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Insulin Stimulates the Synthesis of Plasminogen Activator Inhibitor 1 by the Human Hepatocellular Cell Line Hep G2

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Key words

Insulin – PAI-1 – Hepatocyte – Endothelial cell

Summary

Secretion of plasminogen activator inhibitor 1 (PAI-1) by cultures of human umbilical vein endothelial cells and human hepatocellular cell line Hep G2 was evaluated after insulin stimulation. The secretion of PAI-1 antigen and activity was measured in the conditioned medium and the cellular extracts after incubation of confluent cultures with 1% serum medium for 24 hours.

Insulin induced a dose dependent increase of the PAI-1 secretion by Hep G2 cell line. At 10^{-8} M a two fold increase of PAI-1 antigen and activity were observed whereas α_2 antiplasmin and fibrinogen were not significantly modified. No effect of insulin was observed on PAI-1 antigen and PAI activity production by human endothelial cells whereas endotoxin resulted in a two fold increase in PAI-1 secretion. In recent clinical studies we have demonstrated that the level of plasma insulin correlated with that of PAI-1. Thus we hypothesize that hepatocytes represent a physiological source of plasma PAI-1 which is modulated by plasma insulin level.

Introduction

Increased plasma levels of plasminogen activator inhibitor (PAI) activity occur in various prethrombotic states including coronary artery disease (1), venous thrombosis (2, 3), and the post operative state (4). Elevated plasma PAI activity levels have a predictive value for the recurrence of myocardial infarction (5) which suggests that PAI is of pathophysiological importance.

PAI-1 is synthesized by endothelial cells and by human hepatocytes in culture (6, 7), and occurs in the alpha-granules of blood platelets; PAI-1 seems to be the main physiological inhibitor of t-PA in plasma (8). Several mechanisms which remain poorly understood appear to be involved in the regulation of PAI-1 secretion (8); endotoxin (9) and interleukin 1 (10) stimulate its synthesis in endothelial cells, corticosteroids increase hepatic synthesis (11), whereas the PAI-1 level in blood correlates with the triglyceride level (3, 5, 12).

In recent studies (12, 13) we have demonstrated that the level of plasma insulin correlates with that of PAI-1. In the present study we have therefore compared the effect of insulin on PAI-1 secretion by human endothelial cell and human hepatocyte (Hep G2 line) cultures. It appears that insulin stimulates PAI-1 synthesis by the hepatoma cell line, but not by endothelial cells. Insulin stimulation of hepatic synthesis may explain the increased plasma PAI-1 concentration in hyperinsulinemic subjects (12, 13).

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Materials and Methods

Material

Human insulin was purchased from and proinsulin was a kind gift from Novo industries (Paris). Cycloheximide, Actinomycin D and gelatin were obtained from Sigma Chemical Co (St Louis, MO). Lipopolysaccharide 0111:B4 from *Escherichia coli* was from Osi Laboratories (Paris). Plasminogen, the chromogenic substrate D-Val-Leu-Lys-p-nitroanilide (S2251) and CNBr-digested fibrinogen fragments were from Kabi Vitrum (Amsterdam). The protein assay kit was from BIORAD (Richmond, CA). Fibrinogen, and antiserum against fibrinogen were from Stago (Paris), orthophenylene diamine was from Fluka (Darmstadt, W Germany), and medium 199, Dulbecco's modified eagle medium and fetal calf serum were from Flow Laboratories (Irvine, UK). Human serum was obtained from a pool of healthy subjects. Purified PAI-1, t-PA, α_2 antiplasmin and monoclonal antibodies against PAI-1 and α_2 antiplasmin were prepared as described elsewhere (14, 15). The human hepatoma cell line Hep G2 was provided by Dr B. B. Knowles, Wistar Institute of Anatomy and Biology, Philadelphia, PA. All other reagents were of laboratory grade quality.

Cell Culture

Human endothelial cells were prepared from fresh umbilical cord veins by the method of Jaffe et al. (16). Cells were used after one passage and plated in tissue culture flasks (25 cm^2) coated with gelatin. Cells were grown to confluency at 37° C with atmospheric air and 5% CO_2 in medium 199 supplemented with Hepes 15 mM, $NaHCO_3$ 15 mM, glutamine 2 mM, penicillin 100 IU/ml, streptomycin 100 µg/ml, 10% heat inactivated human serum and 10% heat inactivated fetal calf serum. The cells were identified as endothelial cells by their typical cobblestone pattern, by immunofluorescence staining for von Willebrand factor and by electronic microscopy.

The hepatoma cell line Hep G2 was grown in DMEM supplemented with 10% heat inactivated fetal calf serum and 5% heat inactivated human serum.

Cell cultures were grown in 9.6 cm^2 dishes until confluency. The culture medium was then replaced by 2 ml culture medium containing 1% serum. Endotoxin, insulin or proinsulin were dissolved in the same medium and added in the cell cultures. After incubation, the conditioned medium was harvested and centrifuged for 5 min at 3,500 × g. The samples were either immediately assayed or kept frozen at −20° C until use. Inhibition studies with Cycloheximide (2.5 µM) and Actinomycin D (2 µM) were performed by addition of inhibitors together with insulin to the cell cultures and incubation for 20 hours. Cell extracts were prepared by incubation of cell monolayers with 1 ml 0.5% Triton X100 for 1 hour at room temperature.

The concentrations of PAI-1 and α_2 antiplasmin were measured with specific ELISA's (14, 15). PAI activity was determined by the method of Verheijen et al. (17); the results were expressed in units equivalent to the amount of IU of t-PA neutralized. The activity of t-PA was determined by comparison with the international reference preparation (83/517). Fibrinogen was quantified by an ELISA assay purchased from Stago Laboratories. Protein concentration was determined by the method of Bradford (18).

Table 1 PAI activity and PAI-1 antigen levels (mean ± SEM) in conditioned medium and cell extracts of endothelial and hepatocyte cell cultures

		Conditioned medium			Cell extracts		
	n	Free active PAI (ng/ml)*	PAI-1 antigen (ng/ml)	Ratio	Free active PAI (ng/ml)*	PAI-1 antigen (ng/ml)	Ratio
Endothelial cells (HUVEC)	9	66 ± 13	2300 ± 300	0.03 ± 0.003	8 ± 0.8	70 ± 5.5	0.11 ± 0.009
Hep G2 cells	10	38 ± 2.9	1500 ± 200	0.03 ± 0.003	47 ± 7.4	160 ± 21	0.34 ± 0.06

* Calculated on the basis of 1.4 ng per unit PAI (cf text).

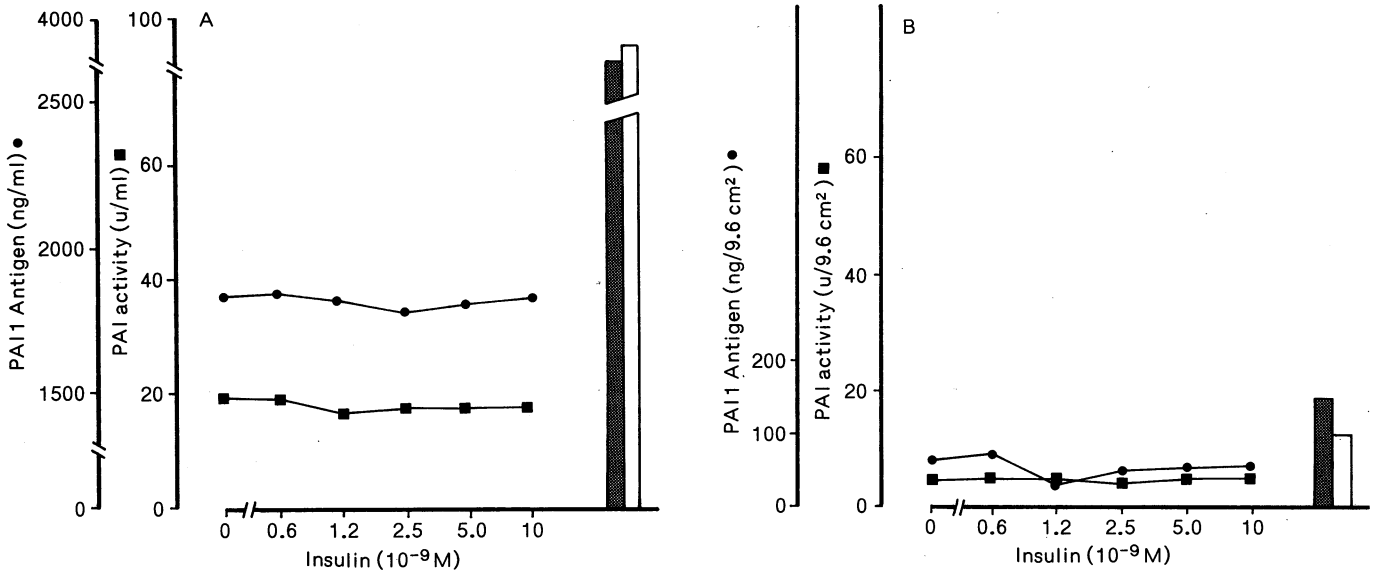

Fig. 1 Effect of insulin (0.6 to 10 nM) on PAI-1 production by human umbilical vein endothelial cells. A: Conditioned medium; B: Cell extracts; ● PAI-1 antigen; ■ PAI activity. Columns represent ■ PAI-1 Ag and □ PAI activity after endotoxin stimulation (10 μg/ml). The values represent the mean of duplicate determinations for 3 experiments

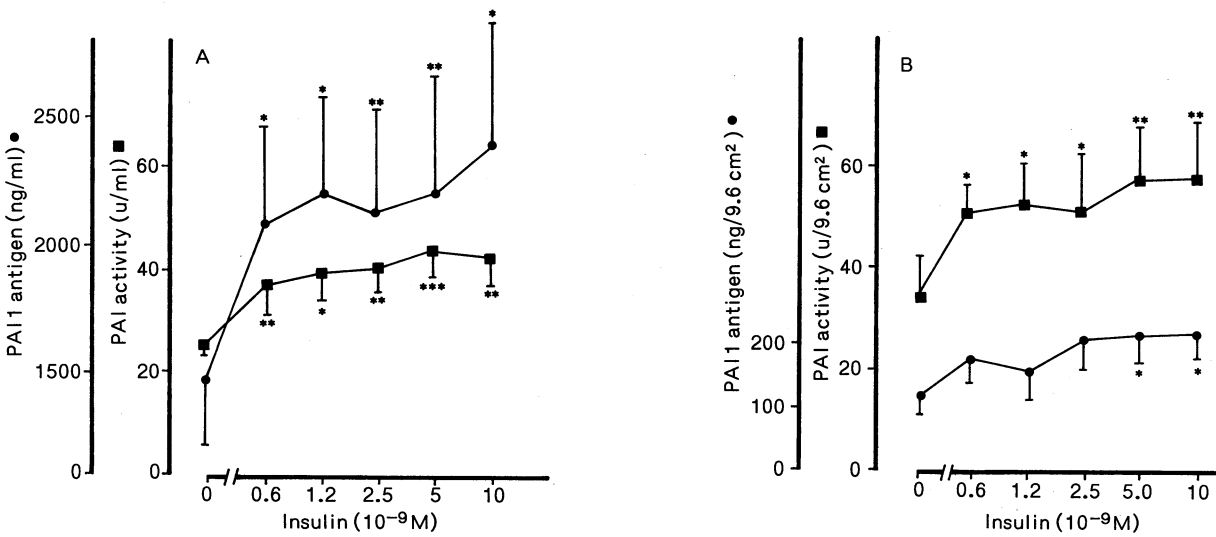

Fig. 2 Effect of insulin (0.6 to 10 nM) on PAI production by the hepatoma cell line Hep G2. A: Conditioned medium; B: Cell extracts; ● PAI-1 antigen; ■ PAI activity. The values represent the mean and the vertical bars SEM of duplicate determinations for 6 experiments. Statistical significances were tested with Student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

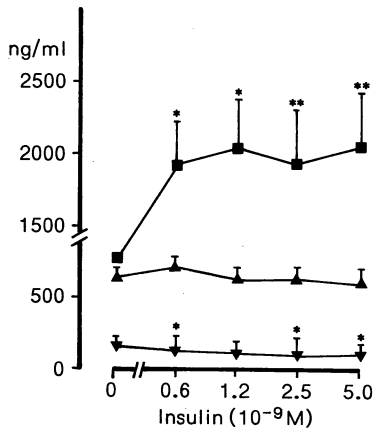

Fig. 3 Insulin effect on PAI-1 antigen, fibrinogen and α_2 antiplasmin production by hepatoma cell line Hep G2 in conditioned medium. ■ PAI-1 antigen; ▼ Fibrinogen; ▲ α_2 antiplasmin. The values represent the mean and the vertical bars SEM of duplicate determinations of 6 experiments. Statistical significances were tested with Student's t test.
* $p < 0.05$, ** $p < 0.01$

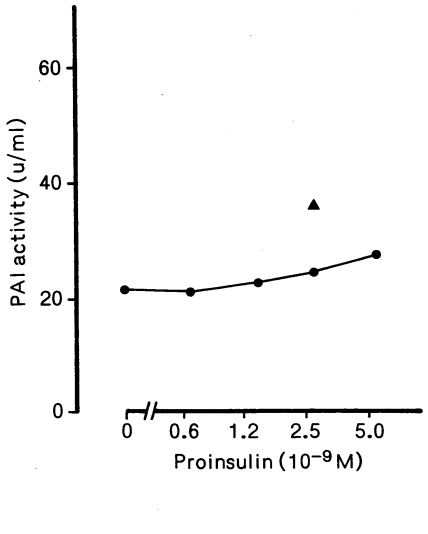

Fig. 4 Effect of proinsulin on PAI activity production by the hepatoma cell line Hep G2. Conditioned medium. ▲ represents insulin effect (2.5 nM) on PAI activity production by hepatoma cell line Hep G2 conditioned medium. The values represent the mean of duplicate determinations with a variability of less than 20%

Table 2 Effect of insulin, cycloheximide and actinomycin D on the secretion of PAI-1 by hepatocyte cell cultures. The data represent means of two experiments

	Free active PAI-1 (ng/ml)	PAI-1 Ag (ng/ml)
Baseline	14	1,600
Insulin (5×10^{-9}M)	28	4,100
Insulin + actinomycin D (2×10^{-6}M)	6	1,000
Insulin + cycloheximide (2.5×10^{-6}M)	3	600

Results

Basal values of PAI activity and PAI-1 antigen in conditioned medium and cellular extracts from once passaged human umbilical vein endothelial cells (HUVEC), and from the hepatoma cell line (Hep G2) are summarized in Table 1. PAI activity was converted to free active PAI-1 concentration assuming a 1:1 complex formation between t-PA and PAI-1, the specific activity of t-PA of 1 IU per 2 ng protein and molecular weights of 70,000 for t-PA and 50,000 for PAI-1. Thus 1 unit of PAI is equivalent to 1.4 ng PAI-1.

The fraction of active PAI-1 was significantly higher in cellular extracts (0.11 and 0.34 for HUVEC and Hep G2) than, in conditioned medium (0.03) which is in agreement with the hypothesis that PAI-1 is synthesized in active form which is inactivated in the conditioned medium after secretion.

Insulin in concentrations up to 10^{-8} M did not change the concentration of PAI-1 in conditioned medium and cell extracts of HUVEC whereas endotoxin resulted in a two fold increase in PAI-1 secretion (Fig. 1). A dose dependent increase of PAI-1 activity and PAI-1 antigen was however observed in conditioned medium and cellular extracts of hepatocytes (Fig. 2) with a maximal increase of 170% over the control value. Insulin did not significantly alter the synthesis of fibrinogen, α_2 antiplasmin (Fig. 3). Proinsulin showed no effect on PAI secretion in endothelial cells (data not shown), whereas a minor effect was demonstrated in hepatocytes (Fig. 4). To verify whether insulin had a direct effect on the fibrinolytic components, insulin was added to the standard curve of the different assays and to the conditioned medium. Insulin did not modify the reactivity of the assays used. Total protein concentration was similar in cellular extracts from both non stimulated cells and cells stimulated by 2.5×10^{-9} M insulin. With endothelial cells (n = 10) values (m ± SEM) without insulin were 321 ± 41.4 µg/9.6 cm^2 as compared to 339 ± 47.5 µg/9.6 cm^2, after insulin stimulation. With the hepatoma cell line (n = 10) corresponding values were 1,680 ± 65.8 µg/9.6 cm^2 and 1,692 ± 97.8 µg/9.6 cm^2 respectively. Inhibition of protein synthesis with Cycloheximide (2.5 µM) and of DNA dependent RNA synthesis, with Actinomycin D (2 µM) abolished the appearence or the increase of PAI activity and PAI-1 antigen in the conditioned medium (Table 2) of hepatocytes after induction with insulin.

Discussion

PAI-1 in plasma may originate from endothelial cells, hepatocytes, platelets. Because hyperinsulinemia is associated with high plasma PAI-1 concentrations (12, 13) we have in the present study investigated the effect of insulin on hepatocyte and endothelial cell cultures.

A dose dependent effect of insulin on the production of PAI-1 by hepatocyte-type cells was observed at a concentration (10^{-9}M) similar to that formed in the portal vein after a meal (19). PAI-1 secretion was blocked by Cycloheximide and Actinomycin D indicating that it depends on de novo protein and RNA synthesis.

The direct effect of insulin shown on hepatocyte PAI-1 synthesis is unlikely to represent a non specific mitogenic action of the hormone. Indeed total protein concentration was similar before and after stimulation, α_2 antiplasmin production remained unchanged whereas fibrinogen production decreased slightly. Proinsulin was a 4 to 5 times less efficient stimulator of PAI-1 synthesis than insulin, which is consistent with the results of receptor binding studies on liver membranes that showed that the insulin receptor had a 20 fold lower affinity for proinsulin (20).

We could not detect any effect of insulin on the secretion of PAI-1 by human umbilical vein endothelial cells (HUVEC). Among other causes this could be due to a discrepancy affecting the number (21) and the affinity of receptors for insulin between hepatocytes and endothelial cells. However, the origin of the endothelial cells has to be considered. Thus HUVEC have fewer insulin receptors than capillary endothelial cells. The metabolic activity of the cells (glucose uptake) and the growth promoting effect of insulin differs according to the cells origin (22–25). In general microvascular endothelium appears to be more susceptible to many effects of insulin, so an effect of insulin on microvascular endothelium PAI-1 synthesis has to be looked for.

The production of PAI-1 by cells was measured both using functional and immunological assays. The ratio of active PAI-1/total PAI-1 indicate that the majority of PAI-1 was found in an

inactive form, however, this ratio was higher in cell extracts than in conditioned medium. These results confirm that PAI-1 is an unstable molecule which following secretion is further inactivated (27). The values of the ratio obtained in cell extracts were lower than expected as compared to Levin's findings (26); an inactivation of PAI-1 during cell extracts preparation could explained these results. As shown in Fig. 2, no modification of the ratio was observed after 24 hours insulin stimulation of hepatocyte type cells indicating that the insulin effect does not modify the stability of PAI-1 or the relative ratio of active versus inactive molecules.

Hyperinsulinemia is considered to be a risk factor for the development of macrovascular disease manifesting as coronary heart disease, peripheral vascular disease (28–30). In two clinical conditions with hyperinsulinemia, obesity (12) and non insulin dependent diabetes (unpublished data), which represent risk factors for atherosclerosis, we found that the increased plasma insulin concentration correlated with increased PAI-1 level. The stimulation by chronic hyperinsulinemia of hepatic PAI-1 production leading to high plasma PAI-1 concentration could explain these findings. The high plasma PAI-1 concentration secondary to hyperinsulinemia should be one of the factors responsible for the development of athero-thrombosis in risk population as was proposed by Hamsten et al. (5).

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