Platelet Tissue Factor Synthesis in Type 2 Diabetic Patients Is Resistant to Inhibition by Insulin

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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 diabetic patients and matched control subjects were adhered to different surface-coated proteins, and TF premRNA splicing, TF protein, and TF procoagulant activity were measured.

RESULTS—Different adhesive proteins induced different levels of TF synthesis. A mimetic of active clopidogrel metabolite (AR-C69931 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 A_2 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 phosphatidylinositol 3-kinase/protein kinase B. Insulin is known to raise cAMP in platelets and inhibited collagen III–induced TF premRNA splicing and reduced TF activity by 35 ± 5 and 47 ± 5% at 1 and 100 nmol/l. Inhibition by insulin was reduced in type 2 diabetes platelets resulting in an ~1.6-fold higher TF synthesis than in matched control subjects.

CONCLUSIONS—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 diabetic patients produce more TF than platelets from matched control subjects. ***Diabetes*** **59:1487–1495, 2010**

Type 2 diabetic patients have a two- to eightfold higher risk of cardiovascular disease than healthy individuals, and 80% 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-antithrombin complexes, are increased. Also, the elevated levels of fibrinogen; factors (F) VII, VIII, and 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 glycemic 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 diabetic patients show better adhesion to a prothrombotic surface, make larger aggregates, have an increased procoagulant surface, and have an elevated cytosolic Ca^{2+} concentration compared with platelets from healthy control subjects (7). Intensive insulin treatment partly normalizes platelet aggregation (8). Indeed, insulin inhibits platelets from healthy individuals but not from type 2 diabetic 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 (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 $G_i\beta\gamma$, 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 hemostatic plug is TF exposure. TF is an integral transmembrane protein (43 kDa) 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 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 premRNAs for a number

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TABLE 1
Characteristics of the study population

	Control group	Type 2 diabetic patients	*P*
n	8	8	
Age (years)	52 ± 3	58 ± 3	0.2
BMI (kg/m^2)	25 ± 0.9	28 ± 2	0.2
Systolic blood pressure (mmHg)	140 ± 7	131 ± 3	0.2
Diastolic blood pressure (mmHg)	86 ± 4	80 ± 4	0.3
HbA1C (%)	5.5 ± 0.09	7.3 ± 0.5	0.002
Cholesterol (mmol/l)	6.1 ± 0.4	4 ± 0.3	0.001
Triglycerides (mmol/l)	1.7 ± 0.3	1.9 ± 0.4	0.6
HDL cholesterol	1.3 ± 0.07	1.1 ± 0.11	0.2
Creatinine (μmol/l)	95 ± 6.4	104 ± 6	0.3
Management with insulin		8	
Duration of diabetes (years)		16 ± 2	
C-peptide (nmol/l)	0.74 ± 0.14 (0.34–1.64)	0.72 ± 0.07 (0.39–1.05)	0.9
Smoking		3	
Medication			
Aspirin		0	
Statin		5	
ACE inhibitor or angiotensin receptor blocker		8	
Cardiovascular disease		2	

Data are means ± SE or means ± SE (range).

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

RESEARCH DESIGN AND METHODS

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); LPS (*E. coli* 0111:B4); indomethacin; puromycin; collagen types I and III; ADP from Sigma (St. Louis, MO); Akt inhibitor 1701-1 from Biovision (Mountain View, CA); iloprost from Schering (Berlin, Germany); forskolin, BAPTA(AM), ionophore A23187, Tg003, and cantharidin from Calbiochem (La Jolla, CA); LY294002 from Biomol (Plymouth Meeting, PA, USA); cycloheximide from MP Biochemicals (Santa Ana, 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); and U-44619 from Cayman Chemicals (Ann Arbor, MI). Plasma-derived VWF was purified from VWF/FVIII concentrate (Hemate P; Behring, Marburg, Germany) (18). Factor X (FX) was purified from fresh-frozen plasma (19). Recombinant FVIIa (rFVIIa) was from Novo Nordisk (Bagsværd, Denmark), and recombinant TF (Innovin) was from Dade Behring (Liederbach, Germany). Antibodies against the horseradish peroxidase–labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA), anti–phospho-Ser polyclonal antibody was from Upstate Biotechnology (Bucks, U.K.), peroxidase-linked goat anti-mouse antibody was from DAKO (Glostrup, Denmark), anti–splicing factor 2 (SF2)/–alternative splicing factor (ASF) antibody was from Abcam (Cambridge, U.K.), anti-TF antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) for immunoprecipitation and Affinity Biologicals (Ancaster, ON, Canada) for Western blotting, and anti-TF pathway inhibitor (TFPI) antibody from American Diagnostica (Stamford, U.K.) 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, U.K.). All other chemicals used were of analytical grade.

Isolated platelet suspensions were made monocyte free and adhered to different surface-coated adhesive proteins. Splicing of TF premRNA 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.

The study had been approved by the medical ethical review board. Type 2 diabetic subjects were recruited from the outpatient clinics of the Utrecht Medical University Hospital (Utrecht, the Netherlands). All patients were anti-GAD negative and had plasma C-peptide levels >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 continuous subcutaneous insulin infusion. 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 diabetic 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 *1*) incubation of a platelet suspension (2×10^8 platelets/ml) with either anti-CD14 antibody– or anti-CD45 antibody–coupled beads (15 min, 22°C, 35 μl anti-CD14 or anti-CD45 beads per 40×10^8 platelets) and subsequent magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and *2*) a double incubation of a concentrated platelet suspension (~15×10^8 platelets/ml) with a cocktail of anti-CD14 antibody– and anti-CD45 antibody–coupled beads (15 min, 22°C, 35 μl anti-CD14 and 35 μl anti-CD45 beads per 40×10^8 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), and RT-PCR was performed using the forward primer 5′-AAAGCACTTCCAGAGCCTGC-3′ and the reverse primer 5′-TC GAGCGTCAGTTCCTTGAGG-3′ on a PTC-200 apparatus (MJ Research, Waltham, MA).

TF expression. To investigate TF synthesis in platelets, an aliquot of suspension (2×10^8 platelets) was incubated for static adhesion experiments in six-well plates (Corning ultra-low attachment surface; Corning, Acton, MA) or kept in suspension for the indicated times at 37°C. Wells were coated with 50 μg/ml horm collagen, 50 μg/ml collagen type I, 50 μg/ml collagen type III, 1 unit/ml thrombin, 100 μg/ml fibrinogen, and 10 μg/ml VWF for 2 h at 37°C. For analysis of TF procoagulant activity, adhered platelets, collected in 250 μl HEPES/Tyrode buffer, or suspensions were lysed by freeze/thawing three times and centrifuged (16200*g*, 2 min, 22°C). Pellet fractions were dissolved in 50 μl HEPES/Tyrode buffer. Samples were incubated with 2 μg/ml anti-TFPI antibody (15 min, 22°C) and thereafter with 10 μg/ml FX, 5 units/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 premRNA, 12×10^8 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) using primers that overlapped intron 4, with forward primer 5′-CTCGGACAGCCAA CAATTCAG-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 2×10^8 platelets were adhered to a coated surface in six-well plates or kept in suspension (1 h, 37°C). Adhered platelets and suspensions were collected in lysis buffer (1% NP40, 0.5% octylglucoside, 0.1% SDS, and 5 mmol/l EDTA in PBS) supplemented with 10% protease inhibitor cocktail, 1 mmol/l $NaVO_3$, and 10 μmol/l 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 three times and dissolved in reducing Laemmli sample buffer.

Proteins were analyzed by SDS-PAGE and Western blotting. After blocking with Odyssey buffer (1 h, 22°C), membranes were incubated (16 h, 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) and Alexa-labeled antibodies according to the manufacturer's instructions. The intensity of the bands was quantified with ImageJ software.

Statistics. Data are expressed as means ± SE, with n observations, and was analyzed with Student t 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. 1*A*, *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. 1*A*, *bottom panel*). Upon stimulation with LPS, CD14-mRNA–containing suspensions adhering to fibrinogen synthesized TF activity detected in the FXa assay, but CD14-mRNA–negative preparations did not (Fig. 1*B*). 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 premRNA. TF synthesis and TF premRNA splicing were studied on fibrinogen- and collagen-coated surfaces. Platelets adhering to a fibrinogen-coated surface (4 h, 37°C) synthesized 0.24 ± 0.04 ng TF/2×10^8 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. 1*C*), confirming earlier work (17,21). To demonstrate splicing of TF premRNA, an RT-PCR with primers overlapping intron 4 was performed on platelets adhered to collagen type I (2 h, 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. 1*D*). Regulation of TF premRNA splicing was examined by measuring the Ser phosphorylation of SF2, an effector of Cdc2-like kinase (Clk-1) (Fig. 1*E*). 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 h, 37°C). Tg003 reduced the phosphorylation, confirming its dependence on Clk-1. These results confirm that adhering platelets synthesize TF through splicing of premRNA.

Different splicing at different adhesive surfaces. The question was addressed regarding which adhesive surface induced the highest TF premRNA 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. 2*A*). 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 (~125×10^6 platelets), whereas adhesion to thrombin, fibrinogen, and VWF was ~30% lower (Fig. 2*B*). Expression per platelet and correction for background TF (defined as ΔTF activity), indicated that horm collagen, thrombin, and collagen I induced the highest TF synthesis followed by fibrinogen, collagen III, and VWF (Fig. 2*C*). 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 h, 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/2.0×10^8 platelets, respectively, $n = 3$), indicating that splicing of TF premRNA is strongly facilitated by adherence to a surface (Fig. 2*D*), in contrast with an earlier finding (17,21).

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 coincubation with AR-C69931 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 (Fig. 3*A*, *insert*). AR-C69931 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. 3*A*). Thus, platelet TF synthesis strongly depends on the extracellular feedback loops mediated by secretion of granule ADP and release of thromboxane A_2 (TxA_2).

There was little effect of metabolic inhibitors on adhesion (not shown). The adenylyl cyclase activator forskolin and the stable prostacyclin mimetic 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^{2+} increases in activated platelets, inhibited TF synthesis almost completely. Incubation with ionophore A23187 alone did not change collagen III–induced TF synthesis, but coincubation with 1 mmol/l 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%, indicating that in the absence of inhibitors, the PI3-K/PKB pathway signals to TF premRNA

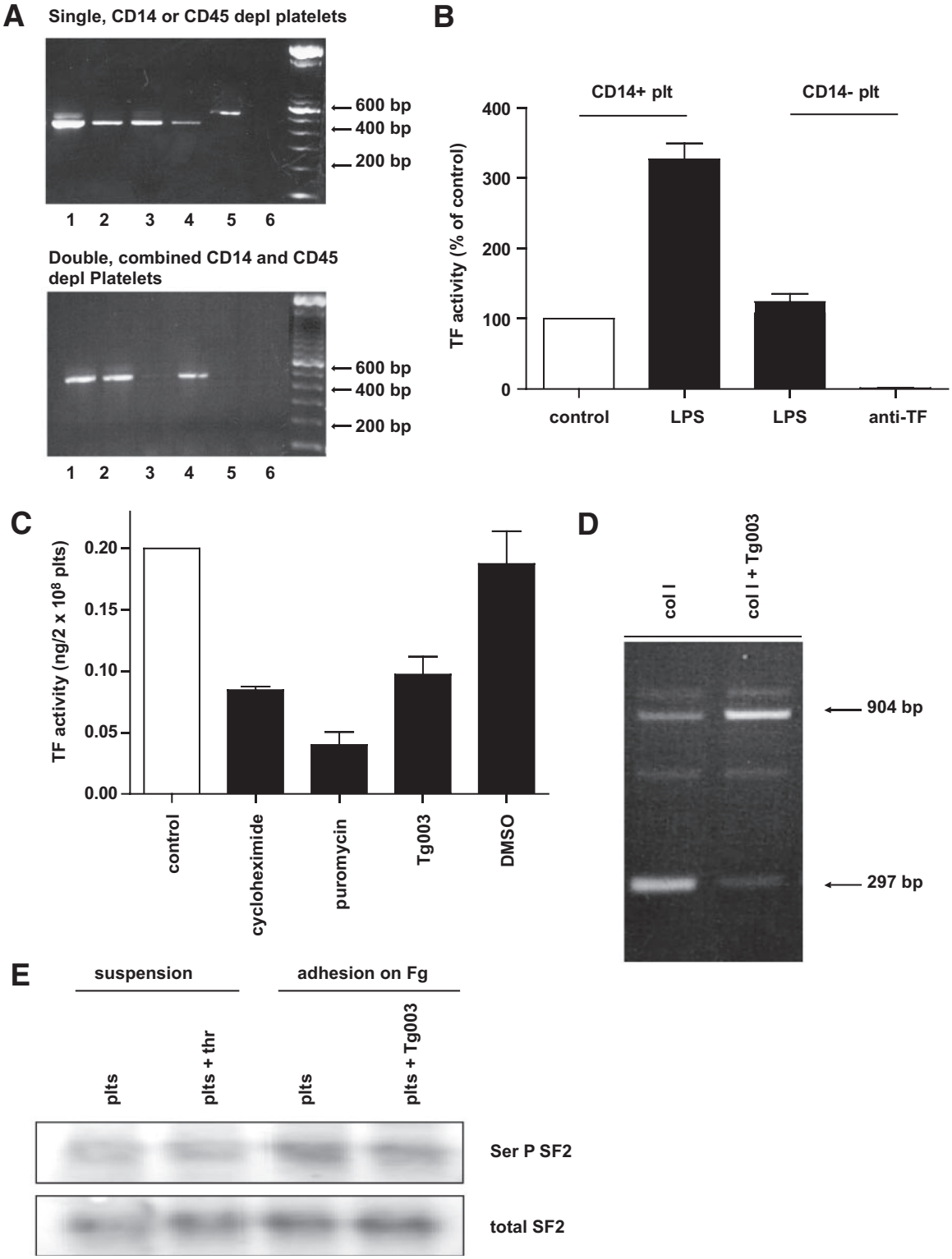

FIG. 1. Platelet-specific splicing of TF premRNA and TF synthesis. *A*: Detection of leukocyte-specific CD14 RNA (422 bp) in monocytes (*lane 1*), platelet suspensions prepared by conventional centrifugation/resuspension (*lane 2*), leukocyte-depleted (depl) platelet suspensions obtained by incubation with either anti-CD14 or anti-CD45 magnetic beads (*lane 3*, *top panel*), or double incubation with anti-CD14 and anti-CD45 magnetic beads (*lane 3*, *bottom panel*) and elution (*lane 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 (plt) suspensions without (□) and with (■) 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 h, 37°C, □) is inhibited by the translation blockers cycloheximide and puromycin and the splicing blocker Tg003 (in DMSO, ■). *D*: Splicing by platelets adhering to collagen I (col I) (4 h, 37°C) demonstrating a shift from premRNA (904 bp) to mature mRNA (297 bp). *E*: Negative SF2 phosphorylation in resting and thrombin (thr)-stimulated platelet suspensions and positive SF2 phosphorylation in fibrinogen-adhered platelets, which is inhibited by Tg003. Data are means ± SE. *n* = 3–12. *A*, *D*, and *E*: representative examples for *n* = 3.

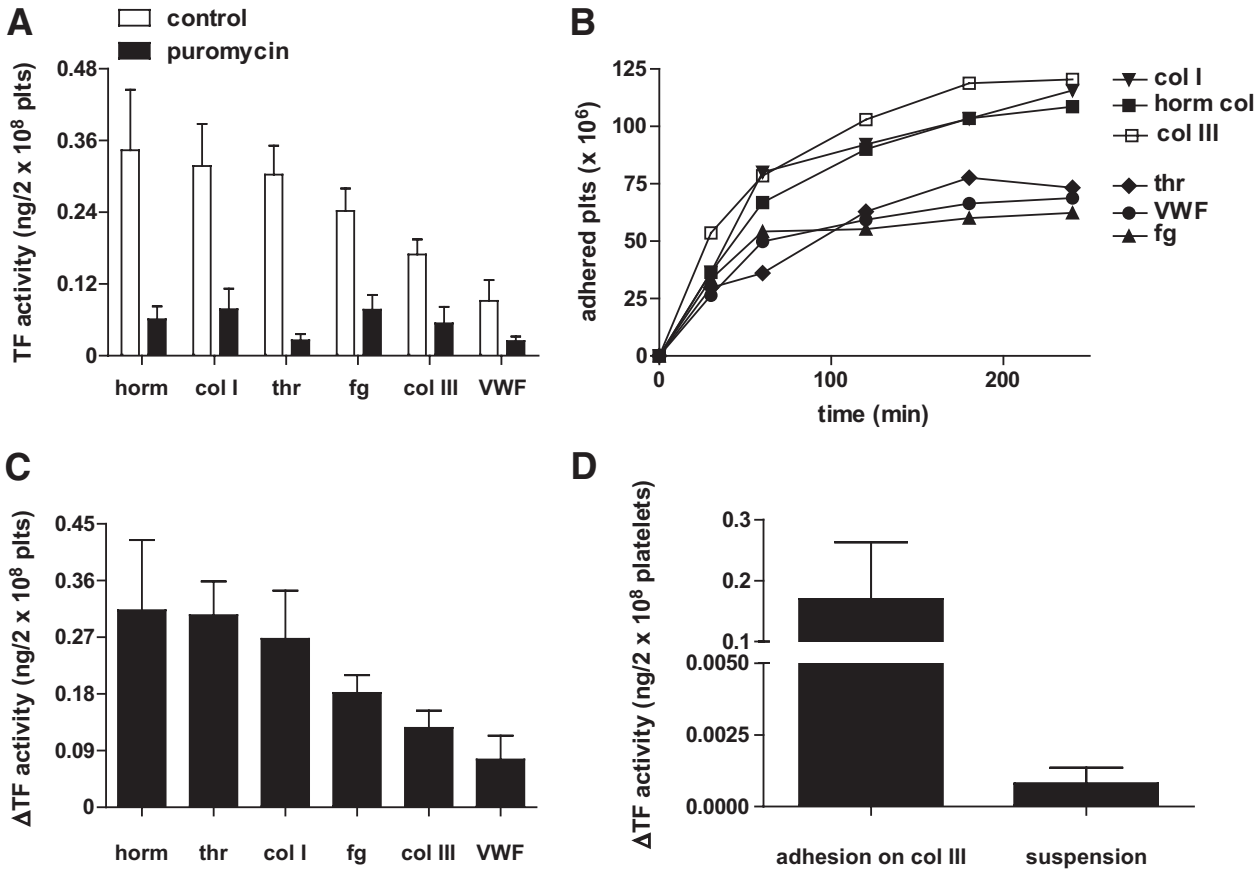

FIG. 2. Surface-dependent platelet TF activity. *A*: TF synthesis in platelets (plts) adhering to surface-coated horm collagen (horm, 50 μg/ml), collagen I (col I, 50 μg/ml), thrombin (thr, 1 unit/ml), fibrinogen (fg, 100 μg/ml), collagen III (col III, 50 μg/ml), and VWF (10 μg/ml, each per well) (4 h, 37°C). TF activity in fibrinogen-adhered platelets was set at 100%. Preincubation 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 h, 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 ± SE. $n = 3–4$.

splicing (Fig. 3*B*). To investigate whether the inhibitors interfere with release of ADP/TxA_2 and/or with signaling from ADP/TxA_2 receptors to the spliceosome, experiments were repeated in the presence of excess of ADP (50 μmol/l) and of the stable TxA_2 analog U-46619 (2 μmol/l). Inhibition of TF synthesis by the metabolic inhibitors was preserved, indicating that in addition to the known suppression of ADP/TxA_2 release, these blockers interfere with the control of TF premRNA splicing (Fig. 3*C*). Western blot analysis confirmed that iloprost inhibited synthesis of TF protein (Fig. 3*D*). 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. Platelets were preincubated with 100 nmol/l 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, and 41 ± 7% ($n = 5$) for horm collagen, thrombin, collagen I, fibrinogen, and collagen III, respectively. On VWF, no inhibition by insulin was found (Fig. 4*A*). 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 premRNA splicing induced by ADP and TxA_2 receptors (Fig. 4*B*). Collagen III–adhered platelets were used to further evaluate the inhibition by insulin. Adhered platelets showed a dose-dependent inhibition between 1 and 100 nmol/l insulin (Fig. 4*C*). Inhibition of premRNA splicing by insulin was clearly detectable, showing decreased levels of mature mRNA compared with platelets in the absence of insulin (Fig. 4*D*). In type 2 diabetic 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 with matched control subjects (59 ± 12%, $n = 8$; $P < 0.05$ with Mann-Whitney U test; Fig. 4*E*).

DISCUSSION

Novel findings in the present study are *1*) the identification of the extra- and intracellular mechanisms that couple surface activation to initiation of TF synthesis in platelets, *2*) the inhibition of TF synthesis by insulin in normal platelets, and *3*) the loss of insulin inhibition in platelets

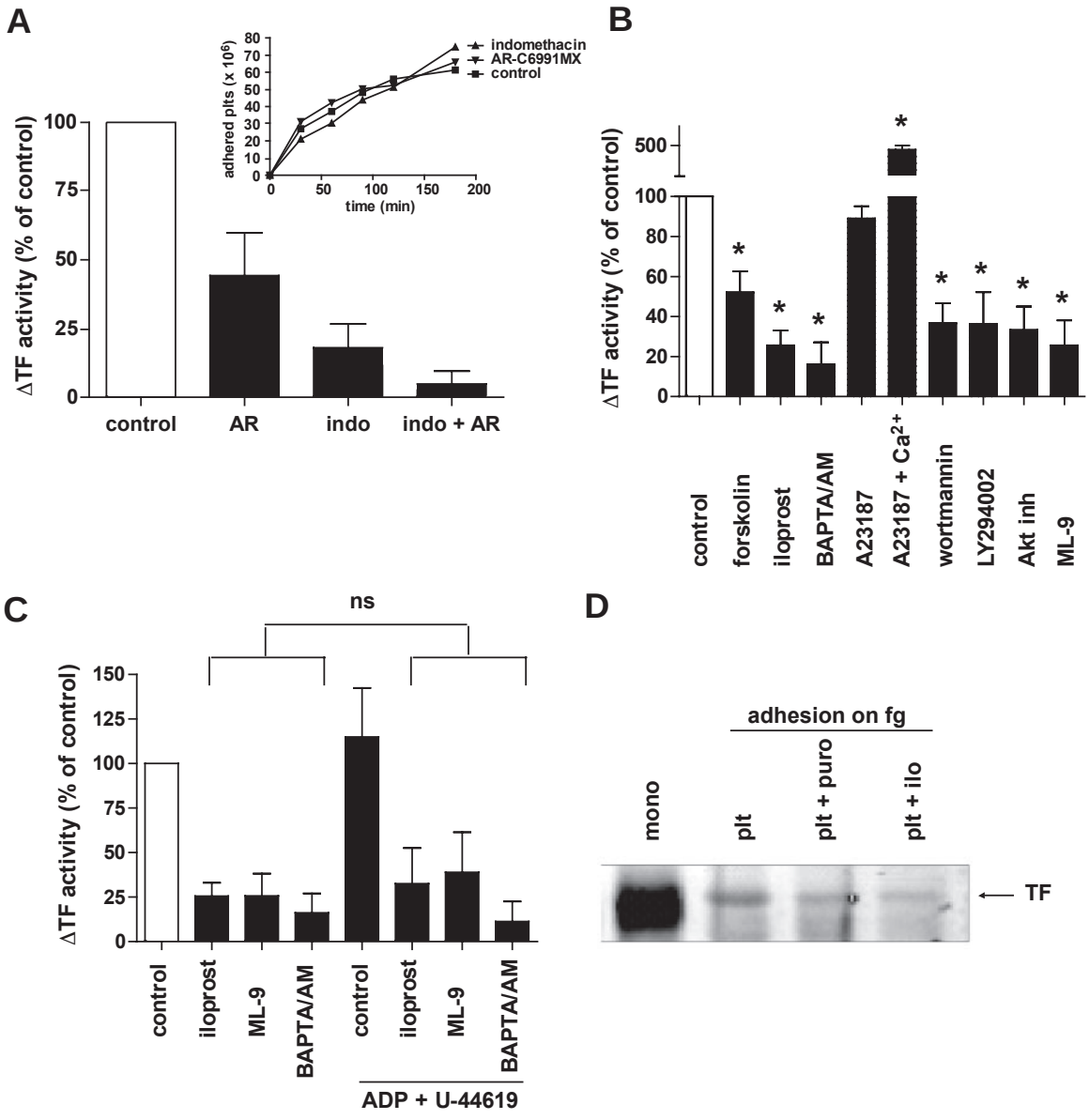

FIG. 3. Effect of inhibitors of signaling pathways on TF synthesis in platelets. *A*: Platelets were preincubated with a mimetic of active clopidogrel metabolite (AR-C69931 MX, AR, 250 nmol/l), the aspirin-like inhibitor indomethacin (Indo, 100 μmol/l) or the combination (15 min, 37°C). AR-C69931 MX and indomethacin did not affect platelet adhesion (*insert*) but reduced surface-induced TF synthesis (4 h, 37°C). *B*: Platelets were preincubated with the adenylyl cyclase activator forskolin (40 μmol/l), the stable prostacyclin mimetic iloprost (1 μg/ml), the Ca^{2+} quencher BAPTA/AM (25 μmol/l), ionophore A23187 (12 μmol/l) in the absence and presence of $CaCl_2$ (1 mmol/l), the PI3-K/PKB inhibitors wortmannin (0.5 μmol/l) and LY294002 (10 μmol/l), the Akt inhibitor 1701-1 (2 μmol/l), and ML-9 (100 μmol/l) (15 min, 37°C). The inhibitors reduced the surface-induced TF synthesis (4 h, 37°C). Addition of vehicle alone did not change surface-induced TF levels (as shown in Fig. 1*C*). *C*: Platelets in the presence of excess of ADP and the TxA_2 mimetic U-44619 were preincubated with iloprost, BAPTA/AM, wortmannin, LY294002, the Akt inhibitor 1701-1, and ML-9 before platelets were adhered to collagen III (4 h, 37°C) and TF activity was determined. *D*: Platelets were adhered to fibrinogen (4 h, 37°C). The blot shows the 60-kDa band (in the presence of DTT) of monocyte (mono) TF, surface-induced platelet TF protein, the inhibition by puromycin (puro), and by the cAMP-raising agent iloprost (ilo). *A*, *insert* and *D*: Representative example for $n = 3$. *A–C*: TF activity in collagen III–adhered platelets was expressed as 100%. Data are means ± SE. $n = 3–4$. *Denotes a significant difference, $P < 0.05$. ns, not significant.

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 diabetic 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 (22–24). 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 premRNA splicing upon stimulation with LPS, which is a potent inducer of TF synthesis in monocytes (16). Apparently, splicing of TF premRNA is not under control of the platelet LPS receptor, which contrasts with other studies that show LPS-induced splicing of interleukin (IL)-1β and cyclooxygenase (COX)-2 premRNA in platelets (25).

Platelets adhering to surface-coated adhesive proteins start splicing of TF premRNA and synthesis of TF protein and induce the capacity to initiate coagulation, confirming earlier observations (17,21). The anucleate platelet has

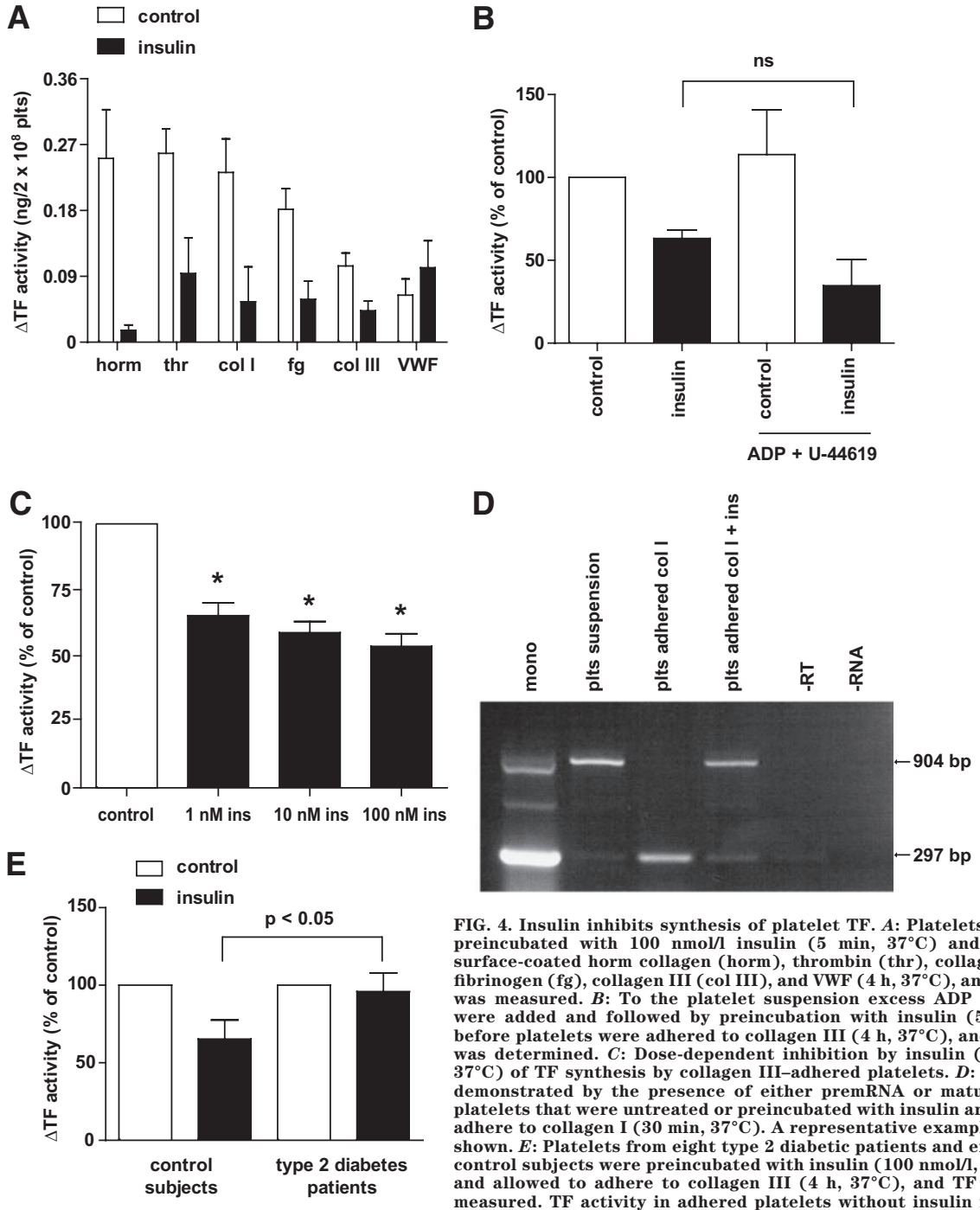

FIG. 4. Insulin inhibits synthesis of platelet TF. *A*: Platelets (plts) were preincubated with 100 nmol/l insulin (5 min, 37°C) and adhered to surface-coated horm collagen (horm), thrombin (thr), collagen I (col I), fibrinogen (fg), collagen III (col III), and VWF (4 h, 37°C), and TF activity was measured. *B*: To the platelet suspension excess ADP and U-46619 were added and followed by preincubation with insulin (5 min, 37°C) before platelets were adhered to collagen III (4 h, 37°C), and TF activity was determined. *C*: Dose-dependent inhibition by insulin (ins) (5 min, 37°C) of TF synthesis by collagen III–adhered platelets. *D*: Splicing was demonstrated by the presence of either premRNA or mature mRNA in platelets that were untreated or preincubated with insulin and allowed to adhere to collagen I (30 min, 37°C). A representative example of $n = 4$ is shown. *E*: Platelets from eight type 2 diabetic patients and eight matched control subjects were preincubated with insulin (100 nmol/l, 5 min, 37°C) and allowed to adhere to collagen III (4 h, 37°C), and TF activity was measured. TF activity in adhered platelets without insulin from control subjects and type 2 diabetic patients were not significant different (0.17 ± 0.05 vs. 0.28 ± 0.13 ng TF/2.0 × 10^8 platelets, respectively). *A–C* and *E*: F activity in fibrinogen or collagen III–adhered platelets was set at 100%. Data are means ± SE. $n = 3–8$. mono, monocytes; ns, not significant. *Denotes a significant difference, $P < 0.05$.

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 (26). Unexpectedly, the platelet cytoplasm SF2/ASF, a regulator of constitutive and alternative splicing (27) together with a number of premRNAs with message for IL-1β, B-cell lymphoma 3, plasminogen activator inhibitor-1, COX-2, and TF (28–30). Platelets stimulated with soluble agonists or adhering to fibrinogen start formation of mature RNAs and translation into functional proteins. Splicing of TF premRNA results in formation of TF protein and the capacity to start coagulation and is under control of dual specificity Clk-1, which contains an NH_2-terminal region enriched in serine/arginine dipeptides (SR) that interacts with SF2/ASF (31). 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 premRNA 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-C69931 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 TxA_2 formation and subsequent activation of the P2Y12 receptor (ADP) and TPα/β receptors (TxA_2). The combination of the blockers reduced TF synthesis almost by 100%. This observation might indicate that in vivo signaling to TF premRNA 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 TxA_2, indicating that surface receptors that trigger TF premRNA splicing predominantly do so by inducing these positive feedback pathways. Collagen I, horm collagen, and thrombin had the strongest capacity to induce splicing of TF premRNA. Fibrinogen and collagen III induced less splicing, and VWF was the weakest activator. These differences might reflect the contribution of ADP/TxA_2 release in platelet activation by these agonists, which is strong in horm collagen (32) and thrombin (33) activation, weak in activation by surface-coated fibrinogen (34), and virtually absent in platelet activation by coated VWF (35).

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/TxA_2, inhibition of TF activity also takes place after the released products start further signaling through the P2Y12 and TxA_2 receptors. Protein kinase A (PKA) α 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 (36). PKB contributes to phosphorylation of SR proteins, specifically SF2 (37), 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 (16,38). Here, PKB inhibits nuclear factor-κB activity (38), such in contrast to anucleated platelets where PKB regulates the splicing mechanism.

The induction of ADP/TxA_2 release followed by signaling from ADP/TxA_2 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 $G_i\alpha_2$, thereby blocking G_i-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 Ca^{2+} 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 ~100 s 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 (39). A key event in the pathogenesis of type 2 diabetes 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 (40). The elevated TF synthesis in platelets form type 2 diabetic patients might result in a more stable clot inducing occlusion of an artery at sites where atherosclerotic lesions have developed.

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