Insulin Acutely Increases Fibrinogen Production in Individuals With Type 2 Diabetes but Not in Individuals Without Diabetes

Rocco Barazzoni, Edward Kiwanuka, Michela Zanetti, Michela Cristini, Monica Vettore, and Paolo Tessari

Fibrinogen is an acute-phase reactant and an independent cardiovascular risk factor. Insulin without amino acid replacement acutely suppressed fibrinogen production in non-diabetic and type 1 diabetic individuals. Fibrinogen production and plasma concentration increase in insulin-resistant type 2 diabetes. It is not known whether altered response to insulin contributes to hyperfibrinogenemia in type 2 diabetes. Fibrinogen fractional (FSR) and absolute (ASR) synthesis rates were measured using a leucine isotopic model in type 2 diabetic men (n = 7; age = 51 ± 3 years; BMI = 26.7 ± 1 kg/m²) compared with matched nondiabetic subjects under basal conditions and following a 4-h euglycemic-, euaminoacidemic-hyperinsulinemic clamp. Basal fibrinogen concentration (+35%, P < 0.05) and ASR (+35%, P < 0.05) were greater in the diabetic subjects. Following clamp, fibrinogen FSR and ASR were unchanged in the control subjects. In contrast, fibrinogen FSR and ASR increased by 41 and 43%, respectively (P < 0.05), in the diabetic subjects. Thus, fibrinogen production is acutely increased by insulin when euglycemia and euaminoacidemia are maintained in type 2 diabetic individuals but not in nondiabetic individuals. Enhanced fibrinogen production by insulin is likely to be a key alteration contributing to hyperfibrinogenemia and therefore cardiovascular risk in type 2 diabetes. Unchanged fibrinogen production in nondiabetic individuals suggests a role of plasma amino acids in regulating fibrinogen production in humans. Diabetes 52:1851–1856, 2003

RESEARCH DESIGN AND METHODS

Subjects. Seven male type 2 diabetic patients and eight male age- and weight-matched nondiabetic control subjects were studied (Table 1). Presence or absence of diabetes was defined according to American Diabetes Association diagnostic criteria. None of the study participants were severely

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ASR, absolute synthesis rate; D-leu, L-[5,5,5-2H]-leucine; FSR, fractional synthesis rate; H-leu, L-[4,5-3H]leucine; KIC, ketoisocapraote; MPE, molar percent enrichment.

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Clinical and anthropometric characteristics of the subjects studied

<table>
<thead>
<tr>
<th>Type 2 diabetic</th>
<th>Nondiabetic</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.7 ± 1</td>
</tr>
<tr>
<td>Diabetes (years)</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>&lt;5.7</td>
</tr>
<tr>
<td>Microalbuminuria (µg/min)</td>
<td>15.5 ± 4</td>
</tr>
<tr>
<td>Fibrinogen concentration (µg/dl)</td>
<td>344 ± 42*</td>
</tr>
<tr>
<td>Fibrinogen pool (g)</td>
<td>10.8 ± 1.3*</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.89 ± 0.32*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.60 ± 0.36</td>
</tr>
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</table>

*P < 0.05 vs. nondiabetic subjects.

Obese (BMI <30 kg/m²); ranges of BMI were 23.6–29.8 kg/m² (type 2 diabetic) and 22.0–29.2 kg/m² (nondiabetic). Diabetic patients were selected according to strict noninvasive criteria to exclude the presence of clinical vascular disease and renal as well as other diabetic complications with potential independent effects on fibrinogen concentration and turnover (11). Medical history and clinical examination revealed no signs of vascular or kidney disease. Electrocardiogram and carotid artery ultrasound were normal in all diabetic patients, except for one showing 30% carotid stenosis. Retinal examination performed by an expert oculist following standard procedures was negative for diabetic retinopathy, and urinary albumin excretion rate was 0.57. No participants had clinical or biochemical (i.e., erythrocyte sedimentation rate and leukocyte count) signs of concurrent inflammatory conditions that might directly affect fibrinogen metabolism.

Five patients were treated with oral hypoglycemic agents (two with metformin, two with glibenclamide, and one with glipizide), and two with diet. Two diabetic and two nondiabetic subjects also had mild essential hypertension; all hypertensive subjects were treated with ACE-inhibitors, and one of the diabetic patients was also treated with a diuretic drug. No other drugs had been taken by any of the participants for at least 3 months before the study.

The mean duration of diabetes was 8 years, and metabolic control at the time of the study was poor, as reflected by HbA₁c (Table 1).

Experimental design. The experimental protocol was approved by the Ethical Committee of the University of Padova, and it complied with the Helsinki Declaration. The study was carried out according to the recommendations of the Radiation Safety Officer. Each subject provided a written consent after the aims and potential risks of the study were explained in detail. Antidiabetic medications were withdrawn 24 h before the study, and no drug was administered to any subject on the morning of the study. All subjects were admitted to the Metabolic Unit of the Department of Metabolic Diseases at 7:00 A.M. on the study day, at their spontaneous glycemic levels under postabsorptive conditions after an overnight fast. An 18-gauge polyethylene catheter was placed in an antecubital vein of the right arm for insulin, insulin, and amino acid infusion. A contralateral wrist vein was then cannulated in a retrograde fashion, and the hand was placed in a box heated to 55°C for venous-arterialized blood sampling throughout the study, which was divided into two experimental periods as follows.

Basal period. After baseline blood samples were taken for determination of natural isotopic enrichments, a primed (60-fold the constant infusion rate per h) continuous infusion of $L^-[4,5-3H]$leucine ($L^-[4,5-3H]leu; 0.5 \times 10^9$ dpm $\cdot$ m⁻¹ $\cdot$ h⁻¹) (Amersham, Buckinghamshire, U.K.) or $L^-[5,5,5-2H]$-leucine ($D^3$-leu; 0.8 µmol $\cdot$ kg⁻¹ $\cdot$ h⁻¹) (Mass Trace, Woburn, MA) was started at 7:30 A.M. (t = −240 min). $L^-[4,5-3H]leu$ was infused in four diabetic and four nondiabetic subjects, while the remaining subjects received the $D^3$-leu isotope. The use of two leucine isotopes was due to a change of regulations forbidding the use of radioactive isotopes at our institution after the first studies with $L^-[4,5-3H]leu$. Data from all subjects in each group were pooled, since results in both subgroups were superimposable (data not shown). Venous-arterialized blood samples were drawn every 30 min for 180 min, then every 15 min from time −60 min to 0 min for measurement of plasma substrates, hormones and isotopic concentrations, and enrichment specific activities, as well as for fibrinogen isolation.

Clamp period. At t = 0 min, a euglycemic, euinaoacidemic hyperinsulinemic clamp was started. Insulin was diluted in saline solution with the addition of 2 ml of each subject's own blood and infused at a rate of 0.05 units $\cdot$ m⁻² $\cdot$ h⁻¹. Plasma glucose concentration was monitored every 10 min thereafter (Beckman Glucose Analyzer 2, Beckman Instruments, Fullerton, CA). After stabilization of plasma glucose concentration, a glucose infusion aimed at maintaining plasma glucose concentration at baseline levels in the nondiabetic control subjects and at a comparable level in type 2 diabetic subjects. A balanced l-amino acid solution (Isopuramin 10%, Bieffe Medical, Sondrio, Italy) was infused at a constant rate to maintain plasma amino acid concentrations at near-basal levels and prevent hypoaminoacidemia during the clamp. The rate of amino acid infusion was chosen based on preliminary studies as well as on data from the literature, and it was the same in the two groups (type 2 diabetic, 0.90 ± 0.11 ml/min; nondiabetic, 0.89 ± 0.15). The composition of the amino acid solution was as follows: alanine 7.0 g/l; arginine 11.70 g/l; lysine 11.50 g/l; phenylalanine 7.90 g/l; tyrosine 0.30 g/l; histidine 5.10 g/l; threonine 9.50 g/l; tryptophan 3.20 g/l; methionine 7.20 g/l; glycine 6.04 g/l; leucine 10.60 g/l; isoleucine 8.00 g/l; valine 11.60 g/l. Blood samples were again drawn every 30 min for 150 min, then every 15 min in the last 90 min of the clamp period (i.e., between 150 and 240 min) for the new steady-state measurements and kinetic calculations. Total blood drawn throughout the study was ~160 ml, excluding that drawn for blood glucose determinations, and the blood draw was well tolerated by all study subjects.

Analytical methods. Fibrinogen was isolated from plasma as previously described (11,21,22). In short, 100 µl of a 1 mol/l CaCl₂ solution and 10 IU thrombin in 100 µl deionized water were added to 2 ml plasma to activate the reactions leading to the formation of a fibrin clot. After 1-h incubation at room temperature, the samples were centrifuged, and the clot was removed from the sample, gently washed on filtered paper with deionized water, and lyophilized for 48 h at 114 N HCl. The fibrinogen-derived free amino acids were then purified through cation exchange AG 50X8 columns (11,21,22). $D^3$-leu specific activity in hydrolyzed fibrinogen samples as well as plasma $L^-[4,5-3H]$leucine (KIC) specific activity were measured by HPLC as previously described (20). $D^3$-leu enrichment in fibrinogen-derived leucine, as well as plasma $D^3$-leu and KIC enrichment, was measured by gas chromatography-mass spectrometry as tert-butyldimethyl-silyl derivatives (22) using a Hewlett-Packard 5988 MS, capillary column, and electron impact ionization. Plasma fibrinogen concentration was determined by nephelometric assay (11). Plasma amino acid concentrations were determined from samples taken at times 0, 0.5, 1, 2, and 4 h after clamp initiation using a Beckman Amino Acid Analyzer. Plasma concentrations of insulin and glucagon were determined by radioimmunoassay as previously described (21).

Calculations. Fibrinogen fractional synthesis/secretion rate (FSR, expressed as percentage of pool per day) was calculated in the last hour of the basal and in the last 90 min of the clamp periods under steady-state conditions for the plasma precursor pool specific activity or mole percent enrichment (MPE, i.e., $D^3$-leu/$L^-[5,5,5-2H]$leucine; KIC specific activity), plasma precursor pool, fibrinogen synthesis (20,21,23,24) and it has been recently confirmed as a reliable surrogate for the true precursor pool of hepatic protein synthesis, i.e., leucyl-tRNA (25). At least five time points were used for the regression analysis of the changes in fibrinogen-bound leucine specific activity versus time in each subject.

The following standard equation (21) was used:

$$\text{FSR} = \frac{\text{SA(MPE)}_{t_2} - \text{SA(MPE)}_{t_1}}{\text{SA(MPE)}_{t_{\text{recurrent}}}} \times 1,440 \times 100$$

where $\text{SA(MPE)}_{t_2}$ and $\text{SA(MPE)}_{t_1}$ are fibrinogen-bound leucine specific activities (disintegrations per minute per nanomole) or MPEs at time points $t_2$ and $t_1$, respectively; the ratio of these differences represents the slope of their linear regression analysis (11). $\text{SA(MPE)}_{t_{\text{recurrent}}}$ is plasma $L^-[4,5-3H]$KIC specific activity or MPE at steady state, and a factor of 1.4 is used to express synthesis rates per day (i.e., 24 h, or 1,440 min). Fibrinogen absolute synthesis rate (ASR; expressed in grams per day) was calculated by multiplying the FSR by the plasma (i.e., intravascular) fibrinogen pool. The intravascular fibrinogen pool was estimated by multiplying plasma fibrinogen concentration (milligrams per liter) by plasma volume (liters). Plasma volume was determined from the distribution of 125iodine (infraclot; SERB, Paris, France) measured in the basal period (26). Leucyl-tRNA intracellular rate of appearance, reflecting whole-body protein turnover, was calculated by dividing the isotope rate of infusion by the steady-state plasma KIC specific activity or MPEs in the last hour of each experimental period (27). Endogenous leucine rate of appearance during the clamp period was calculated by subtracting the exogenous leucine infusion rate from the fibrinogen-specific activity.

Statistical analysis. Single sets of data (mean ± SE) within each experimental period from the two groups were compared by the two-tailed Student’s t test for unequal data. Insulin effects, i.e., changes from basal to clamp, were
TABLE 2
Plasma glucose, insulin, and glucagon concentrations and exogenous intravenous glucose infusion rate to maintain euglycemia in the last 90 min of the clamp period (M)

<table>
<thead>
<tr>
<th></th>
<th>Basal Type 2 diabetic</th>
<th>Basal Nondiabetic</th>
<th>Clamp Type 2 diabetic</th>
<th>Clamp Nondiabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>9.3 ± 0.6*</td>
<td>4.7 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>13 ± 1.4*</td>
<td>8.8 ± 1.5</td>
<td>116 ± 10</td>
<td>122 ± 8</td>
</tr>
<tr>
<td>Glucagon (ng/ml)</td>
<td>121 ± 14</td>
<td>101 ± 12</td>
<td>141 ± 19</td>
<td>112 ± 20</td>
</tr>
<tr>
<td>M (ng · kg⁻¹ · min⁻¹)</td>
<td>—</td>
<td>—</td>
<td>3.6 ± 0.6*</td>
<td>8.6 ± 1.4</td>
</tr>
</tbody>
</table>

*P < 0.05 or less vs. nondiabetic subjects.

RESULTS
Plasma substrates and hormone concentrations and insulin sensitivity. Basal plasma glucose, insulin (Table 2), and triglyceride (Table 1) concentrations were greater (P < 0.05 or less) in type 2 diabetic than in nondiabetic subjects, as expected. Plasma glucagon (Table 2) and total cholesterol (Table 1) concentrations were comparable in the two groups. Following the clamp, insulin was increased to similar steady-state values in type 2 diabetic and control subjects (Table 2). Plasma glucose concentrations decreased to normal values in the subjects in the last 90 min of the clamp period (data not shown) and were then maintained at near-euglycemia by the exogenous glucose infusion for at least the final 120 min of the clamp (Table 2). The exogenous glucose infusion rate to maintain euglycemia (M value, index of insulin-sensitivity) was substantially lower in type 2 diabetic than in control subjects, confirming a marked insulin-resistance (Table 2).

Plasma amino acid concentrations, isotopic enrichments, and whole-body leucine turnover. Plasma leucine and total amino acid concentrations were maintained at near-basal values during the clamp in both type 2 diabetic and control subjects by the exogenous amino acid infusion (Table 3). The slope of changes over time in the ratio between labelled-to-unlabelled leucine bound to fibrinogen, reflecting rate of incorporation into newly synthesized fibrinogen molecules, increased by 61% over basal values during the clamp in the type 2 diabetic group, whereas it was unchanged in the nondiabetic group (P = 0.033, diabetic vs. control group) (Table 3). Steady state in the specific activity or MPE ratio in plasma precursor pool KIC was observed in the two groups in both experimental periods (Fig. 1). In the basal period, leucine Rₐ was comparable in the type 2 diabetic and nondiabetic subjects. During the clamp, total leucine Rₐ was maintained at near-basal values in both groups. Endogenous leucine Rₐ (index of endogenous proteolysis during the euaminoacidic clamp) was suppressed to the same extent in the diabetic and nondiabetic groups (NS) (Table 3).

Fibrinogen concentration and turnover. Plasma fibrinogen concentration and pool (+42%) were higher in diabetic than in control subjects (Table 1). Basal fibrinogen FSR in the diabetic patients was comparable to control values (type 2 diabetic group, 24.5 ± 3.5; control group, 21.1 ± 2.1% of pool per day; NS) (Fig. 2) while ASR was greater than in control subjects (2.45 ± 0.25 vs. 1.8 ± 0.2 g/day, P < 0.03) (Fig. 3). Following the euglycemic, euaminoacidic insulin clamp, FSR did not change in the control subjects, but it increased by 41% in the patients (P < 0.04) (Fig. 2). Similarly, ASR did not change compared with basal in the control subjects, but it increased by 43% in the diabetic subjects (P < 0.05 vs. basal and versus changes in C) (Fig. 3). Changes in fibrinogen FSR during the clamp were inversely correlated with insulin-induced glucose utilization (M), an index of insulin sensitivity (r = −0.56, P < 0.05).

DISCUSSION
The current study demonstrates substantial differences in the effect of insulin on fibrinogen production in type 2 diabetic and nondiabetic subjects. During maintenance of basal plasma glucose and amino acid concentrations, physiological hyperinsulinemia markedly increased fibrinogen production in type 2 diabetic but not in nondiabetic control subjects. We recently showed increased postabsorptive fibrinogen production in hyperglycemic type 2
diabetic patients without vascular complications (11), a finding confirmed by the current study. The current data further indicate that the increased fibrinogen production is associated with altered insulin regulation of fibrinogen production in this disease. Notably, increased plasma insulin and normal amino acid concentrations are commonly observed in type 2 diabetic individuals both without and during insulin treatment (28,29). These observations suggest that increased insulin concentration may contribute to the increased plasma fibrinogen concentration often observed in type 2 diabetes.

Insulin-induced stimulation of fibrinogen production in type 2 diabetic subjects is remarkably consistent with a previous in vitro report of stimulated fibrinogen production following prolonged hepatocyte exposure to insulin (30). The referenced study (30) strongly supports a potential role of chronic hyperinsulinemia in postabsorptive fibrinogen overproduction in type 2 diabetes. Chronic activation of the fibrinogen synthetic pathway (10) may in turn result in differential responses to further acute increments of insulin concentration, as was indeed observed in the current study. Insulin resistance may underlie, at least in part, the observed alterations of fibrinogen production, as indirectly suggested by the inverse correlation between insulin-stimulated glucose utilization and changes in fibrinogen FSR during the clamp. Evidence is emerging for an association between insulin resistance and subclinical inflammatory response (8–10), and high plasma levels of inflammation mediators with potential to stimulate acute-phase response have been shown in insulin-resistant states and type 2 diabetes (8–10). Insulin has been reported to have differential effects on acute-phase reactant secretion in vitro, with no effect on some and reduction of others, not including fibrinogen (14). Interestingly, acute increments of plasma concentrations of proinflammatory cytokines following euglycemic insulin infusion were recently demonstrated in insulin-resistant critically ill patients (31), providing support for an additional stimulatory effect of
insulin on acute-phase response under conditions of pre-existing inflammatory response, as is usually observed in critical illness. The current data indicating a role of insulin in fibrinogen overproduction in vivo in type 2 diabetic subjects suggest links among insulin resistance, hyperinsulinemia, and altered acute-phase reactant production in type 2 diabetes.

Increased fibrinogen production in type 2 diabetic subjects occurred during insulin-induced near-normalization of plasma glucose concentration. While the effects of acute changes in plasma glucose per se remain to be defined, previous studies in the general as well as in smaller populations have reported positive correlations between plasma fibrinogen and glucose concentrations (3, 10). It is therefore possible that spontaneous hyperglycemia contributed to fibrinogen synthesis under basal conditions, but it appears unlikely that acute reduction of plasma glucose concentration played a role in the stimulation of fibrinogen synthesis associated with insulin infusion. The effects of long- or mid-term improvement of glycemic control on plasma fibrinogen in type 2 diabetic individuals yielded conflicting results (32–34). Available literature suggests that the effect of blood glucose control on plasma fibrinogen and other acute-phase reactants may be related to choice of hypoglycemic treatment and resulting plasma insulin levels. Treatment with insulin or sulfonylureas that stimulate insulin secretion leads to no substantial change in fibrinogen and other acute-phase reactants, despite reduced HbA1c (32). Interestingly, and consistent with current findings, intensive insulin treatment in the first year was associated with increased plasma fibrinogen concentration when compared with conventional insulin therapy (33). On the other hand, treatment with the insulin-sensitizer troglitazone for 4 months reduced insulin requirement and presumably concentrations, resulting in reduced plasma concentrations of some acute-phase reactants (34). In agreement with the current data, these studies support a contribution of insulin resistance and increased insulin levels to high fibrinogen and acute-phase reactant levels even during improved glycemic control in type 2 diabetes.

Previous studies have suggested an inhibitory effect of insulin on fibrinogen production in nondiabetic (13) and type 1 diabetic individuals (12). In both reports, however, insulin infusion was associated with acute reductions of plasma amino acid concentrations. Insulin treatment of insulin-deficient type 1 diabetic patients normalized increased plasma concentrations of essential amino acids (12), while insulin infusion in healthy people reduced circulating amino acid levels, as expected (13). The current study shows that insulin does not inhibit basal fibrinogen production in healthy humans when plasma amino acids are maintained at near-basal concentrations by exogenous intravenous infusion, thus suggesting a role of amino acids in maintenance of postabsorptive fibrinogen production in nondiabetic humans. The stimulatory role of amino acids in regulating liver protein synthesis in vitro has been previously demonstrated (17–19). An important role of circulating amino acid levels in maintaining splanchnic (i.e., liver and gut) protein synthesis in humans is strongly supported by the decline of splanchnic protein synthesis during insulin infusion with plasma amino acid fall in both type 1 (35) and nondiabetic (36) subjects.

In agreement with previous data (11, 28), there were no differences between the two study groups in whole-body protein turnover in either experimental period. The suppression of whole-body protein breakdown following insulin and amino acid infusions was also comparable in type 2 diabetic and nondiabetic subjects. The profound differences in fibrinogen production both under basal conditions and following insulin infusion indicate the need to investigate specific protein turnover rates whose changes might be masked by opposite changes occurring in different protein pools during whole-body measurements.

In conclusion, the current paper shows that acute physiological plasma insulin increments with maintained plasma glucose and amino acid concentrations have differential effects on fibrinogen production in type 2 diabetic and nondiabetic individuals. The sharp stimulation of fibrinogen production in type 2 diabetic individuals represents a novel alteration likely to substantially contribute to increased fibrinogen concentrations in this disease. Unchanged fibrinogen production in nondiabetic individuals following euglycemic hyperinsulinemia suggests a role of plasma amino acid concentration in the regulation of fibrinogen production in humans.

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