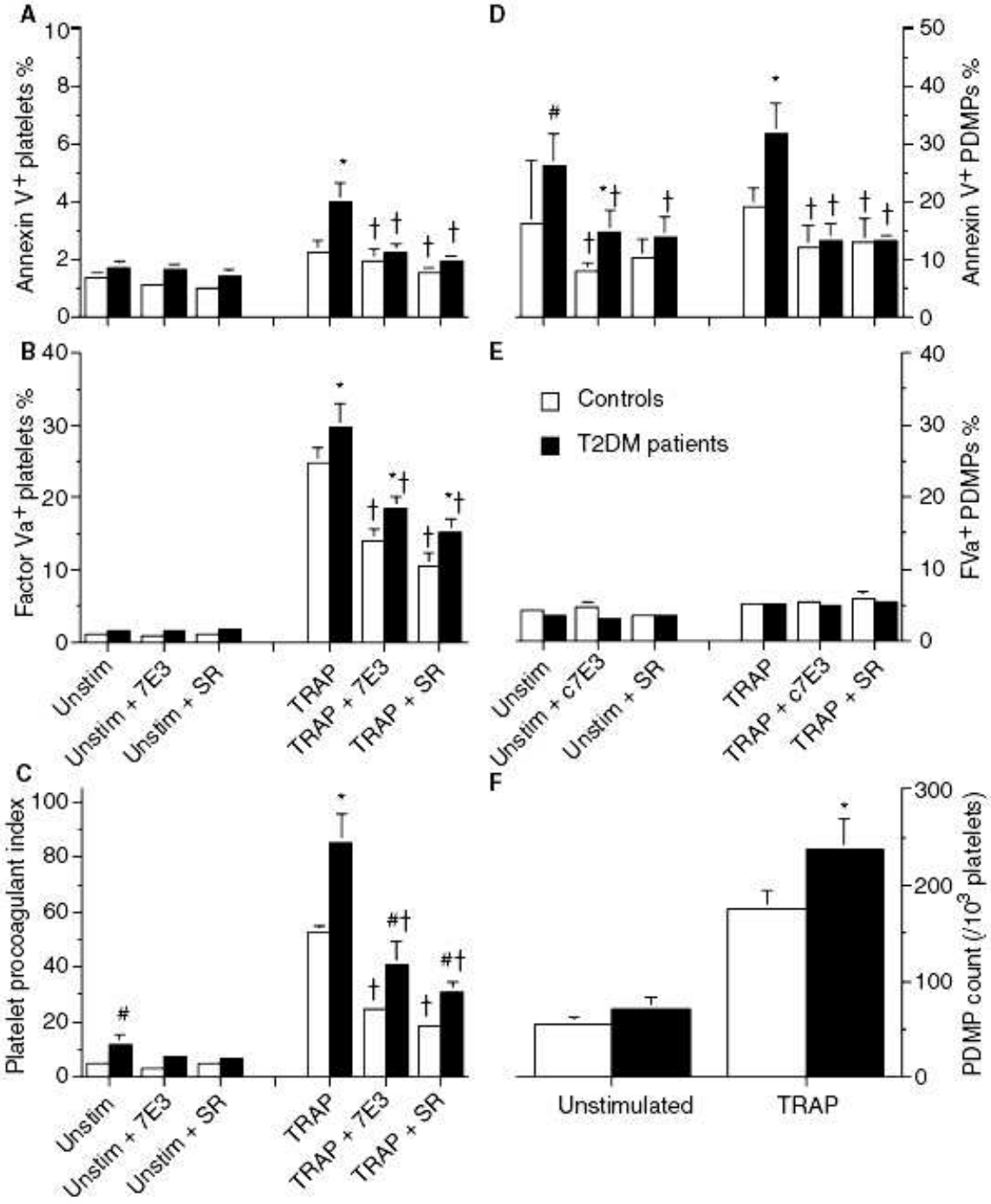

Figure 12. Enhanced platelet procoagulant activity and platelet-derived microparticle (PDMP) generation in T2DM patients**.** Hirudinized whole blood from healthy controls (open bars) and T2DM patients (filled bars) was incubated without (unstimulated) or with 10 μM TRAP in the absence or presence of 20 μg/ml c7E3 or 50 μM SR121566 at 37°C for 20 min. Platelets (panels A-C) and PDMPs (D-F) were defined by their GPIX-positive staining and light scattering characteristics using whole blood flow cytometry. The percentages of phosphotidylserine expressing (Annexin V positive; panels A and D) and FVa binding (B and E) platelets and PDMPs are shown. The calculated platelet procoagulant index (PPI) is shown in panel C. PDMP data are presented as PDMP counts per 1000 platelets (pane F). Data are means±SEM from 12 subjects. **P*<0.05, # 0.05<*P*<0.10 for T2DM patients vs healthy controls; †*P*<0.05 for GPIIb/IIIa blockade compared to corresponding data without GPIIb/IIIa blockade.

GPIIb/IIIa blockade has been shown to attenuate Annexin V binding, i.e. platelet PS exposure [71] (paper III), and FVa binding [71]. In agreement with these previous results, we found that GPIIb/IIIa inhibition by c7E3 or SR121566 decreased TRAP-enhanced platelet PS exposure and FVa binding in patients and controls, leading to marked reductions of the calculated PPIs (fig 12A-C). Interestingly, the inhibition seemed to be more profound in T2DM patients. Thus, c7E3 reduced TRAP-enhanced PS exposure by more than 70%, and SR121566 almost abolished the enhancement in the patients, and concealed the difference in TRAP-enhanced PS exposure between patients and controls. This favorable effect is of interest in view of the greater short-term (30 days) and long-term (one year) survival benefits of GPIIb/IIIa inhibition in diabetic patients with acute coronary syndrome undergoing percutaneous coronary intervention than in non-diabetic patients [124,125]. The effect may also contribute importantly to the risk reduction of restenosis in diabetic patients receiving bare metal stents [126], which is closely related to platelet thrombus formation [126]. Therefore, our findings may explain in part the beneficial effects of GPIIb/IIIa inhibition therapy in diabetic patients undergoing percutaneous coronary intervention.

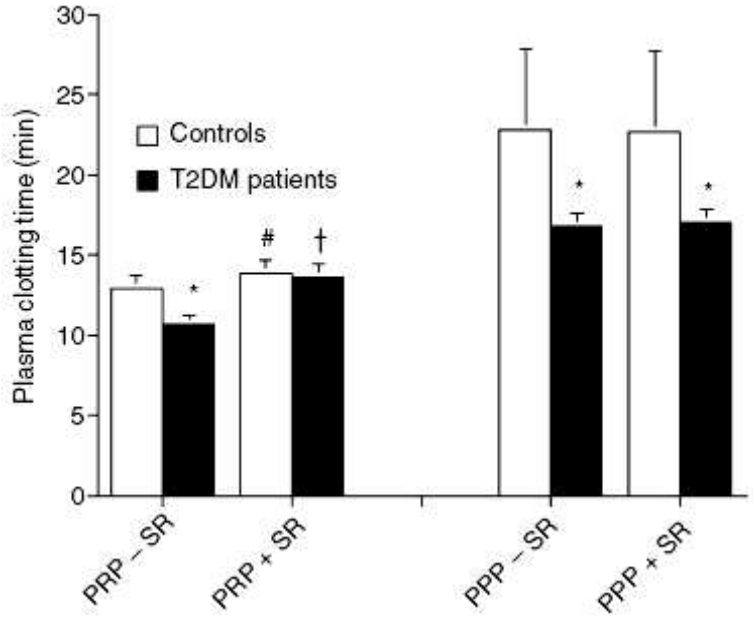

Figure 13. Influence of GPIIb/IIIa blockade on plasma clotting time. $CaCl_2$ was added to the citrated platelet rich plasma (PRP) or platelet poor plasma (PPP) for recalcification. The plasma samples were incubated in the absence or presence SR121566, and the time between plasma recalcification and full plasma clotting was recorded. Data plotted are the means±SEM from six experiments. *P<0.05, T2DM patients vs healthy controls; †P<0.05, #P=0.06, GPIIb/IIIa blockade compared to the corresponding data without GPIIb/IIIa blockade.

PDMPs are the principle source of microparticles in the circulating blood, and they are highly procoagulant [39,93,95,127]. We found that TRAP stimulation induced more marked PDMP generation in T2DM patients than in controls (fig 12F). The PS exposure of circulating PDMPs was higher in T2DM patients than in controls, and the TRAP-enhanced PS exposure of PDMPs was markedly inhibited by GPIIb/IIIa blockade (fig 12D) in the patients. Therefore, attenuation of the PS exposure of PDMPs may contribute to the anticoagulant properties of GPIIb/IIIa inhibition. Furthermore, we found that GPIIb/IIIa blockade significantly inhibited thrombin generation also in PPP from T2DM patients but not from the controls. These data imply that elevated circulating procoagulant PDMPs (which are not removed with the present procedure for PPP preparation), together with elevated plasma levels of coagulation factors [115,116] contribute to the hypercoagulant activity in T2DM patients, and that GPIIb/IIIa inhibition can modify the procoagulant activity of circulating microparticles.

GENERAL DISCUSSION

The present work investigated the influences of high blood glucose and GPIIb/IIIa blockade on platelet function in T2DM patients and healthy subjects. We found that high glucose enhanced platelet reactivity in vitro in healthy subjects. The enhancement of platelet activation by high glucose was shown to be agonist-specific and involve multiple signalling mechanisms. We also found that intake of standardized carbohydrate-rich meal augmented agonist-induced platelet activation, especially responses to thromboxane receptor stimulation, in T2DM patients, but not in healthy controls. This difference in the platelet response to food intake may be due to the postprandial hyperglycemia which was seen among the diabetic patients only. GPIIb/IIIa blockade inhibited platelet PS exposure, as assessed by the surface binding of annexin V to negatively charged phospholids, by enhancing aminotranslocase and attenuating scramblase activity. This leads to reduced platelet procoagulant activity. Interestingly, GPIIb/IIIa blockade counteracted the platelet hyperprocoagulant activity that was seen among T2DM patients.

DM is an important risk factor for cardiovascular disease, and cardiovascular complications are the principal cause of mortality in diabetic patients [53,54,105]. Platelet dysfunction is considered to play an important role in diabetic cardiovascular complications, and antiplatelet treatment is therefore an important component of the management of diabetic patients at high risk [53,54,100]. However, clinical trials have revealed poorer effects of antiplatelet treatment with aspirin in the presence of diabetes [101,102]. This may be related to interference with the irreversible acetylation of platelet COX-1 in DM [105] and/or to insufficient dosing, too long a dose interval, or a shortened platelet turnover in the diabetic patients [106]. In contrast, GPIIb/IIIa blockade seems to provide additional survival benefits in diabetic compared to non-diabetic patients with acute coronary syndrome undergoing percutaneous coronary intervention [124,125] and to reduce restenosis in diabetic patients receiving bare metal stents [126]. Therefore, a good understanding of the mechanisms underlying platelet dysfunction in diabetic patients will be helpful for the optimization of antiplatelet treatment in DM.

Several mechanisms appear to contribute to hyperglycemia-associated platelet hyperreactivity. Hyperglycemia may cause non-enzymatic glycation of proteins and increase aldose reductase activity, leading to the formation of advanced glycation end products (AGEs) and sorbitol accumulation, respectively [91,128]. These mechanisms require prolonged exposure to hyperglycemia, and are thus unlikely to be involved in the short term effects of hyperglycemia

seen in the present studies. It has been shown that hyperglycemia-induced PKC activation and elevation of diacylglycerol (DAG) are associated with cardiovascular complications [129], and that acute hyperglycemia increases platelet activation through an alteration of PKC activity [130]. Acute hyperglycemia in vivo has previously been demonstrated to increase platelet PKC activity [81]. The present work provides new information with regard to the involvement of PKC in hyperglycemia-induced platelet hyperreactivity. We found that hyperglycemia enhanced platelet activation only in the presence of platelet agonists, and that the PKC-dependent enhancement of platelet reactivity was agonist-specific. Thus, hyperglycemia enhanced TRAP- but not ADP-induced platelet aggregability, and the enhancement was completely abolished by PKC blockade.

In agreement with a recent report [131], we found that acute hyperglycemia exerted its effect on platelet activation via elevated osmolarity, as other carbohydrates at the same concentration produced identical effects as D-glucose. Apart from PKC, high osmolality appears to influence other intracellular signalling pathways during platelet activation, such as oxidative stress. Hyperglycemia may induce the production of reactive oxygen species (ROS) via direct effects of glucose metabolism, and ROS may in turn trigger the activation of other signalling molecules such as PKC [132]. The present study showed that superoxid anion scavenging by SOD reduced the hyperglycemic enhancement of platelet secretion (platelet P-selectin expression) induced by both ADP and TRAP. Other signalling mechanism(s) not identified in our study were probably also involved, as superoxide oxide scavenging only inhibited the effect partially. Using different experimental settings, it has also been demonstrated that acute hyperglycemia may attenuate platelet NO synthesis [131]. Taken together, the results suggest that multiple signalling mechanisms are involved in the hyperglycemic enhancement of platelet reactivity, and that intervention against a single signalling pathway may have limited effects in preventing the platelet hyperreactivity induced by short-term hyperglycemia.

Postprandial hyperglycemia caused by a deficient insulin response to food intake is an early abnormality in T2DM [133]. Accumulating evidence indicates that postprandial hyperglycemia is a risk factor for, and contributes to the development of cardiovascular disease [53,54,134-136]. Thus, postprandial hyperglycemia appears to be more strongly associated with atherosclerosis than fasting glucose and HbA1c levels [137]. A recent study showed that reduction of postprandial hyperglycemia by treatment with the α-glucosidase inhibitor acarbose is associated

with a marked reduction of cardiovascular events in subjects with impaired glucose tolerance [138]. A previous study from our laboratory showed that an oral glucose tolerance test induced signs of platelet activation in vivo in T2DM patients [139]. In the present study, food intake induced postprandial hyperglycemia and increased platelet reactivity in T2DM patients, but not in healthy controls. Our finding highlights the possibility that blood glucose spikes after food intake may be an important contributor to diabetic platelet hyperreactivity.

As noted above, diabetic patients appear to benefit less from treatment with aspirin than non-diabetic patients [101,102]. It has, however, been shown that a dual inhibitor of TxA2 synthesis and its receptor was associated with a reduction of vascular death among diabetic patients when compared to aspirin alone [140]. This suggests inadequate platelet inhibition by aspirin, and that thromboxane mediated platelet activation indeed is of importance in diabetic patients. It is therefore of particular interest that it was the platelet reactivity to TxA2 receptor stimulation that was most markedly enhanced after the carbohydrate-rich meal in our T2DM patients.

We investigated platelet inhibitory mechanisms related to GPIIb/IIIa blockade in light of the additional protection afforded by GPIIb/IIIa inhibition in diabetic compared to non-diabetic patients with acute coronary syndrome undergoing percutaneous interventions. We confirmed that GPIIb/IIIa blockade inhibits platelet PS exposure and FVa binding, and reduces thrombin generation and prolongs the clotting time of recalcified citrated plasma in healthy subjects [71] (papers III and IV). Furthermore, we found that the anticoagulant effects of GPIIb/IIIa blockade were even more marked in T2DM patients, and that the GPIIb/IIIa blockers effectively counteracted the platelet hyperprocoagulant activity that was found in these patients (paper IV). Hence, our findings suggest that chronic interference with GPIIb/IIIa receptor function would be an interesting antiplatelet strategy for the optimization of T2DM magagement. However, clinical trials of oral GPIIb/IIIa inhibitor treatment for chronic secondary prevention have all failed to protect the patients, as such treatment actually increased the mortality of acute coronary syndrome patients [125]. This probably has multiple explanations, including subtherapeutic platelet inhibition [125,141] and instrinsic platelet activating properties of moderately dosed oral GP IIb/IIIa inhibition [142]. Nevertheless, our findings encourage the clarification of whether signalling mechanisms underlying the attenuation of platelet procoagulant activity by GPIIb/IIIa blockade, which might be useful targets for the development of novel oral antiplatelet agents. Thus, GPIIb/IIIa blockade inhibits platelet tenase activity [141], reduces PKC activity [109],

reduces FVa and aminophospholipid exposure [115,116] (papers III and IV), and decreases platelet prothrombinase activity [143]. We demonstrated in the present study that these changes resulted in more marked reduction in thrombin generation and plasma clotting time in T2DM patients, and that GPIIb/IIIa blockade reduces aminophospholipid exposure by potentiating translocase and attenuating scramblase activity. Further efforts should be made, for instance, to elucidate the signalling links between GPIIb/IIIa blockade and enhanced translocase/reduced scramblase activity, and to demonstrate how GPIIb/IIIa blockade reduces platelet FVa binding.

PDMPs play important roles in thrombosis and inflammation, such as to enhance thrombin generation and promote the recruitment of leukocytes at sites of inflammation [95]. GPIIb/IIIa blockade has previously been reported to inhibit agonist- and shear-induced PDMP generation [50,51]. This used to be claimed as an attractive characteristic of the GPIIb/IIIa inhibitors besides blockade of fibrinogen/fibrin-GPIIb/IIIa ligation, and is widely cited in literature. However, the present data show that GPIIb/IIIa blockade does not inhibit PDMP generation when expressed as microparticles generated per million incubated platelets. Microparticle generation was estimated as the ratio of microparticles over single platelets in the previous studies [50,51], but agonist stimulation and shear stress induce platelet adhesion and microaggregation, and therefore reduce the single platelet counts. Since GPIIb/IIIa blockade inhibits agonist- or shear-induced platelet adhesion/aggregation the single platelet numbers will increase, and the microparticle/single platelet ratio will give a false impression that GPIIb/IIIa blockade inhibits PDMP generation. Activation of calpain and caspase-3, and elevation of cytosolic calcium are involved in platelet vesiculation [144], but we found that thrombin-induced platelet calpain activation was not altered by GPIIb/IIIa blockade, and that platelet caspase-3 activity was little enhanced by thrombin stimulation, as previously also shown by Wolf et al [120]. Furthermore, agonist-induced elevations of intracellular calcium levels were not influenced by GPIIb/IIIa blockade, in agreement with a previous report from our group [109]. Therefore, our findings do not support an involvement of GPIIb/IIIa in PDMP generation. However, GPIIb/IIIa blockade definitely inhibits other aspects of platelet procoagulant activity. Besides the inhibition of PS exposure and FVa binding discussed above, GPIIb/IIIa blockade inhibits prothrombin binding to GPIIb/IIIa receptors [145] and prothrombinase assembly [143].

Taken together, the platelet dysfunction in mild/moderate T2DM is primarily manifested as platelet hyperreactivity to agonist stimulation. Acute hyperglycemia enhances platelet activation

via multiple intracellular signalling pathways, as demonstrated by the present in vitro studies. Intake of a carbohydrate rich meal causes postprandial hyperglycemia and enhances especially TxA2-receptor mediated platelet activation in T2DM patients. Furthermore, platelets from T2DM patients are hyperprocoagulant, but this alteration may be counteracted by GPIIb/IIIa inhibition. Our findings highlight that the combination of improved glycemic control, especially prevention of postprandial hyperglycemia, and antiplatelet treatment that effectively counteracts TxA2-related platelet activation may be of considerable importance for improving the platelet dysfunction and the cardiovascular risk in T2DM patients.

CONCLUSIONS

- T2DM is associated with platelet hyperreactivity that is manifested as enhanced platelet aggregability (fibrinogen binding) and secretion (P-selectin expression), as well as increased platelet-related procoagulant activity.

- Hyperglycemia enhances platelet activation differently with different stimuli. The enhancement is exerted via elevated osmolality, and affects multiple intracellular signalling pathways. Hyperglycemia increases platelet secretion via both PKC signalling and oxidative stress, and platelet aggregability via PKC signalling.

- Postprandial hyperglycemia is associated with enhanced platelet reactivity, especially the responsiveness to TxA2-receptor stimulation, in T2DM patients. Improved control of postprandial hyperglycemia and effective blockade of TxA2-mediated platelet activation may be beneficial from the standpoint of athero-thrombotic risk in DM.

- Platelet PS exposure is cell-cell contact dependent, and is inhibited by GPIIb/IIIa blockade by enhancing amiophospholipid translocase but attenuating scramblase activity.

- GPIIb/IIIa blockade does not inhibit platelet-derived microparticle generation.

- The platelet hyperprocoagulant activity in T2DM patients, seen as enhanced platelet PS exposure, FVa binding, and PDMP generation, is counteracted by GPIIb/IIIa blockade.

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REFERENCES

1 Gale EA. The rise of childhood type 1 diabetes in the 20th century. *Diabetes*. 2002; **51**: 3353-61.

2 Aronson D. Hyperglycemia and the pathobiology of diabetic complications. *Adv Cardiol*. 2008; **45**: 1-16.

3 Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998; **15**: 539-53.

4 McGovern PG, Pankow JS, Shahar E, Doliszny KM, Folsom AR, Blackburn H, Luepker RV. Recent trends in acute coronary heart disease-mortality, morbidity, medical care, and risk factors. *N Engl J Med*. 1996; **334**: 884-90.

5 Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC, Jr., Sowers JR. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. *Circulation*. 1999; **100**: 1134-46.

6 Ruggeri ZM. Platelets in atherothrombosis. *Nat Med*. 2002; **8**: 1227-34.

7 Carr ME. Diabetes mellitus: a hypercoagulable state. *J Diabetes Complications*. 2001; **15**: 44-54.

8 Coughlin SR. How the protease thrombin talks to cells. *Proc Natl Acad Sci U S A*. 1999; **96**: 11023-7.

9 Siess W. Molecular mechanisms of platelet activation. *Physiol Rev*. 1989; **69**: 58-178.

10 Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. *J Thromb Haemost*. 2003; **1**: 1602-12.

11 Molino M, Bainton DF, Hoxie JA, Coughlin SR, Brass LF. Thrombin receptors on human platelets. Initial localization and subsequent redistribution during platelet activation. *J Biol Chem*. 1997; **272**: 6011-7.

12 Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*. 1999; **103**: 879-87.

13 Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000; **407**: 258-64.

14 Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proc Natl Acad Sci U S A*. 1999; **96**: 11189-93.

15 Mills DC. ADP receptors on platelets. *Thromb Haemost*. 1996; **76**: 835-56.

16 Gachet C, Hechler B, Leon C, Vial C, Leray C, Ohlmann P, Cazenave JP. Activation of ADP receptors and platelet function. *Thromb Haemost*. 1997; **78**: 271-5.

17 Offermanns S. The role of heterotrimeric G proteins in platelet activation. *Biol Chem*. 2000; **381**: 389-96.

18 Ruoslahti E. Integrins. *J Clin Invest*. 1991; **87**: 1-5.

19 Ro HK, Tembe V, Favus MJ. Evidence that activation of protein kinase-C can stimulate 1,25-dihydroxyvitamin D3 secretion by rat proximal tubules. *Endocrinology*. 1992; **131**: 1424-8.

20 Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. *Blood*. 1988; **71**: 831-43.

21 Lefkovits J, Plow EF, Topol EJ. Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med*. 1995; **332**: 1553-9.

22 Bhatt DL, Topol EJ. Scientific and therapeutic advances in antiplatelet therapy. *Nat Rev Drug Discov*. 2003; **2**: 15-28.

23 Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004; **104**: 1606-15.

24 Ulmer TS, Calderwood DA, Ginsberg MH, Campbell ID. Domain-specific interactions of talin with the membrane-proximal region of the integrin beta3 subunit. *Biochemistry*. 2003; **42**: 8307-12.

25 Shattil SJ. Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thromb Haemost*. 1999; **82**: 318-25.

26 Topol EJ, Byzova TV, Plow EF. Platelet GPIIb-IIIa blockers. *Lancet*. 1999; **353**: 227-31.

27 Kasirer-Friede A, Kahn ML, Shattil SJ. Platelet integrins and immunoreceptors. *Immunol Rev*. 2007; **218**: 247-64.

28 Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem*. 1982; **122**: 429-36.

29 Ling JC, Freeman AF, Gharib AM, Arai AE, Lederman RJ, Rosing DR, Holland SM. Coronary artery aneurysms in patients with hyper IgE recurrent infection syndrome. *Clin Immunol*. 2007; **122**: 255-8.

30 Zhou Q, Zhao J, Stout JG, Luhm RA, Wiedmer T, Sims PJ. Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem*. 1997; **272**: 18240-4.

31 Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood*. 1997; **89**: 1121-32.

32 Tracy PB, Eide LL, Bowie EJ, Mann KG. Radioimmunoassay of factor V in human plasma and platelets. *Blood*. 1982; **60**: 59-63.

33 Beth A. Bouchard, Saulius Butenas, Kenneth G. Mann, Paula B. Tracy. Interaction betwwen platelet and coagulation system. In: Michelson A, ed. Platelets, 2nd edition. *San Diego: Elsevier/Academic Press;*. 2007; pp377-402.

34 Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. *J Biol Chem*. 2002; **277**: 1662-8.

35 Schroit AJ, Zwaal RF. Transbilayer movement of phospholipids in red cell and platelet membranes. *Biochim Biophys Acta*. 1991; **1071**: 313-29.

36 Sims PJ, Ginsberg MH, Plow EF, Shattil SJ. Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. *J Biol Chem*. 1991; **266**: 7345-52.

37 Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Rev*. 2007; **21**: 131-42.

38 Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol*. 1999; **30**: 111-42.

39 Nomura S. Function and clinical significance of platelet-derived microparticles. *Int J Hematol*. 2001; **74**: 397-404.

40 Croall DE, DeMartino GN. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol Rev*. 1991; **71**: 813-47.

41 Saido TC, Sorimachi H, Suzuki K. Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J*. 1994; **8**: 814-22.

42 Suzuki K, Sorimachi H, Yoshizawa T, Kinbara K, Ishiura S. Calpain: novel family members, activation, and physiologic function. *Biol Chem Hoppe Seyler*. 1995; **376**: 523-9.

43 Tsujinaka T, Shiba E, Kambayashi J, Kosaki G. Purification and characterization of a low calcium requiring form of Ca2+-activated neutral protease from human platelets. *Biochem Int*. 1983; **6**: 71-80.

44 Ariyoshi H, Oda A, Salzman EW. Participation of calpain in protein-tyrosine phosphorylation and dephosphorylation in human blood platelets. *Arterioscler Thromb Vasc Biol*. 1995; **15**: 511-4.

45 White GC, 2nd. Calcium-dependent proteins in platelets: response of calcium-activated protease in normal and thrombasthenic platelets to aggregating agents. *Biochim Biophys Acta*. 1980; **631**: 130-8.

46 Fox JE, Reynolds CC, Phillips DR. Calcium-dependent proteolysis occurs during platelet aggregation. *J Biol Chem*. 1983; **258**: 9973-81.

47 Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH. Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. *J Biol Chem*. 1995; **270**: 26146-51.

48 Banno Y, Nakashima S, Hachiya T, Nozawa Y. Endogenous cleavage of phospholipase C-beta 3 by agonist-induced activation of calpain in human platelets. *J Biol Chem*. 1995; **270**: 4318-24.

49 Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y. Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). *J Biol Chem*. 1989; **264**: 4088-92.

50 Gemmell CH, Sefton MV, Yeo EL. Platelet-derived microparticle formation involves glycoprotein IIb-IIIa. Inhibition by RGDS and a Glanzmann's thrombasthenia defect. *J Biol Chem*. 1993; **268**: 14586-9.

51 Haga JH, Slack SM, Jennings LK. Comparison of shear stress-induced platelet microparticle formation and phosphatidylserine expression in presence of alphaIIbbeta3 antagonists. *J Cardiovasc Pharmacol*. 2003; **41**: 363-71.

52 Holme PA, Solum NO, Brosstad F, Egberg N, Lindahl TL. Stimulated Glanzmann's thrombasthenia platelets produced microvesicles. Microvesiculation correlates better to exposure of procoagulant surface than to activation of GPIIb-IIIa. *Thromb Haemost*. 1995; **74**: 1533-40.

53 Ryden L, Standl E, Bartnik M, et al. Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. *Eur Heart J*. 2007; **28**: 88-136.

54 American Diabetes Association. Standards of medical care in diabetes 2009. *Diabetes Care*. 2009; **32 Suppl 1**: S13-61.

55 Fava S, Azzopardi J, Agius-Muscat H. Outcome of unstable angina in patients with diabetes mellitus. *Diabet Med*. 1997; **14**: 209-13.

56 Henry P, Makowski S, Richard P, Beverelli F, Casanova S, Louali A, et al. Increased incidence of moderate stenosis among patients with diabetes: substrate for myocardial infarction? *Am Heart J*. 1997; **134**: 1037-43.

57 El Haouari M, Rosado JA. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. *Blood Cells Mol Dis*. 2008; **41**: 119-23.

58 Stratmann B, Tschoepe D. Pathobiology and cell interactions of platelets in diabetes. *Diab Vasc Dis Res*. 2005; **2**: 16-23.

59 Kluft C, Jespersen J. Diabetes as a procoagulant condition. *Br J Diabetes Vasc Dis*. 2002; **2**: 358-62.

60 Gerstein HC, Yusuf S. Dysglycaemia and risk of cardiovascular disease. *Lancet*. 1996; **347**: 949-50.

61 Skyler JS, Bergenstal R, Bonow RO, Buse J, Deedwania P, Gale EA, et al. Intensive glycemic control and the prevention of cardiovascular events: implications of the ACCORD, ADVANCE, and VA diabetes trials. *Diabetes Care*. 2009; **32**: 187-92.

62 Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, et al. Glucose Control and Vascular Complications in Veterans with Type 2 Diabetes. *N Engl J Med*. 2009; **360**: 129-39.

63 Patel A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, et al. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N Engl J Med*. 2008; **358**: 2560-72.

64 Gerstein HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, Buse JB, Cushman WC, et al. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med*. 2008; **358**: 2545-59.

65 Quinn MJ, Byzova TV, Qin J, Topol EJ, Plow EF. Integrin alphaIIbbeta3 and its antagonism. *Arterioscler Thromb Vasc Biol*. 2003; **23**: 945-52.

66 Gurm HS, Lincoff AM, Lee D, Tang WH, Jia G, Booth JE, Califf RM, Ohman EM, Topol EJ, et al. Outcome of acute ST-segment elevation myocardial infarction in diabetics treated with fibrinolytic or combination reduced fibrinolytic therapy and platelet glycoprotein IIb/IIIa inhibition: lessons from the GUSTO V trial. *J Am Coll Cardiol*. 2004; **43**: 542-8.

67 Reverter JC, Beguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis". *J Clin Invest*. 1996; **98**: 863-74.

68 Jilma B, Fasching P, Ruthner C, Rumplmayr A, Ruzicka S, Kapiotis S, Wagner OF, Eichler HG. Elevated circulating P-selectin in insulin dependent diabetes mellitus. *Thromb Haemost*. 1996; **76**: 328-32.

69 Kopp HP, Hopmeier P, Schernthaner G. Concentrations of circulating P-selectin are increased in patients with newly diagnosed insulin-dependent diabetes mellitus. *Exp Clin Endocrinol Diabetes*. 1998; **106**: 41-4.

70 Li N, Wallen NH, Savi P, Herault JP, Herbert JM. Effects of a new platelet glycoprotein IIb/IIIa antagonist, SR121566, on platelet activation, platelet-leukocyte interaction and thrombin generation. *Blood Coagul Fibrinolysis*. 1998; **9**: 507-15.

71 Furman MI, Krueger LA, Frelinger AL, 3rd, Barnard MR, Mascelli MA, Nakada MT, Michelson AD. GPIIb-IIIa antagonist-induced reduction in platelet surface factor V/Va binding and phosphatidylserine expression in whole blood. *Thromb Haemost*. 2000; **84**: 492-8.

72 Yngen M, Ostenson CG, Hu H, Li N, Hjemdahl P, Wallen NH. Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia*. 2004; **47**: 537-40.

73 Davi G, Catalano I, Averna M, Notarbartolo A, Strano A, Ciabattoni G, Patrono C. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med*. 1990; **322**: 1769-74.

74 Betteridge DJ, El Tahir KE, Reckless JP, Williams KI. Platelets from diabetic subjects show diminished sensitivity to prostacyclin. *Eur J Clin Invest*. 1982; **12**: 395-8.

75 De La Cruz JP, Moreno A, Guerrero A, de La Cuesta FS. Antiplatelet effects of prostacyclin and nitric oxide in patients with type I diabetes and ischemic or edematous retinopathy. *Platelets*. 2001; **12**: 210-7.

76 Varughese GI, Tomson J, Lip GY. Type 2 diabetes mellitus: a cardiovascular perspective. *Int J Clin Pract*. 2005; **59**: 798-816.

77 Hasegawa Y, Suehiro A, Higasa S, Namba M, Kakishita E. Enhancing effect of advanced glycation end products on serotonin-induced platelet aggregation in patients with diabetes mellitus. *Thromb Res*. 2002; **107**: 319-23.

78 Watala C. May the alterations in lipid fluidity-mediated platelet hypersensitivity contribute to accelerated aging of platelets in diabetes mellitus? *Med Hypotheses*. 1991; **36**: 142-5.

79 Winocour PD. Platelet abnormalities in diabetes mellitus. *Diabetes*. 1992; **41 Suppl 2**: 26-31.

80 Mazzanti L, Mutus B. Diabetes-induced alterations in platelet metabolism. *Clin Biochem*. 1997; **30**: 509-15.

81 Assert R, Scherk G, Bumbure A, Pirags V, Schatz H, Pfeiffer AF. Regulation of protein kinase C by short term hyperglycaemia in human platelets in vivo and in vitro. *Diabetologia*. 2001; **44**: 188-95.

82 Tomaselli L, Cerletti C, de Gaetano G, Notarbartolo A, Davi G, Pupillo M. Normal platelet function, but increased platelet activation in vivo in diabetic patients. *Thromb Haemost*. 1990; **64**: 604.

83 Davi G, Gennaro F, Spatola A, Catalano I, Averna M, Montalto G, Amato S, Notarbartolo A. Thrombin-antithrombin III complexes in type II diabetes mellitus. *J Diabetes Complications*. 1992; **6**: 7-11.

84 Nagai T. Change of lipoprotein (a) and coagulative or fibrinolytic parameters in diabetic patients with nephropathy. *J Atheroscler Thromb*. 1994; **1**: 37-40.

85 Aoki I, Shimoyama K, Aoki N, Homori M, Yanagisawa A, Nakahara K, Kawai Y, Kitamura SI, Ishikawa K. Platelet-dependent thrombin generation in patients with diabetes mellitus: effects of glycemic control on coagulability in diabetes. *J Am Coll Cardiol*. 1996; **27**: 560-6.

86 Lupu C, Calb M, Ionescu M, Lupu F. Enhanced prothrombin and intrinsic factor X activation on blood platelets from diabetic patients. *Thromb Haemost*. 1993; **70**: 579-83.

87 Erem C, Hacihasanoglu A, Celik S, Ovali E, Ersoz HO, Ukinc K, Deger O, Telatar M. Coagulation and fibrinolysis parameters in type 2 diabetic patients with and without diabetic vascular complications. *Med Princ Pract*. 2005; **14**: 22-30.

88 Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. *Diabetes*. 2006; **55**: 202-8.

89 Boden G, Vaidyula VR, Homko C, Cheung P, Rao AK. Circulating tissue factor procoagulant activity and thrombin generation in patients with type 2 diabetes: effects of insulin and glucose. *J Clin Endocrinol Metab*. 2007; **92**: 4352-8.

90 Schneider DJ, Nordt TK, Sobel BE. Attenuated fibrinolysis and accelerated atherogenesis in type II diabetic patients. *Diabetes*. 1993; **42**: 1-7.

91 Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost*. 2004; **2**: 1282-91.

92 Nomura S, Suzuki M, Katsura K, Xie GL, Miyazaki Y, Miyake T, Kido H, Kagawa H, Fukuhara S. Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis*. 1995; **116**: 235-40.

93 VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. *Cardiovasc Res*. 2003; **59**: 277-87.

94 Freyssinet JM. Cellular microparticles: what are they bad or good for? *J Thromb Haemost*. 2003; **1**: 1655-62.

95 Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol*. 2006; **26**: 2594-604.

96 Wolfs JL, Comfurius P, Rasmussen JT, Keuren JF, Lindhout T, Zwaal RF, Bevers EM. Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol Life Sci*. 2005; **62**: 1514-25.

97 Barry OP, Pratico D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest*. 1998; **102**: 136-44.

98 Pfister SL. Role of platelet microparticles in the production of thromboxane by rabbit pulmonary artery. *Hypertension*. 2004; **43**: 428-33.

99 Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. *Cardiovasc Res*. 2005; **67**: 30-8.

100 Colwell JA. Aspirin therapy in diabetes. *Diabetes Care*. 2003; **26 Suppl 1**: S87-8.

101 Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002; **324**: 71-86.

102 Sacco M, Pellegrini F, Roncaglioni MC, Avanzini F, Tognoni G, Nicolucci A. Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. *Diabetes Care*. 2003; **26**: 3264-72.

103 Ceriello A, Motz E. Prevention of vascular events in diabetes mellitus: which "antithrombotic" therapy? *Diabetologia*. 1996; **39**: 1405-6.

104 Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ, 2nd. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci U S A*. 1990; **87**: 9383-7.

105 Watala C, Boncler M, Gresner P. Blood platelet abnormalities and pharmacological modulation of platelet reactivity in patients with diabetes mellitus. *Pharmacol Rep*. 2005; **57 Suppl**: 42-58.

106 Perneby C, Wallen NH, Rooney C, Fitzgerald D, Hjemdahl P. Dose- and time-dependent antiplatelet effects of aspirin. *Thromb Haemost*. 2006; **95**: 652-8.

107 American Diabetes Association. Standards of medical care in diabetes 2007. *Diabetes Care*. 2007; **30 Suppl 1**: S4-S41.

108 Li N, Hu H, Lindqvist M, Wikstrom-Jonsson E, Goodall AH, Hjemdahl P. Platelet-leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol*. 2000; **20**: 2702-8.

109 Hu H, Zhang W, Li N. Glycoprotein IIb/IIIa inhibition attenuates platelet-activating factor-induced platelet activation by reducing protein kinase C activity. *J Thromb Haemost*. 2003; **1**: 1805-12.

110 Mousa SA, Khurana S, Forsythe MS. Comparative in vitro efficacy of different platelet glycoprotein IIb/IIIa antagonists on platelet-mediated clot strength induced by tissue factor with use of thromboelastography: differentiation among glycoprotein IIb/IIIa antagonists. *Arterioscler Thromb Vasc Biol*. 2000; **20**: 1162-7.

111 Gibson CM, de Lemos JA, Murphy SA, Marble SJ, McCabe CH, Cannon CP, Antman EM, Braunwald E. Combination therapy with abciximab reduces angiographically evident thrombus in acute myocardial infarction: a TIMI 14 substudy. *Circulation*. 2001; **103**: 2550-4.

112 Dangas G, Badimon JJ, Coller BS, Fallon JT, Sharma SK, Hayes RM, Meraj P, Ambrose JA, Marmur JD. Administration of abciximab during percutaneous coronary intervention reduces both ex vivo platelet thrombus formation and fibrin deposition: implications for a potential anticoagulant effect of abciximab. *Arterioscler Thromb Vasc Biol*. 1998; **18**: 1342-9.

113 Palmerini T, Nedelman MA, Scudder LE, Nakada MT, Jordan RE, Smyth S, Gordon RE, Fallon JT, Coller BS. Effects of abciximab on the acute pathology of blood vessels after arterial stenting in nonhuman primates. *J Am Coll Cardiol*. 2002; **40**: 360-6.

114 Collet JP, Montalescot G, Lesty C, Weisel JW. A structural and dynamic investigation of the facilitating effect of glycoprotein IIb/IIIa inhibitors in dissolving platelet-rich clots. *Circ Res*. 2002; **90**: 428-34.

115 Roffi M, Chew DP, Mukherjee D, Bhatt DL, White JA, Heeschen C, Hamm CW, Moliterno DJ, Califf RM, White HD, Kleiman NS, Theroux P, Topol EJ. Platelet glycoprotein IIb/IIIa inhibitors reduce mortality in diabetic patients with non-ST-segment-elevation acute coronary syndromes. *Circulation*. 2001; **104**: 2767-71.

116 Lincoff AM. Important triad in cardiovascular medicine: diabetes, coronary intervention, and platelet glycoprotein IIb/IIIa receptor blockade. *Circulation*. 2003; **107**: 1556-9.

117 Li N, Soop A, Sollevi A, Hjemdahl P. Multi-cellular activation in vivo by endotoxin in humans-limited protection by adenosine infusion. *Thromb Haemost*. 2000; **84**: 381-7.

118 Li N, Hallden G, Hjemdahl P. A whole-blood flow cytometric assay for leukocyte CD11b expression using fluorescence signal triggering. *Eur J Haematol*. 2000; **65**: 57-65.

119 Li N, Goodall AH, Hjemdahl P. Efficient flow cytometric assay for platelet-leukocyte aggregates in whole blood using fluorescence signal triggering. *Cytometry*. 1999; **35**: 154-61.

120 Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, Green DR. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood*. 1999; **94**: 1683-92.

121 Connor J, Pak CH, Zwaal RF, Schroit AJ. Bidirectional transbilayer movement of phospholipid analogs in human red blood cells. Evidence for an ATP-dependent and protein-mediated process. *J Biol Chem*. 1992; **267**: 19412-7.

122 Li N, Goodall AH, Hjemdahl P. A sensitive flow cytometric assay for circulating platelet-leucocyte aggregates. *Br J Haematol*. 1997; **99**: 808-16.

123 Yngen M, Ostenson CG, Hjemdahl P, Wallen NH. Meal-induced platelet activation in Type 2 diabetes mellitus: effects of treatment with repaglinide and glibenclamide. *Diabet Med*. 2006; **23**: 134-40.

124 Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. *Diabetes Care*. 2003; **26**: 2181-8.

125 Tang WH, Lincoff AM. Diabetes, coronary intervention, and platelet glycoprotein IIb/IIIa blockade: the triad revisited. *Circulation*. 2004; **110**: 3618-20.

126 Mehilli J, Kastrati A, Schuhlen H, Dibra A, Dotzer F, von Beckerath N, Bollwein H, Pache J, Dirschinger J, Berger PP, Schomig A. Randomized clinical trial of abciximab in diabetic

patients undergoing elective percutaneous coronary interventions after treatment with a high loading dose of clopidogrel. *Circulation*. 2004; **110**: 3627-35.

127 Zwaal RF, Comfurius P, Bevers EM. Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. *Biochim Biophys Acta*. 1992; **1180**: 1-8..

128 Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. *Diabetes Care*. 2001; **24**: 1476-85.

129 Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL. Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes*. 1994; **43**: 1122-9.

130 Pirags V, Assert R, Haupt K, Schatz H, Pfeiffer A. Activation of human platelet protein kinase C-beta 2 in vivo in response to acute hyperglycemia. *Exp Clin Endocrinol Diabetes*. 1996; **104**: 431-40.

131 Massucco P, Mattiello L, Russo I, Traversa M, Doronzo G, Anfossi G, Trovati M. High glucose rapidly activates the nitric oxide/cyclic nucleotide pathway in human platelets via an osmotic mechanism. *Thromb Haemost*. 2005; **93**: 517-26.

132 Ha H, Lee HB. Oxidative stress in diabetic nephropathy: basic and clinical information. *Curr Diab Rep*. 2001; **1**: 282-7.

133 Polonsky KS, Given BD, Hirsch LJ, Tillil H, Shapiro ET, Beebe C, Frank BH, Galloway JA, Van Cauter E. Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus. *N Engl J Med*. 1988; **318**: 1231-9.

134 Coutinho M, Gerstein HC, Wang Y, Yusuf S. The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. *Diabetes Care*. 1999; **22**: 233-40.

135 Bonora E, Muggeo M. Postprandial blood glucose as a risk factor for cardiovascular disease in Type II diabetes: the epidemiological evidence. *Diabetologia*. 2001; **44**: 2107-14.

136 Gaede P, Vedel P, Parving HH, Pedersen O. Intensified multifactorial intervention in patients with type 2 diabetes mellitus and microalbuminuria: the Steno type 2 randomised study. *Lancet*. 1999; **353**: 617-22.

137 Temelkova-Kurktschiev TS, Koehler C, Henkel E, Leonhardt W, Fuecker K, Hanefeld M. Postchallenge plasma glucose and glycemic spikes are more strongly associated with atherosclerosis than fasting glucose or HbA1c level. *Diabetes Care*. 2000; **23**: 1830-4.

138 Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M. Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: the STOP-NIDDM trial. *Jama*. 2003; **290**: 486-94.

139 Yngen M, Ostenson CG, Li N, Hjemdahl P, Wallen NH. Acute hyperglycemia increases soluble P-selectin in male patients with mild diabetes mellitus. *Blood Coagul Fibrinolysis*. 2001; **12**: 109-16.

140 Neri Serneri GG, Coccheri S, Marubini E, Violi F. Picotamide, a combined inhibitor of thromboxane A2 synthase and receptor, reduces 2-year mortality in diabetics with peripheral arterial disease: the DAVID study. *Eur Heart J*. 2004; **25**: 1845-52.

141 Peter K, Schwarz M, Ylanne J, Kohler B, Moser M, Nordt T, Salbach P, Kubler W, Bode C. Induction of fibrinogen binding and platelet aggregation as a potential intrinsic property of various glycoprotein IIb/IIIa (alphaIIbbeta3) inhibitors. *Blood*. 1998; **92**: 3240-9.

142 Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials. *Circulation*. 2001; **103**: 201-6.

143 Pedicord DL, Thomas BE, Mousa SA, Dicker IB. Glycoprotein IIb/IIIa receptor antagonists inhibit the development of platelet procoagulant activity. *Thromb Res*. 1998; **90**: 247-58.

144 Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thromb Haemost*. 2002; **88**: 186-93.

145 Byzova TV, Plow EF. Networking in the hemostatic system. Integrin alphaiibbeta3 binds prothrombin and influences its activation. *J Biol Chem*. 1997; **272**: 27183-8.

146 Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med*. 1988; **318**: 1315–21.

147 Kowluru RA, Heidorn DB, Edmondson SP, Bitensky MW, Kowluru A, Downer NW, Whaley TW, Trewhella J. Glycation of calmodulin: chemistry and structural and functional consequences. *Biochemistry*. 1989; **28**: 2220–8.

148 Hu H, Li N, Yngen M, Östenson CG, Walle´n NH, Hjemdahl P. Enhanced leukocyte-platelet cross-talk in type 1 diabetes mellitus: relationship to microangiopathy. *J Thromb Haemost*. 2004; **2**: 58–64.

149 Watala C, Boncer M., Golanski J, Koziolkiewcz W, Trojanowski Z, Walkowiak B. Platelet membrane lipid fluidity and intraplatelet calcium mobilization in type 2 diabetes mellitus. *Eur J Haematol.* 1998; **61:** 319–26.

150 Gawaz M, Ott I, Reininger AJ, Neumann FJ. Effects of magnesium on platelet aggregation and adhesion. Magnesium modulates surface expression of glycoproteins on platelets in vitro and ex vivo. *Thromb Haemost*. 1994; **72**: 912-18.