

Effects of Hyperglycemia and Hyperinsulinemia on Circulating Tissue Factor Procoagulant Activity and Platelet CD40 Ligand

Vijender R. Vaidyula,¹ A. Koneti Rao,¹ Maria Mozzoli,² Carol Homko,² Peter Cheung,² and Guenther Boden²

Individuals with chronically elevated glucose and/or insulin levels, i.e., most patients with type 2 diabetes, have accelerated atherosclerosis and are prone to acute vascular events. We have tested the hypothesis that hyperglycemia and/or hyperinsulinemia singly or combined may increase tissue factor, the primary initiator of blood coagulation. We have determined changes in circulating tissue factor procoagulant activity (PCA) and other procoagulation proteins in healthy volunteers exposed to 24 h of selective hyperinsulinemia, selective hyperglycemia, or combined hyperinsulinemia and hyperglycemia. Combined elevations of plasma insulin and glucose levels for 24 h produced a ninefold increase in tissue factor PCA, which was associated with an increase in monocyte tissue factor protein (flow cytometry) and mRNA (RT-PCR), increases in plasma thrombin-antithrombin complexes, prothrombin fragment 1.2, factor VIII coagulant activity, and platelet CD40 ligand as well as decreases in factor VIIa, factor VII coagulant activities, and factor VII antigen. Effects of selective hyperinsulinemia and selective hyperglycemia were less striking but appeared to be additive. We conclude that hyperinsulinemia and hyperglycemia but particularly the combination of both create a prothrombotic state and in addition may be proinflammatory and proatherogenic because of the proinflammatory actions of CD40 ligand and tissue factor. *Diabetes* 55:202–208, 2006

Compared with nondiabetic control subjects, patients with type 2 diabetes have a two- to fivefold increase in premature atherosclerotic vascular disease (ASVD), including myocardial infarction, cerebral vascular accidents, and peripheral vascular disease (1–4). Rupture of atherosclerotic plaques

and subsequent acute thrombosis are key events in the mortality associated with acute coronary syndromes (5). Whereas not all factors responsible for the increased risk for ASVD in type 2 diabetes are known, there are several studies that have reported abnormalities in plasma proteins involved in blood coagulation, fibrinolysis, and platelet function in patients with diabetes (6–9). Moreover, there is evidence suggesting that plasma levels of several coagulation factors may be modulated by hyperglycemia and/or hyperinsulinemia (3,10–12).

The tissue factor pathway is the primary physiological mechanism of initiation of blood coagulation (13,14). Binding of native coagulation factor VII (FVII) to tissue factor converts FVII to the activated form (FVIIa). The resulting tissue factor–FVIIa complex then activates factors IX and X to factors IXa and Xa, respectively, leading to the formation of the prothrombinase complex and thrombin generation. Tissue factor, which is present in the adventitia of normal blood vessels and is highly expressed in atherosclerotic plaques, has been recognized to initiate coagulation and thrombus formation when the vessel wall is injured or plaques are fissured (15). More recently, it has been shown that there is, in addition, a circulating pool of tissue factor in blood that is associated with cells and microparticles, is thrombogenic (13,14,16–18), and is elevated in type 2 diabetes (19,20). Because premature ASVD risk depends partially on glycemic control (21,22), we hypothesized that hyperglycemia and/or hyperinsulinemia contribute to the rise in circulating tissue factor and to the prothrombotic state in these patients. It was, therefore, our objective to determine changes in circulating tissue factor plasma procoagulant activity (PCA) and other components of the blood coagulation system in healthy volunteers exposed to 24 h of hyperinsulinemia or hyperglycemia or combined hyperinsulinemia and hyperglycemia. In addition, we have examined their effect on platelet expression of CD40 ligand (CD40L), a proinflammatory member of the tumor necrosis superfamily (23–25), previously shown to be increased in diabetes (26,27).

From the ¹Section of Hematology, Department of Medicine and the Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania; and the ²Section of Endocrinology/Diabetes/Metabolism, Department of Medicine and the Clinical Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania.

Address correspondence to Guenther Boden, MD, Temple University Hospital, 3401 North Broad St., Philadelphia, PA 19140. E-mail: bodengh@tuhs.temple.edu.

Received for publication 9 August 2005 and accepted 20 September 2005. V.R.V. and A.K.R. contributed equally to the study.

ASVD, atherosclerotic vascular disease; CD40L, CD40 ligand; FITC, fluorescein isothiocyanate; FVII, native coagulation factor VII; FVIIa, activated FVII; FVIIAg, FVII antigen; FVIIc, FVII activity; FVIII, native coagulation factor VIII; PCA, procoagulant activity; TAT, thrombin-antithrombin; TFPI, tissue factor pathway inhibitor.

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

RESEARCH DESIGN AND METHODS

A total of 29 healthy volunteers participated in four different studies. Each volunteer was studied once. The subjects' ages, weights, heights, and BMIs are shown in Table 1. None of the subjects had a family history of diabetes or any other endocrine disorder, and none were taking any medication. Informed written consent was obtained from each participant after explanation of the nature, purpose, and potential risks of these studies. The study protocol was approved by the institutional review board of Temple University Hospital. Subjects were admitted to Temple University Hospital's General Clinical Research Center on the evening before the studies.

TABLE 1
Characteristics of study subjects

	High glucose/ high insulin	Normal glucose/ high insulin	High glucose/ normal insulin	Normal glucose/ normal insulin
Sex (men/women)	7/3	4/3	6/1	4/1
Age (years)	38.3 ± 2.7	31.0 ± 2.6	37.3 ± 2.9	39.6 ± 2.0
Height (cm)	164.8 ± 11.1	175.7 ± 4.3	173.2 ± 3.5	173.0 ± 4.8
Weight (kg)	90.1 ± 5.5	78.6 ± 6.7	84.7 ± 5.4	86.4 ± 6.0
BMI (kg/m ²)	29.0 ± 1.4	23.9 ± 2.0	27.6 ± 2.0	28.9 ± 1.8

Data are means ± SE.

The studies began at ~8:00 A.M. after an overnight fast with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another catheter was placed into the contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket (~70°C) to arterialize venous blood. The following four studies were performed.

High glucose/high insulin clamps. A 20% glucose solution was infused intravenously at variable rates that were adjusted to maintain plasma glucose at ~200 mg/dl (~11 mmol/l). Small blood samples (0.25 ml) were collected every 30–60 min initially and every 1–2 h later for measurement of blood glucose concentrations. Subjects were fasting but were allowed to drink water ad libitum and remained at bed rest for the duration of the study. Plasma electrolytes were monitored every 6 h; body weight every 12 h; and fluid balances every 6 h. Potassium (20 mg) and magnesium (400 mg) were given orally every 12 h.

Selective hyperinsulinemic (normal glucose/high insulin) clamps. Regular human insulin (Humulin; Eli Lilly, Indianapolis, IN) was infused intravenously at a rate of 12 pmol · kg⁻¹ · min⁻¹ (2 mU · kg⁻¹ · min⁻¹). Glucose was maintained at ~5.5 mmol/l (100 mg/dl) by feedback controlled glucose infusions. Glucose concentrations were determined every 15–30 min at the beginning and at 1–2 h intervals later with a glucose analyzer, and the glucose infusion rates were adjusted as needed. Plasma electrolyte and fluid balances were monitored as described above.

Selective hyperglycemia (high glucose/normal insulin) clamps. A 10% glucose infusion was infused at variable rates to maintain blood glucose levels at ~11 mmol/l. Stimulation of endogenous insulin secretion was prevented by continuous infusion of somatostatin (305 nmol/h). Basal plasma insulin and glucagon levels were maintained by continuous intravenous infusion of insulin (0.33 pmol · kg⁻¹ · min⁻¹) and glucagon (0.25 ng · kg⁻¹ · min⁻¹).

Normal glucose/normal insulin (control) clamps. A 5% glucose solution was infused intravenously at variable rates to maintain blood glucose concentrations at ~100 mg/dl (~5.5 mmol/l) for 24 h. The rest of the study was as described above.

Assays. Blood samples were collected from antecubital veins without tourniquet-induced venostasis at 0, 6, 12, 18, and 24 h. Plasma glucose was measured with a glucose analyzer using the glucose oxidase method and serum insulin by radioimmunoassay using an antiserum with minimal (<0.2%) cross-reactivity with proinsulin (Linco Research, St. Charles, MO). Serum triglycerides were measured enzymatically. Electrolytes were measured at the Temple University Hospital Chemistry Laboratory.

All of the following were measured in plasma harvested from blood collected into 0.1 volume 3.8% sodium citrate. FVIIa activity was measured by a clotting assay using recombinant soluble tissue factor (Diagnostics Stago, Parsippany, NJ). FVII antigen (FVIIAg) was measured by an enzyme-linked immunosorbent assay (Diagnostics Stago). FVII activity (FVIIc) was measured by a one-stage clotting assay using a mechanical timer Fibrometer (BBL Microbiology System, Cocksville, MD), Simplastin Excel (Biomérieux, Durham, NC), and FVII-deficient plasma (George King Biomedical, Overland Park, KS). Native coagulation factor VIII (FVIII) coagulant activity was measured with an activated partial thromboplastin time-based one-stage clotting assay using the activated partial thromboplastin time reagent from Biomérieux. FVIII-deficient plasma and pooled normal plasma were purchased from George King Biomedical. Tissue factor pathway inhibitor (TFPI) antigen levels were measured by the Imubind total TFPI ELISA from American Diagnostica (Greenwich, CT). Thrombin generation was assessed by determination of prothrombin fragment 1.2 and thrombin-antithrombin (TAT) complexes in plasma using ELISAs (Enzygnost; Dade Behring, Marburg, Germany).

Whole-blood tissue factor PCA. Tissue factor PCA was measured in whole-blood cell lysates with a two-stage clotting assay using recombinant FVIIa (American Diagnostica), Factor X (Enzyme Research Laboratories, South Bend, IN), and normal human plasma containing phospholipid vesicles,

according to the method described by Key et al. (17). This assay measures cell-bound and microparticle-associated tissue factor in lysed whole blood. Blood samples (1 ml) were drawn into 0.1 volume 3.8% sodium citrate as anticoagulant were frozen at -70°C until assayed. Blood lysates and cellular components were collected from aliquots of whole blood subjected to three cycles of freezing and thawing followed by centrifugation and washing, and they were finally suspended in HBSA buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 5.38 mmol/l KCl, 5.55 mmol/l glucose, and 0.1% bovine serum albumin, pH 7.5) as described previously (17). The final membrane pellet resuspended in HBSA buffer was used as a source of tissue factor. Tissue factor PCA was measured using a two-stage clotting assay. In the first stage, 20 µl sample, 5 nmol/l FVIIa, and 250 nmol/l human factor X were incubated for 5 min at 37°C. CaCl₂ (8.3 mmol/l) was added and incubated for 3 min. In the second stage, 100 µl normal human plasma containing phospholipid vesicles (75% phosphatidylcholine:25% phosphatidylserine prepared by weight; Avanti Polar Lipids, Alabaster, AL) and 100 µl 25 mmol/l CaCl₂ were added, and the clotting time was recorded in a mechanical fibrometer (BBL Microbiology System). Recombinant human tissue factor Ortho Recombiplastin (Ortho Diagnostic Systems, Raritan, NJ) was used as a standard. The tissue factor concentration in the standard was determined by using IMUBIND Tissue Factor antigen ELISA (American Diagnostica). Repeated measurements of 1 arbitrary unit/ml Ortho Recombiplastin gave 1.34 pg/ml. A log plot of tissue factor versus clotting time was linear in the 1–1,000 units/ml range (*r* = 0.99). The interassay coefficient of variation (CV) was 8.6%, the intra-assay CV was 7.0%.

Tissue factor expression on circulating monocytes. Tissue factor expression on monocyte surface was measured by flow cytometry. Blood samples collected into 0.1 volume 3.8% sodium citrate were diluted 1:3 with modified HEPES-Tyrodes buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 2.8 mmol/l KCl, 1 mmol/l MgCl₂, 12 mmol/l NaHCO₃, 0.4 mmol/l Na₂HPO₄, 0.35% BSA, and 5.5 mmol/l glucose, pH 7.4) and incubated (20 min, at room temperature) with fluorescein isothiocyanate (FITC)-labeled anti-human CD14 (monocytes) and phycoerythrin-labeled anti-human CD142 (tissue factor) antibodies. As controls, corresponding matched isotype antibodies FITC anti-mouse IgG_{2a} and phycoerythrin mouse IgG_{1K} were used. All monoclonal antibodies were purchased from BD Biosciences, Pharmingen (San Diego, CA). The samples were fixed with 1.1% formaldehyde (Sigma, St. Louis, MO) for 10 min at room

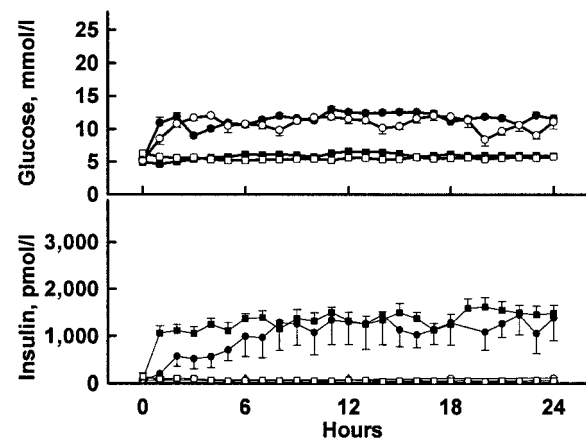


FIG. 1. Plasma glucose concentrations (top panel) and serum insulin concentrations (bottom panel) during 24 h of high glucose/high insulin (●) clamping (*n* = 10), 24 h of normal glucose/high insulin (■) clamping (*n* = 7), 24 h of high glucose/normal insulin (○) clamping (*n* = 7), and 24 h of normal glucose/normal insulin (□) clamping (*n* = 5). Data are means ± SE.

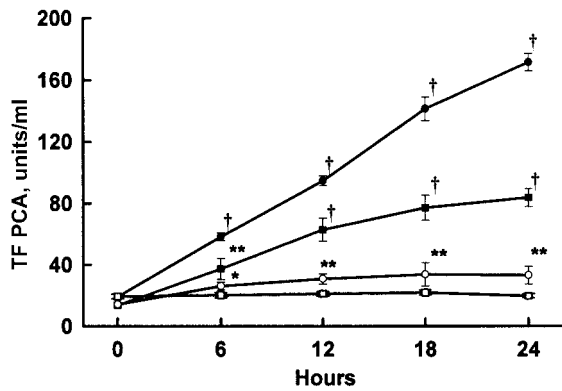


FIG. 2. Circulating tissue factor PCA (TF PCA) during 24 h of high glucose/high insulin (●) clamping ($n = 10$), 24 h of normal glucose/high insulin (■) clamping ($n = 7$), 24 h of high glucose/normal insulin (○) clamping ($n = 6$), and 24 h of normal glucose/normal insulin (□) clamping ($n = 5$). Comparing 0 h versus other time points: * $P < 0.02$, ** $P < 0.01$, † $P < 0.001$. Comparing different studies: control subjects (normal glucose/normal insulin) vs. high glucose/high insulin: $P < 0.001$ at 6, 18, and 24 h; $P < 0.05$ at 12 h. Control subjects vs. normal glucose/high insulin: $P = 0.05$ at 6 h, $P < 0.001$ at 18 and 24 h; high glucose/high insulin vs. normal glucose/high insulin: $P = 0.02$ at 6 h, $P < 0.001$ at 18 and 24 h; high glucose/normal insulin vs. normal glucose/high insulin: $P < 0.001$ at 18 and 24 h.

temperature, diluted fourfold with distilled water, and analyzed using a BD FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) within 2 h of sample preparation. The monocyte population was identified by gating CD14-positive events and tissue factor expression was determined as percent CD142-positive cells. Four thousand monocytes were analyzed. The positive delineator for tissue factor-expressing monocyte population was determined by gating ~1% background staining on both FITC- and phycoerythrin-labeled isotype controls.

CD40 ligand expression on platelets. Platelet-rich plasma was obtained from citrated whole blood by centrifugation at 200g for 15 min. Platelet-rich plasma was diluted threefold with modified HEPES-Tyrodes buffer and incubated with phycoerythrin-labeled anti-human CD154 (CD40 ligand [CD40L]) or corresponding matched isotype control phycoerythrin mouse IgG_{1k} antibody for 20 min. Cells were fixed in 1% formaldehyde for 10 min at room temperature. The samples were diluted 10-fold with HEPES-Tyrodes buffer and analyzed (10,000 platelets) within 2 h in BD FACScan flow cytometer. The CD40L-positive platelets were determined by gating on 1% of isotype control-positive cells.

Real time RT-PCR analysis of tissue factor mRNA in human monocytes.

Mononuclear cells were isolated from 20 ml freshly heparinized whole blood by the Ficol-Paque TM Plus gradient method according to the manufacturer's instructions (Amersham, Piscataway, NJ). Monocytes were positively selected using 20 μ l CD14 microbeads per 10^7 mononuclear cell according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA).

Total RNA isolation and one-step SYBR Green real-time RT-PCR were performed as described previously (28). The following primer sequences were used. For tissue factor (gene ID NM 001993): forward, 5'-CAGTGATTCCTCCCGAACA-3' and reverse 5'-TGCCCTTCTACACTT GTGTAGAG-3' (amplicon

size 173 bp). For 18sRNA: Competimer Primer Set (catalog no. 5103G; Ambion, Austin, TX).

A standard curve of cycle threshold versus concentration was obtained using serial dilutions of control total RNA isolated from tissue factor-expressing human breast cancer cells (MDA-MB-231) (29). Relative concentrations of the tissue factor amplicon were determined from the standard curve, and the after-to-before ratio was normalized with the after-to-before ratio of the internal standard (18sRNA).

Statistical analysis. A one-way ANOVA was used to test for significant differences between studies with Student-Newman-Keuls post hoc analysis. If data were not normally distributed, the Kruskal-Wallis one-way ANOVA with Dunn's post hoc analysis was used. To test for differences across time, a one-way repeated-measures ANOVA with Student-Newman-Keuls post hoc analysis was used. If data were not normally distributed, the Friedman repeated-measures ANOVA on ranks with Student-Newman-Keuls post hoc analysis was used. Statistical differences between day 1 (baseline) and day 2 (24 h) were determined with a paired t test and, if not normally distributed, with the Wilcoxon Signed Rank test. Statistical analyses were performed using SigmaStat for Windows (version 2.0; SPSS, Chicago, IL). Statistical significance was defined as $P < 0.05$. All results are presented as means \pm SE.

RESULTS

Glucose and insulin (Fig. 1). Plasma glucose concentrations remained basal (~5.5 mmol/l or ~100 mg/dl) for 24 h in the selective hyperinsulinemia (normal glucose/high insulin) and the control (normal glucose/normal insulin) clamps and were raised by infusion of glucose to ~11 mmol/l (~200 mg/dl) for 24 h in the selective hyperinsulinemia (high glucose/normal insulin) and the high glucose/high insulin clamps.

Serum insulin concentrations remained basal (~50 pmol/l or ~8 μ U/ml) for 24 h in the selective hyperglycemia and control clamps and were raised by infusion of insulin to ~1,200 pmol/l (~200 μ U/ml) in the selective hyperinsulinemia and high glucose/high insulin clamps.

Circulating tissue factor PCA (Fig. 2). During the control study (normal glucose/normal insulin), basal tissue factor PCA remained unchanged at ~20 units/ml for 24 h. During selective hyperglycemia, tissue factor PCA increased approximately twofold (from 14.2 ± 1.5 to 33.3 ± 5.7 units/ml, $P < 0.05$); during selective hyperinsulinemia, tissue factor PCA rose approximately sixfold (from 13.6 ± 1.3 to 83.9 ± 5.7 , $P < 0.001$); and during combined hyperglycemia and hyperinsulinemia, it rose approximately ninefold (from 19.7 ± 1.1 to 172.4 ± 5.9 , $P < 0.001$). In all groups, except control subjects, significant increases were observed starting at 6 h.

Tissue factor expression on monocytes (Fig. 3). Because much of circulating tissue factor PCA is associated with monocytes, tissue factor expression on monocytes was assessed by flow cytometry at baseline and at 24 h.

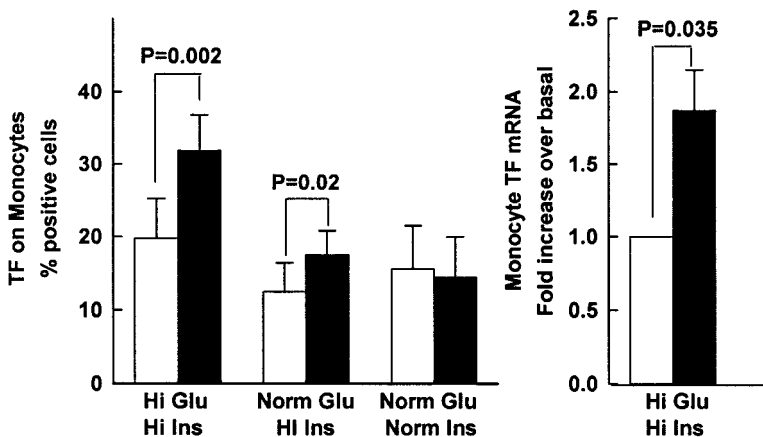


FIG. 3. Left panel: Tissue factor expression on monocytes (percent tissue factor-positive cells) before (□) and 24 h after (■) high glucose/high insulin ($n = 8$), selective hyperinsulinemia (Norm Glu/Hi Ins, $n = 7$) and normal glucose/normal insulin clamping (control subjects, $n = 5$). Data are means \pm SE. Right panel: Tissue factor mRNA expression in monocytes before and 24 h after high glucose/high insulin clamping ($n = 3$). The normalized (by 18sRNA) after-to-before ratio is presented.

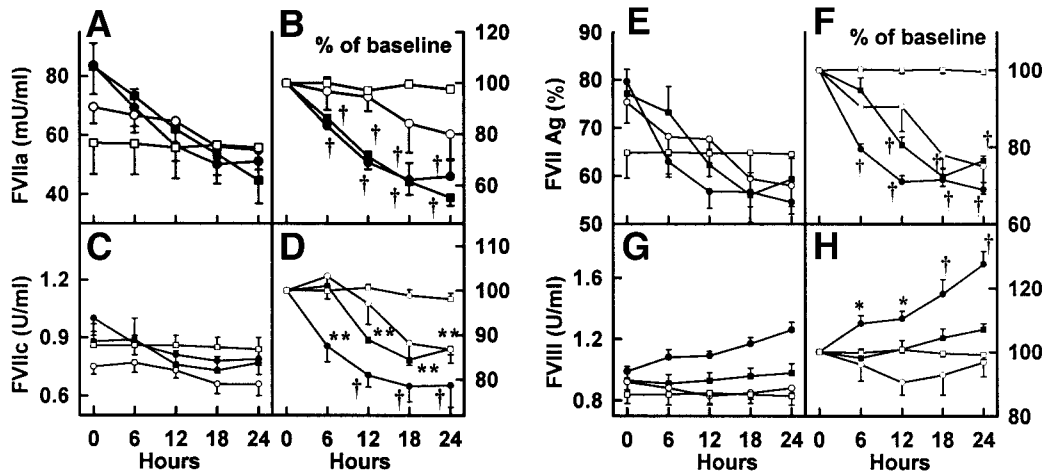


FIG. 4. FVIIa (A and B), FVIIc (C and D), FVIIAg (E and F), and FVIII (G and H) presented as absolute values or as percentage of basal values during 24 h of high glucose/high insulin (●) ($n = 10$), normal glucose/high insulin (■) ($n = 7$), high glucose/normal insulin (○) ($n = 7$), and normal glucose/normal insulin (□) ($n = 5$) clamping. Comparing 0 h with other time points: * $P < 0.05$, ** $P < 0.02$, and † $P < 0.001$ in B, D, F, and H. A: In FVIIa: high glucose/high insulin, $P = 0.002$ at 6 h, $P < 0.001$ at 12, 18, and 24 h; normal glucose/high insulin, $P < 0.001$ at 6, 12, 18, and 24 h. C: In FVIIc: high glucose/high insulin, $P < 0.001$ at 6, 12, 18, and 24 h; normal glucose/high insulin, $P < 0.03$ at 12 and 18 h, $P < 0.05$ at 24 h. E: In FVIIAg: high glucose/high insulin, $P = 0.02$ at 6 h, $P < 0.001$ at 12, 18, and 24 h; normal glucose/high insulin, $P < 0.001$ at 6, 12, 18, and 24 h. G: In FVIII: high glucose/high insulin, $P < 0.05$ at 6 h, $P = 0.002$ at 18 h, $P < 0.001$ at 24 h.

During high glucose/high insulin, tissue factor-positive monocytes increased from 19.8 ± 5.5 to $31.9 \pm 5.0\%$ ($P < 0.002$). There was no increase in the mean fluorescence intensity of tissue factor expression on monocytes. During selective hyperinsulinemia, tissue factor-positive monocytes increased from 12.5 ± 3.9 to $17.5 \pm 3.3\%$ ($P < 0.02$). In contrast, there was no change in the control study (normal glucose/normal insulin). Tissue factor-positive monocytes were not determined during selective hyperglycemia. Tissue factor mRNA in monocytes obtained from three volunteers increased 1.9 ± 0.3 -fold between 0 and 24 h of high glucose/high insulin ($P < 0.04$) (Fig. 3).

FVIIa, FVIIc, FVIIAg, and FVIII (Fig. 4). FVIIa, the activated form of FVII, remained unchanged for 24 h during the control study. Selective hyperinsulinemia and high glucose/high insulin were both associated with large decreases in FVIIa, whereas FVIIa decreased less during selective hyperglycemia (Fig. 4A and B). Plasma FVIIc decreased most during high glucose/high insulin and to a lesser degree during selective hyperinsulinemia and selective hyperglycemia and did not change during the control studies (Fig. 4C and D). Similarly, FVIIAg levels declined during the high glucose/high insulin studies, selective

hyperinsulinemia, and selective hyperglycemia but remained unchanged during the control studies (Fig. 4E and F). Factor VIII increased continuously during the high glucose/high insulin studies but did not change significantly during the other three studies (Fig. 4G and H).

There were statistically significant inverse relationships between tissue factor PCA and FVIIa in the high glucose/high insulin ($r = -0.52$, $P < 0.001$), the selective hyperinsulinemia ($r = -0.51$, $P = 0.002$), and the control ($r = -0.52$, $P = 0.007$) studies. FVIII levels correlated positively with tissue factor PCA ($r = 0.57$, $P < 0.0001$) during the high glucose/high insulin studies.

TAT complexes and prothrombin fragment 1.2 (Fig. 5). TAT, a sensitive indicator of thrombin formation, did not change during the control study. There were, however, large and significant increases in TAT during the selective hyperinsulinemia and during the high glucose/high insulin studies (Fig. 5A and B). The TAT levels during these three studies correlated positively with tissue factor PCA levels ($r = 0.49$, $P = 0.006$; $r = 0.35$, $P = 0.04$; and $r = 0.57$, $P < 0.0001$, respectively). Fragment 1.2, another indicator of thrombin formation, also remained unchanged during the

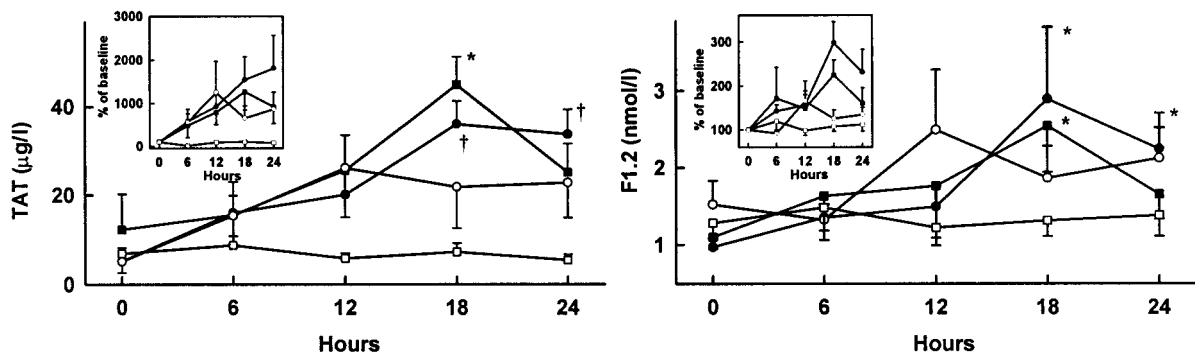


FIG. 5. TAT complexes and fragment 1.2 (F 1.2) presented as absolute values or as percentage of basal values (insets) during 24 h of high glucose/high insulin (●) ($n = 10$), normal glucose/high insulin (■) ($n = 7$), high glucose/normal insulin (○) ($n = 7$), and normal glucose/normal insulin (□) ($n = 5$) clamping. Comparing 0 h with other time points (inset): * $P < 0.5$, ** $P < 0.02$, † $P < 0.001$. Left and right panels: TAT: high glucose/high insulin, $P < 0.001$ at 18 and 24 h; normal glucose/high insulin, $P < 0.05$ at 18 h. F 1.2: high glucose/high insulin, $P < 0.05$ at 6, 12, 18, and 24 h.

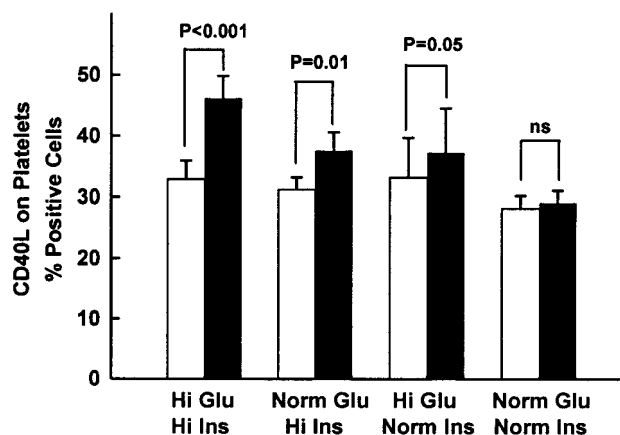


FIG. 6. CD40L expression on platelets determined with flow cytometry before (□) and after 24 h (■) of high glucose/high insulin ($n = 8$), selective hyperinsulinemia ($n = 7$), selective hyperglycemia ($n = 5$), and control subjects ($n = 5$). Data are means \pm SE.

control studies but rose during selective hyperinsulinemia and during high glucose/high insulin (Fig. 5C and D).

TFPI. Plasma TFPI antigen levels tended to decline in all four studies, but these changes reached statistical significance only in the selective hyperinsulinemia group, where the levels declined from 86.0 ± 9.6 to 69.6 ± 7.4 ng/ml at 18 h ($P < 0.001$) and to 65.7 ± 3.0 ng/ml at 24 h ($P < 0.05$).

CD40L expression on platelets (Fig. 6). No change occurred during the control study (28.1 ± 2.1 vs. $29.0 \pm 2.1\%$, NS). In contrast, during high glucose/high insulin, CD40L-positive platelets increased from 32.9 ± 3.0 to $46.1 \pm 3.7\%$, ($P < 0.001$). CD40L-positive platelets also increased during selective hyperinsulinemia (from 31.2 ± 2.0 to $37.5 \pm 3.1\%$, $P = 0.01$) and during selective hyperglycemia (from 33.2 ± 6.5 to $37.2 \pm 7.4\%$, $P < 0.05$), indicating that platelets had been activated. There was no significant increase in the mean fluorescence intensity of platelet CD40L expression in these groups.

DISCUSSION

Tissue factor is the physiological initiator of blood coagulation (13). The earlier concept that blood coagulation is initiated after vascular damage when blood is exposed to “extrinsic” tissue factor has been broadened by the observations that tissue factor is also present in circulating blood (13,14,16,17) and that hematopoietic cell-associated tissue factor is important in thrombogenesis (18). For instance, circulating tissue factor has been associated with increased blood thrombogenicity in patients with cardiovascular disease (30), sickle cell disease (17), antiphospholipid antibody syndrome (31), hyperlipidemia (20), and disseminated intravascular coagulation (32). In addition, we (19) and others (20) have reported that patients with type 2 diabetes have increased blood levels of tissue factor PCA. This is of considerable clinical interest because these patients are known to be at greatly increased risk for ASVD and acute arterial events. In the current study, we have examined in healthy subjects the hypothesis that the hyperglycemia and/or hyperinsulinemia, such as is seen in many patients with type 2 diabetes, may increase circulating tissue factor PCA. Exposure for 24 h to selective hyperglycemia resulted in an approximate twofold rise, exposure to selective hyperinsulinemia in an approximate sixfold rise, and exposure to combined high glucose/high insulin in an approximate ninefold rise in circulating tissue

factor PCA (Fig. 2). (Direct comparison of the magnitude of changes observed in the selective hyperglycemia group with the other three groups needs to be made with some caution because of the additional infusion of somatostatin in the selective hyperglycemia protocol.) These results suggested that the effects of selective high glucose and high insulin levels were additive and that the circulating tissue factor PCA may have been even greater had the studies lasted >24 h. They are consistent with our previously reported findings of elevated tissue factor PCA in patients with type 2 diabetes compared with nondiabetic control subjects (72.5 ± 12.6 vs. 23.2 ± 0.2 units/ml, $P < 0.001$) (19). The levels of plasma TAT were also elevated in these patients.

Monocytes are the only hematopoietic cells with established ability to synthesize tissue factor (13,14). During high glucose/high insulin and during selective hyperinsulinemia, tissue factor-positive monocytes increased significantly (Fig. 3). In addition, monocyte tissue factor mRNA increased approximately twofold during high glucose/high insulin, indicating increased tissue factor transcription.

The modest increases in monocyte surface expression of tissue factor and mRNA may not be sufficient to explain the marked increase in circulating tissue factor PCA. A possible explanation for this discrepancy is that tissue factor-containing microparticles may have been released from activated monocytes on exposure to high glucose and/or high insulin and may have accumulated in the blood during the 24-h experiments. They are measured in the tissue factor PCA assay together with tissue factor on lysed cell membranes but are not detected by flow cytometry, which measured only monocyte surface tissue factor.

Several lines of evidence point to an important role of blood-borne tissue factor in thrombus formation. Circulating tissue factor is biologically active in converting factor X to Xa (16). Tissue factor-bearing microparticles are highly procoagulant and have been shown to transfer tissue factor to platelets and to propagate further thrombus growth (13,16,18,33). Even where thrombus initiation was mediated by vessel wall tissue factor, the propagation of the initial thrombus depended on the recruitment of blood-borne tissue factor (13,14,16,18,34). Also, enhanced thrombus formation occurred when blood from patients with type 2 diabetes and elevated circulating tissue factor levels was perfused over a collagen-coated surface (20). Moreover, in patients undergoing angioplasty or stent implantation, elevated whole-blood tissue factor PCA present before the procedure predicted restenosis (30). We therefore evaluated effects of increases in circulating tissue factor PCA on other coagulation markers. We found that TAT and fragment 1.2, two sensitive indicators of thrombin generation, rose continuously during selective hyperinsulinemia and more so during the combined high glucose/high insulin studies (Fig. 5). Plasma factor VIII also rose in the latter group (Fig. 4). It needs to be noted that at least some of the monocyte membrane-bound tissue factor is encrypted, i.e., it can bind FVIIa without leading to activation of coagulation (35). This and the inhibitory effect of TFPI may have limited thrombin generation in our studies.

Plasma levels of FVIIa, FVIIc, and FVIIAg paradoxically declined during the high glucose/high insulin-stimulated rise of tissue factor PCA (Fig. 4), reflecting a decrease in total plasma FVII. Similar decreases in FVIIa have been reported after bolus infusions of endotoxin (36) and in sepsis (37). Because tissue factor is the principal ligand for

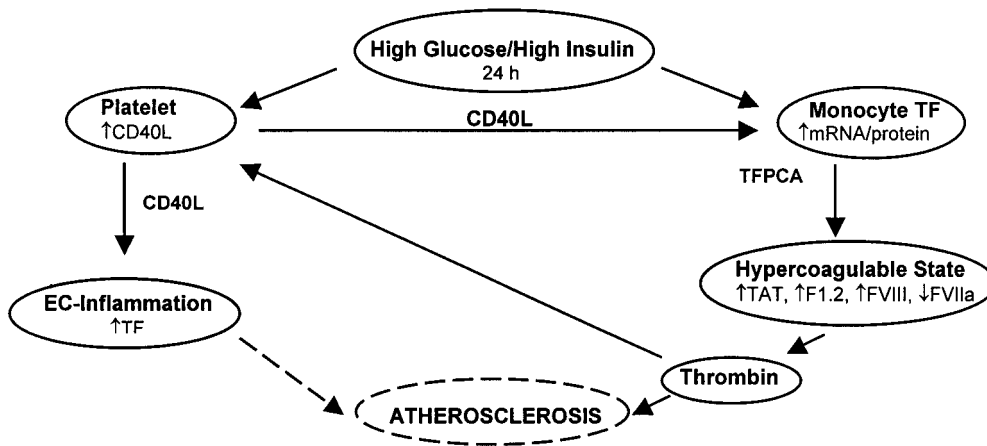


FIG. 7. Proposed scheme mechanisms to explain changes in tissue factor PCA, FVII, FVIII, TAT, F 1.2, and CD40L in normal subjects in response to high glucose and/or high insulin exposure. Key events are increases in tissue factor protein and mRNA in monocytes and in circulating tissue factor PCA. This results in a procoagulant state and the generation of thrombin. Thrombin activates platelets and promotes atherosclerosis. High glucose and/or high insulin is also associated with platelet activation and expression of CD40L, which induces tissue factor expression on monocytes and promotes endothelial cell inflammation and atherosclerosis. The decreases in plasma FVII is considered to reflect increased binding to monocyte tissue factor, which is upregulated.

FVIIa, we believe that the decrease in plasma FVII levels reflect accelerated clearance as a result of binding to an increased number of available tissue factor sites.

Thus, our studies provided evidence of increased circulating tissue factor PCA and FVIII associated with increased thrombin generation during hyperglycemia/hyperinsulinemia. These studies were performed in healthy subjects, but comparable findings have been reported in diabetic patients (19). The implications are that hyperglycemia/hyperinsulinemia induced a hypercoagulable state with the potential for enhanced thrombus formation, particularly in the context of plaque rupture. This is likely to play a role in the development of acute vascular events in patients with diabetes who are known to have a higher incidence of thrombus formation than nondiabetic patients (5,38).

CD40L is a 39-kDa glycoprotein belonging to the tumor necrosis factor superfamily (23–25). Most (>95%) of CD40L in circulation is stored in platelets, is expressed rapidly on platelet surfaces after activation, and is then released into the circulation (25). CD40L expressed on platelets interacts with cells that constitutively display its receptor CD40, including endothelial cells, monocytes, and macrophages, and incites a cascade of inflammatory responses, including expression of adhesion factors (including e-selectin and vascular cell adhesion molecule-1), expression of tissue factor, and release of chemokines and cytokines (including monocyte chemoattractant protein-1, interleukin-6, and interleukin-8) (23–25).

In our studies, CD40L-bearing platelets increased during the combined high glucose/high insulin studies with a somewhat lesser increase during selective hyperglycemia and selective hyperinsulinemia (Fig. 6). These findings provide strong evidence of *in vivo* platelet activation. They are relevant for patients with type 2 diabetes because CD40L triggers inflammatory reactions and is a major contributor to atherogenesis (24,39,40). Serum CD40L levels are elevated in diabetic patients (41). The upregulation of CD40L in platelets in our studies also suggested a mechanism for the increased monocyte tissue factor expression. Tissue factor is by itself proinflammatory (42) and in turn stimulates thrombosis and platelet release of CD40L, thereby sustaining a vicious cycle of inflammation and thrombosis (43). The sequence of events is summarized in Fig. 7.

In summary, combined elevation of plasma insulin and glucose levels for 24 h in healthy volunteers produced 1) a dramatic (ninefold) increase in circulating tissue factor

PCA associated with an increase in monocyte tissue factor surface expression and mRNA and 2) changes in other components of blood coagulation, suggesting that the coagulation system had been activated, including increases in plasma TAT complexes, prothrombin fragment 1.2, and FVIII and decreases in FVIIa and FVIIc activities. In addition, there was an increase in platelet CD40L, indicating platelet activation. Effects of selective hyperinsulinemia and selective hyperglycemia were less dramatic but appeared to be additive. We conclude that hyperinsulinemia and hyperglycemia, but particularly the combination of both, create a prothrombotic state and may in addition be proinflammatory and proatherogenic by virtue of the recognized actions of CD40L and tissue factor.

ACKNOWLEDGMENTS

G.B. has received National Institutes of Health Grants R01-DK-58895, R01-AG-15353, R01-HL-733267, and R01-DK-066003 and a Mentor-Based Training grant from the American Diabetes Association. A.K.R. has received National Institutes of Health Grant R01-DK-58895. The Molecular Core Facility of the Center for Substance Abuse at Temple University has received grant P30-DA-13429.

We thank Karen Kresge for outstanding technical assistance, Mayumi Katoaka for assistance with the flow cytometry studies, and Constance Harris Crews for typing the manuscript. We also thank the Molecular Core Facility of the Center for Substance Abuse at Temple University for letting us use their Roche Light-Cycler for the real-time RT-PCR measurements.

REFERENCES

- Pan WH, Cedres LB, Liu K, Dyer A, Schoenberger JA, Shekelle RB, Stamler R, Smith D, Collette P, Stamler J: Relationship of clinical diabetes and asymptomatic hyperglycemia to risk of coronary heart disease mortality in men and women. *Am J Epidemiol* 123:504–516, 1986
- Uusitupa MI, Niskanen LK, Siitonen O, Voutilainen E, Pyorala K: Five-year incidence of atherosclerotic vascular disease in relation to general risk factors, insulin level and abnormalities in lipoprotein composition in non-insulin-dependent diabetic and nondiabetic subjects. *Circulation* 82: 27–36, 1990
- Kannel WB, D'Agostino RB, Wilson PW, Belanger AJ, Gagnon DR: Diabetes, fibrinogen and risk of cardiovascular disease: the Framingham experience. *Am Heart J* 120:672–676, 1990
- Laakso M, Lehto S: Epidemiology of macrovascular disease in diabetes. *Diabetes Rev* 5:294–315, 1997
- Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM: Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20: 1262–1275, 2000

6. Osterman H, van de Loo J: Factors of the hemostatic system in diabetic patients. *Haemostasis* 16:386–416, 1986
7. Kwaan HC: Changes in blood coagulation, platelet function, and plasminogen-plasmin system in diabetes. *Diabetes* 41 (Suppl. 2):32–35, 1992
8. Kario K, Mastuo T, Kobayashi H, Matsuo M, Sakata T, Miyata T: Activation of tissue factor-induced coagulation and endothelial cell dysfunction in non-insulin-dependent diabetic patients and microalbuminuria. *Arterioscler Thromb Vasc Biol* 15:1114–1120, 1995
9. Morishita E, Asakura H, Jokaji H, Saito M, Uotani C, Kumabashiri I, Yamazaki M, Aoshima K, Hashimoto T, Matsuda T: Hypercoagulability and high lipoprotein (a) levels in patients with type II diabetes mellitus. *Atherosclerosis* 120:7–14, 1996
10. Jones RL: Fibrinopeptide-A in diabetes mellitus. *Diabetes* 34:836–843, 1985
11. Ceriello A, Giacomello R, Colatutto A, Taboga C, Gonano F: Hyperglycemia-induced thrombin formation in diabetes: the possible role of oxidative stress. *Diabetes* 44:924–928, 1995
12. Rao AK, Chouhan V, Chen X, Sun L, Boden G: Activation of the tissue factor pathway of blood coagulation during prolonged hyperglycemia in young healthy men. *Diabetes* 48:1156–1161, 1999
13. Rauch U, Nemerson Y: Tissue factor, the blood, and the arterial wall. *Trends Cardiovasc Med* 10:139–143, 2000
14. Mackman N: Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arterioscler Thromb Vasc Biol* 24:1015–1022, 2004
15. Wilcox JN, Smith KM, Schwartz SM, Gordon D: Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A* 86:2839–2843, 1989
16. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ, Hember J, Riederer MA, Nemerson Y: Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci U S A* 96:2311–2315, 1999
17. Key NS, Slungaard A, Dandele L, Nelson SC, Moertel C, Styles LA, Kuypers FA, Bach RR: Whole blood tissue factor procoagulant activity is elevated in patients with sickle cell disease. *Blood* 91:4216–4223, 1998
18. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B: Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood* 104:3190–3197, 2004
19. Abdel-Hafiz E, Vaidyula VR, Bagga S, London FS, Boden G, Rao AK: Elevated whole blood tissue factor procoagulant activity in diabetes mellitus: vitamin E inhibits glucose induced tissue factor activity in vitro (Abstract). *Blood* 100:496a, 2002
20. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ: Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. *Circulation* 107:973–977, 2003
21. Gerstein HC, Yusuf S: Dysglycaemia and risk of cardiovascular disease. *Lancet* 347:949–950, 1996
22. Diabetes Control and Complications Trial (DCCT) Research Group: Effect of intensive diabetes management on macrovascular event and risk factors in the diabetes control and complications trial. *Am J Cardiol* 75:894–903, 1995
23. Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczeck RA: CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391:591–594, 1998
24. Phipps RP: Atherosclerosis: the emerging role of inflammation and the CD40-CD40 ligand system. *Proc Natl Acad Sci U S A* 97:6930–6932, 2000
25. André P, Nannizzi-Alaimo L, Prasad SK, Phillips DR: Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation* 106:896–899, 2002
26. Jinchuan Y, Zongui W, Jimming C, Li L, Xintao K: Upregulation of CD40-CD40 ligand system in patients with diabetes mellitus. *Clin Chim Acta* 339:85–90, 2004
27. Harding SA, Sommerfiled AJ, Sarma J, Twomey PJ, Newby DE, Frier BM, Fox KA: Increased CD40 ligand and platelet-monocyte aggregates in patients with type 1 diabetes mellitus. *Atherosclerosis* 176:321–325, 2004
28. Boden G, Homko C, Mozzoli M, Showe LC, Nichols C, Cheung P: Thiazolidinediones upregulated fatty acid uptake and oxidation in adipose tissue of diabetic patients. *Diabetes* 54:880–885, 2005
29. Zhou JN, Lijungdahl S, Shoshan MC, Swedengorg J, Linder S: Activation of tissue-factor gene expression in breast carcinoma cells by stimulation of the RAF-ERK signaling pathway. *Mol Carcinog* 21:234–243, 1998
30. Tutar E, Ozcan M, Kilickap M, Gulec S, Aras O, Pamir G, Oral D, Dandele V, Key NS: Elevated whole-blood tissue factor procoagulant activity as a marker of restenosis after percutaneous transluminal coronary angioplasty and stent implantation. *Circulation* 108:1581–1584, 2003
31. Amengual O, Atsumi T, Khamashta MA, Bertolaccini ML, Hughes GR: The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb Haemost* 79:276–281, 1998
32. Asakura H, Kamikubo Y, Goto A, Shiratori Y, Yamazaki M, Jokaji H, Saito M, Uotani C, Kumabashiri I, Morishita E, Aoshima K, Nakamura S, Matsuda T: Role of tissue factor in disseminated intravascular coagulation. *Thromb Res* 80:217–224, 1995
33. Rauch U, Bonderman D, Bohrmann B, Badimon JJ, Hember J, Riederer MA, Nemerson Y: Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood* 96:170–175, 2000
34. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y: Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med* 9:458–462, 2003
35. Bach RR, Moldow CF: Mechanism of tissue factor activation on HL-60 cells. *Blood* 89:3270–3276, 1997
36. Taylor FB, Haddad PA, Hack E, Chang AC, Peer GT, Morrissey JH, Li A, Allen RC, Wada H, Kinasewitz GT: Two-stage response to endotoxin infusion into normal human subjects: correlation of blood phagocyte luminescence with clinical and laboratory markers of the inflammatory, hemostatic response. *Crit Care Med* 29:326–334, 2001
37. Mesters RM, Mannucci PM, Coppola R, Keller T, Osterman H, Kienast J: Factor VIIa and antithrombin III activity during severe sepsis and septic shock in neutropenic patients. *Blood* 88:881–886, 1996
38. Moreno PR, Murcia AM, Palacios IF, Leon MN, Bernardi VH, Fuster V, Fallon JT: Coronary composition and macrophage infiltration in atherectomy specimens from patients with diabetes mellitus. *Circulation* 102:2180–2184, 2000
39. Lutgens E, Cleutjens KB, Heeneman S, Koteliansky VE, Burkly LC, Daemen MJ: Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc Natl Acad Sci U S A* 7:7464–7469, 2000
40. Schonbeck U, Sukhova GK, Shimizu K, Mach F, Libby P: Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. *Proc Natl Acad Sci U S A* 97:7458–7463, 2000
41. Marx N, Imhof A, Froehlich J, Siam L, Ittner J, Wierse G, Schmidt A, Maerz W, Hombach V, Koenig W: Effect of rosiglitazone treatment on soluble CD40L in patients with type 2 diabetes and coronary artery disease. *Circulation* 107:1954–1957, 2003
42. Bokarewa MI, Morrissey JH, Tarkowski A: Tissue factor as a proinflammatory agent. *Arthritis Res* 4:190–195, 2002
43. Libby P, Simon DI: Inflammation and thrombosis: the clot thickens. *Circulation* 103:1718–1720, 2001