No Influence of Proinsulin and Insulin on Plasma Levels of Plasminogen Activator Inhibitor Type 1 and Tissue Plasminogen Activator in Young Women Before and During Intake of Contraceptive Steroids

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Clinical observations in patients predisposed to cardiovascular disorders and recent experimental observations suggest that proinsulin and insulin participate in the regulation of fibrinolysis in vivo. In the present study, we examined if proinsulin and insulin affect the constitutive (fasting) secretion of plasminogen activator inhibitor type 1 (PAI-1) and tissue plasminogen activator (t-PA) in young healthy women (N = 17). We also measured the antigen concentrations of PAI-1 and t-PA during slow and fast changes in proinsulin and insulin levels induced by oral (OGTT) and intravenous (IVGTT) glucose tolerance tests. The assessments were performed before and after 6 months of treatment with contraceptive steroids, which have a well-defined influence on the fibrinolytic variables. We observed no consistent correlations between fasting values of proinsulin, insulin, PAI-1, and t-PA either before or during hormonal treatment. Before hormonal treatment, PAI-1 and t-PA antigen levels decreased ($P < .05$) during the hyperproinsulinemia and hyperinsulinemia induced by the OGTT and IVGTT. After hormonal intake for 6 months, a decrease only in t-PA concentrations during the OGTT was observed despite similar proinsulin and insulin responses to the glucose loads. Our findings suggest that proinsulin and insulin have no influence on the regulation of plasma levels of PAI-1 and t-PA in young healthy women, irrespective of intake of contraceptive steroids.

PATIENTS WITH elevated plasma insulin levels measured with conventional radioimmunoassay (immunoreactive insulin) have an increased incidence of clinical manifestations of premature atherosclerosis resulting from plaque formation and subsequent thrombosis.[1-4] Fibrin participates not only in the formation of thrombi but also in the growth of the atherosclerotic plaque by the binding and accumulation of low-density lipoprotein and stimulation of cell proliferation.[3,5] Removal of fibrin is therefore essential for the prevention of atherothrombosis, and impaired fibrinolysis has been reported to play a prominent role in the pathogenesis of atherosclerotic coronary disease.[6-9] The systemic hypofibrinolysis observed in such patients primarily seems to be a result of increased plasma levels of plasminogen activator inhibitor type 1 (PAI-1), the main physiological inhibitor of tissue plasminogen activator (t-PA).

Plasma levels of insulin seem to be positively correlated with PAI-1, and the high atherosclerotic morbidity in patients with increased levels of immunoreactive insulin may therefore be caused by a PAI-1–mediated decrease in fibrinolytic activity.[10-12] However, recent observations from cell cultures and animal models have indicated that not only insulin, but also its precursor molecules, ie, proinsulin, may be involved in the regulation of PAI-1 levels.[13-15] It is not known if proinsulin is involved in the regulation of PAI-1 levels in man, but observations in survivors of myocardial infarction and in patients with non–insulin-dependent diabetes mellitus (NIDDM) support the possibility.[16,17] However, the dynamic interaction between proinsulin, insulin, and fibrinolytic variables described in populations prone to atherosclerotic morbidity[10-12,16,17] may not be extended to normal individuals.

In a group of young healthy women, we studied the influence of proinsulin and insulin on the constitutive (fasting) secretion of PAI-1 and t-PA. To evaluate acute changes in the mass concentrations (antigen) of PAI-1 and t-PA induced by slow and fast changes in proinsulin and insulin levels, the women were additionally subjected to oral (OGTT) and intravenous (IVGTT) glucose tolerance tests. The assessments were performed before and during intake of contraceptive steroids, which have a well-defined effect on plasma levels of PAI-1 and t-PA and also may influence the metabolism of insulin.[18,19]

SUBJECTS AND METHODS

Study Population

Seventeen young (median age, 23 years; range, 21 to 26) non-obese women without a family history of abnormalities in carbohydrate metabolism or evidence of actual or past thromboembolic or hepatic disease were recruited to the study. All participants had regular menstrual cycles (interval, 26 to 30 days), and none were lactating or had been pregnant during the past 3 months; in cases of previous intake of oral contraceptives, a washout period of three menstrual cycles was required. Women with a constant daily consumption of less than 10 cigarettes were included, due to the paired study design and because light smoking does not seem to influence the t-PA/PAI-1 system or plasma levels of insulin in young healthy women.[20,21] The participants were treated with a triphasic combination of ethinyl estradiol (EE) and norgestimate (NGT) for six consecutive cycles (day 1 to 7, 35 μg EE + 180 μg NGT; day 8 to 14, 35 μg EE + 215 μg NGT; day 15 to 21, 35 μg EE + 250 μg NGT). Clinical and metabolic evaluations were performed within the last 10 days of the cycle preceding hormone intake and during the last 7 days of the sixth treatment period.

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Blood Collection and Analysis

Endogenous secretion of proinsulin and insulin was stimulated by OGTT and IVGTT. After 12 hours of fasting and abstinence from smoking, a 3-hour OGTT with 75 g glucose was performed in the morning between 9 and 12 AM after at least 20 minutes of supine rest. Plasma concentrations of proinsulin, insulin, PAI-1 antigen, and t-PA antigen were measured 15 minutes before (fasting values) and 40, 60, 90, and 180 minutes after the glucose load.

Two days later, an IVGTT with glucose 300 mg/kg body weight was performed under similar circumstances; this test included injection of 300 mg tolbutamide (Orinase Diagnostic; Upjohn, Kalamazoo, MI) 20 minutes after the glucose injection.[22] Concentrations of proinsulin, insulin, PAI-1 antigen, and t-PA antigen were determined 15 minutes before (fasting values) and 5, 25, 40, and 180 minutes after the glucose injection.

Blood samples were drawn from an antecubital vein through an indwelling plastic catheter (Venflon 2, 17-gauge; Vigo, Helsingborg, Sweden) without the use of a tourniquet. The catheter was flushed with 5 mL isotonic saline before each sampling, and the first 2 mL blood was discarded. The samples were collected in precooled bathed tubes and centrifuged at 4°C and 2,000 × *g* for 20 minutes. The separated plasma was pipetted into plastic vials and then rapidly frozen within 2 hours and stored at −80°C.

Blood samples for determination of plasma concentrations of proinsulin and insulin were drawn in 5-mL glass tubes containing 125 μL 0.15-mol/L EDTA. The proinsulin concentration (reference interval, 4 to 12 pmol/L) was determined by a previously described enzyme-linked immunosorbent assay (ELISA) method based on monoclonal murine antibodies.[23] The detection limit of the assay is 0.25 pmol/L in serum and plasma, and there is no cross-reactivity of C-peptide or insulin at concentrations of 10,000 and 5,000 pmol/L, respectively. The assay measured total proinsulin, ie, intact proinsulin and the four major conversion mediates.

Insulin (reference interval, 9 to 94 pmol/L) level was measured with an ELISA method with a detection limit of 3.0 pmol/L; murine monoclonal antibodies (HUI018 as catching antibody and OXI005 as detecting antibody) without cross-reactivity with intact proinsulin, split 32-33-, or des(31,32)proinsulin were used.[24] Split 65-66- and des(64,65) cross-reacted 44% and 66%, respectively; however, these components are found in minimal concentrations in normal individuals and do not interfere with the assay. Intraserial coefficients of variation of the proinsulin and insulin assays were 6.7% and 5.0%, respectively.

Plasma mass concentrations (antigen) of t-PA and PAI-1 were derived from blood collected in 5-mL siliconized tubes stabilized with sodium citrate (0.5 mL 0.109-mol/L sodium citrate and theophylline, adenosine, and dipyridamole [Diatube H; Diagnostica Stago, Asnieres, France]). The total plasma antigen concentration of t-PA (reference interval, 1.2 to 10.2 ng/mL) was determined with an ELISA technique using goat antihuman t-PA IgG as catching antibody and goat antihuman t-PA IgG labeled with horseradish peroxidase (HRP) as detecting antibody (Imulyse; Biopool, Umeå, Sweden).[25] The total antigen concentration of PAI-1 in plasma (reference interval, 0.0 to 14.1 ng/mL) was measured similarly using murine monoclonal antibodies against human PAI-1 (MA-7D4 as catching antibody and HRP-labeled MA-7F5 as detecting antibody, Imulyse; Biopool).[26] Intraserial coefficients of variation ranged from 8% for t-PA to 5% for PAI-1.

Arterial blood pressure was measured with a standard clinical sphygmomanometer (cuff size, 12 cm × 25 cm). Mean arterial blood pressure was calculated as the diastolic pressure [⅓× (systolic pressure − diastolic pressure)]. Body weight is expressed as body mass index (normal range, 19 to 25 kg/m^2). The waist to hip ratio was determined by measuring the smallest waist circumference and the circumference at the level of the greater trochanters.

Statistical Analysis

Because of skewed distributions of most variables, nonparametric statistics were applied.[27] Results are therefore expressed as medians and ranges. The median values of each variable shown in the tables are calculated from the mean value of two measurements of each variable in individual plasma samples, ie, all samples of each subject were analyzed in duplicate and in one series. The values obtained during glucose tolerance tests were evaluated by Friedmann's ANOVA, and in case of significant results, differences between the sample periods were evaluated by Wilcoxon's signed rank-sum tests. Wilcoxon's test was also used in the comparison between values obtained before and after 6 months of hormone intake. The Spearman test was used in the evaluation of covariation. Two-tailed *P* values less than .05 (2 α) were considered statistically significant.

RESULTS

The baseline characteristics of the women before and after 6 months of hormone intake are summarized in Table 1. Smoking habits of the three smokers were unchanged during the study period. Mean arterial blood pressure decreased in 11 women ($P = .016$); a concomitant weight reduction was observed in only three of these women. No other differences of statistical significance were noted in the variables of Table 1.

Fasting concentrations of proinsulin, PAI-1, and t-PA measured during the OGTT and IVGTT (performed with an interval of 2 days within the same cycle) did not differ either before or during hormone intake. However, fasting levels of insulin were significantly lower during the IVGTT versus the OGTT performed before hormone intake (median, 24.7 *v* 33.1 pmol/L, $P = .02$; Tables 2 and 3). There was no difference between fasting levels of insulin measured after 6 months.

OGTT

Fasting values (0 minutes) and OGTT concentrations of proinsulin and insulin were similar before and after 6 months of hormone intake. When compared with pretreatment values, decreased concentrations of both t-PA and PAI-1 were observed in the fasting state and during the OGTT after 6 months of hormone intake. The decrease in fasting values was proportionate, since an unchanged ratio between t-PA and PAI-1 was noted. Before hormone intake, concentrations of PAI-1 were stable during the first

Table 1. Baseline Characteristics of 17 Healthy Women Before and After 6 Months of Treatment With a Triphasic Combination of EE and NGT

Characteristic	Before		After 6 Months	
	Median	Range	Median	Range
BMI (kg/m^2)	20.3	17.7-23.9	20.5	18.1-22.9
Waist to hip ratio	0.74	0.69-0.80	0.71	0.68-0.79
Mean arterial blood pressure (mm Hg)	85	72-93	82*	77-83
Smokers (n)	3		3	

*$P < .05$.

part of the OGTT, but decreased levels were observed after 180 minutes; decreased levels of t-PA were noted at the first measurement after the glucose load. After 6 months, only t-PA decreased after the glucose intake.

IVGTT

As observed during the OGTT, proinsulin concentrations before and during the IVGTT were similar before and after 6 months of hormone intake. Increased fasting insulin concentration ($P = .0038$) was observed after hormone intake, but the insulin response to glucose injection did not differ. After 6 months of hormone intake, plasma levels of both PAI-1 and t-PA were significantly reduced before and after glucose injection. The reduction in fasting levels was proportionate, as during the OGTT. The observed decrease in PAI-1 and t-PA concentrations after intravenous glucose before hormone treatment was not found after 6 months, where increased PAI-1 and unchanged t-PA levels were observed.

Correlations

We observed a negative correlation ($r_s = -.517, P < .05$) between fasting levels of proinsulin and PAI-1 at the IVGTT after 6 months, and also when proinsulin was adjusted for BMI and the waist to hip ratio. At the corresponding OGTT, r_s was .482, yielding a P value between .1 and .05. No other significant correlations between fasting concentrations of proinsulin or insulin and PAI-1 or t-PA antigen could be demonstrated before or after 6 months of hormone intake.

DISCUSSION

Observations indicating that insulin may influence the t-PA/PAI-1 system originate primarily from studies of individuals at risk for cardiovascular disease due to hypertension, obesity, or NIDDM.[10-12,28-30] These patients often have increased levels of immunoreactive insulin, but the relationship has also been described in healthy normoinsulinemic subjects.[30] However, the radioimmunoassay used in these studies may overestimate the actual plasma insulin levels because of cross-reactivity with proinsulin and split products. These entities can be distinguished only with specific monoclonal antibody–based assays such as the one used in the present study.[23,24,31,32]

Evidence from studies of cell cultures suggests that both insulin and proinsulin affect the t-PA/PAI-1 system. Insulin has been reported to stimulate PAI-1 secretion from cells of hepatic origin, whereas proinsulin seems to have a pronounced and dose-related effect on the PAI-1 secretion of both endothelial and hepatic cells, the two main sources of PAI-1 in vivo.[13,14] However, studies in intact rabbits have shown a similar effect of insulin and proinsulin on PAI-1, since infusion of either increased PAI activity in plasma to the same degree; in both cases, the increase was attributable to accelerated de novo synthesis of PAI-1.[15]

The observation that exogenous insulin is able to induce a parallel decrease in PAI-1 and proinsulin levels in patients with NIDDM without changes in glycemic control or insulin resistance suggests that proinsulin may also affect fibrinolysis in man.[17] Recent observations by Gray et al,[16] who demonstrated that insulin precursors measured with specific methods are more strongly correlated with PAI activity than insulin in survivors of myocardial infarction, also indicate a regulatory role of proinsulin in fibrinolysis. Moreover, it has been demonstrated that patients with insulin-dependent diabetes mellitus without endogenous production of insulin (or proinsulin) tend to have normal or low levels of PAI-1, whereas patients with NIDDM with elevated levels of insulin precursors invariably have high PAI-1 levels.[10,33,34]

We observed a proportionate decrease in fasting antigen concentrations of PAI-1 and t-PA during hormonal intake, which is in accordance with previous findings.[18,35] The corresponding fasting levels of proinsulin, PAI-1, and t-PA before and during intake of contraceptive steroids do not indicate that proinsulin increases the plasma levels of these variables in young lean nondiabetic women. On the contrary, we found indications of a negative relation between fasting levels of proinsulin and PAI-1 during intake of contraceptive steroids. Although significant values were noted in only one of two evaluations, the finding is in contrast to earlier reports of a positive relation between

Table 2. Plasma Concentrations of Proinsulin, Insulin, PAI-1, and t-PA Before and During an OGTT With 75 g Glucose Performed Before and After Hormone Intake (N = 17)

	Proinsulin (pmol/L)		Insulin (pmol/L)		PAI-1 (ng/mL)		t-PA (ng/mL)	
Time	Median	Range	Median	Range	Median	Range	Median	Range
Before								
0 min (fasting values)	4.5	1.6-37.4	31.1	10.6-44.2	5.0	1.5-14.4	3.1	1.9-13.9
40 min	21.4*	6.4-71.0	356.9*	55.0-1,406.2	5.7	1.5-13.0	2.8*	1.1-5.7
60 min	26.4*	9.0-85.0	251.6*	96.0-944.4	5.1	1.4-37.9	3.1*	1.4-4.7
90 min	26.2*	6.3-106.6	175.5*	67.3-756.4	4.6	1.2-42.4	3.1*	1.2-5.3
180 min	18.6*	4.2-48.3	73.1*	9.9-165.1	3.8*	0-31.2	2.9*	1.1-5.0
After								
0 min (fasting values)	5.1	1.6-8.2	31.3	11.7-50.0	2.3	0-11.5	2.4	0.4-3.1
40 min	23.0*	7.8-57.5	299.0*	116.5-730.7	2.5	0.3-11.5	1.9*	0.3-2.6
60 min	25.7*	12.1-65.0	286.8*	128.8-714.3	2.7	0.4-21.5	1.9*	0.2-7.0
90 min	29.7*	13.0-87.7	216.7*	45.9-982.9	1.8	0-17.8	1.9*	0.1-2.9
180 min	19.0*	6.8-53.9	88.5*	11.7-245.2	1.7	0.3-19.6	2.0*	0-3.1

*$P < .05$.

Table 3. Plasma Concentrations of Proinsulin, Insulin, PAI-1, and t-PA Before and During an IVGTT With Glucose 0.3 g/kg Body Weight Performed Before and After Hormone Intake (N = 17)

	Proinsulin (pmol/L)		Insulin (pmol/L)		PAI-1 (ng/mL)		t-PA (ng/mL)	
Time	Median	Range	Median	Range	Median	Range	Median	Range
Before								
0 min (fasting values)	3.9	1.4-7.7	24.7	6.8-42.6	6.8	2.6-26.1	4.5	2.6-6.0
5 min	13.5*	3.4-26.9	269.6*	57.9-573.7	6.6	1.9-23.3	3.7*	1.7-5.9
25 min	28.0*	7.7-48.8	356.2*	80.5-712.0	6.8	2.1-16.3	3.5*	0.8-5.3
40 min	19.7*	7.8-49.9	133.3*	54.0-331.3	7.5	1.8-12.5	3.6*	0.7-5.6
180 min	5.9*	1.8-16.2	25.1	8.6-45.0	3.7*	0-8.8	3.2*	1.7-5.0
After								
0 min (fasting values)	4.7	1.2-10.5	33.1	18.2-72.1	1.5	0-18.1	2.0	0-4.0
5 min	13.2*	3.9-29.2	253.4*	67.4-599.6	1.7	0-18.3	1.7	0-4.0
25 min	21.4*	5.8-57.0	341.8*	81.7-670.6	2.1*	0-61.2	1.6	0-3.0
40 min	21.8*	6.4-46.0	154.4*	60.1-259.8	2.1*	0-12.3	1.7	0-3.4
180 min	5.1*	2.2-15.8	26.6	11.5-55.9	1.4	0-42.1	2.1	0-3.3

*$P < .05$.

these variables in patients with NIDDM or coronary heart disease.[16,17] We were not able to confirm previous observations of a positive correlation between fasting insulin and PAI-1.[10-12]

Before hormone treatment, the observed changes in plasma concentrations of PAI-1 and t-PA during the 3-hour glucose tolerance tests were not different from those previously described in normal subjects under fasting conditions during the morning hours.[36,37] PAI-1 and t-PA concentrations have not been previously studied in women taking oral contraceptives subjected to glucose challenges. Our findings suggest that the fluctuations in antigen concentrations during the sampling period of 3 hours are attenuated by the steroids through mechanisms independent of the changed concentrations of proinsulin and insulin.

The effects of endogenous hyperinsulinemia have previously been studied only in males, either obese or suffering from coronary heart disease. In these individuals, hyperinsulinemia induced by oral glucose caused a decrease in both PAI-1[38,39] and t-PA.[39] Apart from unchanged PAI-1 concentrations during the OGTT after 6 months of hormone intake, our findings are in accordance with these reports. The acute increase in insulin and proinsulin after the glucose load had no instant effect on antigen levels of PAI-1 either before or during hormone intake. The effects of induced endogenous hyperproinsulinemia on PAI-1 and t-PA antigen concentrations have not previously been evaluated in humans.

If proinsulin and insulin have an effect on PAI-1, two mechanisms are possible: (1) release of stored intracellular deposits of PAI-1 molecules, or (2) increased gene transcription followed by de novo protein synthesis. Acute release of intracellular stored fibrinolytic factors has been described for t-PA, where release into the extracellular phase has been reported to occur in two phases: one reaching its maximum a few minutes after stimulation, and a second peaking after 15 to 30 minutes.[40,41] Our results do not indicate that insulin or proinsulin stimulate the release of t-PA. Should a similar mechanism exist for PAI-1, which is not known at present, our findings do not suggest that increased levels of insulin or proinsulin elicit acute release of PAI-1 into the circulation. Induction of PAI-1 and t-PA de novo synthesis requires at least 3 hours, so our study cannot definitively exclude the possibility that increased levels of proinsulin or insulin observed after the glucose load may stimulate the synthesis after the sampling period of our study.[15,41,42]

In conclusion, we found no consistent relations between fasting values of proinsulin, insulin, PAI-1, and t-PA and no indication that acute elevations of the endogenous secretion of proinsulin and insulin are accompanied by an increase in plasma levels of PAI-1 or t-PA. Therefore, irrespective of intake of contraceptive steroids, proinsulin or insulin do not seem to be involved in the regulation of plasma levels of PAI-1 and t-PA in young healthy women.

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