

# Activation of the Tissue Factor Pathway of Blood Coagulation During Prolonged Hyperglycemia in Young Healthy Men

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Patients with diabetes have an increased prevalence of premature atherosclerotic vascular disease, and alterations in plasma coagulation proteins have been incriminated as a possible cause. The roles of hyperglycemia and hyperinsulinemia in the pathogenesis of these changes are unknown. To examine the effects of prolonged hyperglycemia and of selective hyperinsulinemia on the tissue factor pathway of blood coagulation, nine healthy young men were infused with glucose to maintain levels at 11.1 mmol/l (~200 mg/dl) for 18–72 h (hyperglycemia-hyperinsulinemia group). Five normal men were infused with regular insulin to maintain levels comparable to that in the previous group (900 pmol/l, ~150 pU/ml) and with glucose to maintain levels at 5.6 mmol/l (~100 mg/dl) (euglycemia-hyperinsulinemia group). Measured were plasma activated factor VII activity (FVIIa), FVII coagulant (FVIIC) activity, FVIII coagulant (FVIIC) activity, tissue factor pathway inhibitor (TFPI) antigen, and thrombin markers; and serum glucose, insulin, and electrolytes. Plasma FVIIa, FVIIC, FVIIC, and TFPI rose during hyperglycemic-hyperinsulinemia but not during euglycemic-hyperinsulinemia. Markers of thrombin generation rose transiently and inconsistently during hyperglycemia-hyperinsulinemia. We concluded that in normal subjects, hyperglycemia-hyperinsulinemia induced activation of the tissue factor pathway, reflected by increases in plasma FVIIa, FVIIC, and TFPI. This activation was independent of hyperinsulinemia, hypertriglyceridemia, and hyperosmolality. The elevations in plasma coagulation factors during hyperglycemia-hyperinsulinemia, characteristic of type 2 diabetes, may constitute a potential for enhanced thrombin generation and thrombosis when triggered by exposure of tissue factor, such as during arterial plaque rupture. *Diabetes* 48:1156–1161, 1999

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APTT, activated partial thromboplastin time; ELISA, enzyme-linked immunosorbent assay; F1.2, fragment 1.2; FVII, factor VII; FVIIa, activated factor VII; FVIIC, factor VII coagulant activity; FVIII, factor VIII; FVIIC, factor VIII coagulant activity; GIR, glucose infusion rate; TAT, thrombin-antithrombin complexes; TFPI, tissue factor pathway inhibitor.

Patients with type 2 diabetes have an increased prevalence of atherosclerotic vascular disease and morbidity and mortality from myocardial infarction, cerebrovascular accidents, and peripheral vascular disease (1–4). Hyperglycemia is a major contributor to vascular disease (5,6); however, the mechanisms by which this occurs are uncertain. Numerous studies have documented alterations in plasma of proteins involved in blood coagulation and fibrinolysis, and platelet function in patients with diabetes (7–10). The mechanisms leading to these changes are not well understood. There is evidence suggesting that plasma levels of several coagulation factors may be modulated by hyperglycemia and/or hyperinsulinemia. For example, glycemic control has been shown to correlate with plasma fibrinogen (3) and plasma and urinary fibrinopeptide A levels (11,12). In other studies, acute elevation of plasma glucose concentrations during oral glucose tolerance testing or intravenous glucose infusion increased plasma factor VII coagulant (FVIIC) activity and plasma prothrombin fragment 1.2 (F1.2) (an indicator of thrombin generation) (13,14). It is not clear, however, whether these effects were due to the increase in plasma glucose or the accompanying hyperinsulinemia. This distinction may be important because hyperinsulinemia has been identified as an independent risk factor for coronary artery disease and mortality (15–18). Moreover, in these studies glucose infusions extended for only 1–2 h, and we are not aware of studies on the relative effects of prolonged hyperglycemia and hyperinsulinemia on blood coagulation. We have, therefore, examined in young healthy volunteers the effect of prolonged (18–72 h) hyperglycemic and hyperinsulinemic clamping on the tissue factor pathway of blood coagulation, the primary physiological mechanism for the initiation of coagulation (19–21).

## RESEARCH DESIGN AND METHODS

**Subjects and study design.** A total of 14 healthy men between 24 and 45 years of age volunteered for the studies. Nine men (mean  $\pm$  SE; age:  $30 \pm 2$  years; height:  $176 \pm 3$  cm; weight:  $82 \pm 3.5$  kg; fasting plasma glucose:  $4.9 \pm 0.2$  mmol/l, range 4.1–5.7 mmol/l) underwent hyperglycemic-hyperinsulinemic clamping (hyperglycemia group), and five men (age:  $39 \pm 2$  years; height:  $179 \pm 3$  cm; weight:  $71.8 \pm 5.4$  kg; fasting plasma glucose  $5.0 \pm 0.3$  mmol/l, range 4.2–5.7 mmol/l) underwent euglycemic-hyperinsulinemic clamping (hyperinsulinemia group) for up to 72 h. Thus, both groups were characterized by hyperinsulinemia, while the serum glucose levels were either elevated or normal. All subjects were admitted to the Temple University Hospital General Clinical Research Center on the evening before the studies. The studies began between 8:00 and 10:00 A.M. after an overnight fast, with the subjects reclining in bed during the entire study. A short polyethylene catheter was inserted into an antecubital vein for infusion of glucose,

insulin, and electrolytes. Another catheter was placed into a contralateral forearm vein for blood sampling. This arm was wrapped with a heating blanket ( $-70^{\circ}\text{C}$ ) to arterialized venous blood.

**Hyperglycemic-hyperinsulinemic clamping (hyperglycemia group).** A 20% glucose solution was infused intravenously at variable rates, which were adjusted to maintain plasma glucose at 216–234 mg/dl (12–13 mmol/l) for up to 72 h. The study participants did not eat during the entire duration of the study, but they were allowed to drink water ad libitum. Plasma electrolytes and fluid balance were monitored every 6 h and body weight every 12 h. Fluid balance was maintained with infusion of normal saline. Potassium and magnesium were added to the glucose infusion as needed to maintain normal plasma concentrations.

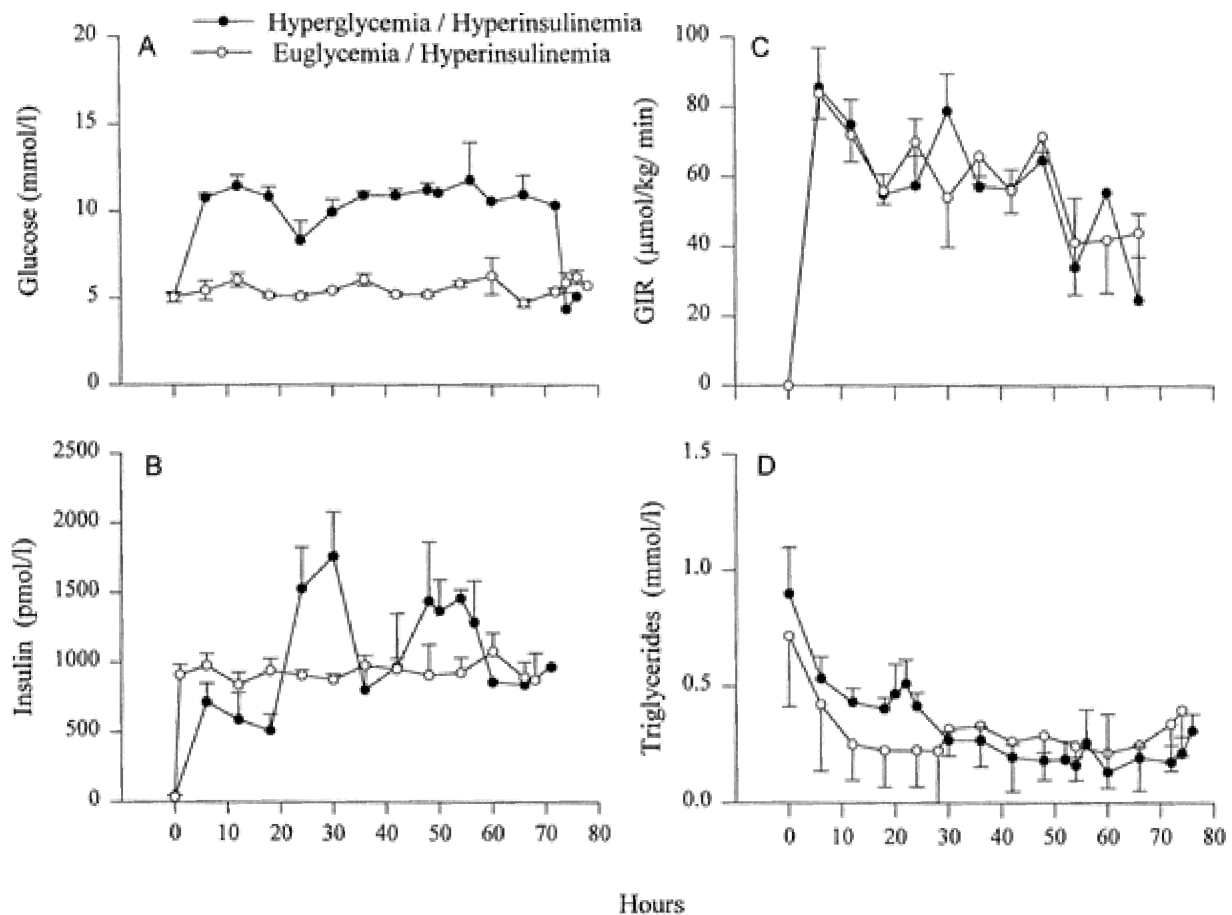
**Euglycemic-hyperinsulinemic clamping (hyperinsulinemia group).** Regular human insulin (Humulin; Eli Lilly, Indianapolis, IN) was infused intravenously at a rate of  $12 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  ( $2 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). Glucose was maintained at  $\sim 4.7 \text{ mmol/l}$  (85 mg/dl) by feedback-controlled glucose infusions. Glucose concentrations were determined every 15–30 min at the beginning and at 1- to 2-h intervals later with a glucose analyzer, and the glucose infusion rates (GIRs) were adjusted as needed. Testing of plasma electrolytes, fluid balance, body weight, and potassium and magnesium replacement were done as described for the hyperglycemia group (see above). In several studies, there was early cessation of glucose infusions because of loss of venous access, nausea, general fatigue, and irritability, the latter presumably due to loss of normal sleep. In the hyperglycemia group, all nine men completed 18 h, four completed 56 h, two completed 66 h, and one completed 72 h. In the hyperinsulinemia group, all five men completed 24 h, four completed 48 h, three completed 66 h, and two completed 72 h. All adverse effects disappeared within 1–2 h after discontinuation of the glucose infusion.

**Sample collection.** Blood samples were collected from antecubital veins without tourniquet-induced venostasis. Blood samples were drawn at baseline, at 6-h intervals up to 72 h, and at 1, 2, and 4 h after discontinuation of the clamps. Blood

was collected in plastic tubes containing one-tenth volume of 3.8% sodium citrate as an anticoagulant. Plasma was separated by centrifugation at  $2,500g$  for 20 min within 30 min of collection. Aliquots of platelet-poor plasma were stored at  $-80^{\circ}\text{C}$  until assayed.

**Assays.** Plasma activated FVII (FVIIa) activity was measured by a clotting assay using recombinant soluble tissue factor (American BioProducts, Parsippany, NJ) (22). FVII antigen was measured by an enzyme-linked immunosorbent assay (ELISA) (American BioProducts). FVII activity was measured by a one-stage clotting assay using a mechanical timer Fibrometer (BBL Microbiology System, Cockville, MD), Simplastin Excel (Organon Technika, Durham, NC), and FVII-deficient plasma (George King Biomedical, Overland Park, KS). FVIII coagulant (FVIIIc) activity was measured with an activated partial thromboplastin time (APTT)-based one-stage clotting assay using the APTT reagent from Organon Technika. 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 F1.2 and thrombin-antithrombin (TAT) complexes in plasma using ELISAs (Enzygnost; Behringwerke, Marburg, Germany). Plasma glucose was measured with a glucose analyzer using the glucose oxidase method, and serum insulin was determined 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.

**Statistical analysis.** Comparisons of the values obtained within each group at different time points (baseline, 18 h, and 48 h) were performed using the Student's *t* test for comparison of means. Experimental protocols were approved by the human research review committee of Temple University School of Medicine. An informed consent to participate in the study was obtained from all subjects.



**FIG. 1.** Serum glucose, insulin, and triglyceride levels, and GIR during hyperglycemic-hyperinsulinemic clamping (●, hyperglycemia group) and euglycemic-hyperinsulinemic clamping (○, hyperinsulinemia group) in normal subjects. Means  $\pm$  SE are shown. In the hyperglycemia group, circadian changes are also observed in the insulin levels (21).

TABLE 1  
Changes in plasma during hyperglycemia-hyperinsulinemia and euglycemia-hyperinsulinemia

	Hyperglycemia-hyperinsulinemia group			Euglycemia-hyperinsulinemia group		
	Baseline	18 h	48 h	Baseline	18 h	48 h
<i>n</i>	9	9	4	5	5	4
Glucose (mmol/l)	4.9 ± 0.2	10.8 ± 0.6‡	11.3 ± 0.4‡	5.1 ± 0.3	5.1 ± 0.2	5.8 ± 0.2
Insulin (pmol/l)	66 ± 23	533 ± 93‡	1,522 ± 589*	44 ± 9.0	850 ± 87‡	947 ± 79‡
Triglycerides (mmol/l)	0.91 ± 0.22	0.41 ± 0.05*	0.18 ± 0.03*	0.77 ± 0.30	0.25 ± 0.18	0.30 ± 0.19
FVIIa (mU/ml)	16.7 ± 1.12	38.6 ± 8.5*	80.3 ± 17.7‡	31.3 ± 3.4	31.6 ± 3.1	34.2 ± 2.9
FVIIC (U/ml)	0.89 ± 0.03	1.12 ± 0.03‡	1.64 ± 0.16‡	0.81 ± 0.06	0.82 ± 0.05	0.80 ± 0.08
FVII antigen (%)	67.1 ± 1.6	65.3 ± 1.3	62.1 ± 0.70	69.6 ± 4.7	67.5 ± 3.6	70.3 ± 4.1
TFPI (ng/ml)	77.0 ± 5.2	99.6 ± 7.8*	105.0 ± 16.0‡	74.2 ± 5.3	69.2 ± 5.6	74.2 ± 5.4
FVIIIc (U/ml)	1.09 ± 0.30	1.22 ± 0.27‡	1.37 ± 0.02‡	1.08 ± 0.58	1.10 ± 0.06	1.10 ± 0.07

Data are means ± SE. \**P* < 0.05, †*P* < 0.01, ‡*P* < 0.001 compared with baseline values within each group.

RESULTS

Figure 1 and Table 1 show that plasma glucose levels were consistently higher while insulin and triglyceride levels and GIRs (needed to maintain the glucose clamps) were comparable in the hyperglycemia and hyperinsulinemia groups. In the hyperglycemia group, there were circadian changes in insulin concentrations (Fig. 1) that we have recently described (23). Areas under the insulin curves of the two groups, however, were not significantly different from each other (3.03 ± 0.6 vs. 2.12 ± 0.6 μmol · min · l<sup>-1</sup>). Triglyceride levels (Fig. 1) declined in both groups.

Plasma FVIIa levels, the activated form of FVII, rose in all volunteers in the hyperglycemia group and continued to increase in most for several hours after the blood glucose had returned to normal (Fig. 2). FVIIa levels were significantly

higher at 18 (*P* < 0.05) and 48 h (*P* < 0.01) than at baseline (Table 1). In contrast, FVIIa levels did not change in the hyperinsulinemia group (Fig. 2). Plasma FVIIC levels, which reflect the combined activity of the FVII zymogen and FVIIa, also rose in the hyperglycemia group but not in the hyperinsulinemia group (Fig. 2), with significant elevations over baseline at 18 and 48 h (Table 1). In contrast, plasma FVII antigen levels did not increase in either group (Fig. 2).

Plasma FVIIIc and TFPI levels rose in all subjects in the hyperglycemia group but not in the hyperinsulinemia group (Fig. 3). For both proteins, the mean levels in the hyperglycemic group at 18 and 48 h were significantly higher compared with baseline (Table 1).

To assess whether the elevations in FVII and FVIII resulted in thrombin generation, levels of plasma prothrombin F1.2 and TAT complexes were determined. The pattern of changes in

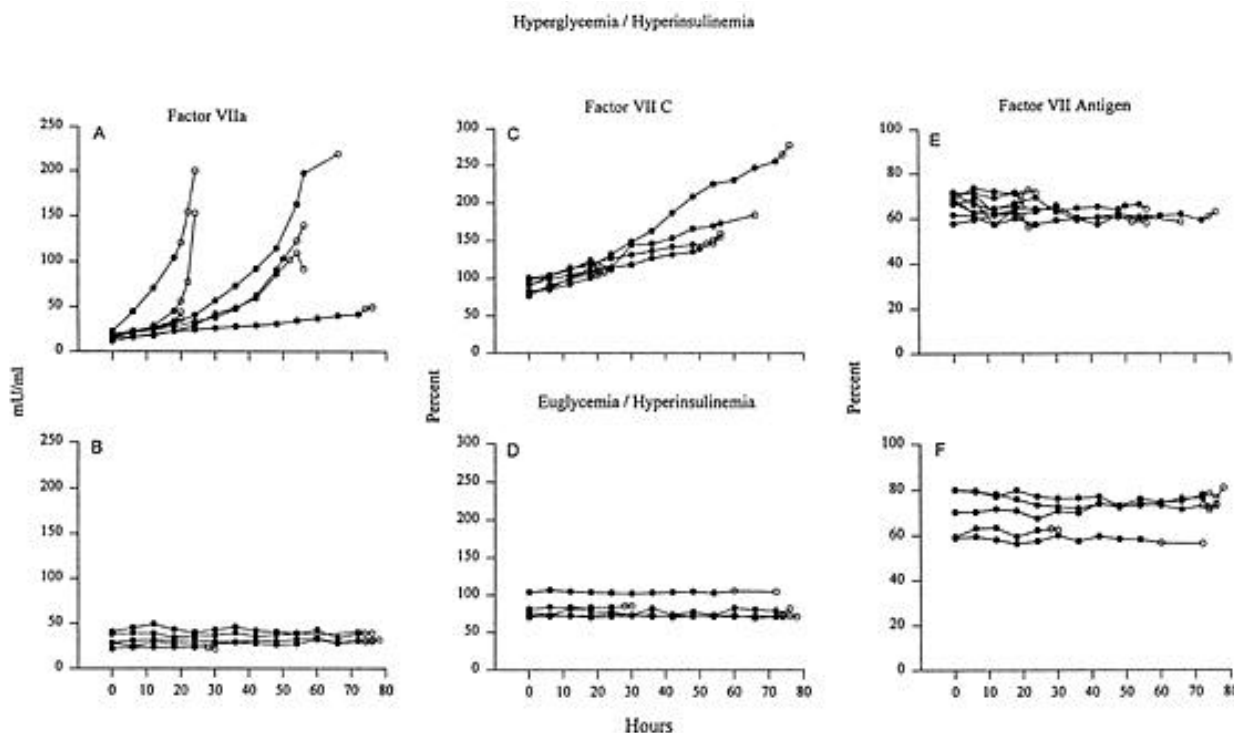


FIG. 2. Plasma levels of FVIIa, FVIIC activity, and FVII antigen during hyperglycemic-hyperinsulinemic clamping (A, C, and E, hyperglycemia group) and euglycemic-hyperinsulinemic clamping (B, D, and F, hyperinsulinemia group) in normal volunteers. ○, Levels after discontinuation of the glucose infusion.

plasma F1.2 (Fig. 3) and TAT complexes (not shown) showed transient and inconsistent fluctuations in individual subjects. In general, the fluctuations in plasma F1.2 levels appeared to be higher in the hyperglycemic than in the hyperinsulinemic group (Fig. 3). At 48 h, the mean levels of F1.2 or TAT were not significantly elevated over baseline in either group.

## DISCUSSION

The tissue factor pathway is the primary physiological mechanism of initiation of blood coagulation (19–21). Binding of native coagulation FVII to tissue factor converts native FVII to the activated form, FVIIa. The resultant tissue factor–FVIIa complex then activates factors IX and X to factors IXa and Xa, respectively, resulting in the formation of the prothrombinase complex and thrombin generation. The major inhibitor of the tissue factor–FVIIa complex is TFPI (19–21). The main finding in this study was that raising blood glucose levels from 5.6 to 11.1 mmol/l (100 to 200 mg/dl) for 18–72 h in young healthy volunteers resulted in activation of the tissue factor pathway of blood coagulation. Specifically, plasma FVIIa and FVIIC rose continuously, along with elevations in plasma FVIIIc, in all nine volunteers during 18–72 h of hyperglycemic-hyperinsulinemic clamping. In contrast, selective hyperinsulinemia, comparable in degree to the hyperinsulinemia that occurred in the hyperglycemia group, had no effect on factors VIIa, VIIC, and VIIIc or on TFPI. Interestingly, there was no striking thrombin generation even in the hyperglycemia group, as evidenced by unimpressive and inconsistent changes in plasma F1.2 (Fig. 3) or TAT levels, two indicators of thrombin generation. A likely reason for this is the simultaneous increase in the major inhibitor TFPI (19–21) (Fig. 3). We postulate that hyperglycemia activated the tissue factor pathway; however, this is rapidly controlled by the TFPI, thereby preventing substantial FX activation and

thrombin generation. It is also conceivable that a localized increase in thrombin generation is not reflected in an increase in peripheral levels of the thrombin markers.

Elevated plasma FVIIa, FVIIC, FVIIIc, and other coagulation proteins have previously been reported in patients with type 2 diabetes (7–10). Many of these patients, however, had preexisting atherosclerotic disease in addition to hyperglycemia (7,9,10). Ceriello et al. (13,14) have reported elevations in plasma FVIIC and F1.2 levels in response to brief elevations of plasma glucose in normal subjects. It is difficult to compare their results with ours because of differences in study duration (2 vs. 48 h) and blood sampling intervals (every 10 min vs. every 6 h). For instance, in one study (14), FVIIC rose in normal subjects by ~100% within 10 min, compared with a similar increase after ~48 h in this study. In another study (13), plasma F1.2 as well as glucose concentrations rose in all seven normal subjects, whereas in our study, no consistent pattern of increase was noted in plasma F1.2. Moreover, in neither study (13,14) was the effect of hyperinsulinemia addressed. The current study provides new evidence that prolonged hyperglycemia, but not hyperinsulinemia, induces activation of the tissue factor pathway of blood coagulation in young healthy individuals apparently free of preexisting atherosclerotic disease.

High plasma levels of triglyceride and free fatty acid have been shown to increase FVIIC in normal subjects (24–26). This mechanism could be excluded in our study, since plasma triglyceride and free fatty acid levels declined in all subjects under the influence of hyperinsulinemia (Fig. 1). Moreover, hyperosmolar stress could not be incriminated because the amounts of glucose infused (GIR) to maintain either hyperglycemia or hyperinsulinemic-euglycemia were similar. Therefore, we believe that the prolonged hyperglycemia per se was responsible for the observed changes in the coagula-

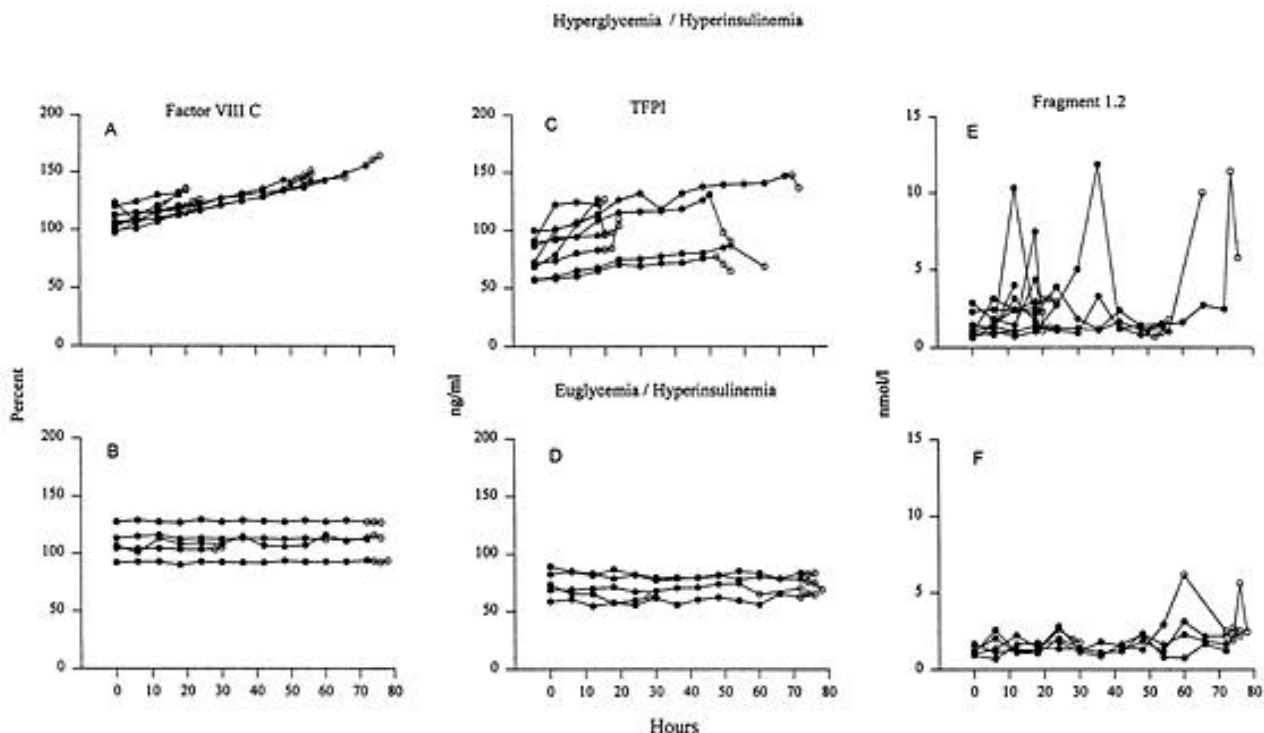


FIG. 3. Plasma levels of FVIIC activity, TFPI, and prothrombin F1.2 during hyperglycemic-hyperinsulinemic clamping (A, C, and E, hyperglycemia group) and euglycemic-hyperinsulinemic clamping (B, D, and F, hyperinsulinemia group) in normal volunteers. ○, Levels after discontinuation of the glucose infusion.

tion system, although we cannot completely exclude the possibility that the activation of coagulation resulted from the combined effect of hyperglycemia and hyperinsulinemia.

The mechanisms for the observed activation of the tissue factor pathway need to be delineated. This may occur via activation of monocytes or endothelial cells that can be induced to express tissue factor (27,28). Prolonged exposure of cultured human endothelial cells to hyperglycemia increases tissue-factor gene expression (29). Moreover, perturbation of the endothelium and exposure of tissue factor may be secondary to hyperglycemia-induced oxidative stress (30,31). In our studies, plasma FVIIIC levels rose during hyperglycemia while FVII antigen levels, which reflect total FVII protein in plasma, remained unchanged. The latter finding suggests that the increase in FVIIIC is not due to increased hepatic FVII synthesis but to an enhanced conversion of zymogen FVII to FVIIa (32). Plasma FVIIIC levels also rose in the hyperglycemia group but not in the hyperinsulinemia group (Fig. 3). Elevated plasma FVIIIC without changes in FVIII antigen have been reported in diabetic children without vascular disease and attributed to increased intravascular activation of FVIII (33).

Most of the FVII in plasma circulates in the inactive zymogen form, and the picomolar amounts of circulating FVIIa normally present serve an important priming function in triggering blood coagulation on exposure to tissue factor (19,22). Thus, the high levels of circulating FVIIa, FVIIIC, and FVIII observed during hyperglycemia provide an exaggerated potential for brisk thrombin generation given appropriate initiating stimuli, as for example, the intense exposure of tissue factor during atherosclerotic plaque rupture or erosion (34,35). They may, thus, play an amplifying role in precipitating acute thrombosis and vascular events once the mechanisms are triggered. Angiographic studies in patients with unstable angina show a higher incidence of intracoronary thrombosis and of plaque ulceration in diabetic than in nondiabetic patients (36). Moreover, in epidemiological studies, FVIIIC has been found to be predictive of the risk of fatal but not nonfatal myocardial infarction (37,38). The assay for FVIIIC used in one of these studies (the Northwick Park Heart Study) was particularly sensitive to FVII (32). More recently, certain polymorphisms of the FVII gene have been shown to be associated with increased risk of familial myocardial infarction as well as with high plasma FVII levels (39), and the increased incidence of myocardial infarction has been attributed to elevated FVII levels (39).

In conclusion, our studies provide evidence for activation of the tissue factor pathway of blood coagulation in normal volunteers subjected to hyperglycemia-hyperinsulinemia at levels commonly encountered in type 2 diabetes, but not during selective hyperinsulinemia with euglycemia. The elevated levels of FVIIa, FVIIIC, and FVIIIC may set the stage for acute thrombosis when triggered by a potent stimulus and thereby contribute to the increased incidence of acute vascular events in patients with diabetes (1-4). Thus, a heretofore unrecognized beneficial effect of strict control of blood glucose in diabetic subjects is prevention of the hypercoagulable state that accompanies hyperglycemia.

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