PLATELET TISSUE FACTOR SYNTHESIS IN TYPE 2 DIABETES PATIENTS IS RESISTANT TO INHIBITION BY INSULIN

Anja J Gerrits,¹ Cornelis A Koekman,¹ Timon W van Haeften²
and Jan Willem N Akkerman¹

¹Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology,
²Department of Internal Medicine,
University Medical Center Utrecht, Utrecht, the Netherlands

Correspondence:
Prof.Dr. J.W.N. Akkerman
Email: j.w.n. akkerman@umcutrecht.nl

Submitted 10 July 2009 and accepted 18 February 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
**Objective** Patients with type 2 diabetes have an increased risk of cardiovascular disease and show abnormalities in the coagulation cascade. We investigated whether increased synthesis of Tissue Factor (TF) by platelets could contribute to the hypercoagulant state.

**Research Design and Methods** Platelets from type 2 diabetes patients and matched controls were adhered to different surface-coated proteins and TF-pre-mRNA splicing, -protein and -procoagulant activity were measured.

**Results** Different adhesive proteins induced different levels of TF-synthesis. A mimetic of active clopidogrel metabolite (AR-C699331 MX) reduced TF synthesis by 56 ± 10%, an aspirin-like inhibitor (indomethacin) by 82 ± 9% and the combination by 96 ± 2%, indicating that ADP release and thromboxane A₂ production followed by activation of P2Y12- and thromboxane receptors mediate surface-induced TF-synthesis. Interference with intracellular pathways revealed inhibition by agents that raise cAMP and interfere with PI3-K/PKB. Insulin is known to raise cAMP in platelets and inhibited collagen-III induced TF-pre-mRNA splicing and reduced TF activity by 35±5 and 47±5% at 1 and 100 nM. Inhibition by insulin was reduced in type 2 diabetes platelets resulting in a ~1.6-fold higher TF synthesis than in matched controls.

**Discussion** We characterized the extra- and intracellular mechanisms that couple surface activation to TF-synthesis in adhering platelets. In healthy individuals, TF-synthesis is inhibited by insulin but in patients with type 2 diabetes inhibition is impaired. This leads to the novel finding that platelets from type 2 diabetes patients produce more TF than platelets from matched controls.
Type 2 diabetes patients have a 2–8-fold higher risk of cardiovascular disease than healthy individuals and eighty percent will die of arterial thrombosis-related disorders (1). Type 2 diabetes induces a prothrombotic state caused by imbalance of the hemostatic mechanism with evidence of hypercoagulation, decreased fibrinolysis, platelet hyperaggregability and endothelial dysfunction (2;3). Circulating markers for activated coagulation such as prothrombin fragment 1 + 2 and thrombin-anti-thrombin complexes are increased. Also the elevated levels of fibrinogen, factors (F) VII, VIII, XI and von Willebrand factor (VWF) might contribute to the prothrombotic tendency (4). In particular, circulating tissue factor (TF) is increased (5), which, as the main initiator of the coagulation cascade, might contribute to hypercoagulability. Interestingly, improvement of glycaemic control lowers plasma TF (6).

The hyperaggregability of type 2 diabetes platelets might be caused by loss of sensitivity to insulin. Under experimental conditions, platelets of type 2 diabetes patients show better adhesion to a prothrombotic surface, make larger aggregates, have an increased pro-coagulant surface and an elevated cytosolic Ca\(^{2+}\) concentration compared to platelets from healthy controls (7). Intensive insulin treatment partly normalizes platelet aggregation (8). Indeed, insulin inhibits platelets from healthy individuals but not from type 2 diabetes subjects (9;10).

Normally, platelet activators start signaling pathways that initiate aggregation and secretion while concurrently reducing the level of the intracellular inhibitor cAMP through the G-protein subunit G\(_i\alpha_2\). Insulin inactivates G\(_i\alpha_2\) through binding of insulin receptor substrate-1 (IRS-1) to G\(_i\alpha_2\) (7,11). In type 2 diabetes platelets, this property is lost presumably due to a defect in IRS-1. In addition to signaling through G\(_i\alpha_2\), insulin releases G\(_{\beta\gamma}\), activating the phosphatidylinositol 3-kinase (PI3-K) / protein kinase B (PKB) pathway and increasing glucose uptake through GLUT3 transporters (12;13).

A key step in the initiation of a haemostatic plug is TF exposure. TF is an integral transmembrane protein (43 kD) located on the plasma membrane (14). TF binds activated FVII and the complex activates FVII, FIX and FX, generating thrombin and inducing clotting. TF is constitutively expressed by vascular smooth muscle cells and vessel damage rapidly starts coagulation. Also monocytes and endothelial cells produce TF following a shift to a prothrombotic phenotype induced by inflammatory factors such as lipopolysaccaride (LPS). Studies in mice with a general and a bone marrow-specific TF suppression show that fibrin propagation depends exclusively on blood-born TF. The source of blood-born TF is sought in microparticles. They deliver TF in an encrypted state which upon contact with the thrombus changes to an active conformation that initiates clotting (15). We showed recently that TF production by LPS-stimulated monocytes is suppressed by insulin via a mechanism similar but not identical to insulin’s inhibition of platelet functions (16). Preliminary data indicate that in type 2 diabetes monocytes have lost sensitivity to insulin and produce more TF than their normal counterparts.

Recent findings suggest a new source for TF expression (17). The anucleate platelet has long been considered incapable of synthesizing proteins, but the presence of a splicing machinery together with pre-mRNAs for a number of proteins including TF make them a source for TF synthesis.

In the present study we investigated how contact with adhesive proteins initiates platelet TF synthesis and studied a possible
Inhibition of platelet TF synthesis by insulin

interference by insulin in healthy individuals and type 2 diabetes patients.

RESEARCH DESIGN AND METHODS
Materials - We obtained human recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mmol/L acetic acid, 100 mmol/L NaCl, and 0.01% BSA to a stock concentration of 100 µmol/L), wortmannin, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), lipopolysaccharide (LPS, E. coli 0111:B4), indomethacin, puromycin, collagen type I and III, ADP from Sigma (St. Louis, MO, USA), Akt inhibitor 1701-1 from Biovision (Mountain View, CA, USA), iloprost from Schering AG (Berlin, Germany), forskolin, BAPTA(AM), ionophore A23187, Tg003 and cantharidin from Calbiochem (La Jolla, CA, USA), LY294002 from Biomol (Plymouth Meeting, PA, USA), cycloheximide from MP Biochemicals (Santa Cruz, CA, USA), collagen reagent Horm from Nycomed Pharma, a mixture of 95% type I and 5% type III collagen (Munich, Germany), thrombin and fibrinogen from Enzyme Research Laboratories (South Bend, IN, USA), U-44619 from Cayman Chemicals (Ann Arbor, MI, USA). Plasma-derived VWF was purified from VWF/FVIII concentrate (Haemate P, Behring, Marburg, Germany) (18). Factor X (FX) was purified from fresh-frozen plasma (19). Recombinant Factor VIIa (rFVIIa) was from Novo Nordisk (Bagsvaerd, Denmark) and recombinant TF (Innovin) from Dade Behring, Liederbach, Germany. Antibodies against the horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA), anti-phospho-Ser polyclonal antibody from Upstate Biotechnology (Bucks, UK), peroxidase-linked goat anti-mouse antibody from DAKO (Glostrup, Denmark), anti-SF2/ASF antibody from Abcam (Cambridge, UK), anti-TF antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for immunoprecipitation and Affinity Biologicals (Ancaster, ON, Canada) for Western blotting and anti-TF pathway inhibitor (TFPI) antibody from American Diagnostica (Stamford, UK). An inhibitory antibody against TF was a generous gift from Dr M Kjalke (Hemostasis Biology, Novo Nordisk, Malov, Denmark). The ADP receptor P2Y12 antagonist, N6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK). All other chemicals used were of analytical grade.

Study design – Isolated platelet suspensions were made monocyte-free and adhered to different surface-coated adhesive proteins. Splicing of TF pre-mRNA and synthesis of TF- protein and TF-procoagulant activity were measured. Pharmacologic interference with extra- and intracellular signaling was investigated, focusing specifically on the role of insulin in healthy individuals and patients with type 2 diabetes.

Subjects - The study had been approved by the Medical Ethical Review Board. Type 2 diabetes subjects were recruited from the outpatient clinics of the Utrecht Medical University Hospital (Utrecht, Netherlands). All patients were anti-GAD negative, and had plasma C-peptide levels higher than 0.30 nmol/L. We recruited insulin-using patients in order to circumvent the possibility that oral hypoglycemic agents interfere with the study; only metformin use was acceptable (it was stopped the evening for the study). Six patients used four injections with insulin per day, one used CSII. Their further characteristics are given in Table 1. Patients and matched control subjects gave their informed consent prior to participation in the study.

Platelet isolation - Freshly drawn venous blood from healthy volunteers and type 2 diabetes patients was collected into 0.1
volume of 130 mmol/L Na$_3$ citrate. Platelets were isolated as described (11). Attempts to remove contaminating monocytes included (i) incubation of a platelet suspension ($2 \times 10^8$ platelets/mL) with either anti-CD14 or CD45 antibody coupled beads (15 min, 22°C, 35 µL anti-CD14 or anti-CD45 beads per 40 $\times 10^8$ platelets) and subsequent magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and (ii) a double incubation of a concentrated platelet suspension (~15 $\times 10^8$ platelets/mL) with a cocktail of anti-CD14 and anti-CD45 antibody coupled beads (15 min, 22°C, 35 µL anti-CD14 and 35 µL anti-CD45 beads per 40 $\times 10^8$ platelets) and magnetic separation. Contamination with monocytes was measured by RT-PCR of CD14 mRNA. To this end, total RNA was isolated using the kit from RNA-Bee (Tell Tech, Friendswood, TX, USA) and RT-PCR was performed using the forward primer 5’-AAAGCACTTCCAGACGCTGC-3’ and the reverse primer 5’-TCGAGCGTCAGTTCCTTGAGG-3’ on a PTC-200 apparatus (MJ Research, Waltham, MA, USA).

**TF expression** – To investigate TF synthesis in platelets, an aliquot of suspension ($2 \times 10^8$ platelets) was incubated for static adhesion experiments in 6-wells plates (Corning Ultra-Low Attachment surface, Corning, Acton, MA, USA) or kept in suspension for the indicated times at 37°C. Wells were coated with 50 µg/mL hrom collagen, 50 µg/mL collagen type I, 50 µg/mL collagen type III, 1 U/mL thrombin, 100 µg/mL fibrinogen and 10 µg/mL VWF for 2 hrs at 37°C. For analysis of TF procoagulant activity, adhered platelets, collected in 250 µL HEPES/Tyrde buffer, or suspensions were lysed by 3 times freeze/ thawing and centrifuged (16200 x g, 2 min, 22°C). Pellet fractions were dissolved in 50 µL HEPES/Tyrde buffer. Samples were incubated with 2 µg/mL anti-TFPI antibody (15 min, 22°C) and thereafter with 10 µg/mL FX, 5 U/mL rFVIIa and 5 mmol/L CaCl$_2$ (45 min, 37°C). FXa generation was measured in a fluorescence reader at 405 nm after addition of FXa substrate (Pentapharm, Basel, Switzerland). Procoagulant activity was determined based on a standard curve derived from serial dilutions of recombinant TF.

For analysis of splicing of TF pre-mRNA, 12 $\times 10^8$ platelets were adhered to a coated surface or kept in suspension, as indicated. Total RNA was isolated (RNA-Bee, Tell Tech) and RNA levels adjusted for variations per sample (by measuring the optical density OD260 on a NanoDrop spectrophotometer) and RT-PCR was performed on a PCR apparatus (PTC-200, MJ Research, Waltham, MA, USA) using primers that overlapped intron four, with forward primer 5’-CTCGGACAGCCAACAATTGCAG-3’ and reverse primer 5’-CGGGCTGTCTGTACTCTTCC-3’.

**Alkaline phosphatase assay** – The number of adhered platelets was inferred from the content of alkaline phosphatase in collected samples, as published (20). Alkaline phosphatase activity was related to platelet number using a serial dilution of platelets in suspension.

**Immunoprecipitation, SDS-PAGE and Western blotting** – To determine levels of TF protein and the Ser phosphorylation of SF2, aliquots of $2 \times 10^8$ platelets were adhered to a coated surface in 6-wells plates or kept in suspension (1 hr, 37°C). Adhered platelets and suspensions were collected in lysis buffer (1% NP40, 0.5% octylglucoside, 0.1% SDS, 5 mmol/L EDTA in PBS) supplemented with 10% protease inhibitor cocktail, 1 mmol/L NaVO$_3$ and 10 µM cantharidin. Proteins were precipitated with protein G-sepharose in combination with 1 µg/mL anti-TF or anti-SF2 antibody overnight at 4°C. Precipitates were washed 3 times and dissolved in reducing Laemmli sample buffer.
Proteins were analyzed by SDS-PAGE and Western blotting. After blocking with Odyssey buffer (1 hr, 22°C), membranes were incubated (16 hrs, 4°C) with 1 µg/mL anti-TF, anti-phospho-Ser or anti-SF2 antibody. Immunoblots were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and Alexa-labelled antibodies according to the manufacturer’s instructions. The intensity of the bands was quantified with ImageJ software.

Statistics - Data is expressed as mean ± S.E.M. with n observations and was analyzed with Student’s test for unpaired observations or Mann Whitney U test, as indicated. Differences were considered significant at p<0.05.

RESULTS

Preparation of pure platelet suspensions - Since monocytes have a much higher capacity to synthesize TF than platelets, it was essential to prepare pure platelet suspensions. Platelet preparations prepared by conventional centrifugation/resuspension (7;11) without and with treatment with anti-CD45 magnetic beads were positive for the monocyte-marker CD14 detected by RT-PCR (Fig. 1a, top panel). Pure preparations were obtained by a double incubation of concentrated suspensions with anti-CD14/CD45 magnetic beads and monocyte removal by magnetic sorting. After 35 cycli, RT-PCR of CD14 was negative in the platelet preparation and positive in the eluate (Fig. 1a, bottom panel). Upon stimulation with LPS, CD14-mRNA containing suspensions adhering to fibrinogen synthesized TF activity detected in the Factor Xa assay, but CD14-mRNA negative preparations did not (Fig. 1b). To ensure purity of platelet suspensions, a sample of adhered platelets with LPS was included in all experiments and series with LPS-induced TF synthesis were discarded. Addition of anti-TF antibody (0.5 mg/mL, 15 min, 22°C) completely blocked TF activity in activated platelets confirming specificity of the FXa assay.

Activated platelets express TF by splicing of pre-mRNA - TF synthesis and TF pre-mRNA splicing were studied on fibrinogen- and collagen-coated surfaces. Platelets adhering to a fibrinogen-coated surface (4 hr, 37°C) synthesized 0.24 ± 0.04 ng TF/ 2·10⁸ platelets (n=10). TF synthesis in adhered platelets was inhibited by the translation blockers cycloheximide (58 ± 2%, n=3) and puromycin (76±6%, n=12) and the splicing blocker Tg003 (51 ± 7%, n=6; Fig. 1c), confirming earlier work (17;24). To demonstrate splicing of TF pre-mRNA, a RT-PCR with primers overlapping intron four was performed on platelets adhered to collagen type I (2 hr, 37°C). The result showed the expected band for spliced mRNA at 297 bp and for unspliced mRNA at 904 bp in the presence of Tg003 (Fig. 1d). Regulation of TF pre-mRNA splicing was examined by measuring the Ser phosphorylation of SF2, an effector of Cdc2-like kinase (Clk-1; Fig. 1e). The low levels of SF2-Ser phosphorylation in platelets in suspension were unchanged after stimulation with thrombin and increased when platelets had adhered to fibrinogen (4 hrs, 37°C). Tg003 reduced the phosphorylation, confirming its dependence on Clk1. These results confirm that adhering platelets synthesize TF through splicing of pre-mRNA.

Different splicing at different adhesive surfaces - The question was addressed which adhesive surface induced the highest TF pre-mRNA splicing. Puromycin was used to separate translation-dependent TF activity from background TF. TF synthesis on horm collagen, collagen I and thrombin was 0.34 ± 0.10, 0.32 ± 0.07 and 0.30 ± 0.05%, respectively. A lower TF activity was found on fibrinogen (0.24 ± 0.04), collagen III (0.17 ± 0.02%) and VWF (0.09 ± 0.03%) (n=4; Fig. 2a). Since different adhesive surfaces induced different extents of adhesion/aggregation,
bound platelets were quantified on the basis of alkaline phosphatase content. The highest adhesion/aggregation was found with collagen III, collagen I and horm collagen (~125 x 10^6 platelets), whereas adhesion to thrombin, fibrinogen and VWF was about 30% lower (Fig. 2b). Expression per platelet and correction for background TF (defined as ∆TF activity), indicated that horm collagen, thrombin and collagen I induced the highest TF synthesis followed by fibrinogen, collagen III and VWF (Fig. 2c). Further experiments were based on collagen III, because it induced the highest platelet adhesion and is present in the vasculature. We examined whether adhered platelets produce more TF than platelets in suspension. Collagen III-adhered platelets (4 hrs, 37°C) produced 600 fold more TF than platelets in suspension stimulated with collagen III (0.17 ± 0.09 and 0.0003 ± 0.0001 ng TF/ 2.0 x 10^8 platelets respectively, n=3), indicating that splicing of TF pre-mRNA is strongly facilitated by adherence to a surface (Fig. 2d), such in contrast with an earlier finding (17;24).

Inhibition of platelet TF synthesis- To examine the mechanisms that couple surface activation to TF synthesis, platelets were adhered to collagen III without and with co-incubation with AR-C699331 MX (which mimics blockade of the P2Y12-receptor by the active metabolite of clopidogrel), with indomethacin, an easily soluble COX-1 inhibitor which mimics the action of aspirin, or a combination. The inhibitors did not change platelet adhesion during prolonged contact with collagen III (insert Fig. 3a). AR-C699331 induced a 56 ± 16% decrease in TF synthesis, indicating that in its absence TF synthesis is enhanced by ADP secretion and P2Y12 receptor-mediated signaling. Indomethacin induced an even steeper reduction and inhibited TF synthesis by 82 ± 9%. The combination of the two inhibitors reduced TF synthesis by 96 ± 2% (n=4, Fig. 3a). Thus, platelet TF synthesis strongly depends on the extracellular feed-back loops mediated by secretion of granule ADP and release of TxA2.

There was little effect of metabolic inhibitors on adhesion (not shown). The adenylyl cyclase activator forskolin and the stable prostacyclin mimic iloprost raise cAMP and inhibited TF synthesis on collagen III by 51 ± 14 and 82 ± 6% respectively. BAPTA/AM, which lowers the cytosolic Ca^{2+} concentration and quenches Ca^{3+} increases in activated platelets, inhibited TF synthesis almost completely. Incubation with ionophore A23187 alone did not change collagen III-induced TF synthesis, but co-incubation with 1 mM Ca^{2+} increased TF synthesis to 443 ± 57%. Inhibitors of PI3-K (wortmannin, LY294002) and PKB (Akt inhibitor 1701-1, ML-9) decreased TF synthesis by 60% or more, indicating that in the absence of inhibitors, the PI3-K/PKB pathway signals to TF pre-mRNA splicing (Fig. 3b). To investigate whether the inhibitors interfere with release of ADP/TxA2 and/or with signaling from ADP/TxA2 receptors to the spliceosome, experiments were repeated in the presence of excess of ADP (50 µM) and of the stable TxA2 analog U-46619 (2 µM).

Inhibition of TF synthesis by the metabolic inhibitors was preserved, indicating that in addition to the known suppression of ADP/TxA2 release, these blockers interfere with the control of TF pre-mRNA splicing (Fig. 3c). Western blot analysis confirmed that iloprost inhibited synthesis of TF protein (Fig. 3d). Together, these data reveal that surface-induced TF synthesis is inhibited by a rise in cAMP, strongly depends on cytosolic Ca^{2+} and is induced by signaling through the PI3-K/PKB pathway.

Insulin inhibits collagen-induced TF synthesis in platelets- Since surface-induced TF synthesis was sensitive to agents that raise cAMP or inhibit the PI3-K/PKB pathway, we addressed the question whether insulin interferes with adhesion-induced TF synthesis.
Inhibition of platelet TF synthesis by insulin

Platelets were pre-incubated with 100 nM insulin (5 min, 37°C) and adhered to different adhesive surfaces. The degree of inhibition differed between adhesive proteins and reached 94 ± 6, 64 ± 27, 76 ± 27, 67 ± 14, 41 ± 7% (n=5) for hrom collagen, thrombin, collagen I, fibrinogen and collagen III respectively. On VWF no inhibition by insulin was found (Fig. 4a). To elucidate whether insulin interferes with splicing before or after release of ADP/TxA$_2$, experiments were repeated in the presence of excess of ADP and TxA$_2$ mimetic. Inhibition of collagen III-induced TF synthesis was preserved, indicating that insulin inhibits TF-synthesis by interfering with the control of pre-mRNA splicing induced by ADP and thromboxane A$_2$ receptors (Fig. 4b). Collagen III-adhered platelets were used to further evaluate the inhibition by insulin. Adhered platelets showed a dose-dependent inhibition between 1 and 100 nM insulin (Fig. 4c). Inhibition of pre-mRNA splicing by insulin was clearly detectable, showing decreased levels of mature mRNA compared to platelets in the absence of insulin (Fig. 4d). In type 2 diabetes patients, collagen III-induced TF activity inhibition by insulin was lost, resulting in higher levels of collagen III-induced TF activity (96 ± 12%, n=8) compared to matched controls (59 ± 12%, n=8; p<0.05 with Mann Whitney U test; Fig 4e).

DISCUSSION

Novel findings in the present study are (i) the identification of the extra- and intracellular mechanisms that couple surface activation to initiation of TF synthesis in platelets, (ii) the inhibition of TF synthesis by insulin in normal platelets and, (iii) the loss of insulin inhibition in platelets from patients with type 2 diabetes resulting in a 1.6-fold higher TF synthesis. The increased TF synthesis might be a cause of the hypercoagulant state observed in type 2 diabetes patients and contribute to the increase in thrombotic risk.

The presence of TF synthesis in platelets is controversial. Some investigators attributed a positive outcome to contamination with monocytes which have a many fold higher translation capacity (21-23). Therefore, we included a purity check in every experiment to demonstrate unequivocally that platelets synthesize TF. The CD14 mRNA-free platelet suspensions showed no TF pre-mRNA splicing upon stimulation with LPS, which is a potent inducer of TF synthesis in monocytes (16). Apparently, splicing of TF pre-mRNA is not under control of the platelet LPS receptor which contrasts with other studies that show LPS-induced splicing of IL-1β and COX-2 pre-mRNA in platelets (26).

Platelets adhering to surface-coated adhesive proteins start splicing of TF pre-mRNA, synthesis of TF protein and induce the capacity to initiate coagulation, confirming earlier observations (17;24). The anucleate platelet has long been considered incapable of synthesizing proteins. Intron removal was thought to be confined to the nucleated megakaryocyte and platelet proteins to be the exclusive result of transcription and translation in this progenitor cell (27). Unexpectedly, the platelet cytoplasm contains splicing factor 2 /alternative splicing factor (SF2/ASF), a regulator of constitutive and alternative splicing (28) together with a number of pre-mRNAs with message for IL-1β, B-cell lymphoma 3, plasminogen activator inhibitor-1, cyclo-oxygenase-2 (COX-2) and TF (29-31). Platelets stimulated with soluble agonists or adhering to fibrinogen, start formation of mature RNAs and translation into functional proteins. Splicing of TF pre-mRNA results in formation of TF protein and the capacity to start coagulation and is under control of dual specificity Cdc2-like kinase (Clk)1, which contains an N-terminal region enriched in serine/arginine dipeptides (SR) that interacts...
with SF2/ASF (32). Inhibition of splicing by Tg003 and of protein synthesis by puromycin confirm that platelets are capable of transcription/translation-dependent TF synthesis (17). However, the inhibition was incomplete, suggesting that platelets contain a small store of encrypted TF that becomes active when the cells adhere to an adhesive surface.

Splicing of TF pre-mRNA was 600-fold more efficient in adhering platelets than in platelet suspensions, suggesting that close contact between platelets accelerates this process. In adhered platelets, the P2Y12-receptor blocker AR-C699331 MX inhibited TF synthesis by 56% and the COX-1 blocker indomethacin by 82%, illustrating that without inhibitors, TF synthesis is strongly enhanced by ADP secretion and TxA₂ formation and subsequent activation of the P2Y12 receptor (ADP) and TPαβ receptors (TxA₂). The combination of the blockers reduced TF synthesis almost by 100%. This observation might indicate that in vivo signaling to TF pre-mRNA splicing is inhibited by the active clopidogrel metabolite as well as aspirin and that the combination abolishes the major part of platelet TF production. Apparently, there is little TF synthesis in the absence of feedback activation by released ADP and TxA₂, indicating that surface receptors that trigger TF pre-mRNA splicing predominantly do so by inducing these positive feedback pathways. Collagen I, horn collagen and thrombin had the strongest capacity to induce splicing of TF pre-mRNA. Fibrinogen and collagen III induced less splicing and VWF was the weakest activator. These differences might reflect the contribution of ADP/TxA₂ release in platelet activation by these agonists, which is strong in horn collagen (33) and thrombin (34) activation, weak in activation by surface-coated fibrinogen (35) and virtually absent in platelet activation by coated VWF (36).

Iloprost, BAPTA-AM and inhibitors of PI3-K / PKB inhibited surface-induced TF synthesis, indicating that negative control through cAMP-dependent mechanisms and positive control through the PI3-K/PKB route determine the rate of TF synthesis. In addition to a direct effect of these inhibitors on the release of ADP/TxA₂, inhibition of TF activity also takes place after the released products start further signaling through the P2Y12- and TxA₂ receptors. PKAα and PKAβ are activated by an increase in cAMP concentration induced by forskolin or 8-CPT-cAMP and phosphorylate SR proteins in vitro and change splice site selection in vivo (37). PKB contributes to phosphorylation of SR proteins, specifically SF2 (38), which potentiate their binding to recently transcribed mRNA and/or directly stimulate SR protein activities. Interestingly, in monocytes TF expression is under negative control by the PKB pathway (16;39). Here PKB inhibits NF-kB activity (39), such in contrast to anucleated platelets where PKB regulates the splicing mechanism.

The induction of ADP/TxA₂ release followed by signaling from ADP/TxA₂ receptors to the spliceosome is under control of insulin. In normal platelets, insulin interferes with the P2Y12 pathway by inducing the association of IRS-1 with Gᵢα₂, thereby blocking Gᵢ-mediated cAMP suppression, which attenuates platelet functions. In addition, insulin stimulates the PI3-K – PKB pathway, initiating glucose uptake through upregulation of GLUT3 transporters (7;13). Interference with cAMP suppression by insulin impairs agonist-induced Ca²⁺ rises, aggregation, secretion and the generation of a procoagulant surface and the interference with TF synthesis presented here adds to the list of platelet functions under negative control by insulin.

Platelet TF does not seem to play a role in acute injury, since it takes long to express active TF. An animal model of laser induced...
vessel wall injury (14) shows the first appearance of TF at about 100 sec after induction of vessel wall damage, which is well before the first sign of platelet TF synthesis. Diabetes increases the risk of developing lower extremity arterial disease, which is one of the first signs of a generalized atherosclerotic disease (40). A key event in the pathogenesis of diabetes type 2 is loss of atherosclerotic plaque stability. When the fibrous cap is disrupted, a platelet plug forms, which continuously increases in size until the lumen is completely obstructed. In the chronic process of arterial occlusive disease, the slow but consistent production of TF by platelets may well contribute to the propagation and stabilization of a thrombus. Indeed, previous studies have detected TF antigen and activity in the atherosclerotic plaque (41). The elevated TF synthesis in platelets form type 2 diabetes patients might result in a more stable clot inducing occlusion of an artery at sites where atherosclerotic lesions have developed.

ACKNOWLEDGEMENTS
This work was supported by the Dutch Diabetes Research Foundation (no. 2004.00.029) and the Netherlands Thrombosis Foundation (2006-1). The authors acknowledge the cooperation of the patients and control subjects.
REFERENCES


Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=8)</th>
<th>Type 2 diabetes mellitus patients (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52 ± 3</td>
<td>58 ± 3</td>
<td>0.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 0.9</td>
<td>28 ± 2</td>
<td>0.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140 ± 7</td>
<td>131 ± 3</td>
<td>0.2</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>86 ± 4</td>
<td>80 ± 4</td>
<td>0.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 ± 0.09</td>
<td>7.3 ± 0.5</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>6.1 ± 0.4</td>
<td>4 ± 0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.3 ± 0.07</td>
<td>1.1 ± 0.11</td>
<td>0.2</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>95 ± 6.4</td>
<td>104 ± 6</td>
<td>0.3</td>
</tr>
<tr>
<td>Management with insulin</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>16 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-peptide (nmol/L)</td>
<td>0.74 ± 0.14 (0.34-1.64)</td>
<td>0.72 ± 0.07 (0.39–1.05)</td>
<td>0.9</td>
</tr>
<tr>
<td>Smoking</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medication – Aspirin</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medication – Statin</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medication – ACE-inhibitor or ARB</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (range)

Figure legends

Figure 1. Platelet-specific splicing of TF pre-mRNA and TF synthesis
(a) Detection of leukocyte-specific CD14 RNA (422 bp) in monocytes (lanes 1), platelet suspensions prepared by conventional centrifugation/resuspension (2), leukocyte-depleted platelet suspensions obtained by incubation with either anti-CD14 or anti-CD45 magnetic beads (3, top panel) or double incubation with anti-CD14 and anti-CD45 magnetic beads (3, bottom panel) and elution (4, leukocyte-rich fraction). Controls -RT and –RNA show the presence of genomic DNA and the specificity of the PCR (5,6). (b) Lack of LPS induced TF synthesis in CD14 mRNA-negative suspensions. CD14 mRNA-positive and CD14 mRNA-negative platelet suspensions without (open bars) and with (closed bars) 1 μg/mL LPS. Incubation with a blocking antibody against TF completely inhibited TF activity in CD14 mRNA-negative suspensions. TF activity in fibrinogen-adhered platelets without LPS stimulation was expressed as 100%. (c) TF activity in fibrinogen-adhered platelets (4 hr, 37°C; open bar) is inhibited by the translation blockers cycloheximide and puromycin and the splicing blocker Tg003 (in DMSO); closed bars. (d) Splicing by platelets adhering to collagen I (4 hr, 37°C) demonstrating a shift from pre-mRNA (904 bp) to mature mRNA (297 bp). (e) Negative SF2 phosphorylation in resting and thrombin-stimulated platelet suspensions and positive SF2 phosphorylation in fibrinogen-adhered platelets which is inhibited by Tg003. Data are means ± SEM; n=3-12 (a), (d) and (e) representative examples for n=3.

Figure 2. Surface-dependent platelet TF activity
(a) TF synthesis in platelets adhering to surface-coated horm collagen (horm, 50 μg/mL), collagen I (col I, 50 μg/mL), thrombin (thr, 1 U/mL), fibrinogen (fg, 100 μg/mL), collagen III (col III, 50 μg/mL) and VWF (10 μg/mL, each per well) (4 hr, 37°C). TF activity in fibrinogen-
Inhibition of platelet TF synthesis by insulin

adhered platelets was set at 100%. Pre-incubation with puromycin (15 min, 37°C) inhibited TF activity to background. (b) Platelets were adhered to different adhesive proteins and at different times alkaline phosphatase was measured and the number of adhered platelets determined. A representative example of n=4 is shown. (c) For different adhesive proteins surface-induced TF activity was expressed per platelet, corrected for background TF and defined as ΔTF activity. (d) Platelets adhering to collagen III (4 hr, 37°C) produce 600-fold more TF activity than the same platelet number in suspension stimulated with a same concentration of collagen III. (a) and (c) TF activity in fibrinogen-adhered platelets was expressed as 100%. Data are means ± SEM; n=3-4.

Figure 3. Effect of inhibitors of signaling pathways on TF synthesis in platelets
(a) Platelets were pre-incubated with a mimetic of active clopidogrel metabolite (AR-C699331 MX, AR, 250 nM), the aspirin-like inhibitor indomethacin (Indo, 100 µM) or the combination (15 min, 37°C). AR-C699331 MX and indomethacin did not affect platelet adhesion (insert) but reduced surface-induced TF synthesis (4 hr, 37 °C). (b) Platelets were preincubated with the adenylyl cyclase activator forskolin (40 µM), the stable prostacyclin mimetic iloprost (1 µg/mL), the Ca²⁺ quencher BAPTA/AM (25 µM), ionophore A23187 (12 µM) in the absence and presence of CaCl₂ (1 mM), the PI3-K/PKB inhibitors wortmannin (0.5 µM) and LY294002 (10 µM), the Akt inhibitor 1701-1 (2 µM) and ML-9 (100 µM) (15 min, 37°C). The inhibitors reduced the surface-induced TF synthesis (4 hr, 37 °C). Addition of vehicle alone did not change surface-induced TF levels (as shown in Fig. 1c). (c) Platelets in the presence of excess of ADP and the TxA₂ mimic U-44619 were pre-incubated with iloprost, BAPTA/AM, wortmannin, LY294002, the Akt inhibitor 1701-1 and ML-9 before platelets were adhered to collagen III (4 hr, 37°C) and TF activity was determined. (d) Platelets were adhered to fibrinogen (4 hr, 37°C). The blot shows the 60 kD band (in presence of DTT) of monocyte TF, surface-induced platelet TF protein, the inhibition by puromycin and by the cAMP raising agent iloprost. (a, insert) and (d) representative example for n=3. (a), (b) and (c) TF activity in collagen III-adhered platelets was expressed as 100%. Data are means ± SEM; n=3-4.

Figure 4. Insulin inhibits synthesis of platelet TF
(a) Platelets were pre-incubated with 100 nM insulin (5 min, 37°C) and adhered to surface-coated hrom collagen, thrombin, collagen I, fibrinogen, collagen III and VWF (4 hr, 37°C) and TF activity was measured. (b) To the platelet suspension excess ADP and U-46619 were added and followed by pre-incubation with insulin (5 min, 37°C) before platelets were adhered to collagen III (4 hr, 37°C) and TF activity was determined. (c) Dose-dependent inhibition by insulin (5 min, 37°C) of TF synthesis by collagen III-adhered platelets. (d) Splicing was demonstrated by the presence of either pre-mRNA or mature mRNA in platelets that were untreated or pre-incubated with insulin and allowed to adhere to collagen I (30 min, 37°C). A representative example of n=4 is shown. (e) Platelets from 8 type 2 diabetes patients and 8 matched controls were pre-incubated with insulin (100 nM, 5 min, 37°C), allowed to adhere to collagen III (4 hr, 37°C) and TF activity was measured. TF activity in adhered platelets without insulin from controls and type 2 diabetes patients were not significant different (0.17 ± 0.05 versus 0.28 ± 0.13 ng TF/2.0 x 10⁸ platelets respectively) (a), (b), (c) and (e) TF activity in fibrinogen or collagen III-adhered platelets was set at 100%. Data are means ± SEM; n=3-8.
Inhibition of platelet TF synthesis by insulin

Figure 1

A. Single, CD14 or CD45 depl platelets

B. TF activity (% of control)

C. TF activity (ng/2 × 10^8 plt)

D. col I + Tg003

E. suspension and adhesion on Fg
Figure 2

A

B

C

D

Inhibition of platelet TF synthesis by insulin
Figure 3

**A**

Inhibition of platelet TF synthesis by insulin

**B**

**C**

**D**

Adhesion on fg

TF
Figure 4

A

![Graph showing inhibition of platelet TF synthesis by insulin](image)

B

![Bar graph comparing control and insulin effects](image)

C

![Bar graph comparing different insulin concentrations](image)

D

![ agarose gel analysis](image)

E

![Bar graph comparing control subjects and type 2 diabetes patients](image)