Genotype-Specific Transcriptional Regulation of PAI-1 Gene by Insulin, Hypertriglyceridemic VLDL, and Lp(a) in Transfected, Cultured Human Endothelial Cells

Hernan E. Grenett, Raymond L. Benza, Gunther M. Fless, Xin-Nong Li, Glenda C. Davis, Francois M. Booyse

Abstract—Plasminogen activator inhibitor-1 (PAI-1) has been shown to be an independent risk factor for coronary artery disease. Variations in plasma PAI-1 levels have been attributed to variations in the PAI-1 gene, and associations between PAI-1 levels and PAI-1 genotypes suggest that PAI-1 expression may be regulated in a genotype-specific manner by insulin, hypertriglyceridemic (HTG) very low density lipoprotein (VLDL), or lipoprotein(a) [Lp(a)]. Polymerase chain reaction–amplified 1106-bp fragments of the promoter of the 1/1 and 2/2 PAI-1 genotypes were sequenced and showed 5 regions of small nucleotide differences in the 1/1 versus 2/2 PAI-1 promoters that consistently occurred with high frequency. These fragments were ligated into the luciferase reporter gene, and 1/1 and 2/2 PAI-1 genotype human umbilical vein endothelial cell (HUVEC) cultures were transiently transfected with their respective p1PAI110/luc and p2PAI110/luc constructs and vice versa. Insulin induced an ~12- to 16-fold increase in luciferase activity in both the 1/1 and 2/2 PAI-1 genotype HUVEC cultures transfected with the p1PAI110/luc construct. HTG-VLDL and Lp(a) induced luciferase activity by ~14- to 16- and ~8- to 11-fold, respectively, in both the 1/1 and 2/2 PAI-1 genotype HUVEC cultures transfected with the p2PAI110/luc construct. The positive control interleukin-1 showed an ~7- to 12-fold response in the 1/1 and 2/2 PAI-1 genotype HUVEC cultures transfected with either of the constructs. These cross-over results demonstrate that regulation of either the 1/1 or 2/2 PAI-1 genotype by its respective inducer is due to the promoter itself and not to some factor(s) expressed differently in the 1/1 or 2/2 PAI-1 genotype HUVEC cultures. (Arterioscler Thromb Vasc Biol. 1998;18:1803-1809.)

Key Words: plasminogen activator inhibitor-1 ■ transfection ■ insulin ■ hypertriglyceridemic VLDL ■ lipoprotein(a)

Impaired fibrinolysis and hypercoagulability have been suggested as predisposing factors for coronary artery thrombosis, which may have an important role in the pathogenesis of coronary artery disease (CAD) and myocardial infarction (MI). Young survivors of MI have reduced fibrinolytic activity, increased plasminogen activator inhibitor-1 (PAI-1) levels, and an “impaired release” of tissue plasminogen activator, suggesting that reduced fibrinolytic activity, presumably endothelial cell (EC) related, may have pathogenic importance in MI. Certain risk factors for CAD, including hyperinsulinemia and hypertriglyceridemic (HTG) VLDL, have been associated with an increase in plasma PAI-1 antigen and activity levels. Because PAI-1 is generally considered to be a major regulator of fibrinolysis through its interaction with plasminogen activators, it is conceivable that the elevated blood levels of PAI-1 in hyperinsulinemic and hyperlipoproteinemic subjects may in part explain their increased thrombotic risk and CAD prevalence that are unexplained by conventional risk factors. Recent studies have shown that variations in the PAI-1 gene sequence may be closely associated with the regulation of PAI-1 expression and therefore, the increased risk for thrombosis. In addition, several studies have indicated that regulation of PAI-1 at the transcriptional level plays an important role in determining the amount of active PAI-1 in plasma.

Investigation into the regulation of PAI-1 gene expression has confirmed the existence of 3 different polymorphic variations in the human PAI-1 gene: a 4G/5G polymorphism in the promoter region; an allelic variation at a (C-A) n dinucleotide repeat polymorphism in the fourth intron; and an HindIII restriction fragment length polymorphism (RFLP) due to a base change at the 3’ end of the PAI-1 gene. With the use of the HindIII RFLP as a marker for genetic variation in the PAI-1 gene, a higher plasma PAI-1 antigen level was observed in the 1/1 PAI-1 genotype than in the 1/2 and 2/2 genotypes of young post-MI patients and population-based controls. Functional studies utilizing several DNA-mediated gene transfer approaches have localized a number of inducible elements in the PAI-1 gene promoter. Deletion fragments of the PAI-1 gene promoter and the 5’ flanking region have
been used to demonstrate transcriptional regulation of the PAI-1 gene promoter by various inducers in different cell culture systems. These observations have demonstrated that the PAI-1 promoter is precisely regulated by a variety of effector molecules and further suggest that the promoter is highly reactive to various trans-acting pathways. In these studies, we now additionally demonstrate and confirm the genotype-specific transcriptional regulation of the 1/1 and 2/2 PAI-1 genotypes by insulin and HTG-VLDL/lipoprotein(a) [Lp(a)], respectively, in cultured human umbilical vein ECs (HUVECs) transiently transfected with the p1PAI110/luc and p2PAI110/luc constructs, with lipofectamine as the liposome-mediated delivery system.

**Methods**

**Materials**

Collagenase (type I, CLS) was obtained from Boehringer Mannheim Biochemicals; FBS from Intergen Corp; heparin (porcine intestinal mucosa), BSA, and insulin from Sigma Chemical Co; [α-3P]dCTP (3000 Ci/mmol) from Amer sham; KpnI, BglII, HindIII, calf intestinal phosphatase, T4 DNA ligase, GeneLight vector pGL2-basic expression vector, pSV–β-galactosidase, luciferase enzyme assay kit, β-galactosidase enzyme assay system, and agarose from Promega Inc; pfu DNA polymerase from Stratagene; and the Sequenase II kit from UBI. Lipofectamine, Opti-MEM 1 reduced serum medium, and 0.025 mol/L HEPES buffer, pH 7.4; 0.002 mol/L fresh L-glutamine; 100 U/mL penicillin; 100 µg/mL streptomycin; 10% heat-deactivated FCS; 90 µg/mL heparin; and 50 µg/mL partially purified EC growth factor.  

**Cell Culture**

HUVECs derived from fresh (discarded) umbilical cords by mild collagenase treatment were seeded individually into human fibronectin–coated plastic T-25 or Petri dishes (960 mm²) and grown to confluency in complete culture medium consisting of M199 (GIBCO; powder medium containing L-glutamine and Earl's salts); 0.025 mol/L HEPES buffer, pH 7.4; 0.002 mol/L fresh L-glutamine; 100 µM penicillin; 100 µg/mL streptomycin; 10% heat-deactivated FCS; 90 µg/mL heparin; and 50 µg/mL partially purified EC growth factor.  

**DNA Isolation**

Genomic DNA was isolated from human umbilical cords according to Maniatis et al.  In brief, pieces of tissue (300 to 500 mg) were incubated in digestion buffer containing proteinase K (100 µg/mL) in the presence of 0.02 mol/L EDTA and 0.5% SDS for 16 hours at 55°C. Digested samples were then extracted with phenol saturated with 0.5 mol/L Tris-HCl, pH 8.0, and the DNA was recovered by ethanol precipitation. DNA resuspended in 0.01 mol/L Tris-HCl, pH 8.0, and 0.001 mol/L EDTA was then incubated with 1 µg/mL DNase-free RNase for 30 minutes at 37°C, followed by extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation. The DNA was washed once with 75% ethanol and resuspended in a buffer of 0.089 mol/L Tris-HCl, 0.89 mol/L boric acid, and 0.002 mol/L EDTA, pH 8.3.  

**Southern Blot Hybridization and Identification of HindIII Polymorphism**

Genomic DNA (3 µg) was digested with HindIII (3 U/µg DNA) for 16 hours at 37°C and then electrophoresed overnight in 0.7% agarose in a buffer of 0.089 mol/L Tris-HCl, 0.89 mol/L boric acid, and 0.002 mol/L EDTA, pH 8.3.  

**Amplification and Sequencing of the Promoter and 5’ Flanking Regions of the 1/1 and 2/2 PAI-1 Genotypes**

A 1106-bp fragment of the promoter and the 5’ flanking region from different 1/1 and 2/2 PAI-1 individual subjects containing the start site of transcription at +1 as well as the TATA box at –23 to –28 was amplified by polymerase chain reaction (PCR) with pfu DNA polymerase. The PCR was carried out using an upstream primer (5’ CGATCGTGTTTTGGCTACGACCCGC 3’), identical to positions 2193 to 2214, and a downstream primer (5’ CGATCAAGGTGGTGCTGGATTG 3’), complementary to positions 3207 to 3237 of the human PAI-1 gene.  

**Cell Culture**

Each primer was also digested with a GATC clamp and a KpnI site in the upstream primer (underlined) and a BglII site in the downstream primer (underlined) to aid in the subsequent cloning into the luciferase reporter gene (luc, pGL2-basic expression vector; Promega). PCR was carried out with 100 ng of genomic DNA and 150 pmol of each primer by using pfu polymerase (2.5 U) in a DNA thermal cycler PTC-100-96 (MJ Research Inc). Conditions for the PCR reactions were as follows: template denaturation, 94°C, 45 seconds; primer annealing, 58°C, 45 seconds; primer extension, 72°C, 45 seconds, all for 30 cycles; and an additional 5-minute extension at 72°C. PCR-amplified fragments (1106 bp) were purified by electrophoresis on 1% agarose. The pGL2 vector was linearized upstream of the luc gene by double digestion with KpnI and BglII and treated with calf intestinal phosphatase. PCR promoter fragments were digested with KpnI and BglII and ligated into the KpnI/BglII sites of the promoterless and enhancerless luciferase reporter gene,  to generate the p1PAI110/luc (from 1/1 PAI-1 promoter) and p2PAI110/luc (from 2/2 PAI-1 promoter) constructs. Ligation mixtures were transformed into JM109-competent cells for sequencing analysis. Detailed sequencing analyses were carried out with duplicate PCR clones from single PCR amplifications of 7 individual 1/1 (14 sequences) and 8 individual 2/2 (16 sequences) PAI-1 genotype promoter fragments to rule out errors due to PCR cloning or sequencing errors. Sequencing was carried out on both strands by the university’s Automated DNA Sequencing Core Facility.

**Transient Transfection Experiments and Measurement of Luciferase and β-Galactosidase Activities**

Constructs (p1PAI110/luc, p2PAI110/luc, and pSV–β-galactosidase) were purified by ultracentrifugation through a CsCl/ethidium bromide gradient before transient transfection into genotyped HUVECs. Transfection experiments were carried out on semi-confluent (40% to 50%), cultured (third or fourth passage) HUVECs in 6-well tissue culture plates (960 mm²/well, 4.5×10⁴ cells/well). DNA (pSV–β-galactosidase)-lipofectamine complexes

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were performed by incubation of varying combinations of DNA (1, 2, and 3 µg/well) and lipofectamine (2.5 to 20 µg/well) in Opti-MEM 1 medium (containing only 0.25% BSA) for 45 minutes. To determine the conditions for optimal transfection efficiency into cultured HUVECs, cultures were washed twice with Opti-MEM 1 medium and then transiently transfected with the various preformed β-galactosidase DNA–lipofectamine complexes in Opti-MEM 1 medium for 4 to 24 hours before measurement of β-galactosidase activity (see below). Once optimal transfection conditions were determined, 1/1 and 2/2 PAI-1 genotyped HUVEC cultures were transiently transfected with their respective 1/1 and 2/2 PAI-1 promoter regions with the published PAI-1 sequences of Nygren et al.25 and Bosma et al.24 showed homology both with 5 gaps for the 1/1 PAI-1 promoter and 7 and 9 gaps for the 2/2 PAI-1 promoter, respectively. Detailed sequence analyses of the 1/1 (upper sequence) versus 2/2 PAI-1 (bottom sequence) promoter fragments shows that these minor alterations occur consistently and with high frequency.

**Results**

**Determination of PAI-1 Genotypes (HindIII RFLPs) in Individual Human Umbilical Cords**

Southern blot hybridization of individual HindIII-digested DNAs, using a 32P-labeled, full-length, 2.2-kb PAI-1 cDNA probe, identified the presence of the HindIII RFLPs, designated 1/1 (22-kb fragment), 1/2 (18- plus 22-kb fragments), and 2/2 (18-kb fragment only; contains the mutation site), as shown in Figure 1. Figure 1. Southern blot hybridization of PAI-1 (HindIII RFLP) genotypes. Genomic DNA was isolated from individual cords and digested with HindIII. Digested DNA was blotted to a nylon membrane, which was then hybridized with a 32P-labeled, full-length, 2.2-kb PAI-1 cDNA probe, followed by autoradiography, as described in Methods. Restriction fragment bands were used to identify the 3 different PAI-1 genotypes, designated in these studies as 1/1 (22-kb fragment only), 1/2 (18- plus 22-kb fragments), and 2/2 (18-kb fragment only; contains the mutation site).

**Sequence Analysis of the Promoter and 5' Flanking Regions of the 1/1 and 2/2 PAI-1 Genotypes**

Alignment and comparison of the 1/1 versus 2/2 PAI-1 promoter regions with the published PAI-1 sequences of Nygren et al.25 and Bosma et al.24 showed homology both with 5 gaps for the 1/1 PAI-1 promoter and 7 and 9 gaps for the 2/2 PAI-1 promoter, respectively. Detailed sequence analyses of the 1/1 (upper sequence) versus 2/2 (lower sequence) PAI-1 promoters show that these minor alterations occur consistently and with high frequency (Figure 2). These sequence variations have been designated as follows: Box A (a 2-bp difference at positions 840 and 841; AA for GG); box B (a 1-bp deletion at position −557; T); box C (a 1-bp difference at position −241; T for C); box D (a 1-bp deletion at position −216; T); and box E (a 1-bp deletion at position −138; T), as shown in Figure 2.

**Transient Transfection of Cultured HUVECs With Their Respective p1PAI110/luc and p2PAI110/luc Constructs and Vice Versa**

Transient transfections with the varying combinations of preformed pSV–β-galactosidase plasmid DNA–lipofectamine complexes for various incubation times were initially used to determine the conditions for optimal transfection efficiency into cultured HUVECs. These experiments indicated that optimal transfection efficiency was consistently achieved by incubation of monolayer cultured HUVECs (in 960-mm² wells; ~4.5x10⁵ cells/well) for 8 hours at 37°C with preformed DNA-lipofectamine complexes consisting of 2 µg of DNA/well mixed with 10 µg lipofectamine/well. Higher concentrations of lipofectamine (>10 µg) did not significantly increase the uptake of DNA by cultured HUVECs but did affect cell viability and caused significant cell death at >15 µg/well (data not shown). Under the optimized conditions described above, PAI-1 genotyped, cultured HUVECs (1/1 and 2/2) were transiently
transfected with their respective p1PAI110/luc and p2PAI110/luc constructs and then incubated with the various inducers insulin, HTG-VLDL, and Lp(a). Insulin induced an approx 12- to 16-fold increase in luciferase activity in both the 1/1 and 2/2 PAI-1 HUVEC cultures transfected with the p1PAI110/luc construct but essentially no increase (approx 1- to 2-fold) in the 1/1 and 2/2 PAI-1 HUVEC cultures transfected with the p2PAI110/luc construct (Figure 3). Conversely, HTG-VLDL and Lp(a) induced an approx 14- to 16-fold and an approx 8- to 11-fold increase, respectively, in luciferase activity in both the 1/1 and 2/2 PAI-1 HUVEC cultures transfected with the p2PAI110/luc construct but essentially no increase (approx 1- to 2-fold) in the 1/1 and 2/2 PAI-1 HUVEC cultures transfected with the p1PAI110/luc construct (Figure 4). The positive control IL-1 showed an approx 7- to 12-fold increase in luciferase activity in 1/1 and 2/2 PAI-1 HUVEC cultures transfected with either the p1PAI110/luc or p2PAI110/luc constructs, as shown in Figures 3 and 4.

Results from these studies demonstrate that the 1106-bp fragment of the promoter and 5′ flanking region of the 1/1 and 2/2 PAI-1 genotypes contains the regulatory sequence(s) important in the transcriptional regulation of the 1/1 and 2/2 PAI-1 genotypes by insulin, HTG-VLDL, and Lp(a). These cross-over results also demonstrate that transcriptional regulation of either the 1/1 or 2/2 PAI-1 genotype by their respective inducers is due to the promoter itself and not to some factor(s) expressed differently in the 1/1 versus the 2/2 PAI-1 genotype HUVEC cultures.

Discussion
Impaired fibrinolysis has been suggested to predispose to coronary artery thrombosis, which may have an important role in the pathogenesis of CAD and MI.1,2 Well-established risk factors for MI, such as smoking, obesity, hyperlipoproteinemia, and hyperinsulinemia, are commonly associated with impaired fibrinolysis.3,20-31 Impaired fibrinolysis has been associated with elevated PAI-1 levels, and this relation strongly suggests that PAI-1 may have an important pathological role in the development and progression of CAD and eventual MI.3,12 It is conceivable that the elevated blood levels of PAI-1 seen in patients with non-insulin-dependent diabetes mellitus, HTG, and syndrome X3,33-35 may in part explain their increased thrombotic risk and CAD prevalence, which are unexplained by conventional risk factors in these subjects.3,6 Also, elevated levels of PAI-1 are often associated with HTG-VLDL. Several in vitro studies have demonstrated that these specific components, including high levels of insulin or proinsulin-like molecules, hyperglycemia, HTG, and Lp(a), will increase PAI-1 production.3,7,8,12,36,37 Various cell types have been shown to produce PAI-1 in vivo, including hepatocytes and ECs.38 In HepG2 cells, insulin induced a dose-dependent increase in secreted PAI-1 antigen (4.8-fold) and a 2- to 3-fold increase in steady-state PAI-1 mRNA levels.39,40 Similarly, HTG-VLDL induced PAI-1 mRNA expression 2- to 3-fold and PAI-1 antigen secretion by 2-fold.41,42 Lp(a) has also been shown to increase PAI-1 antigen and mRNA levels.43 Epidemiological studies have described an interrelationship between plasma PAI-1 levels, specific PAI-1 genotypes, and risk factors.12,44,45 An HindIII RFLP and a dinucleotide repeat (C-A)n polymorphism have been used as markers for genetic variation in the PAI-1 gene in young post-MI patients...
and population-based controls. Of major interest was the fact that the associations of insulin and VLDL with PAI-1 levels appeared to be genotype specific. Subjects with the HindIII genotype 2/2 showed increased PAI-1 in association with plasma VLDL only, whereas the 1/1 genotype showed increased PAI-1 levels in association with insulin only. These authors hypothesized that the HindIII polymorphism is in linkage disequilibrium with a base change at a site of functional importance in the regulation of PAI-1 and that a relationship exists between PAI-1 levels, PAI-1 genotypes, and regulation by VLDL and insulin. Previous studies using cultured ECs (human umbilical vein or human or porcine aorta) did not demonstrate an insulin-mediated effect on PAI-1 expression. These conclusions are in apparent contradiction with the studies reported here, in which PAI-1 expression may be regulated by insulin in a genotype-specific manner in cultured HUVECs. Because the 1/1 PAI-1 genotype occurs in only \( \approx 20\% \) of the population and most studies use pooled cultures, it is conceivable that the predominant population of ECs in these pooled cultures in fact represents only the low or nonresponsive 1/2 or 2/2 PAI-1 genotypes and, hence shows little or no response to insulin as we report herein. These studies emphasize the newly emerging concept and importance of conducting future experiments with individually genotyped cultured HUVECs to identify and define the responsiveness of human ECs to regulators or inducers of specific protein expression.

Several recent studies have demonstrated that regulation of the PAI-1 gene at the transcription level occurs through specific cis-regulatory regions. Functional studies in which the promoter region of the human PAI-1 gene was attached to a reporter gene have provided reliable information on the ability of different sections of the gene to promote transcriptional responses to different stimuli in cell culture, including glucocorticoids, transforming growth factor-\( \beta \), and phorbol myristate acetate. Recently, tumor necrosis factor-\( \alpha \) was shown to increase cytosolic calcium in cultured U937 cells, and it was concluded that calcium triggers a pathway that upregulates PAI-1 synthesis and positively interacts with the tumor necrosis factor-\( \alpha \)-induced pathway that stimulates PAI-1 synthesis. Previous studies have demonstrated the ability of insulin to transcriptionally regulate the expression of various genes, including \( c-fos \), glucagon, and amylase genes. Insulin also stimulates a serine/threonine kinase in 3T3-L1 adipocytes (mitogen-activated protein kinases). However, the mechanism(s) of signal transduction in the induction of PAI-1 mRNA by insulin is presently unknown. Insulin and insulin growth factor-1 have similar biological activities and have been shown to induce PAI-1 gene expression in HepG2 cells. We have carried out similar experiments with PAI-1 (HindIII RFLP) genotyped, cultured HUVECs and insulin growth factor -1 (10^{-7} mol/L), without any effects on PAI-1 mRNA levels (H.E.G. et al, unpublished data, 1998). A new class of antidiabetic agents, thiazolidinediones, has been shown to affect insulin-induced stimulation of glycogen synthase as well as leptin and lipoprotein lipase gene expression at the transcriptional levels. However, the effect of thiazolidinediones on the regulation of PAI-1 gene expression by insulin has not been reported.

The mechanism by which insulin, HTG-VLDL, or Lp(a) regulates PAI-1 expression in a genotype-specific manner has not yet been clearly identified or defined. Recently, we have demonstrated the genotype-specific transcriptional upregulation of the 2/2 PAI-1 genotype by HTG-VLDL Sf 100 to 400 and Lp(a) in cultured HUVECs by using nuclear transcription run-on assays. Similarly, we have demonstrated the genotype-specific transcriptional upregulation of the 1/1 PAI-1 genotype by insulin in cultured HUVECs. These results are in apparent contradiction with studies in HepG2 cells, in which HTG-VLDL and insulin increased PAI-1 levels by stabilizing the steady-state levels of PAI-1 mRNA rather than by increasing gene transcription. The apparent differences in the regulation of PAI-1 gene expression by HTG-VLDL and insulin in cultured HUVECs versus HepG2 cells is presently unknown and remains to be further elucidated. However, the studies described here demonstrate that the regulation of PAI-1 gene expression by HTG-VLDL and insulin is mediated through specific inducible element(s) contained in the 1106-bp promoter and 5'-flanking region of the 1/1 and 2/2 PAI-1 genotypes. Identification of regulatory elements in the promoter and 5'-flanking region of the 1/1 and 2/2 PAI-1 genotypes, responsive to HTG-VLDL, Lp(a), or insulin, would provide significant new insights into a unique form of regulation of the fibrinolytic system. This genotyped regulation may be important in explaining the increased risk for thrombosis and atherosclerosis in patients with non-insulin-dependent diabetes mellitus and HTG. It may be particularly important in those patients with syndrome X, who have these combined metabolic abnormalities.

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**References**

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