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Research Article

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Plasminogen Activator Inhibitor-1 Synthesis in the Human Hepatoma Cell Line Hep G2

Metformin Inhibits the Stimulating Effect of Insulin

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Abstract

High plasma plasminogen activator inhibitor-1 (PAI-1) activity is associated with insulin resistance and is correlated with hyperinsulinemia. The cellular origin of plasma PAI-1 in insulin resistance is not known. The hepatoma cell line Hep G2 has been shown to synthesize PAI-1 in response to insulin. The aim of this study was to analyze the insulin-mediated response of PAI-1 and lipid synthesis in Hep G2 cells after producing an insulin-resistant state by decreasing insulin receptor numbers. The effect of metformin, a dimethyl-substituted biguanide, known to lower plasma insulin and PAI-1 levels in vivo was concomitantly evaluated. Preincubation by an 18-h exposure of Hep G2 cells to 10^{-7} M insulin aimed at reducing the number of insulin receptors, was followed by a subsequent 24-h stimulation with 10^{-9} M insulin. The decrease in insulin receptors was accompanied as expected, by a reduction in [^{14}C]acetate incorporation, an index of lipid synthesis, whereas PAI-1 secretion and PAI-1 mRNA expression were enhanced. The addition of metformin did not modify the effect of insulin on insulin receptors or [^{14}C]acetate incorporation. In contrast, the drug (10^{-4} M) inhibited insulin-mediated PAI-1 synthesis. The results indicate that PAI-1 synthesis in presence of insulin is markedly increased in down-regulated cells, and that metformin inhibits this effect by acting at the cellular level. These in vitro data are relevant with those found in vivo in insulin-resistant patients. Hep G2 cells may be a suitable model to study PAI-1 regulation in response to hyperinsulinemia. (*J. Clin. Invest.* 1993. 91:2185–2193.) Key words: fibrinolysis • insulin • insulin resistance • metformin • plasminogen activator inhibitor 1

Introduction

Plasminogen activator inhibitor-1 (PAI-1)[1] is the major physiological inhibitor of tissue-type plasminogen activator (t-PA) (1). Increased PAI activity is responsible for impaired fibrinolysis associated with thrombotic diseases. Elevated plasma PAI-1 concentrations have been observed in coronary artery disease and were shown to be predictive of recurrent myocardial infarction (2).

Epidemiological studies have indicated that hyperinsulinemia in the fasting state and after an oral glucose load is associated with an increased risk of developing coronary heart disease (3–6). Hyperinsulinemia is the biological marker of insulin resistance, a multifaceted syndrome. It is characterized by a decrease in the ability of insulin to stimulate in vivo glucose utilization and is associated with hypertension, android obesity, and an abnormal lipid profile including elevated triglyceride and decreased high density lipoprotein cholesterol levels (7, 8). We have previously shown that increased plasma PAI-1 levels are linked to hyperinsulinemia and are found in insulin-resistant states (for review see reference 9). Significant correlations have been established between plasma PAI-1 levels, fasting plasma insulin, body mass index, android obesity, and hypertriglyceridemia. A direct link between PAI-1 levels and insulin resistance has been established by decreasing hyperinsulinemia in vivo. Diet, weight loss, and physical training improve the insulin-resistant state by reducing insulinemia and also lower PAI-1 levels. The oral antidiabetic drug, metformin, a dimethyl substituted biguanide, increases peripheral glucose uptake, reduces gluconeogenesis and decreases plasma insulin and triglyceride levels (10–13). In addition, metformin improves fibrinolysis in cardiovascular disease (14). We have also shown in a previous study that, in obese non diabetic women, recovery of fibrinolytic activity after Metformin treatment was mainly due to decreased PAI-1 levels (15).

Several attempts have been undertaken to explain the link between increased PAI-1 levels and the insulin-resistant state. Insulin resistance is associated with several cell defects including decreased receptor numbers, alterations in insulin receptor tyrosine kinase activity leading to impaired insulin action, and postreceptor defects (16–19). A decrease in insulin receptor numbers may be secondary to a down-regulation of the receptors owing to insulin binding and internalization and may be induced at the cell level by acute or chronic exposure of cells to a high concentration of insulin (20, 21). In vitro PAI-1 is synthesized by several cell types including endothelial cells, hepatocytes (22), and smooth muscle cells (for review see reference 23). The human hepatoma cell line Hep G2 cells have retained several properties of human hepatocytes (24) and possess functional insulin receptors which can be down-regulated in response to chronic or acute exposure to high concentrations of insulin (25), thus leading to insulin desensitization. We have previously shown that insulin increased PAI-1 synthesis by Hep G2 cells (26). The results have been confirmed in other studies using Hep G2 cells (27–29) and fresh human hepatocytes (27).

The molecular events that mediate PAI-1 synthesis in response to insulin are poorly understood. Insulin exerts several metabolic actions in Hep G2 cells including a direct effect on

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1. *Abbreviations used in this paper:* CM, conditioned media; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor-1.

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triacylglycerol synthesis (30). In this study we have examined PAI-1 synthesis and acetate incorporation into lipids after induction of an insulin-resistant state in Hep G2 cells by acute exposure to high insulin concentrations. The data have established that down-regulation of insulin receptors leads to a reduction in acetate incorporation and in contrast, an up-regulation of PAI-1 synthesis. Metformin in the presence of insulin, inhibited the insulin-mediated PAI-1 synthesis but exerted no effect on insulin-mediated acetate incorporation indicating that the two insulin actions are regulated by different mechanisms.

Methods

The human hepatoma cell line Hep G2 was provided by Dr. B. Knowles, Wistar Institute of Anatomy and Biology (Philadelphia, PA). Fetal calf serum, cell culture reagents, cell culture medium, and molecular biology reagents were purchased from Gibco BRL Life Biotechnologies (Cergy Pontoise, France). BSA (cell culture reagent tested), endothelial cell growth supplement, and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Plastic tissue culture flasks and plates were obtained from Falcon Labware (Becton, Dickinson & Co., Meylan, France). Human insulin (solution for injection, 7×10^{-4} M) was purchased from Novo Industries (Copenhagen, DK). Monoclonal antibodies specific for PAI-1 and PAI-1 cDNA human probe were a gift of Prof. D. Collen, Dr. P. J. Declerck, and Dr. L. Nelles (Center for Thrombosis and Vascular Research, Leuven, Belgium). β-actin cDNA human probe was purchased from Clontech (Palo Alto, CA). [^{35}S]Methionine, ^{125}I-insulin, [^{14}C]acetic acid sodium salt, nylon N^+ membranes, and multiprime labeling DNA system were purchased from Amersham International (Amersham, UK). Safe Emulsiefer was from Packard (Meridien USA). Metformin was a gift from Lipha Laboratories (Lyon, France).

Cell culture

Cells from the human hepatoma cell line Hep G2 were grown to confluency in Ham's F12/Eagle's MEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin under a 5% CO_2 atmosphere. For specific experiments Hep G2 monolayers were cultured on 24- or 12-well culture plates. 24 h after reaching confluence, Hep G2 monolayers were cultured overnight in serum-starved medium supplemented with 0.5% BSA followed by incubation with insulin and/or metformin at different concentrations. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins according to the method of Jaffe et al. (31). Cells were used at confluency after one passage. For specific experiments, confluent HUVEC monolayers were passed on 24-well plates. Cell viability was assessed by the trypan blue dye exclusion test. Conditioned media (CM) were harvested, immediately centrifuged at 2,000 *g* to exclude cellular debris, aliquoted, and stored at −20°C. Cells were trypsinized and counted.

PAI-1 antigen assay

PAI-1 antigen (PAI-1 Ag) assay in CM was performed using an ELISA as described elsewhere (32). Results were expressed in terms of PAI-1 Ag per 10^6 cells.

RNA extraction and Northern blot analysis

Total RNA was extracted according to Chomczynski et al. (33) and fractionated on 1.2% agarose gel containing 20% formaldehyde. RNA was transferred to nylon N^+ membranes. Hybridization conditions and processing of the filters were carried out as described by Medcalf et al. (34). The PAI-1 cDNA probe was a 436-bp fragment of human PAI-1 cDNA. The human β-actin probe was a complete 2-kb human β-actin cDNA and was used as control. The PAI-1 cDNA and β-actin cDNA probes were labeled with [α-^{32}P]dCTP using the random priming technique (35).

[^{14}C]Acetate incorporation

Serum-starved Hep G2 monolayers were incubated with [^{14}C]acetic acid sodium salt ([^{14}C]acetate 1 μCi/ml, sp act 56.2 mCi/mM) in presence of insulin and/or metformin. At the end of the incubation the conditioned media were discarded, the monolayers were rinsed three times with cold PBS and the cells were incubated on ice with a lysing buffer (Tris HCl 0.05 M, pH 8.0, NaCl 0.15 M, NP-40 1%, PMSF 1 mM, and aprotinin 1 μg/ml). The cell extracts were centrifuged to eliminate cell debris and an aliquot (50 μl) was used for quantifying the protein content. Incorporation of [^{14}C]acetate in the lipid phase of the cell extracts was assayed as described by Folch et al. (36). An aliquot (150 μl) of the lipid phase was mixed with 10 ml of Safe Emulsifier and counted in a β counter.

Residual binding of ^{125}I-insulin to Hep G2 cells

Residual insulin binding was performed on serum-starved cell monolayers in the presence of ^{125}I-insulin as follows: after a 24-h incubation with insulin and/or metformin, cells were acid-washed three times at 4°C as described by Garvey et al. (18) to remove bound insulin, followed by three rinses in PBS. Cells were then incubated for 3 h at 4°C in MEM Ham's F12 medium 0.5% BSA containing 0.2 ng ^{125}I-insulin per well. Free hormone was removed with five rinses of cold PBS and the cells were again acid-washed with 250 μl of MEM, pH 4.0, to dissociate receptor-bound insulin. The radioactivity recovered in the acid-washed eluate was counted. Nonspecific binding determined with the addition of 200 ng of unlabeled insulin was <5% of total counts bound.

Down-regulation of Hep G2 cells

Down-regulation of Hep G2 cell receptors was performed as described by Blake et al. (25): serum-starved confluent Hep G2 cells were pre-incubated with a high concentration of insulin (10^{-7} M) for a prolonged time (18 h), cooled on ice to avoid receptor recycling, acid-washed in MEM, pH 4.0, to remove bound insulin, and then washed with PBS, and finally with culture medium, each washing being performed three times. When required, cells were preincubated with 10^{-7} M insulin for different times and subsequently treated as described above.

Insulin stimulation of Hep G2 cells

Control cells. Serum-starved Hep G2 cells were incubated for 24 h with doses of insulin ranging between 10^{-10} and 10^{-7} M. Unstimulated cells were used as controls. When required control cells were incubated with 10^{-7} or 10^{-9} M insulin for different times. PAI-1 secretion, PAI-1 RNA expression and [^{14}C]acetate incorporation into lipids were assayed as above.

Down-regulated cells. Down regulation was performed as described above; cells were subsequently stimulated with 10^{-9} M insulin for 24 h. Control unstimulated cells were kept in serum-starved medium during the two steps of this procedure. Control down-regulated cells were down-regulated by 10^{-7} M insulin for 18 h, acid-washed, and kept in serum-starved medium for a further 24-h incubation.

Measurements of insulin remaining in the conditioned media were performed due to insulin degradation. We assayed insulin concentrations during the two incubation periods: (*a*) when 10^{-9} M insulin was added to Hep G2 cells, the insulin content in the medium decreased 12 h after the beginning of the incubation and was not detectable after 18 h; (*b*) when 10^{-7} M insulin was added to the cells, the insulin remaining in the medium after an 18-h incubation represents one third of the initial content. Residual insulin was eliminated by acid washes (25).

Effect of metformin on insulin-mediated PAI-1 synthesis and [^{14}C]acetate incorporation

The effects of metformin on insulin stimulation were studied by incubating serum starved control Hep G2 cells for 24 h with insulin in presence of different concentrations of metformin ranging between 10^{-6} and 10^{-3} M. The time-dependent effect of metformin on insulin-mediated PAI-1 secretion was studied by incubating Hep G2 cells with 10^{-4} M metformin for up to 24 h.

To study the effect of metformin on insulin stimulation in down-regulated cells, 10^{-4} M metformin was added either to 10^{-7} M insulin in the 18-h preincubation step, or to 10^{-9} M insulin in the stimulation step.

When required, Hep G2 cells were stimulated for 24 h by 10^{-7} M PMA (37) in absence or presence of 10^{-4} M metformin.

HUVEC were stimulated for 24 h by IL-1 10 IU/ml (38) in presence or absence of 10^{-4} M metformin.

Statistical analysis

Data are expressed as mean±SE. The significance of differences was examined using unpaired Student's *t* test. *P* values < 0.05 were considered significant.

Results

Effects of insulin on PAI-1 synthesis and acetate incorporation

The effects of insulin on PAI-1 synthesis and acetate incorporation were studied in control or down-regulated cells.

Control cells. Serum-starved Hep G2 cells were cultured for up to 24 h with increasing doses (10^{-10} to 10^{-7} M) of insulin. As previously shown (26) our results confirm that insulin induced a dose-dependent increase in PAI-1 secretion up to 4.8-fold (Fig. 1*A*). The maximal effect occurred with 10^{-8} M insulin (469±39.3 in stimulated cells versus 101±8.3 ng/10^6 cells in control cells, $P < 0.01$). Insulin also induced changes in PAI-1 mRNA expression. The steady-state level of both forms of mature PAI-1 mRNA (3.2 and 2.3 kb) was increased by insulin stimulation. No change in the β-actin gene expression was observed (Fig. 1, *inset*).

Insulin induced a dose-dependent increase from 10^{-10} to 10^{-7} M in [^{14}C]acetate incorporation into Hep G2 cells up to 2.2-fold (1.52±0.12 vs. 0.69±0.09 nM/mg protein [$P < 0.05$]) when the cells were stimulated by 10^{-7} M insulin.

Kinetic studies indicate that insulin at 10^{-7} and 10^{-9} M, induced a time-dependent increase in PAI-1 secretion and [^{14}C]acetate incorporation into Hep G2 cells with a 4-h lag-phase (Fig. 1*B*).

Down-regulated cells. Serum-starved Hep G2 cells were down-regulated by 10^{-7} M insulin for 18 h, then stimulated for 24 h with 10^{-9} M insulin. PAI-1 synthesis and [^{14}C]acetate incorporation were evaluated at the end of the stimulation step. Results in Fig. 2*A* indicated that in down-regulated cells, a stimulation with 10^{-9} M insulin led to an increase in PAI-1 secretion by 1.4-fold over the level induced by the same stimulation of control cells (597±54 vs. 408±39 ng/10^6 cells), and by 5.9-fold over the level of the unstimulated control cells (597±54 vs. 101±8.3 ng/10^6 cells). It should be noted that PAI-1 secretion levels from down-regulated cells kept in serum-starved medium for an additional 24 h after acid wash were not significantly different from those observed with control cells (132±15.9 vs. 101±8.3 ng/10^6 cells, Fig. 2*A*). Stimulation mediated by 10^{-9} M insulin of down-regulated Hep G2 cells induced changes in PAI-1 mRNA expression. This procedure resulted in the stimulation of both forms of mature PAI-1 mRNA over the expression mediated by 10^{-9} M insulin in control cells (Fig. 2, *inset*). No change in β-actin mRNA expression was observed.

In contrast to PAI-1 synthesis, the stimulation with 10^{-9} M insulin in down-regulated cells, resulted in a net decrease in

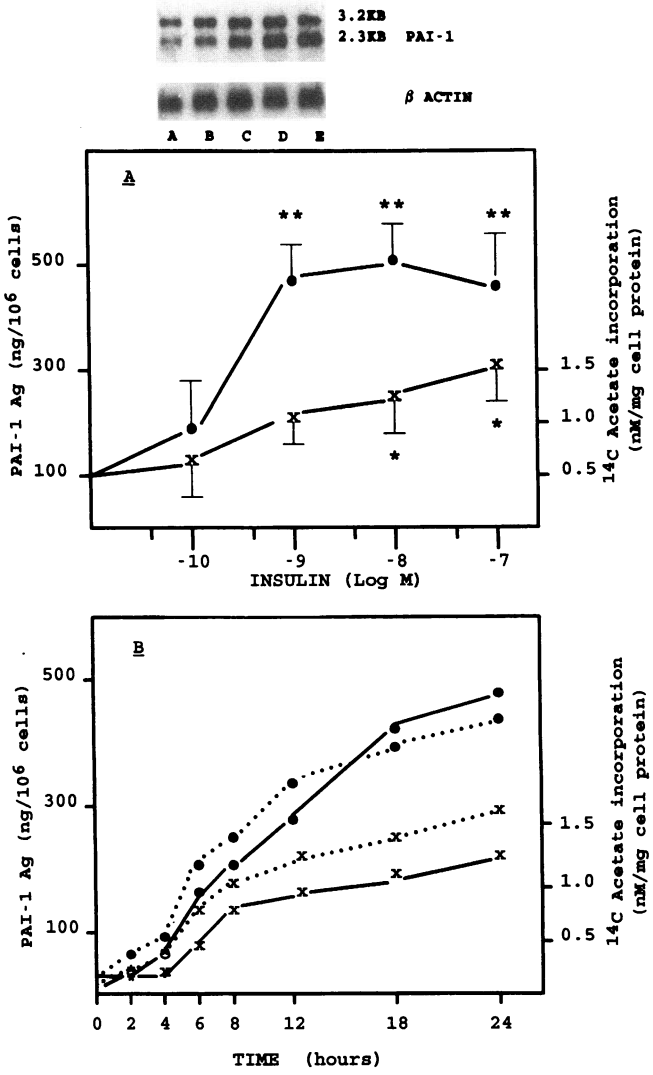

Figure 1. Dose- and time-dependent effects of insulin on PAI-1 secretion (●) and [^{14}C]acetate incorporation (×) in Hep G2 cells. (*A*) Dose-dependent effects of a 24-h insulin stimulation on PAI-1 secretion (●) and [^{14}C]acetate incorporation (x). Results represent means±SE of five experiments performed in triplicate: $*P < 0.05$, $**P < 0.01$. *Inset:* Effects of increasing doses of insulin on PAI-1 and β-actin mRNA expression in Hep G2 cells. Lane *A*, control cells; lane *B*, 10^{-10} M insulin; lane *C*, 10^{-9} M insulin; lane *D*, 10^{-8} M insulin; lane *E*, 10^{-7} M insulin. (*B*) Time-dependent effects of insulin on PAI-1 secretion (●) and [^{14}C]acetate incorporation (x). Hep G2 cells were stimulated up to 24 h by 10^{-9} M (—) or 10^{-7} M (· · ·) insulin. Results represent means of two experiments performed in duplicate.

[^{14}C]acetate incorporation (Fig. 2*B*): it resulted in a 2.4-fold decrease compared with the level obtained with 10^{-9} M insulin in control cells (0.5±0.2 vs. 1.18±0.09 nM/mg protein [$P < 0.05$]) and a 1.4-fold decrease compared with unstimulated control cells (0.51±0.04 vs. 0.69±0.09 nM/mg protein [$P < 0.05$]). Down-regulated cells kept for a further 24-h incubation in serum-starved medium expressed [^{14}C]acetate incorporation identical to control unstimulated cells (0.76±0.1 vs. 0.69±0.09).

The preincubation time required to induce hypersecretion of PAI-1 and to decrease [^{14}C]acetate incorporation was then

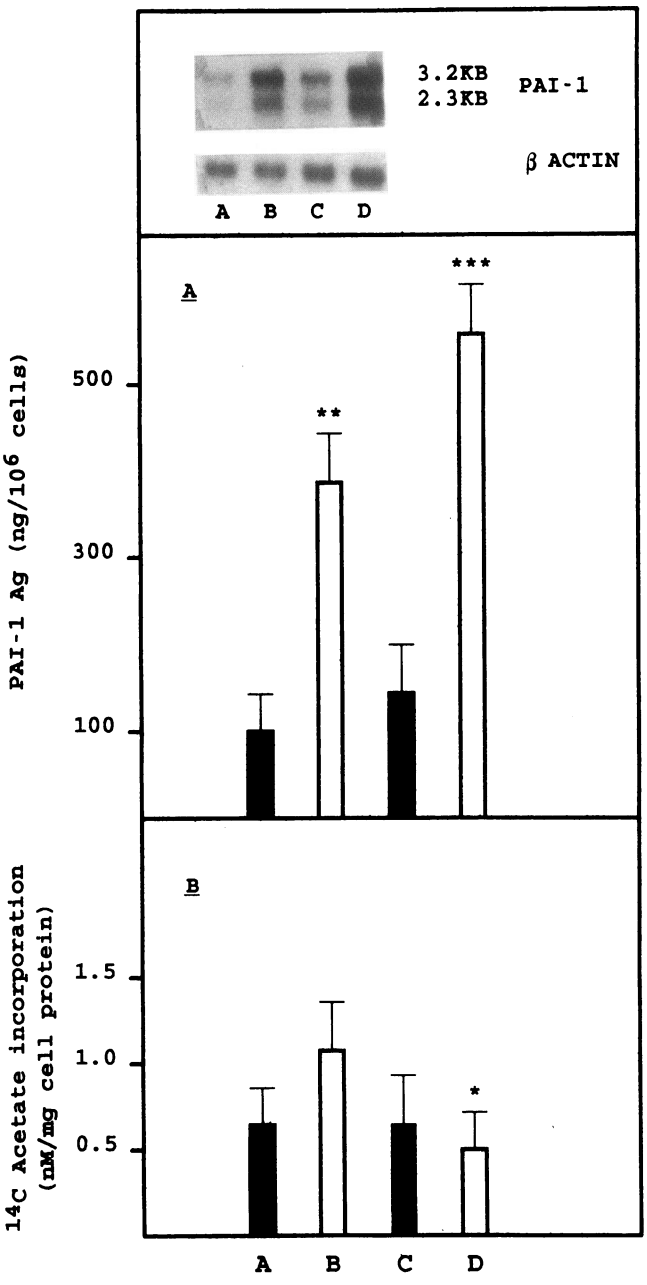

Figure 2. Effects of 10^{-9} M insulin stimulation for 24 h on PAI-1 secretion (*A*), [^{14}C]acetate incorporation (*B*), and PAI-1 and β-actin mRNA expression (*inset*) in control and down-regulated Hep G2 cells. Lane *A*, control unstimulated cells; lane *B*, control cells stimulated by 10^{-9} M insulin; lane *C*, down-regulated cells washed and kept in serum-starved medium for 24 h; lane *D*, down-regulated cells washed and stimulated for 24 h by 10^{-9} M insulin. Results in panels *A* and *B* represent means±SE of five experiments performed in triplicate: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

investigated. Hep G2 cells were preincubated for different times, up to 18 h, with 10^{-7} M insulin and after acid wash, they were stimulated for 24 h with 10^{-9} M insulin. Kinetic studies (Fig. 3) reveal that PAI-1 secretion began to gradually decrease in down-regulated cells during the first 4 h of preincubation and was then progressively enhanced when the preincubation time was increased from 4 to 18 h. In contrast to PAI-1 secretion, a 4 h preincubation with 10^{-7} M insulin was effective in decreasing [^{14}C]acetate incorporation, and this level remained unchanged irrespective of the time used for the preincubation.

Effects of metformin on insulin-mediated PAI-1 synthesis and acetate incorporation

Control cells. The effects of increasing doses (10^{-6} to 10^{-3} M) of metformin on PAI-1 synthesis mediated by 10^{-9} M insulin during a 24-h stimulation of control cells were first investigated. Metformin exerted a dose-dependent inhibition on the insulin-mediated PAI-1 secretion for doses ranging between 10^{-5} and 10^{-3} M (Fig. 4*A*). When cells were stimulated for increasing times with 10^{-9} M insulin in presence of 10^{-4} M metformin, the inhibitory effect of the drug was observed as soon as the stimulating effect of insulin was detectable, i.e., after a 4-h incubation. From the 8th h, a complete inhibition of insulin-mediated PAI-1 synthesis was obtained (Fig. 4*B*).

In the presence of 10^{-4} M metformin, the stimulating effect of 10^{-10} to 10^{-7} M insulin was abolished, PAI-1 levels being identical to unstimulated control cell levels (Fig. 5). The same results were obtained with 10^{-3} M metformin (data not shown). Metformin alone had no effect on PAI-1 constitutive secretion (Table I) or PAI-1 mRNA expression (Fig. 6, *inset*, lane *A*). The inhibitory effect of metformin on insulin-mediated PAI-1 synthesis was also observed at the mRNA level. Metformin inhibited the insulin-mediated increase in both 3.2- and 2.3-kb transcripts of PAI-1 mRNA. No change in the β actin gene expression could be observed after insulin or insulin plus metformin stimulation (Fig. 6, *inset*, lane *B*).

The effects of 10^{-4} M metformin on [^{14}C]acetate incorporation mediated by 10^{-10} M to 10^{-7} M insulin were studied. As illustrated in Fig. 5, incubation of Hep G2 cells with 10^{-4} M metformin had no significant effect on insulin-mediated [^{14}C]-acetate incorporation irrespective of the insulin concentration studied. Therefore, in Hep G2 cells, metformin was able to suppress the insulin-mediated PAI-1 synthesis but not the insulin-mediated acetate incorporation into lipids.

Down-regulated cells. The effects of metformin on insulin-

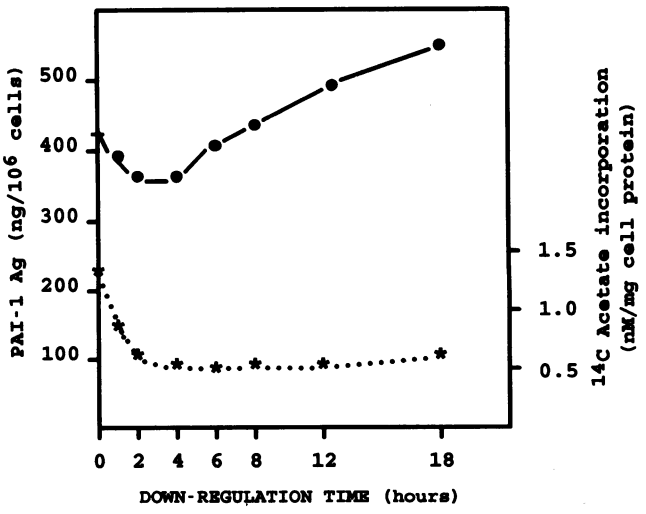

Figure 3. Effects of the down-regulation time on PAI-1 secretion (●) and [^{14}C]acetate incorporation (×). Hep G2 cells were down-regulated for increasing times by 10^{-7} M insulin, and after acid washes, stimulated for 24 h by 10^{-9} M insulin. Results represent means of three experiments performed in duplicate.

mediated PAI-1 synthesis and acetate incorporation in down-regulated cells were then investigated. In the first set of experiments Hep G2 cells were incubated for 18 h with 10^{-7} M insulin in presence of 10^{-4} M metformin, and after washing, were stimulated by 10^{-9} M insulin. Results in Fig. 6 *A* indicated that addition of metformin in the preincubation procedure completely reversed the overproduction of PAI-1 synthesis mediated by 10^{-9} M insulin in down-regulated cells, the levels of PAI-1 secretion being identical to unstimulated control cell levels ($P < 0.001$). In a further set of experiments 10^{-4} M metformin was added to 10^{-9} M insulin in the stimulation step. Results indicated that metformin inhibited the insulin-mediated PAI-1 synthesis, to a similar extent to that obtained

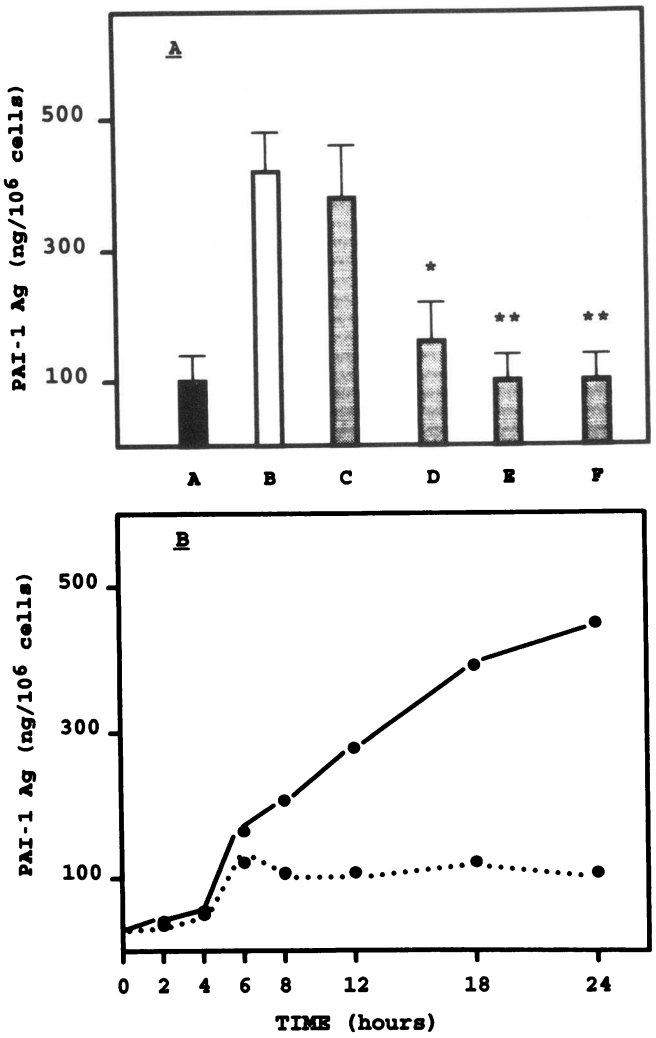

Figure 4. Dose- and time-dependent effects of metformin on insulin-mediated PAI-1 secretion by Hep G2 cells. (*A*) Dose-dependent effects of a 24-h insulin stimulation with increasing doses of metformin (10^{-6} to 10^{-3} M) for 24 h. Lane *A*, control cells; lane *B*, control cells stimulated by 10^{-9} M insulin; lane *C*, control cells stimulated by 10^{-9} M insulin—plus 10^{-6} M metformin; lane *D*, -plus 10^{-5} M metformin; lane *E*, -plus 10^{-4} M metformin; lane *F*, -plus 10^{-3} M metformin. Results represent means±SE of three experiments performed in triplicate: $*P < 0.05$; $**P < 0.01$. (*B*) Time-dependent effects of metformin on insulin-mediated PAI-1 synthesis. Hep G2 cells were incubated with 10^{-9} M insulin in the absence (———) or in presence (· · ·) of 10^{-4} M metformin for up to 24 h. Results represent means of two experiments performed in duplicate.

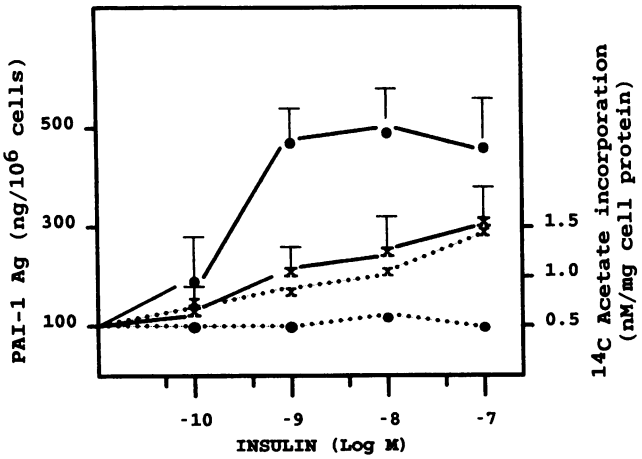

Figure 5. Effects of 10^{-4} M metformin on PAI-1 secretion and [^{14}C]acetate incorporation mediated by increasing doses of insulin in Hep G2 cells. PAI-1 secretion after stimulation with insulin alone (●—●) or with insulin plus 10^{-4} M metformin (●· · ·●). [^{14}C]Acetate incorporation after stimulation with insulin alone (x—x) or with insulin plus 10^{-4} M metformin (x· · ·x). Results represent means±SE of five experiments performed in triplicate.

by addition of metformin in the preincubation step. The suppressive effect of metformin on the insulin-mediated PAI-1 overproduction resulted from an inhibition of the expression of PAI-1 mRNA (Fig. 6, *inset*, lane *C*), without change in the β-actin gene expression.

The effect of 10^{-4} M metformin on incorporation of [^{14}C]-acetate into lipids was tested using the same conditions of culture. Results in Fig. 6 *B* indicate that the addition of metformin to insulin did not modify the insulin-mediated acetate incorporation in down-regulated cells irrespective of the time that the drug was added.

Specificity of metformin effects for insulin-mediated PAI-1 synthesis

To test whether metformin could inhibit PAI-1 synthesis mediated by other agents known to regulate PAI-1 synthesis, we tested the effect of metformin on PMA stimulation. Addition

Table I. Effects of Metformin on PAI-1 Synthesis

Cells	Stimulating agents	Metformin	PAI-1 Ag
			ng/10⁶ cells
Hep G2	—	—	100.6±18.3
	—	10^{-3} M	127.9±15.3
	—	10^{-4} M	113.3±17.2
	—	10^{-5} M	107.5±8.9
	—	10^{-6} M	105.8±12.4
	PMA 10^{-7} M	—	1446.6±186.3
	PMA 10^{-7} M	10^{-4} M	1451.6±201.4
	Insulin 10^{-9} M	—	408.4±39.3
	Insulin 10^{-9} M	10^{-4} M	110.8±17.4
HUVEC	—	—	908.3±10.2
	IL-1 10 IU/ml	—	3537.5±408.4
	IL-1 10 IU/ml	10^{-4} M	3462.5±393.7

Metformin was added alone or in combination with the different stimulating agents. Incubation was performed for 24 h and PAI-1 Ag was assayed in CM. Results represent means of three experiments±SE.

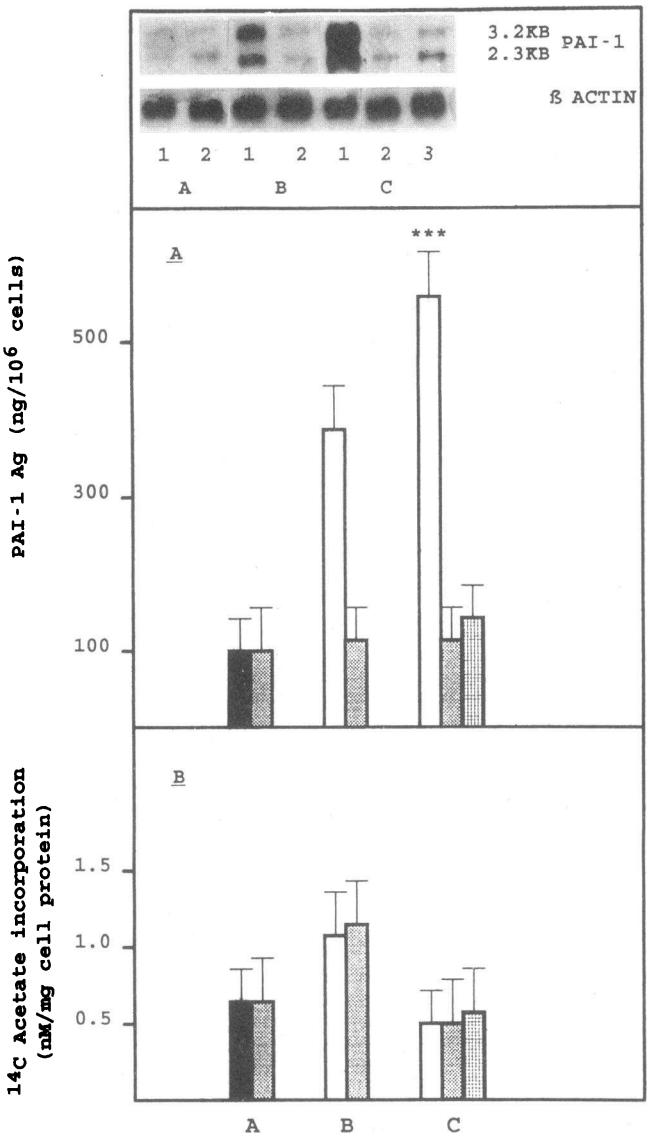

Figure 6. Effects of 10^{-4} M metformin on PAI-1 secretion (*A*), [^{14}C]acetate incorporation (*B*), PAI-1 and β-actin mRNA expression (*inset*) mediated by 10^{-9} M insulin in control and down-regulated Hep G2 cells. Lane *A*, control cells: (*black bar*) lane *1*, without metformin; (*gray bar*) lane *2*, with 10^{-4} M metformin. Lane *B*, control cells stimulated by 10^{-9} M insulin: (*open bar*) lane *1*, without metformin; (*gray bar*) lane *2*, with 10^{-4} M metformin. Lane *C*, down-regulated cells stimulated by 10^{-9} M insulin: (*open bar*) lane *1*, without metformin; (*gray bar*) lane *2*, with 10^{-4} M metformin added in the preincubation step; (*dotted bars*) lane *3*, with 10^{-4} M metformin added in the stimulation step. Results represent means±SE of four experiments performed in triplicate.

of 10^{-4} M metformin to 10^{-7} M PMA did not modify the PMA-induced PAI-1 accumulation in a 24-h CM (Table I). The effect of metformin on IL-1–induced PAI-1 secretion by HUVEC was also tested. HUVEC were incubated for 24 h with IL-1 (10 UI/ml) in presence and absence of metformin 10^{-4} M). Results in Table I indicated that, at this dose, Metformin did not modify the IL-1–induced PAI-1 secretion.

Residual binding of ^{125}I-insulin on Hep G2 cells

Changes in insulin responsiveness in the presence of metformin could result from differences in cell surface insulin binding. We studied the expression of insulin receptors by the binding of tracer amounts (0.2 ng) of ^{125}I-insulin on Hep G2 cells after incubation with insulin and/or metformin. Results are presented in Fig. 7. Stimulation of Hep G2 cells with insulin 10^{-10} to 10^{-7} M for 24 h resulted in a dose-dependent loss of cell surface receptors compared to unstimulated control cells taken as 100%. Stimulation of cells by 10^{-9} M insulin, led to a 40% decrease and stimulation by 10^{-7} M insulin led to a 81% decrease in residual insulin binding.

The effect of insulin stimulation on down-regulated cells resulted, as expected, in a more marked decrease of residual insulin binding: 10^{-9} M insulin added to down-regulated cells induced a decrease of 70% compared to unstimulated control cells taken as 100%.

Addition of 10^{-4} M metformin to unstimulated control cells and to insulin-stimulated control cells had no significant effect on the ^{125}I-insulin binding when compared to cells preincubated without the drug. In the down-regulation procedure, 10^{-4} M metformin exerted no significant effect on the ^{125}I residual insulin binding irrespective of the time at which the drug was added to insulin, i.e., during the preincubation or the stimulation step. These results showed that insulin stimulation induced a loss in cell surface receptors and addition of metformin displayed no significant effect on the expression of insulin receptors.

Discussion

Resistance to insulin-mediated glucose disposal is a well-known phenomenon associated with obesity (7). The syndrome of insulin resistance is characterized by a compensatory hyperinsulinemia, hypertriglyceridemia, low plasma high density lipoprotein cholesterol concentration, and high blood pressure. These features usually cluster in the same individual and have been identified as increasing the risk of coronary heart

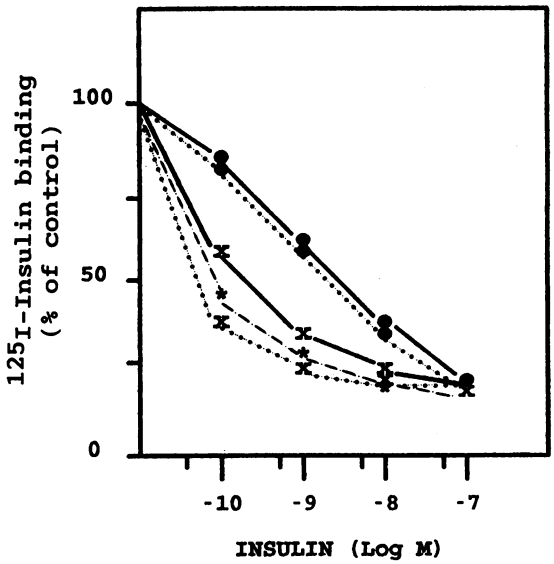

Figure 7. ^{125}I-insulin residual binding in control (●) and down-regulated Hep G2 cells (x) stimulated by increasing concentrations of insulin. Binding in control unstimulated cells was taken as 100%: (—) without metformin; (· · ·) with 10^{-4} M metformin (added in the preincubation step for down-regulated cells); (- - -) with 10^{-4} M metformin added in the stimulation step.

disease (8). High plasma PAI-1 activity has been shown in a cohort of young survivors of myocardial infarction to be an independent risk marker for reinfarction (39) and is associated with insulin resistance (9). Clear relationships have been observed among insulinemia, serum triglycerides, and PAI-1 (40). Interventional measures including weight loss and metformin administration result in a reduction of both insulin resistance and plasma PAI-1 levels (15).

Hepatic insulin resistance is found in obesity and in non–insulin-dependent diabetic subjects (41). Cellular sensitivity to insulin is dependent on the level of functional insulin receptors on the cell surface, and a well-established inverse relationship exists between ambient insulin levels and the number of cell surface insulin receptors (18, 20, 21). Our purpose was to reproduce a hepatic insulin-resistant state by decreasing insulin receptor numbers in the human hepatoma cell line Hep G2, which is known to synthesize PAI-1 in response to insulin (26). Hep G2 cell receptors were down-regulated by an exposure to a high concentration of insulin. It has been shown that exposure of Hep G2 cells to 10^{-7} M insulin for 18 h was efficient to allow internalization of the insulin receptor (25) and to selectively and markedly down-regulate the steady-state level of its mRNA (42, 43). Our results from the residual ^{125}I-insulin trace amount binding as shown in Fig. 7 are in agreement with these data. The residual binding is inversely proportional to both the time and concentration of insulin to which the cells are exposed.

Insulin is a potent stimulator of fatty acid synthesis and lipogenesis (30). Physiological concentrations of insulin have been shown to increase the cellular content of triacylglycerol in hepatocytes from normal and diabetic rats (44, 45). We first confirmed that insulin stimulated PAI-1 synthesis in a dose- and time-dependent manner. The maximal effect was obtained for a concentration of insulin similar to that found in the portal vein after a meal (46). We also confirmed that the stimulation of [^{14}C]acetate incorporation into lipid extract by insulin was dose and time dependent. Nevertheless, the cells were less sensitive to the insulin stimulation as far as lipid synthesis is concerned.

Comparisons between insulin residual binding and acetate incorporation indicated that down-regulated cells expressed low insulin receptor numbers and consequently, [^{14}C]acetate incorporation in response to an insulin stimulation was markedly reduced. These data are in agreement with the development of an insulin-resistant state induced by the down-regulation of the insulin receptors, leading to a reduced responsiveness of the cells to insulin. In contrast to the above-mentioned data, down-regulation of Hep G2 cells did not induce a decrease in the insulin-mediated PAI-1 synthesis in spite of the loss of surface receptors. On the contrary, PAI-1 secretion was increased above the secretion level observed in stimulated non–down-regulated cells. Kinetic analysis showed that the overexpression of insulin-mediated PAI-1 synthesis in down-regulated cells was observed for down-regulation times longer than 4 h. This effect was dependent on the time of down-regulation. This over production of PAI-1 resulted from an insulin-mediated stimulation of PAI-1 mRNA steady state levels. The mechanism leading to this increased synthesis remains unknown.

Insulin resistance is usually expressed in terms of reduced glucose transport (8, 47) associated with other post receptor defects (48, 49) including a loss of tyrosine kinase activity which is important for transducing the biological effects of insulin (50–52). Down-regulated Hep G2 cells expressed multifunctional defects including an increased proportion of tyrosine kinase incompetent insulin receptors leading to a defect of insulin–receptor complex internalization (53). Although there is a general agreement that insulin receptor tyrosine kinase activity mediates many of the actions of insulin, the stimulation of this enzyme may not be a necessary step for all insulin signaling pathways. Some studies now suggest that a non–tyrosine kinase-dependent pathway may also exist (54, 55). The discrepancy between the regulations of lipid and PAI-1 synthesis in our model may be relevant for multiple signal transduction pathways that would explain the biological actions of insulin. Nevertheless, a direct relationship between PAI-1 synthesis and non–tyrosine-dependent pathways remains to be proven.

Metformin has been shown in vivo to lower both basal PAI-1 levels in obese nondiabetic women (15) and postvenous occlusion PAI-1 levels in type II diabetic patients (56). We have previously shown that the decrease in fasting insulinemia was associated with a decrease in plasma PAI-1 (15). Our present study indicates that metformin in therapeutic doses (57) exerts a direct effect on PAI-1 synthesis at the cellular level by suppressing the insulin stimulating effect both in control and down-regulated cells. Metformin added to insulin in down-regulated cells inhibited the over production of PAI-1 synthesis by a direct effect on PAI-1 mRNA expression. Indeed its addition to 10^{-7} M insulin in the preincubation step rendered down-regulated Hep G2 cells unresponsive to a further stimulation by 10^{-9} M insulin, in spite of its elimination from the medium by washings. However, metformin did not display any activity either on the steady-state level of PAI-1 synthesis or on basal [^{14}C]acetate incorporation and residual ^{125}I-insulin binding. The results on ^{125}I-insulin binding in the presence of metformin are in agreement with previous reports indicating that metformin modified neither the insulin binding nor the affinity of insulin receptors (58). The action on lipogenesis by metformin remains unclear. Melin et al. (59) reported a restoration of lipogenesis by metformin in rat insulin-resistant hepatocytes but such differences between our respective results could be explained by the use of different cells and protocols.

The intracellular site of action of metformin presently remains unknown. Usually metformin restores the biological effect of insulin by enhancing the substrate uptake (58). In contrast, in our study, metformin decreased the paradoxical effect of insulin on PAI-1 synthesis to normal levels. Our results clearly indicate that metformin may modify some aspects of the action of insulin in Hep G2 cells but not by increasing the binding of insulin to its receptor. Similarly metformin has been shown to have no effect on insulin-mediated amino butyrate incorporation in rat hepatoma cells whereas it enhanced the insulin-stimulated glucose incorporation into glycogen (60). The data thus suggest that several effects of insulin are mediated by several postreceptor mechanisms and that only some of these were modified by metformin. Metformin exerts several post receptor effects (61, 62) including an action on the restoration of tyrosine kinase activity (63), an improvement of glucose transport, and potentiation of GLUT 4 translocation (64, 65). This latter action is unlikely in our study because Hep G2 cells do not express GLUT 4 transporter (66).

In conclusion, the present work demonstrates the ability of insulin to induce an upregulation of PAI-1 in down-regulated cells. These findings are related to in vivo data showing that in

insulin-resistant patients PAI-1 plasma levels are largely increased (9) whereas insulin receptor numbers are decreased on insulin target cells (48). The results also indicate that metformin acts at a cellular level by inhibiting the insulin-mediated PAI-1 synthesis. This effect could be responsible for the decreased PAI-1 plasma levels observed in vivo after metformin therapy (15). Although extrapolation to clinical circumstances should be done with caution, our results are consistent with the hypothesis that Hep G2 cells which mimic human hepatocytes (24) behave as insulin-resistant cells in terms of PAI-1 synthesis.

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