Original Article

Hyperglycemia Stimulates Coagulation, Whereas Hyperinsulinemia Impairs Fibrinolysis in Healthy Humans

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Type 2 diabetes and insulin resistance syndromes are associated with an increased risk for cardiovascular and thrombotic complications. A disturbed balance between coagulation and fibrinolysis has been implicated in the pathogenesis hereof. To determine the selective effects of hyperglycemia and hyperinsulinemia on coagulation and fibrinolysis, six healthy humans were studied on four occasions for 6 h: *1*) lower insulinemic-euglycemic clamp, *2*) lower insulinemic-hyperglycemic clamp, *3*) hyperinsulinemic-euglycemic clamp, and *4*) hyperinsulinemic-hyperglycemic clamp. In the hyperglycemic clamps, target levels of plasma glucose were 12 versus 5 mmol/l in the normoglycemic clamps. In the hyperinsulinemic clamps, target plasma insulin levels were 400 versus 100 pmol/l in the lower insulinemic clamps. Hyperglycemia exerted a procoagulant effect irrespective of insulin levels, as reflected by mean twofold rises in thrombin-antithrombin complexes and soluble tissue factor, whereas hyperinsulinemia inhibited fibrinolysis irrespective of glucose levels, as reflected by a decrease in plasminogen activator activity levels due to a mean 2.5-fold rise in plasminogen activator inhibitor type 1. The differential effects of hyperglycemia and hyperinsulinemia suggest that patients with hyperglycemia due to insulin resistance are especially susceptible to thrombotic events by a concurrent insulin-driven impairment of fibrinolysis and a glucose-driven activation of coagulation. *Diabetes* 55:1807–1812, 2006

Type 2 diabetes and its antecedents impaired glucose tolerance and syndromes of insulin resistance are associated with a profoundly increased risk for thrombosis. Eighty percent of type 2 diabetic patients die a thrombotic death, and cardiovascular disease (CVD) is by far the leading cause of mortality in this patient population (1–3). Remarkably, although cardiovascular mortality in the general population has declined precipitously in recent years, diabetic patients have not experienced such a favorable decrease (4). Although the prevalence of traditional risk factors for atherosclerosis, such as hypertension and hypercholesterolemia, is increased in type 2 diabetes, these factors account for only half of the observed excess risk for CVD (5). Therefore, additional risk factors have been implicated in the pathogenesis of CVD in type 2 diabetes. In this respect, disturbances in fibrinolysis and coagulation secondary to insulin resistance have emerged as likely mechanisms contributing to the increased cardiovascular risk (2,3,6). Impaired fibrinolysis, in particular due to elevated levels of plasminogen activator inhibitor type 1 (PAI-1), is a consistent finding in type 2 diabetes (7,8). In addition, coagulation activation markers, including thrombin-antithrombin complexes (TATcs), have been found to be elevated in patients with type 2 diabetes (9–11).

The question remains as to how type 2 diabetes and insulin resistance syndromes influence the balance between fibrinolysis and coagulation. Several experimental studies have addressed the question of whether hyperglycemia or hyperinsulinemia per se influences fibrinolysis or coagulation (12–17). These studies, in which plasma glucose and/or insulin levels were artificially increased by exogenous infusion, have been inconclusive, in particular, because in none of these studies were both plasma glucose and insulin concurrently maintained at either normal levels or at levels found in type 2 diabetes. Here, we report on a controlled cross-over study in which healthy humans were exposed during 6 h to hyperglycemia (targeted at 12 mmol/l) in the presence of basal insulin levels, to hyperinsulinemia (targeted at 400 pmol/l) in the presence of normal glucose levels, or to combined hyperglycemia and hyperinsulinemia. We demonstrate for the first time that hyperinsulinemia inhibits fibrinolysis irrespective of glucose concentrations, whereas hyperglycemia stimulates coagulation irrespective of insulin concentrations.
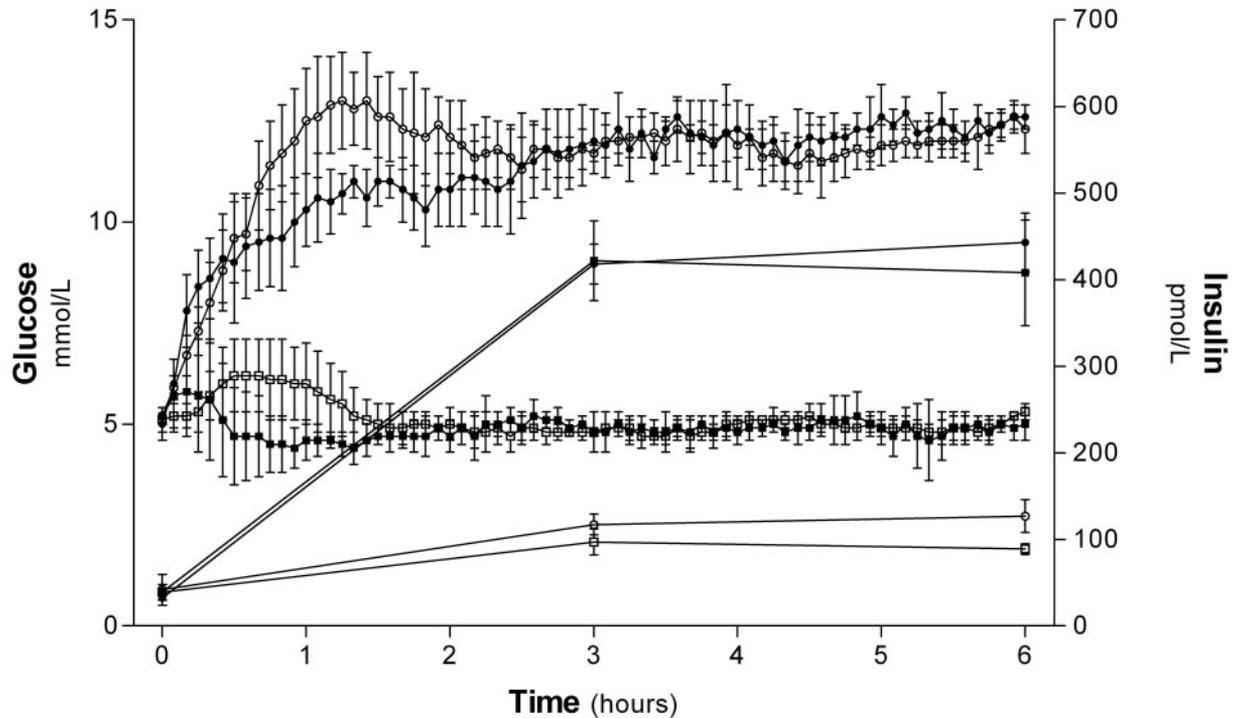
FIG. 1. Plasma concentrations of glucose and insulin. Data are presented as means ± SD from six subjects studied on four separate occasions: during an $L_{insu}E_{gluc}$ clamp (□), an $L_{insu}H_{gluc}$ clamp (○), an $H_{insu}E_{gluc}$ clamp (■), and an $H_{insu}H_{gluc}$ clamp (●). Glucose (*left axis*) was measured every 5 min; insulin (*right axis*) was measured at 0, 3, and 6 h.

RESEARCH DESIGN AND METHODS

Six healthy, nonsmoking, male volunteers (age 21.7 ± 1.2 years; weight 73.2 ± 4.8 kg; BMI 21.8 ± 0.9 kg/m² [means ± SD]) were studied. None of them used medication or had a positive family history of diabetes. All volunteers had normal plasma values of fasting glucose, insulin, erythrocyte sedimentation rate, complete blood count, lipid profile, and renal and hepatic function, and all had a normal oral glucose tolerance test. The study was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam, and all subjects gave written informed consent.

The study protocol had a cross-over design, with a washout period of 4 weeks, and was done in balanced assignment. Each volunteer served as his own control and was studied on four occasions: during a lower insulinemic-euglycemic ($L_{insu}E_{gluc}$) clamp (target insulin level 100 pmol/l; target glucose level 5 mmol/l), a lower insulinemic-hyperglycemic ($L_{insu}H_{gluc}$) clamp (insulin 100 pmol/l; glucose 12 mmol/l), a hyperinsulinemic-euglycemic ($H_{insu}E_{gluc}$) clamp (insulin 400 pmol/l; glucose 5 mmol/l), and a hyperinsulinemic-hyper-

glycemic ($H_{insu}H_{gluc}$) clamp (insulin 400 pmol/l; glucose 12 mmol/l). For 3 days before the study, all volunteers consumed a weight-maintaining diet containing at least 250 g carbohydrates. After an overnight fast, the subjects were admitted to the clinical research unit and confined to bed. The study started at 8:45 A.M. with placement of a catheter into an antecubital vein for infusion of insulin, somatostatin, glucagon, and glucose 10 or 20%. Another catheter was inserted retrogradely into a contralateral hand vein kept in a thermoregulated (60°C) Plexiglas box for sampling of arterialized venous blood. Saline (0.9% NaCl) was infused with a slow drip to keep the catheters patent.

At $t = 0$ (9:00 A.M.), infusions of somatostatin (250 µg/h; Somatostatine-ucb; UCB Pharma, Breda, the Netherlands) to suppress endogenous insulin and glucagon secretion and glucagon (1 ng · kg^{-1} · min^{-1}; Glucagen; Novo Nordisk, Alphen aan den Rijn, the Netherlands) to replace endogenous glucagon concentrations were started; concurrently, infusions of insulin (Actrapid/l; Novo Nordisk) at a rate of 10 or 40 mU/m² body surface area per 1 min (lower or hyperinsulinemic clamp, respectively) and 10 or 20% glucose at a variable

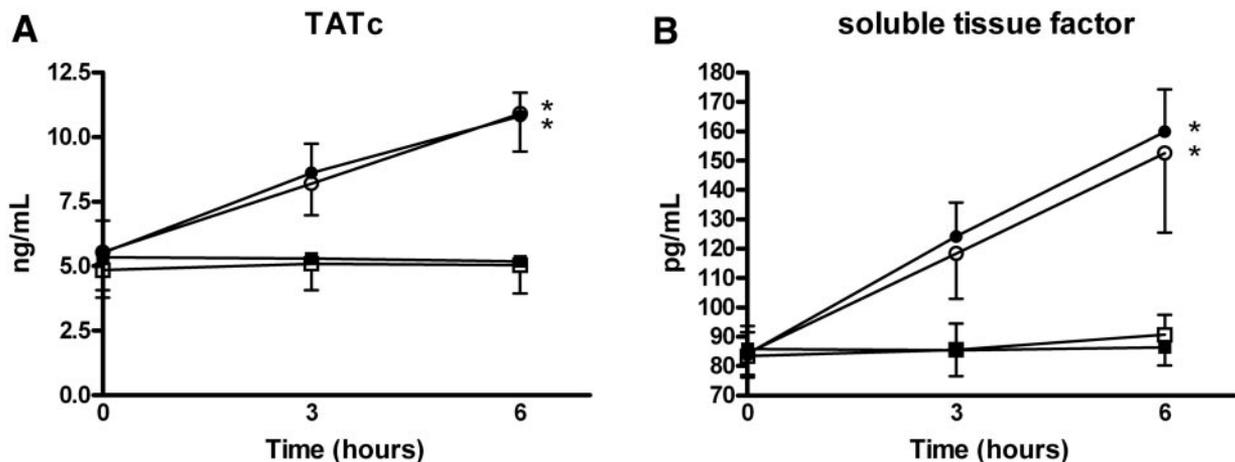**FIG. 2. Hyperglycemia activates coagulation irrespective of insulin levels. Mean ± SD plasma concentrations of TATc (*A*) and soluble tissue factor (*B*) measured in six subjects studied on four separate occasions: during an $L_{insu}E_{gluc}$ clamp (□), an $L_{insu}H_{gluc}$ clamp (○), an $H_{insu}E_{gluc}$ clamp (■), and an $H_{insu}H_{gluc}$ clamp (●). *$P < 0.001$ vs. both euglycemic clamps.**
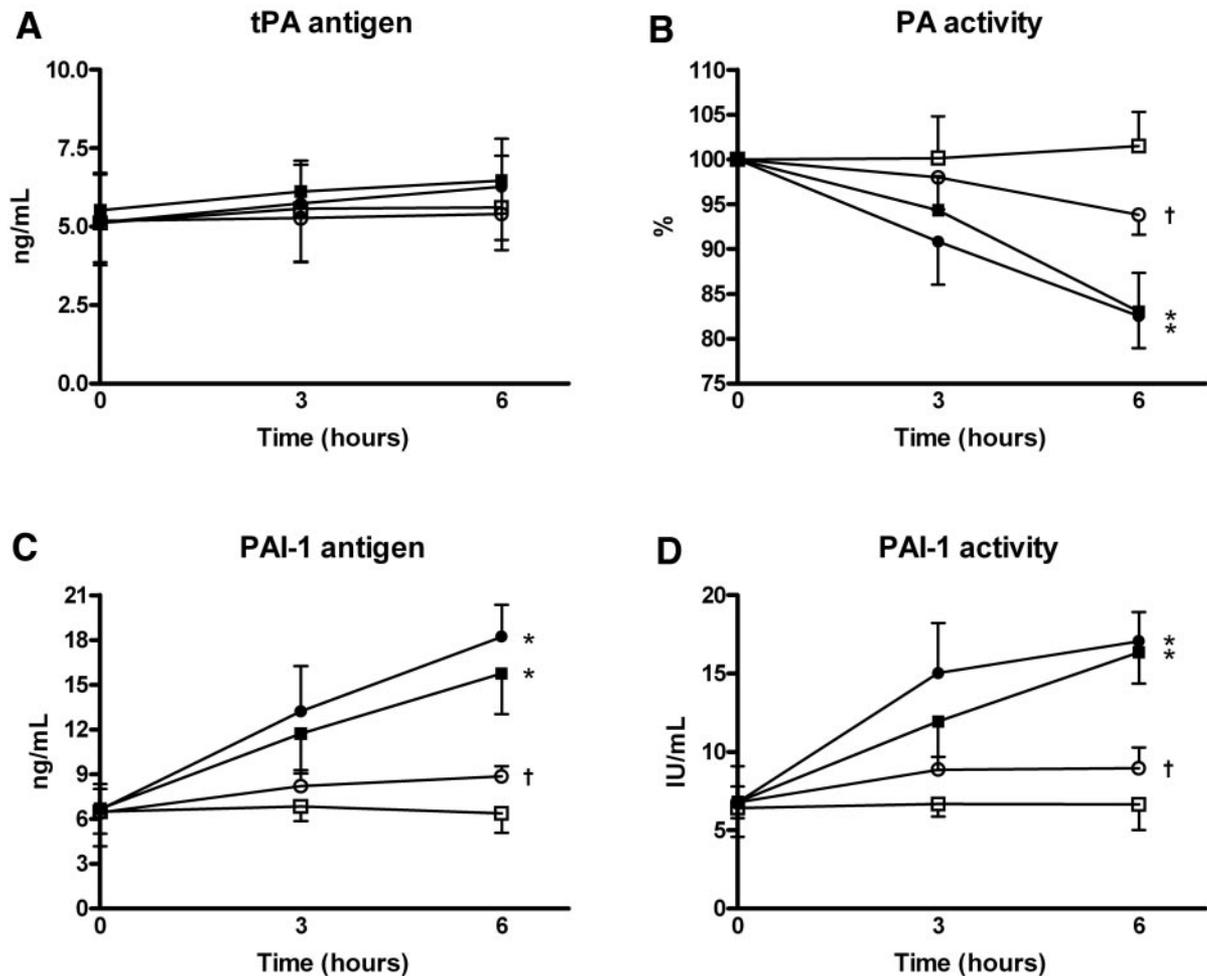
FIG. 3. Hyperinsulinemia inhibits fibrinolysis irrespective of glucose levels. Mean ± SD plasma concentrations of tPA antigen (*A*), plasminogen activator activity (PA activity) (*B*), PAI-1 antigen (*C*), and PAI-1 activity (*D*) measured in six subjects studied on four separate occasions: during an $L_{insu}E_{gluc}$ clamp (□), an $L_{insu}H_{gluc}$ clamp (○), an $H_{insu}E_{gluc}$ clamp (■), and an $H_{insu}H_{gluc}$ clamp (●). *$P < 0.01$ vs. both lower insulinemic clamps; †$P < 0.05$ vs. $L_{insu}E_{gluc}$ clamp.

rate to obtain eu- or hyperglycemia were started. Glucose (20%) was used during the $L_{insu}E_{gluc}$ clamp; in the other clamps, 10% glucose was used to prevent the possibility of phlebitis induced by the high infusion rates that were required. All infusions were administered by calibrated syringe pumps (Perfusor fm; Braun, Melsungen AG, Germany). To clamp glucose at 5 or 12 mmol/l (eu- or hyperglycemic) from $t = 0$ until $t = 6$, every 5 min, bedside plasma glucose concentration was measured on a Beckman Glucose Analyzer 2 (Beckman, Palo Alto, CA). From $t = 2:40$ until $t = 3:00$ and from $t = 5:40$ until $t = 6:00$, blood samples were drawn every 10 min for determination of the concentration of plasma insulin. In RESULTS, the mean values of these three measurements are presented. Blood for measurement of coagulation and fibrinolysis parameters was collected directly before the initiation of the infusions ($t = 0$), 3 h into the infusions ($t = 3$), and at the end of the infusions ($t = 6$) in siliconized vacutainer tubes (Becton Dickinson, Plymouth, U.K.) containing 0.105 mol/l sodium citrate in a 1:9 (vol/vol) anticoagulant-to-blood ratio.

Assays. All measurements in each individual subject were performed in the same run. Plasma insulin concentration was determined by a chemiluminescent immunometric assay (Immulite; Diagnostic Products, Los Angeles, CA). TATc, soluble tissue factor, PAI-1, and tissue-type plasminogen activator (tPA) were measured using enzyme-linked immunosorbent assays (TATc: Behringwerke, Marburg, Germany; soluble tissue factor: American Diagnostics, Greenwich, CT; PAI-1 [TintElize PAI-1]: Biopool, Umea, Sweden; and tPA [Asserachrom tPA]: Diagnostica Stago, Asnieres-sur-Seine, France). PAI-1 activity and plasminogen activator activity were measured by amidolytical assays (18,19). In brief, plasminogen activator activity is measured by incubating the sample with digested fragments of fibrin in the presence of an excess concentration of plasminogen and a synthetic plasmin substrate, which is cleaved into a chromogenic product. For PAI-1 measurements, a

standard amount of tPA is added to the sample and the products described above; and subsequently, residual tPA (the amount of which is dependent on the amount of PAI-1 activity in the sample) can now be detected by measuring the chromogenic activity. By means of a standard curve using the international standard preparation for PAI-1, this chromogenic activity is then recalculated to plasma levels (in IU/ml).

Statistical analysis. To analyze the effect of hyperinsulinemia and/or hyperglycemia, their interactions, and the effect of time, results of the four clamps were compared using a repeated-measures ANOVA. Data were checked for normal distribution and equal variances of the residuals. Depending on the results of these tests, data were analyzed either parametrically or nonparametrically. Results are presented as means ± SD. P values of <0.05 were considered statistically significant. SPSS statistical software version 12.0.1 (SPSS, Chicago, IL) was used to analyze the data.

RESULTS

Glucose and insulin. The clamps were carried out successfully (Fig. 1). In the two hyperglycemic clamps, plasma glucose levels rapidly increased during the 1st h, and from 3 h onward, the extent of hyperglycemia was virtually identical in the $L_{insu}H_{gluc}$ and $H_{insu}H_{gluc}$ clamps. At the end of the 6-h study period, plasma glucose levels were 12.2 ± 0.5 and 12.4 ± 0.1 mmol/l in the $L_{insu}H_{gluc}$ and $H_{insu}H_{gluc}$ clamps, respectively. Plasma glucose levels remained at ~5 mmol/l throughout the euglycemic clamps; at 6 h after the initiation of the study, plasma glucose

concentrations were 5.1 ± 0.1 and 5.0 ± 0.2 mmol/l in the $L_{insu}E_{gluc}$ and $H_{insu}E_{gluc}$ clamps, respectively (both $P < 0.05$ for the difference with either of the two hyperglycemic clamps). In the two hyperinsulinemic clamps, plasma insulin concentrations had reached the target levels at 3 h after the start of the infusion, which were maintained throughout the remainder of the 6-h study. At this time point, plasma insulin levels were 408 ± 61 and 443 ± 34 pmol/l in the $H_{insu}E_{gluc}$ and $H_{insu}H_{gluc}$ clamps, respectively, whereas plasma insulin concentrations were 89 ± 6 and 127 ± 19 pmol/l in the $L_{insu}E_{gluc}$ and $L_{insu}H_{gluc}$ clamps (both $P < 0.05$ for the difference with either of the two hyperinsulinemic clamps). Of note, insulin levels were modestly higher in the $L_{insu}H_{gluc}$ than in the $L_{insu}E_{gluc}$ clamp ($P < 0.05$).

Coagulation. Hyperglycemia resulted in a marked activation of coagulation, as reflected by rises in the plasma levels of the thrombin generation marker TATc and soluble tissue factor, whereas hyperinsulinemia had no effect on these parameters, as demonstrated by the nonsignificant interaction (Fig. 2). Both coagulation activation markers remained constant during the $L_{insu}E_{gluc}$ and $H_{insu}E_{gluc}$ clamps. During the $L_{insu}H_{gluc}$ and the $H_{insu}H_{gluc}$ clamps, however, TATc and soluble tissue factor levels displayed a gradual increase, peaking at the end of the 6-h hyperglycemic period (both $P < 0.05$ for the difference with either of the two euglycemic clamps). At this time point, TATc concentrations had increased 2.1-fold (relative to baseline) in both hyperglycemic clamps, to 10.9 ± 1.5 ng/ml in the $L_{insu}H_{gluc}$ clamp and 10.8 ± 0.9 ng/ml in the $H_{insu}H_{gluc}$ clamp. Plasma levels of soluble tissue factor increased 1.8-fold during the $L_{insu}H_{gluc}$ clamp (to 152.5 ± 27.0 pg/ml) and 1.9-fold during the $H_{insu}H_{gluc}$ clamp (to 159.8 ± 14.5 pg/ml). Hence, these results suggest that hyperglycemia stimulates coagulation regardless of insulin concentrations.

Fibrinolysis. Fibrinolytic activity was profoundly affected by hyperinsulinemia but not by hyperglycemia (Fig. 3). Plasma plasminogen activator activity decreased during the $H_{insu}E_{gluc}$ clamp (to $83.0 \pm 4.3\%$) and the $H_{insu}H_{gluc}$ clamp (to $82.5 \pm 3.6\%$; both $P < 0.05$ for the difference with either of the two lower insulin clamps). The insulin-induced inhibition of plasminogen activator activity was completely due to an increase in PAI-1 levels, whereas tPA antigen levels did not change during any of the four clamps. During the $H_{insu}E_{gluc}$ and the $H_{insu}H_{gluc}$ clamps PAI-1 antigen and activity levels showed a marked increase that peaked at the end of the 6-h hyperinsulinemic period (both $P < 0.05$ for the difference with either of the two lower insulin clamps). At this time point, PAI-1 antigen levels had increased to 15.8 ± 2.7 ng/ml in the $H_{insu}E_{gluc}$ clamp (a mean 2.5-fold increase relative to baseline) and to 18.2 ± 2.1 ng/ml in the $H_{insu}H_{gluc}$ clamp (a mean 2.9-fold increase); peak PAI-1 activity levels had increased to 16.4 ± 2.0 IU/ml in the $H_{insu}E_{gluc}$ clamp (a mean 2.5-fold rise) and to 17.1 ± 1.9 IU/ml in the $H_{insu}H_{gluc}$ clamp (a mean 2.8-fold rise). In contrast, during the $L_{insu}E_{gluc}$ clamp, all fibrinolytic parameters remained constant. During the $L_{insu}H_{gluc}$ clamp, both PAI-1 antigen and PAI-1 activity demonstrated a modest increase when compared with the $L_{insu}E_{gluc}$ clamp, peaking at 8.9 ± 0.7 ng/ml and 9.0 ± 1.3 IU/ml, respectively (corresponding with a mean 1.5- and 1.4-fold rise relative to baseline, respectively), which was accompanied by a slight decrease in plasma plasminogen activator activity, reaching a nadir of $93.8 \pm 2.2\%$ (all $P < 0.05$ vs. the $L_{insu}E_{gluc}$ clamp). Hence,

these data suggest that hyperinsulinemia inhibits fibrinolysis by stimulating PAI-1 secretion regardless of glucose concentrations.

DISCUSSION

Impaired fibrinolysis and increased coagulation have been implicated in the pathogenesis of CVD in type 2 diabetes and insulin resistance syndromes (2,3,6). We here demonstrate in healthy subjects that hyperglycemia and hyperinsulinemia exert differential effects on the coagulation and the fibrinolytic systems, respectively. Moreover, via its design, this study is the first to test the interaction between hyperglycemia and hyperinsulinemia within a subject. The main finding of our study is that hyperglycemia selectively stimulates coagulation irrespective of insulin levels, whereas hyperinsulinemia inhibits fibrinolysis (primarily by enhancing PAI-1 secretion) irrespective of glucose concentrations. Hence, the presence of both hyperglycemia and hyperinsulinemia (such as in type 2 diabetic patients) has a strong procoagulant effect by enhancement of coagulation and simultaneous inhibition of fibrinolysis.

Our results indicate that a 1.5- to 2-fold increase in levels of PAI-1 inhibits endogenous fibrinolysis. This is a consistent finding in comparable studies on this subject (20,21).

Several investigations studied the influence of hyperinsulinemia on the fibrinolytic system. Although in rabbits, infusion of insulin increased PAI-1 activity (15), exogenous insulin administration under euglycemic and hyperglycemic conditions did not influence plasma PAI-1 activity in investigations in human subjects (12–14). Although in these human studies, plasma insulin concentrations were raised to ~575–650 pmol/l, these levels were maintained rather briefly, considering that insulin was infused for only 1–3 h; our investigation clearly shows that alterations in PAI-1 levels occur in a time-dependent way, with the strongest change recorded at the end of the 6-h observation period. In addition, in none of the previous human studies (12–14) was somatostatin used to suppress endogenous glucagon secretion. In the present study, insulin concentrations ~400 pmol/l clearly enhanced PAI-1 antigen and activity levels, and this was associated with a diminished plasminogen activator activity. Of note, during the $L_{insu}H_{gluc}$ clamp, insulin release was not completely prevented, as indicated by the modestly higher insulin levels during this study period when compared with the $L_{insu}E_{gluc}$ clamp. Interestingly, even these slightly elevated insulin levels elicited modest increases in PAI-1 antigen and activity together with a decline in plasminogen activator activity, suggesting that under the tightly controlled conditions of the current study, wherein every subject served as his own control, the effect of circulating insulin on plasma fibrinolytic activity was dose dependent. The production of tPA was not reduced by hyperinsulinemia, indicating that the increased synthesis and release of PAI-1 was responsible for the impaired fibrinolytic response. These insulin effects are in line with laboratory studies demonstrating that both insulin and proinsulin can augment PAI-1 expression in endothelial cells (22), vascular smooth muscle cells (23), and hepatocytes (24) in vitro. In addition, even in subjects with normal glucose tolerance, elevated levels of fasting insulin are associated with increased circulating PAI-1 levels (6), providing further evidence for a link between hyperinsulinemia and impaired fibrinolysis. Of considerable interest, atherosclerotic plaques of type 2 diabetic patients demonstrated

enhanced PAI-1 protein levels compared with plaques from nondiabetic subjects (25), suggesting that in patients, circulating PAI-1 may partially reflect an attenuated intramural fibrinolytic system within arteries. In our experiments, hyperinsulinemia influenced fibrinolysis independently from plasma glucose levels. Elevated insulin levels did not affect coagulation parameters. Hence, our data strongly implicate hyperinsulinemia (and not hyperglycemia) in impairment of fibrinolysis associated with type 2 diabetes and insulin resistance syndromes.

Knowledge of the selective effect of hyperglycemia on hemostasis is quite limited and, in all instances, derived from studies in which endogenous insulin secretion (occurring in response to artificially elevation of plasma glucose levels) was not controlled. In one study, plasma levels of the prothrombin fragment F1 + 2, indicative for thrombin generation, increased during an oral glucose tolerance test in both diabetic and healthy subjects (26). Prolonged hyperglycemia (12 mmol/l during 18–72 h) induced by intravenous infusion of glucose resulted in activation of the tissue factor pathway of coagulation without evidence of enhanced thrombin generation; insulin levels were highly variable in that study (17). In the present investigation, endogenous insulin secretion was inhibited by somatostatin (27), allowing assessment of the specific effect of hyperglycemia on coagulation and fibrinolysis. Our data clearly show that hyperglycemia causes a time-dependent activation of coagulation, here measured as the plasma concentrations of TATc and soluble tissue factor. These alterations were independent of insulin levels, strongly suggesting that glucose, not insulin, triggers coagulation. The mechanisms by which hyperglycemia affects thrombin generation remain to be established. Tissue factor is the main initiator of the coagulation system (28). Prolonged exposure (10–12 days) to high glucose levels (30 mmol/l) of human vascular endothelial cells did not alter tissue factor mRNA or protein expression (29). The previous observation that glycated albumin causes blood monocytes to produce tissue factor mRNA suggests that glucose may indirectly influence tissue factor function (30). Although the precise role of soluble tissue factor is not known, the presence of increased levels of this marker has been shown to correlate well with increased cellular tissue factor expression in various conditions (31).

Recently, lowering blood glucose levels by intensive insulin therapy in patients admitted to a surgical intensive care unit was found to reduce mortality by 34% when compared with patients who were conventionally treated; this reduction was primarily due to a decreased mortality caused by multiple organ failure with a proven infectious focus (32). In patients treated with insulin, blood glucose levels were maintained between 4.4 and 6.1 mmol/l, whereas in the control group, glucose levels were >10 mmol/l. Maintaining normoglycemia with intensive insulin therapy was found to protect the vascular endothelium (33); the effect of intensive insulin therapy on coagulation and fibrinolysis has not been reported thus far. In light of our current findings and the accepted role of coagulation activation in the pathogenesis of critical illness and sepsis (28,34), it would be of considerable interest to determine the effect of reducing glucose levels in the critically ill on activation of the coagulation system.

Our study is the first to provide firm evidence for the distinct effect of elevated glucose and insulin levels on coagulation and fibrinolysis. It should be noted that it is only feasible to investigate the short-term effects of hyperglycemia and/or hyperinsulinemia (here up to 6 h) in healthy humans in a tightly controlled design such as implemented here; obviously, type 2 diabetes and insulin resistance syndromes are more chronic. Nonetheless, the present data strongly suggest that hyperglycemia due to insulin resistance renders the patient more susceptible to thrombotic events by an insulin-driven impairment of fibrinolysis and a glucose-driven activation of coagulation.

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