Original Articles

Transcriptional Regulation of Plasminogen Activator Inhibitor Type 1 Gene by Insulin

Insights Into the Signaling Pathway

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Impairment of the fibrinolytic system, caused primarily by increases in the plasma levels of plasminogen activator inhibitor (PAI) type 1, are frequently found in diabetes and the insulin-resistance syndrome. Among the factors responsible for the increases of PAI-1, insulin has recently attracted attention. In this study, we analyzed the effects of insulin on PAI-1 biosynthesis in HepG2 cells, paying particular attention to the signaling network evoked by this hormone. Experiments performed in CHO cells overexpressing the insulin receptor indicate that insulin increases PAI-1 gene transcription through interaction with its receptor. By using inhibitors of the different signaling pathways evoked by insulin-receptor binding, it has been shown that the biosynthesis of PAI-1 is due to phosphatidylinositol (PI) 3-kinase activation, followed by protein kinase C and ultimately by mitogen-activated protein (MAP) kinase activation and extracellular signal–regulated kinase 2 phosphorylation. We also showed that this pathway is Ras-independent. Transfection of HepG2 cells with several truncations of the PAI-1 promoter coupled to a CAT gene allowed us to recognize two major response elements located in the regions between −804 and −708 and between −211 and −54. Electrophoretic mobility shift assay identified three binding sites for insulin-induced factors, all colocalized with putative Sp1 binding sites. Using supershifting antibodies, the binding of Sp1 could only be confirmed at the binding site located just upstream from the transcription start site of the PAI-1 promoter. A construct comprising four tandem repeat copies of the −93/−62 region of the PAI-1 promoter linked to CAT was transcriptionally activated in HepG2 cells by insulin. These results outline the central role of MAP kinase activation in the regulation of PAI-1 induced by insulin. ***Diabetes* 50:1522–1530, 2001**

Insulin is the most important hormone regulating energy metabolism. Since its discovery, insulin has been subjected to extensive research to elucidate its activity in a variety of metabolic processes, including glucose homeostasis, substrate metabolism, gene transcription, protein synthesis, and protein degradation (1). A considerable body of evidence supports the hypothesis that hyperinsulinemia is a risk factor for the development of macrovascular disease, manifesting mostly as coronary artery disease (2–4). Hyperinsulinemia has been associated with coronary heart disease in nondiabetic people (5). Although many mechanisms are responsible, one factor implicated is impaired fibrinolysis, secondary to overexpression of the primary physiological inhibitor of plasminogen activator, plasminogen activator inhibitor (PAI) type 1 (6). Plasma PAI-1 activity and concentration parallel the extent of hyperinsulinemia in several metabolic conditions, and insulin has been implicated as the major physiological regulator of PAI-1 (7). PAI-1 in blood may derive from endothelial cells, adipocytes, and hepatocytes. A direct effect of insulin in stimulating PAI-1 production has been reported in various cell types (8–10). Moreover, insulin has been shown to induce PAI-1 secretion in human hepatocytes in vitro as well as in human umbilical vein endothelial cells genotyped by a *Hin*dIII restriction fragment–length polymorphism (11).

The signal transduction pathway evoked by insulin has recently been partially disentangled (12). However, no information is yet available that identifies the signaling network as well as the transcriptional machinery evoked by insulin in the induction of PAI-1 biosynthesis. In this study, we have investigated the receptor-mediated mechanisms involved in insulin activation of the PAI-1 gene. The data indicate that PAI-1 induction is the result of phosphatidylinositol (PI) 3-kinase and protein kinase (PK)-C, whereas Ras does not play an obligatory role in this pathway. Mitogen-activated protein (MAP) kinase activation and extracellular signal–regulated kinase (ERK) 2 phosphorylation (a downstream event of PKC stimulation) result in activation of nuclear transcription fac-

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ACF, N-acetyl-S-*trans,trans*-farnesyl-L-cysteine; ELISA, enzyme-linked immunosorbent assay; EMSA, electromobility shift assay; ERK, extracellular signal–regulated kinase; FCS, fetal calf serum; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; IRS, insulin receptor substrate; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MAP, mitogen-activated protein; MEK, MAP/ERK; MEM, minimal essential medium; PAI, plasminogen activator inhibitor; PD98059, 2′-amino-3′-methoxyflavone; PI, phosphatidylinositol; PK, protein kinase; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride.

tors—of which Sp1 is the most likely candidate—leading to PAI-1 gene induction.

RESEARCH DESIGN AND METHODS

Materials. Minimal essential medium (MEM) and fetal calf serum (FCS) were from Gibco, Life Technologies (Milan, Italy). Mycoplasma detection kit and anti-mouse IgG–horseradish-peroxidase antibody were from Boehringer Mannheim (Mannheim, Germany). Plastic ware for cell culture was from Costar (Cambridge, MA). F1–5 enzyme-linked immunosorbent assay (ELISA) for PAI-1 antigen detection was from Monozyme (Copenhagen). [γ-^{32}P]ATP (specific activity 3,000 Ci/mmol) and the ECL detection system were from Amersham (Milan, Italy). Rabbit polyclonal antibody against phosphospecific p44/p42 MAPK was from New England Biolabs, Celbio (Milan, Italy). Antibody anti–AP-1 (sc-44x) and anti-Sp1 (sc-59x), which is not reactive to Sp2 or Sp3, were from Santa Cruz Biotechnology. Other reagents were from Sigma Chemical (St. Louis, MO). Dominant-negative p21^{ras} mutant plasmid, p21(Asn-17)^{Hras}, was kindly provided by Dr. A. Maggi (Milano Molecular Pharmacology Laboratory, University of Milan, Milan, Italy).

Chemicals. 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), genistein, phenylmethylsulfonyl fluoride (PMSF), and phorbol 12-myristate 13-acetate (PMA), manumycin A, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), and N-acetyl-S-*trans*,*trans*-farnesyl-L-cysteine (ACF) were from Sigma; rapamycin and calphostin C were from Biomol Research Laboratories (Plymouth Meeting, PA); and 2′-amino-3′-methoxyflavone (PD98059) was from New England Biolabs (Beverly, MA). Simvastatin in lactone form was from Merck, Sharp & Dohme Research Laboratories (Woodbridge, NJ). H7 was dissolved in sterile distilled water, and all other chemicals were dissolved in DMSO or ethanol.

Cell culture. HepG2 cells were cultured as previously described (13). The cell line was found free of mycoplasma infection. For the experiments, cells were plated at 1.5×10^4 in 12-well plates and used at subconfluency after a 24-h preincubation in serum-free medium. Cells were incubated in the presence or absence of insulin with appropriate chemicals or vehicle additions (DMSO or ethanol, 0.1% vol/vol). Both agents (DMSO and ethanol) did not influence biochemical response or PAI-1 by HepG2 cells and did not induce cytotoxicity, as judged by morphology and trypan blue exclusion.

Transfection of insulin receptor and selection of cell lines. Stable transfected CHO were provided by Dr. Eric Clauser (Institut National de la Santé et de la Recherche Médicale [INSERM] U36, Chaire de Médecine Expérimentale, Collège de France, Paris). The stable transfection of CHO cells with the wild-type human insulin receptor cDNA (TBF4) and the selection of clones were achieved by the double-transfection technique with a neomycin-resistant plasmid (pSV2neo) and by selection with the neomycin analog G418, as previously described (14).

Transfection assay. HepG2 cells were transfected using a calcium phosphate precipitation method as described by Sambrook et al. (15). pRSV-galactosidase control vector (Promega) was cotransfected as an internal control. The construction of the PAI-1 CAT plasmids has been described elsewhere (16). The 4G-PAI-pCAT construct comprises the human PAI-1 sequences −804 to 17. The truncated promoter constructs −708-PAI-pCAT, −609-PAI-pCAT, −502-PAI-pCAT, −400-PAI-pCAT, −302-PAI-pCAT, −211-PAI-pCAT, −103-PAI-pCAT, and −54-PAI-pCAT were constructed from the 4G-PAI-pCAT as previously described (16).

The (HIV)-CAT, 1xIRE-HCAT, and 4xIRE-HCAT vectors were constructed using a set of the double-stranded oligonucleotides 5′GATCCCCAGAGCC-AGTGAGTGGGTGGGGCTGGAACA (upper strand) and 5′-GATCTGTTCCA-GCCCCACCCACTCACTGGCTCTGGG (lower strand) constituting the −93/−62 region of the PAI-1 promoter flanked by *Bam*HI and *Bgl*II ends. One or four double-stranded oligonucleotides were ligated head-to-tail into *Bam*HI-restricted HIV-CAT vector (17). The correct sequence and orientation of the inserts was tested by DNA sequencing. CAT was assayed by ELISA with a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions. β-galactosidase activity was assayed as previously described (15).

Quantification of PAI-1 antigen. The concentration of PAI-1 in conditioned medium of HepG2 cells was assayed by specific ELISA (13). The possible interference of compounds with the assay was excluded by experiments in which PAI-1 antigen was determined in medium containing different concentrations of the compounds.

MAP kinase. For MAP kinase activity, HepG2 cells were incubated in MEM containing 0.1% FCS for 48 h, then with medium alone for 24 h, and finally with 100 nmol/l insulin for different times. Cells were washed and scraped in 0.5 ml of homogenization buffer, and MAP kinase activity was determined as previously described (13,18).

MAP kinase immunoblotting was performed with cell lysates prepared in Laemmli's buffer (19) as previously described (13), using an antibody against phospho-specific p44/p42 MAPK, which detects p42 and p44 MAP kinases (ERK1 and ERK2) only when activated by phosphorylation at tyrosine 204 and threonine 202. After incubation with horseradish-peroxidase–conjugated secondary antibody, the blot was developed using the Amersham enhanced chemiluminescence system.

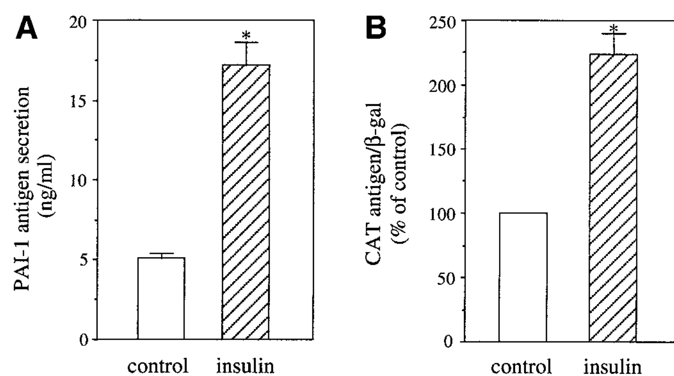

FIG. 1. Effect of insulin on PAI-1 secretion and gene transcription by HepG2. *A*: cells were kept in serum free medium for 24 h and then incubated for 16 h with 100 nmol/l of insulin. PAI-1 antigen levels were determined in cell supernatants. Data are means ± SE of five individual experiments performed in duplicate. *P < 0.01 vs. unstimulated cells. *B*: cells were transiently transfected with 4G-PAI-1-pCAT construct and exposed to 100 nmol/l insulin for 16 h. CAT antigen levels were determined in cell extract by ELISA and normalized for β-galactosidase activity. Values are the means ± SE of eight individual experiments performed in triplicate. *P < 0.01 vs. unstimulated cells.

Electromobility shift assay. Nuclear extracts were prepared essentially as described by Alksnis et al. (20). Protein concentration was determined spectrophotometrically (21). Equal amounts of protein from nuclear extracts (1 μg) were incubated on ice with 2 μg of poly(dI-dC) and 1 μg of acetylated bovine serum albumin in binding buffer (giving the final concentrations stated below) for 10 min (22). The oligonucleotide probe (50,000 cpm in 5 μl) was added, and the reaction mixture (25 μl) was incubated for 25 min at room temperature. Final concentrations in binding reactions were as follows: 10% glycerol and (in mmol/l) 10 HEPES (pH 7.9), 60 KCl, 5 $MgCl_2$, 0.5 EDTA, 1 dithiothreitol, and 1 PMSF. DNA-protein complexes were separated from unbound DNA probe on native 7% polyacrylamide gels (acrylamide:bisacrylamide [wt/wt 80:1] in low ionic-strength buffer containing (in mmol/l) 22.5 Tris, 22.5 borate, and 0.5 EDTA (pH 8) by electrophoresis at 200 V for 2 h. The sequences of the double-stranded oligonucleotide probes (labeled with T4 kinase and [γ-^{32}P]ATP and purified using Pharmacia NICK Sephadex G50 columns) were as follows: 1, GCTTTTACCATGGTAACCCCTGGTCCCGT-TCAGCCAC; 2, TTCAGCCACCACCACCCCACCCAGCACACCTCCAAC; 3, CCTCCAACCTCAGCCAGACAAGGTTGTTGACACAAGAGAGC; 4, CATGC-CTCAGCAAGTCCCAGAGAGGGAGG; 5, CCCAGAGCCAGTGAGTGGGTG-GGGCTGGAAC; 6, GGAACATGAGTTCATCTAT; 7, TCTATTTCCTGCCCA-CATCTG.

For supershift analysis, 1 μg of the relevant antibodies was added after the binding buffer solution, and the incubation time on ice was increased from 10 to 35 min. Unlabeled competitor oligonucleotides were added in 50-fold excess to confirm the specificity of the binding reactions.

Statistical analysis. Data are expressed as means ± SE. Statistical comparison of control with treated groups was performed by ANOVA repeated measures followed by Tukey's test. The accepted level of significance was $P <$ 0.05; n = number of individual experiments performed in duplicate or triplicate, as indicated.

RESULTS

Effect of insulin on PAI-1 biosynthesis. In the present study, insulin was incubated with cells for 16 h, and PAI-1 was determined in conditioned medium. Insulin increased PAI-1 secretion from the cells with maximal effect at 100 nmol/l (Fig. 1*A*); this confirmed previous data (23). Transfection studies performed with a fragment (804 bp) of the PAI-1 promoter coupled to a CAT gene showed that insulin induced transcription of the PAI-1 promoter by 2.2-fold (n = 8, $P <$ 0.01 vs. control) (Fig. 1*B*).

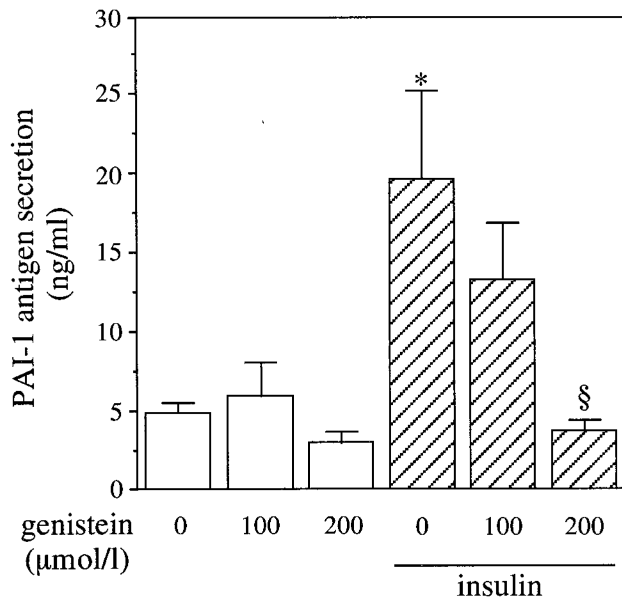

FIG. 2. Effect of the tyrosine phosphorylation suppressor, genistein, on PAI-1 secretion by HepG2. Cells preincubated for 1 h with genistein or vehicle were stimulated for 16 h in the absence (□) or presence (▨) of 100 nmol/l insulin in the continued presence or absence of genistein. PAI-1 antigen levels were determined in cell supernatants. Data are means ± SE of five individual experiments performed in duplicate. $*P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells.

A tyrosine kinase inhibitor, genistein, added to the cells for 1 h before 100 nmol/l insulin inhibited insulin-induced PAI-1 secretion in a concentration-dependent manner, which suggests that tyrosine phosphorylation is required for PAI-1 biosynthesis (Fig. 2). We therefore investigated the possible involvement of the insulin receptor in this process. CHO cells that were stable overexpressing the insulin receptor (TBF4) were transiently transfected with the PAI-1 promoter. Insulin induced a 14-fold increase in PAI-1 transcription in cells overexpressing insulin receptor, whereas in cells stable transfected with the empty vector (pSVNeo) and in normal CHO cells, insulin only doubled PAI-1 transcription (Fig. 3).

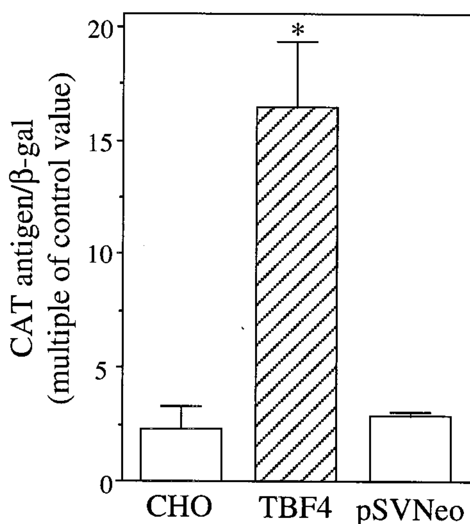

FIG. 3. Role of insulin receptor on PAI-1 secretion in HepG2. Nontransfected CHO and CHO stable transfected with insulin receptor (TBF4) or empty vector (pSVneo) were transiently transfected with 4G-PAI-1-pCAT construct and incubated with 100 nmol/l insulin for 16 h. CAT antigen was then measured in cell lysates and normalized for β-galactosidase activity. Data are expressed as a multiple of control values and are the means ± SE of five experiments. $*P < 0.01$ vs. unstimulated cells.

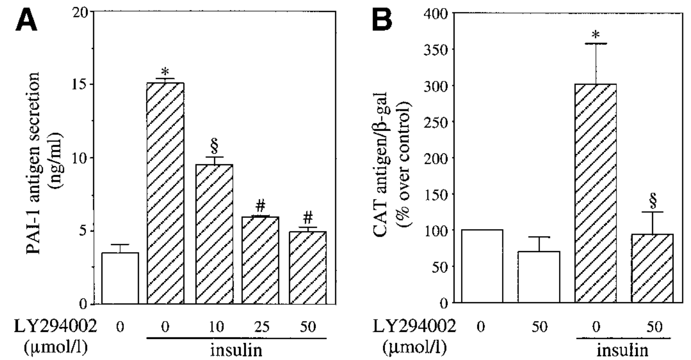

FIG. 4. Effect of the PI 3-kinase inhibitor LY294002 on PAI-1 biosynthesis. *A*: cells were kept in serum-free medium for 24 h, incubated for 1 h with the indicated concentrations of LY294002 or vehicle, and then stimulated for 16 h in the absence (□) or presence (▨) of 100 nmol/l insulin in the continued presence or absence of LY294002. PAI-1 antigen levels were determined in cell supernatants. Each value represents the mean ± SE of four individual experiments performed in duplicate. $*P < 0.01$ vs. unstimulated cells; §$P < 0.05$; #$P < 0.01$ vs. insulin-stimulated cells. *B*: cells were transiently transfected with 4G-PAI-1-pCAT construct, incubated with 50 μmol/l LY294002 for 1 h, and then incubated with 100 nmol/l insulin for 16 h in the continued presence of the inhibitor. CAT antigen was then measured in cell lysates and normalized for β-galactosidase activity. Data are the means ± SE of four experiments performed in triplicate. $*P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells.

The role of the insulin receptor was further supported by experiments in which desensitization of the insulin receptor by prior exposure of HepG2 cells to 100 nmol/l insulin for 24 h led to reduced insulin stimulation (data not shown). These data suggest that insulin increases PAI-1 biosynthesis in HepG2 cells via an interaction with its receptor. The following studies provided insight into the mechanisms participating in the insulin-induced elevation of PAI-1 expression.

Signaling pathways involved in PAI-1 secretion induced by insulin.

Role of PI 3-kinase. PI 3-kinase activation is a critical step in the regulation of the insulin-signaling pathway and glucose uptake (24). We therefore investigated whether insulin would increase PAI-1 through stimulation of PI 3-kinase activation. LY294002, a highly specific PI 3-kinase inhibitor (25), did not influence basal PAI-1 secretion, but it prevented the effect of insulin on secretion and transcription of PAI-1 in a concentration-dependent manner (Fig. 4*A* and *B*).

Role of Ras in insulin regulation of PAI-1. The role of Ras in the insulin regulation of PAI-1 was first investigated by blocking farnesylation of Ras with a farnesyltransferase inhibitor, manumycin A (26). At concentrations up to 5 μmol/l, manumycin A did not affect the ability of insulin to induce PAI-1 secretion; rather, manumycin A significantly increased it (Fig. 5*A*). Moreover, pretreatment of cells with an inhibitor of hydroxymethylglutaryl-CoA-reductase (27) at a concentration known to inhibit farnesylation of Ras proteins (simvastatin 10 μmol/l) did not influence insulin-induced PAI-1 secretion (data not shown). Similar results were obtained with ACF at concentrations known to inhibit Ras carboxyl methylation (10–50 μmol/l) (28) (Fig. 5*B*). PAI-1 transcription was doubled in cells cotrans-

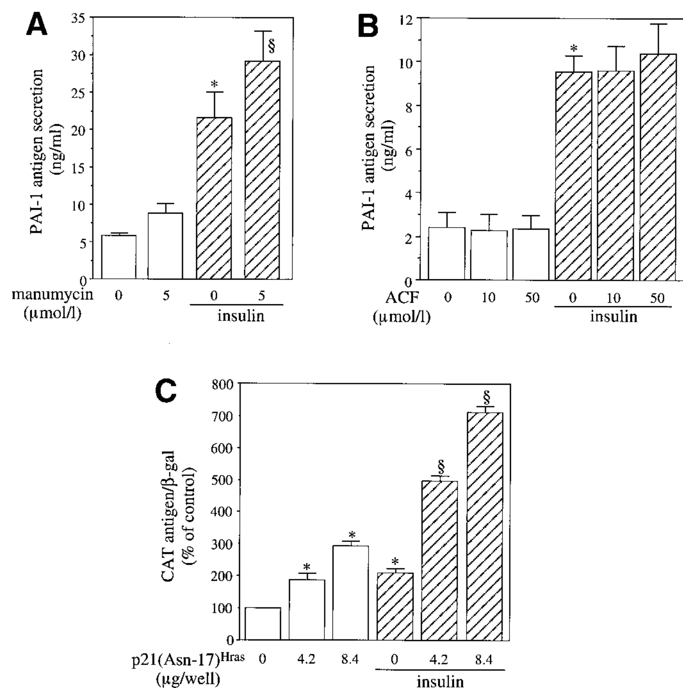

FIG. 5. Role of Ras. HepG2 cells were kept in serum-free medium for 24 h, incubated for 1 h with the indicated concentrations of the farnesylation blocker manumycin A (*A*) and the Ras carboxylmethylation inhibitor ACF (*B*) or vehicle, and then stimulated for 16 h in the absence (□) or presence (▨) of 100 nmol/l insulin in the continued presence or absence of the compounds. PAI-1 antigen levels were determined in cell supernatants. Each value represents the mean ± SE of four individual experiments performed in duplicate. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells. *C*: HepG2 cells were transiently transfected with 4G-PAI-1-pCAT construct in the presence of different concentrations of dominant-negative Ras p21(Asn-17)^{Hras} and empty vector to equalize the amount of plasmid used. The cells were then incubated with 100 nmol/l insulin for 16 h. CAT antigen was measured in cell lysates and normalized for β-galactosidase activity. Data are expressed as the percentage of control and are the means ± SE of five experiments. *$P < 0.01$ vs. control cells; §$P < 0.01$ vs. insulin-stimulated cells.

fected with −804 bp PAI-1 promoter and different concentrations of dominant-negative Ras, p21(Asn-17)^{Hras}, which interferes with the activity of son-of-sevenless (SOS), preventing exchange of GDP for GTP on endogenous Ras and interfering with downstream signaling (29). Insulin further enhanced this increase in PAI-1 transcription (Fig. 5*C*).

Role of S6 kinase in insulin regulation of PAI-1. S6 kinase has been shown to be a downstream target of PI 3-kinase; in several cell types, rapamycin blocks the stimulation of this enzyme by insulin (30). Rapamycin at concentrations up to 10 nmol/l did not reduce insulin-induced secretion or transcription of PAI-1, but rather increased it (Fig. 6, panel A and B). This suggests that PAI-1 biosynthesis by insulin does not involve a rapamycin-sensitive pathway in PAI-1 production.

Insulin-induced PAI-1 release is dependent on PKC. The relationship between PKC activation and PAI-1 secretion was then investigated. The PKC inhibitors H7 (35 μmol/l) and calphostin C (2 μmol/l) completely prevented PAI-1 induction by insulin (Fig. 7*A* and *C*). In additional experiments, HepG2 cells were treated with vehicle or 100 nmol/l PMA for 24 h to downregulate PKC (31). The supernatant was then replaced with serum-free medium or supplemented with 100 nmol/l insulin or 100 nmol/l PMA. In cells preincubated with vehicle, insulin and PMA increased PAI-1 secretion by 217% ± 12 and 700% ± 90 ($n = 4$), respectively. After downregulation of PKC, insulin- and PMA-induced PAI-1 secretion was lowered by 70% ± 10 and 80% ± 9, respectively ($P < 0.01$ vs. control, $n = 4$), further confirming that PKC activation is involved in insulin-induced PAI-1 secretion.

PAI-1 regulation by MAP kinase. Insulin caused MAP kinase activation within 5 min ($P < 0.01$) and a rapid time-dependent phosphorylation of the p42 (ERK2) isoform of MAP kinase (Fig. 8*A*–*C*). Phosphorylation of the ERK2 isoform was reduced in cells preincubated with LY294002 and H7 (Fig. 8*B* and *C*). PD98059, at a concentration known to inhibit MAP/ERK (MEK) kinase >50% (32), inhibited both basal and insulin-stimulated PAI-1 secretion and PAI-1 gene transcription by >75% ($P < 0.01$), which suggests that MAP kinase is necessary for insulin-induced PAI-1 (Fig. 8*D* and *E*).

Mapping of the insulin-responsive element in PAI-1 promoter. To localize the regulatory elements responsible for the induction of PAI-1 transcriptional activation by insulin, HepG2 cells were transfected with several truncations of the promoter coupled to a CAT gene. Successive 100-bp segments were deleted from the initial promoter construct containing 804 bp of the proximal promoter (16). Figure 9 shows schematically the deletions and relative promoter activity of insulin-challenged extracts. The deletion of 100 bp starting from 5′ yielded 60% the activity of the −804 bp probe used as positive control. Further deletions up to −302 bp did not effectively modify PAI-1 transcription, but a further reduction was observed at −103 bp, with minimal promoter activity recovered with the −54 bp probe. We therefore evaluated potential binding regions for any insulin-inducible transcription factors that could mediate transcriptional activation. For this purpose, seven overlapping electromobility shift assay (EMSA) probes were constructed that covered the region from −804 to −708 and from −211 to −54 of the PAI-1 promoter. HepG2 cells were exposed to insulin from 1 to 8 h, and nuclear extracts were prepared. The specificity of the

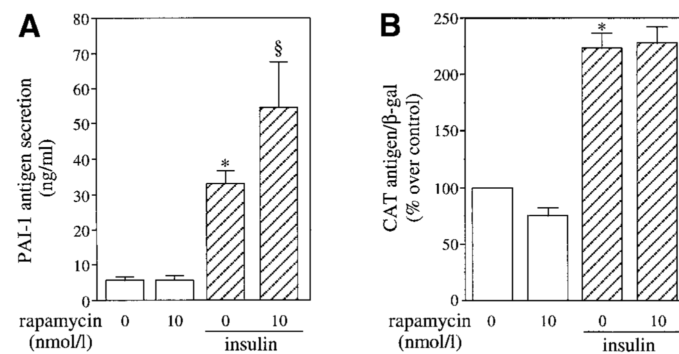

FIG. 6. Effect of the S6 kinase blocker rapamycin on PAI-1 biosynthesis. *A*: HepG2 cells were kept in serum-free medium for 24 h, incubated for 1 h with the indicated concentration of rapamycin or vehicle, and then stimulated for 16 h in the absence (□) or presence (▨) of 100 nmol/l insulin in the continued presence or absence of rapamycin. PAI-1 antigen levels were determined in cell supernatants. Each value represents the mean ± SE of four individual experiments performed in duplicate. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells. *B*: cells were transiently transfected with 4G-PAI-1-pCAT construct, incubated with 10 nmol/l rapamycin for 1 h, and then incubated with 100 nmol/l insulin for 16 h in the continued presence of the inhibitor. CAT antigen was then measured in cell lysates and normalized for β-galactosidase activity. Data are the means ± SE of three experiments performed in triplicate. *$P < 0.01$ vs. unstimulated cells.

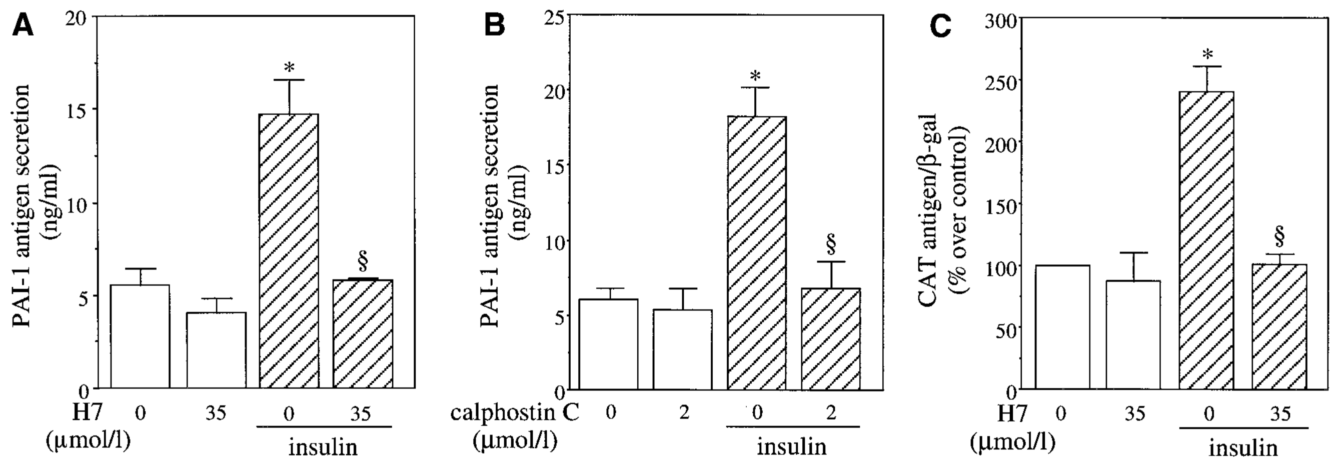

FIG. 7. Effect of the PKC inhibitors H7 and calphostin C on insulin-induced PAI-1 biosynthesis. *A* and *B*: cells preincubated for 1 h with the PKC inhibitors H7 (*A*) and calphostin C (*B*) or with vehicle were stimulated for 16 h in the absence (□) or presence (▨) of 100 nmol/l insulin in the continued presence or absence of the inhibitors. PAI-1 antigen levels were determined in cell supernatants. Data are the means ± SE of five individual experiments performed in duplicate. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells. *C*: cells were transiently transfected with 4G-PAI-1-pCAT construct, incubated with 35 μmol/l H7 for 1 h, and then incubated with 100 nmol/l insulin for 16 h in the continued presence of the inhibitor. CAT antigen was then measured in cell lysates and normalized for β-galactosidase activity. Data are the means ± SE of four experiments performed in triplicate. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells.

binding was analyzed by including unlabeled EMSA probes in excess as competitors. Insulin-induced binding of nuclear proteins was detected with the −777/−741, −157/−128, and −93/−62 probes (Fig. 10).

Because these probes coincide with putative Sp1 response elements, the presence of Sp1 in the insulin-induced complexes was evaluated. Excess unlabeled Sp1 oligonucleotide effectively prevented complex formation only on the −93/−62 construct, and an antibody against Sp1 supershifted the complex (Fig. 10). Jun/Fos antibody did not influence the formation of any complex with the EMSA probes tested. The induction of Sp1 was time-dependent, starting from 4 h and peaking at 8 h (Fig. 10*C*). Interestingly, a consensus double-stranded AP-1 oligonucleotide bound an insulin-induced factor (data not shown).

To determine whether the increased binding of Sp1 to this region was responsible for the increased function of the PAI-1 promoter in response to insulin, experiments with a reporter gene containing one or four repeats of the −93/−62 region were performed. We found a 51% ± 20 and 53% ± 20 ($n = 6$) stimulation of CAT synthesis by insulin in cells transfected with (HIV)-CAT and 1xIRE-HCAT plasmids, respectively. In cells transfected with a 4xIRE-HCAT construct, insulin further increased CAT synthesis (+96% ± 24, $n = 6$; $P < 0.05$ vs. HIV-CAT and 1xIRE-HCAT).

DISCUSSION

High levels of PAI-1 in plasma constitute an independent risk factor for recurrent myocardial infarction and are found elevated in type 2 diabetes, hypertriglyceridemia, and insulin-resistance syndrome. Moreover, the level of fasting insulin correlates positively with PAI-1 concentration (33,34). However, as yet there has been no evidence of a direct effect of insulin on PAI-1 in vivo in humans (35,36). Exploration of the insulin-fibrinolytic axis in vivo is a difficult task, and the negative results may derive from the study design and/or from the fact that because insulin infusion changes several metabolic variables, it is difficult to isolate the effect. Reducing insulin levels and insulin resistance by exercise, weight loss, and drugs such as metformin has, however, been shown to lower PAI-1 activity (37).

Insulin in vitro has been shown to increase PAI-1 antigen and mRNA in several cell systems (8,38), but the mechanism of the relevant signal transduction is as yet unknown. Regulation of gene expression by insulin has been studied for many years, but gaps still remain in our understanding (39).

Insulin was previously shown to increase PAI-1 protein through an effect on PAI-1 mRNA stabilization (40). We show here that insulin increases PAI-1 gene transcription and that this effect requires interaction of the hormone with its receptor. Thus, insulin may operate on two distinct mechanisms to increase the biosynthesis of this antifibrinolytic protein. Downstream of insulin receptor interaction is the activation of PI 3-kinase, which is an essential step for insulin-induced PAI-1 transcription, as we have shown. One protein lying downstream of PI 3-k is pp70 S6 kinase. This protein has been shown to be responsible for insulin-stimulated phosphorylation of the ribosomal protein S6 in vivo (41,42). Rapamycin, which blocks pp70 S6 kinase activation in several cell types, did not reduce insulin-induced PAI-1 transcription by HepG2 cells, which suggests that insulin increases PAI-1 in HepG2 cells via a rapamycin-insensitive pathway. In 3T3-L1 cells, glycogen synthase is inhibited by rapamycin (43), whereas insulin regulation of the same enzyme in PC-12 cells is rapamycin-insensitive (44). The insulin regulation of glycogen synthase kinase-3 (45), gene 33 (46), PEPCK (47), and 6-phospho-fructo-2-kinase (48) have also been shown to be rapamycin-insensitive.

Binding of PI 3-kinase to tyrosine-phosphorylated insulin receptor substrate (IRS) (49) induces its activation, with the concomitant phosphorylation of PI 4-phosphate and PI 4,5-bisphosphate to produce the physiologically significant regulators PI 3,4-diphosphate and PI 3,4,5-trisphosphate. These lipid products activate both protein kinase B and PKC. Binding of phosphoinositide 3,4-bisphosphate and/or phosphoinositide 3,4,5-triphosphate to

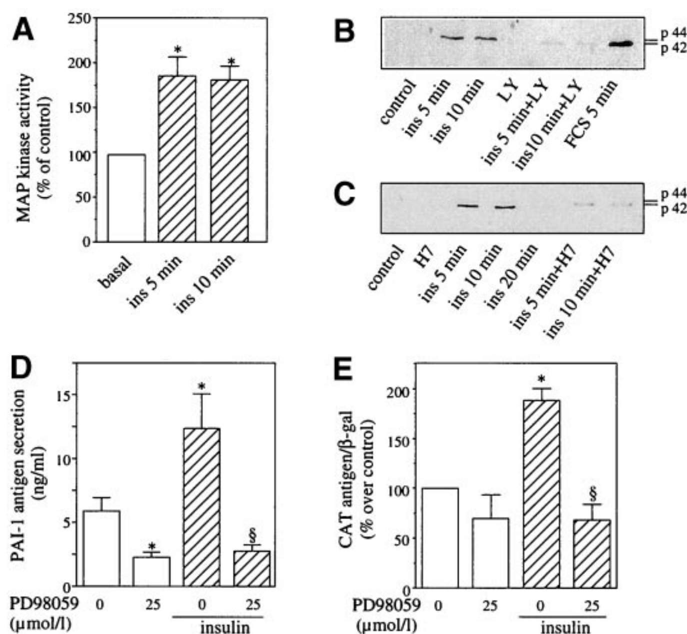

FIG. 8. Effect of insulin on MAP kinase activation in HepG2 cells. *A*: cells were kept in medium containing 0.1% FCS for 48 h and then stimulated with 100 nmol/l insulin for the indicated times. MAP kinase activity was determined in cell lysates. Data are the means ± SE of three individual experiments. *$P < 0.01$ vs. unstimulated cells. *B* and *C*: cells were incubated for 48 h in medium containing 0.1% FCS, incubated for 1 h with 50 μmol/l LY294002 (*B*) and 35 μmol/l H7 (*C*) and then stimulated with 100 nmol/l insulin for the indicated times. Cell lysates (10 μg/lane) were fractionated on a 12% SDS-PAGE followed by immunoblotting with anti-phosphorylated ERK1 and ERK2 antibody. The experiment is representative of three independent experiments. *D*: cells kept in serum-free medium for 24 h were treated with vehicle or with 25 μmol/l PD98059 (inhibitor of MEK) for 1 h, and then they were treated with 100 nmol/l insulin for 16 h in the continued presence of the inhibitor. PAI-1 antigen levels were determined in cell supernatants. Data are the means ± SE of four individual experiments performed in duplicate. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells. *E*: cells were transiently transfected with 4G-PAI-1-pCAT construct, incubated with 25 μmol/l PD98059 for 1 h, and then incubated with 100 nmol/l insulin for 16 h in the continued presence of the inhibitor. CAT antigen was then measured in cell lysates and normalized for β-galactosidase activity. Data are expressed as fold increase over control and are the means ± SE of five experiments. *$P < 0.01$ vs. unstimulated cells; §$P < 0.01$ vs. insulin-stimulated cells. Ins, insulin; LY, LY294002.

different isoforms of PKC (ϵ, η, ζ, and λ) activates them (50,51). In HepG2 cells, inhibition of PKC prevented insulin-induced PAI-1 transcription, which suggests that this process also requires PKC activation, presumably occurring downstream of PI 3-kinase activation.

On the basis of different experimental approaches, the involvement of the Ras signaling pathway in insulin-induced PAI-1 biosynthesis was ruled out. Experiments in HepG2 cells transfected with dominant-negative p21 $(Asn\text{-}17)^{Hras}$ confirm this hypothesis. Thus, insulin-induced PAI-1 transcription probably occurs as the result of PI 3-kinase activated directly via IRS-1 without the need for any additional input by activated Ras. PI 3-kinase activation may thus undergo interaction with PKC, which results in MAP kinase activation and ERK2 phosphorylation. Indeed, inhibition of PKC activation by H7 prevented ERK2 phosphorylation.

Mechanisms have been proposed for Ras-independent activation of MAP kinase (52). It has been reported (53,54) that inhibitors of PI 3-kinase inhibit insulin-induced increases in ERK1/2 activation in many cell types besides hepatocytes, similar to what we have found in our study. Moreover, inhibition of MEK kinase completely prevented insulin-induced PAI-1 transcription, indicating that MAP kinase activation is an obligatory step for this process.

Interestingly, inhibition of PI 3-kinase, PKC, and MAP kinase all resulted in the suppression of the insulin-induced secretion of PAI-1 protein. We have previously shown that with the exception of PI 3-kinase, a similar pathway (13) is responsible for PAI-1 induction by triglyceride-rich lipoproteins, which exert an additive or potentiating effect to that of insulin in inducing PAI-1 (23). Interestingly, it has been shown that when increased simultaneously, three components of the diabetic milieu (hyperglycemia, hyperinsulinemia, and hypertriglyceridemia) augment PAI-1 levels in vivo in healthy individuals (55).

Activated ERKs can translocate to the nucleus and lead to the phosphorylation and activation of transcriptional factors, with subsequent induction of several genes (56). Sequential-deletion reporter constructs of the PAI-1 gene indicated two major responsive regions located between −804 and −708 and between −211 and −54 from the start of the transcription site.

Sequences in the PAI-1 promoter region that are involved in the transcriptional regulation of PAI-1 have been identified previously. These have been shown to bind AP-1–, AP-2–, and Sp1-like proteins (57). Gel-shift experiments using nuclear extracts from HepG2 cells and labeled double-stranded oligomers representing seven regions containing putative binding sites for AP-1 and Sp1 revealed several DNA-protein complexes. Moreover, enhanced binding of nuclear factor was obtained with three of the seven oligonucleotides in cell extracts derived from insulin-treated HepG2 cells. Excess oligonucleotides representing the Sp1 consensus sequence showed competition for nuclear factors binding to the oligonucleotide covering the Sp1 region located at −93/−62 bp only. Supershift experiments confirmed that the Sp1 factor binds to this region. In contrast, the nuclear proteins recognizing oligonucleotides covering regions −777/−741 and −157/−128 could not be identified as Sp1 or AP-1. PMA, serum, and interleukin-1α have been previously reported to induce PAI-1 transcription involving the binding of Jun/Fos to putative AP-1 sites located at −58/−50 bp in PAI-1 promoter in HepG2 cells (57). Surprisingly, we could not identify any

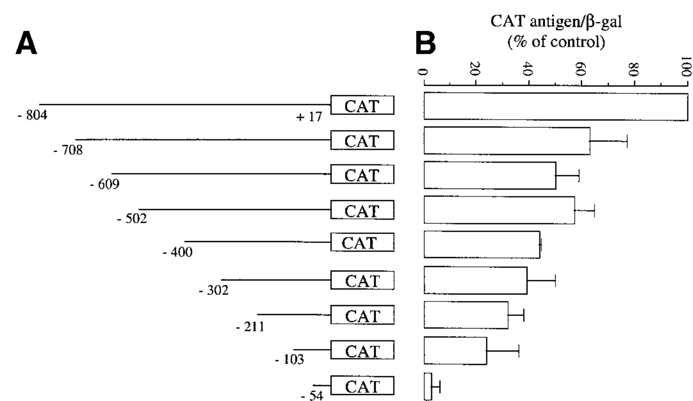

FIG. 9. PAI-1 promoter. *A*: schematic diagram of PAI-1 promoter deletion constructs used in transient transfection assays. *B*: induction of CAT antigen in HepG2 cells transfected with PAI-1 promoter constructs and incubated with 100 nmol/l insulin. Results represent the means ± SE of seven independent experiments performed in triplicate.

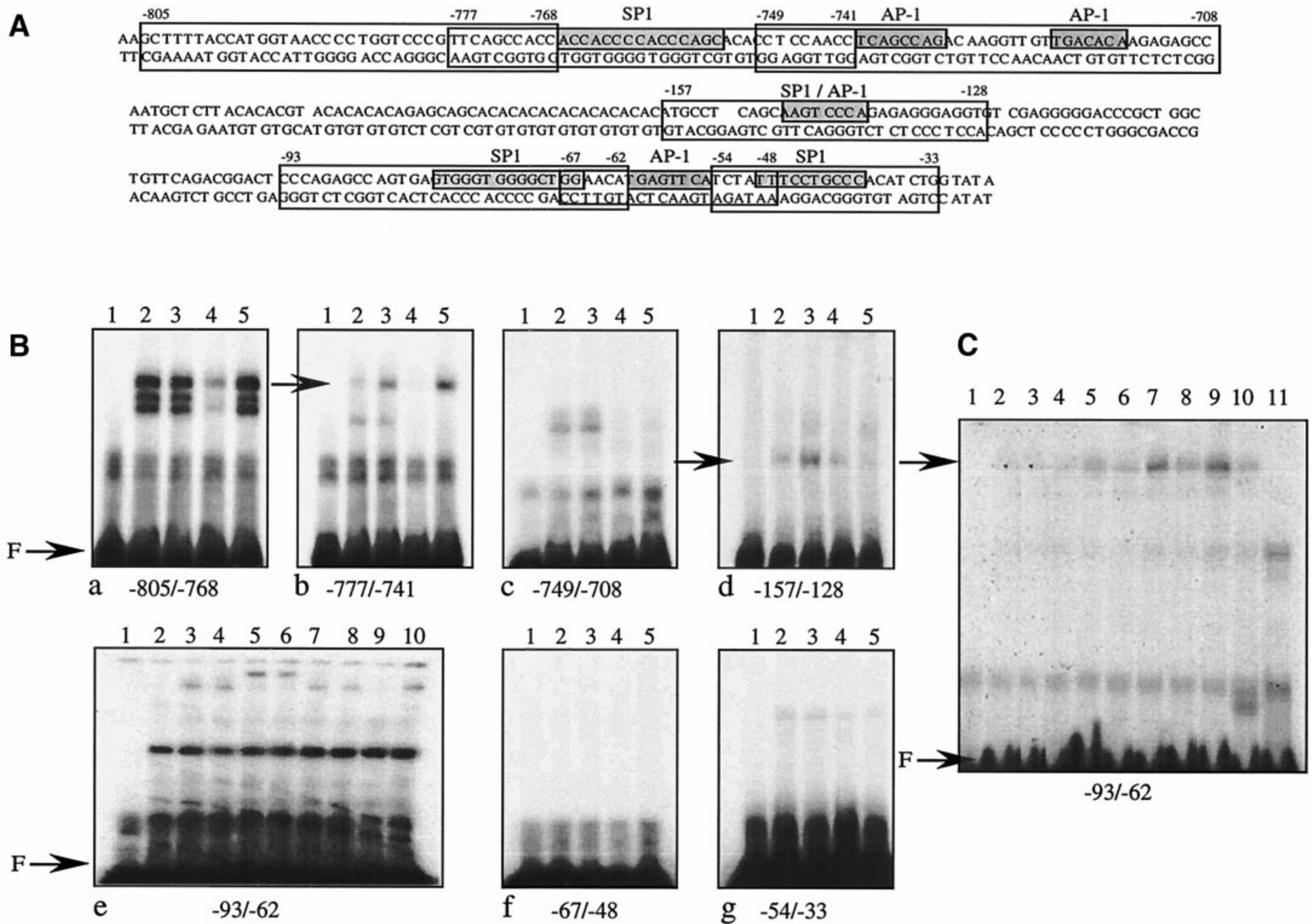

FIG. 10. EMSA. ***A*****: human PAI-1 promoter sequences from −805 to −708, from −207 to −108, and from −107 to −28. Open boxes delineate the different probes and shaded boxes indicate putative nuclear factor binding sites.** ***B*****: EMSA of HepG2 nuclear extracts incubated with insulin for 8 h and bound to the probes −805/−768, −777/−741, −749/−708, −157/−128, −93/−62, −67/−48, and −54/−33. The arrows denote an insulin-induced factor. a, b, c, f, and g:** ***Lane 1*****, no extract;** ***lane 2*****, no insulin;** ***lane 3*****, 100 nmol/l insulin;** ***lane 4*****, 50-fold excess of unlabeled competitor;** ***lane 5*****, excess of unlabeled noncompetitor. d:** ***Lane 1*****, no extract;** ***lane 2*****, no insulin;** ***lane 3*****, 100 nmol/l insulin;** ***lane 4*****, 50-fold excess of unlabeled noncompetitor;** ***lane 5*** **excess of unlabeled competitor. F indicates free DNA. e:** ***Lanes 1-3*****, as above;** ***lane 4*****, anti-Jun/Fos antibody;** ***lane 5*****, anti-Sp1 antibody;** ***lane 6*****, anti-Jun/Fos antibody plus anti-Sp1 antibody;** ***lane 7*****, excess of unlabeled AP-1 oligonucleotide;** ***lane 8*****, excess mutant AP-1 oligonucleotide;** ***lane 9*****, excess Sp1 unlabeled oligonucleotide;** ***lane 10*****, excess unlabeled mutant Sp1 oligonucleotide.** ***C*****: time course effect of insulin on Sp1 binding:** ***lane 1*****, no extract;** ***lane 2*****, no insulin for 2 h;** ***lane 3*****, 100 nmol/l insulin for 2 h;** ***lane 4*****, no insulin for 4 h;** ***lane 5*****, 100 nmol/l insulin for 4 h;** ***lane 6*****, no insulin for 6 h;** ***lane 7*****, 100 nmol/l insulin for 6 h;** ***lane 8*****, no insulin for 8 h;** ***lane 9*****, 100 nmol/l insulin for 8 h;** ***lane 10*****, 50-fold excess of unlabeled noncompetitor;** ***lane 11*****, excess of unlabeled competitor.**

region specifically binding an AP-1 factor with nuclear extracts derived from insulin-treated cells.

Finally, insulin induced transcription was significantly increased in cells transfected with a construct bearing four copies of the −93/−62 region.

Thus, at least one Sp1 nuclear factor binding in the −93/−62 region of PAI-1 promoter may be involved in the upregulation of PAI-1 gene by insulin. Sp1 has been shown to mediate the actions of PKC on several genes. The stimulation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription required an interaction between Sp1 and PKCζ (58). More recently, Sp1 was shown to be essential in the 12-O-tetradecanoyl-phorbol-13-acetate stimulation of human lysosomal acid lipase gene activity in monocytes (59) and in the regulation of apoAI gene expression by PKA and PKC (60).

In conclusion, we have disentangled the signaling pathway evoked by the insulin-receptor interaction responsible for PAI-1 biosynthesis in HepG2 cells and identified PAI-1 promoter sequences required for this effect. We have also identified at least one Sp1 factor that could be involved in this process. Interestingly, high glucose has been shown to increase PAI-1 transcription–inducing Sp1 binding to −85/−65 region of PAI-1 promoter (61,62). Thus, Sp1 may play a pivotal role in mediating the action of both glucose and insulin in inducing PAI-1. In this context it is worth mentioning that hyperglycemia increases PAI-1 in cultured vascular smooth muscle cells, interacting with two Sp1 sites just upstream the transcription start site of PAI-1 (61). These observations may have clinical implications and suggest that a common network of transcription factors may predispose to hypofibrinolysis in hyperinsulinemia as well as in hyperglycemia.

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