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

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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).1,2 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.3 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,4,5 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.6,7 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.8 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.9,10

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.9,10 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.12 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 shown to affect the transcriptional activity of the PAI-1 gene.11,12 Recent studies have shown that variations in the PAI-1 gene sequence may...
been used to demonstrate transcriptional regulation of the PAI-1 gene promoter by various inducers in different cell culture systems.\textsuperscript{1,11-15} 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; \([\alpha-^{32}P]dCTP (3000 Ci/mmol)\) from Amersham; 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; pUC DNA polymerase from Stratagene; and the Sequenase II kit from UBI. Lipofectamine, Opti-MEM 1 reduced serum medium, and medium 199 (M199) were from BRL. Purified HTG-VLDL (5, 100 to 400) was obtained from Drs William A. Bradley and Sandra H. Gianturco, University of Alabama at Birmingham, and was isolated and characterized as described previously.\textsuperscript{16} Purified Lp(a) was obtained from Dr Gunther M. Fless, University of Chicago, Ill, and was also isolated and characterized as described previously.\textsuperscript{17,18}

Cell Culture

HUVECs derived from fresh (discarded) umbilical cords by mild collagenase treatment\textsuperscript{19,20} were seeded individually into human fibroblast–coated plastic T-25 or Petri dishes (960 mm\(^2\)) and grown with 0.5 mol/L Tris-HCl, pH 8.0, and the DNA was recovered by 55°C. Digested samples were then extracted with phenol saturated in the presence of 0.02 mol/L EDTA and 0.5% SDS for 16 hours at 68°C. The filters were washed twice in 2× SSC and 0.5% SDS for 30 minutes at room temperature, once in 1× SSC and 0.5% SDS for 30 minutes at 68°C, and once in 0.1× SSC and 0.1% SDS for 10 minutes at 68°C. The filters were exposed to Fuji film at –70°C for 24 hours by using Fisher Biotech L-Phs intensifying screens. From the Southern blot autoradiograms, the presence of the 18- and/or 22-kb restriction fragment bands was 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). The identification of each PAI-1 genotype was confirmed from Southern blot hybridization autoradiograms by 3 different individuals.

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’ CGATCGTGATCTAAAGCACACCCGTCAAC 3’), identical to positions 2193 to 2214, and a downstream primer (5’ GATCTAAGATCCTGCCTGCGATTG 3’), complementary to positions 3279 to 3278 of the human PAI-1 gene.\textsuperscript{22} Both primers have a CGATC 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 1.0% 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,\textsuperscript{26,27} pGL2-basic, 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.

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.\textsuperscript{22} DNA digests were blotted onto nylon membranes by capillary transfer in 10× SSC (1× SSC is 0.15 mol/L NaCl and 0.015 mol/L sodium citrate buffer, pH 7.0) for 16 hours at room temperature and then UV cross-linked to the nylon membrane. The immobilized DNA was hybridized with a 2.2-kb human PAI-1 cDNA fragment\textsuperscript{23} radiolabeled with [α-\textsuperscript{32}P]dCTP to a specific activity of ~10\(^{6}\) cpm/μg by using a random-primer DNA labeling kit. Prehybridization and hybridization were carried out with Quick Hyb solution in a hybridization oven for 20 minutes and 1 hour, respectively, at 68°C. The filters were washed twice in 2× SSC and 0.5% SDS for 30 minutes at room temperature, once in 1× SSC and 0.5% SDS for 30 minutes at 68°C, and once in 0.1× SSC and 0.1% SDS for 10 minutes at 68°C. The filters were exposed to Fuji film at –70°C for 24 hours by using Fisher Biotech L-Phs intensifying screens. From the Southern blot autoradiograms, the presence of the 18- and/or 22-kb restriction fragment bands was 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). The identification of each PAI-1 genotype was confirmed from Southern blot hybridization autoradiograms by 3 different individuals.

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

Constructs (p1PAI110/luc, p2PAI110/luc, and pSV–β-galactosidase) were purified by ultra-centrifugation through a CsCl-ethidium bromide gradient before transient transfection into genotyped HUVECs. Transfection experiments were carried out on semiconfluent (40% to 50%), cultured (third or fourth passage) HUVECs in 6-well tissue culture plates (960 mm\(^2\)/well, ~4.5×10\(^5\) cells/well). DNA (pSV–β-galactosidase)-lipofectamine complexes
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 pβ-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/luc constructs (p1PAI110/luc and p2PAI110/luc) and vice versa by using lipofectamine. An internal control plasmid, pSV–β-galactosidase (2 μg/well), was cotransfected with the luc plasmids, and β-galactosidase activity was used to correct for differences in DNA uptake. After transient transfection with the p1PAI110/luc and p2PAI110/luc promoter constructs, the media were removed and cultures incubated with fresh, serum-containing M199 containing 0.25% BSA for an additional 12 to 24 hours in the absence or presence of insulin (10 g/mL), HTG-VLDL (20 g/mL), Lp(a) (50 g/well), insulin (10 g/well), HTG-VLDL (20 g/mL), Lp(a) (50 g/well), insulin (10 g/well), or interleukin-1 (IL-1) (50 ng/mL, positive control). Luciferase activity was measured luminometrically in a Turner model TD-20 luminometer, and β-galactosidase activity was measured colorimetrically (A420 nm) in an automated Dynatech model MR 5000 microplate reader. Individual luciferase activities were normalized for transfection efficiencies by dividing relative light units by β-galactosidase activities from cotransfection with pSV–β-galactosidase.

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, contains mutation site), as shown in Figure 1.

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 Ny et al22 and Bosma et al23 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 have reproducibly identified 5 regions of small nucleotide differences that consistently occur 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 semiconfluent cultured HUVECs (in 960-mm2 wells; ∼4.5×104 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 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 (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 14- to 16-fold and an 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 (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 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. Well-established risk factors for MI, such as smoking, obesity, hyperlipoproteinemia, and hyperinsulinemia, are commonly associated with impaired fibrinolysis. 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. It is conceivable that the elevated blood levels of PAI-1 seen in patients with non–insulin-dependent diabetes mellitus, HTG, and syndrome X may in part explain their increased thrombotic risk and CAD prevalence, which are unexplained by conventional risk factors in these subjects. 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. Various cell types have been shown to produce PAI-1 in vivo, including hepatocytes and ECs. 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. Similarly, HTG-VLDL induced PAI-1 mRNA expression 2- to 3-fold and PAI-1 antigen secretion by 2-fold. Lp(a) has also been shown to increase PAI-1 antigen and mRNA levels. Epidemiological studies have described an interrelationship between plasma PAI-1 levels, specific PAI-1 genotypes, and risk factors. An HindIII RFLP and a dinucleotide repeat (C-A), 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 ≈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. 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 different sections of the gene to promote transcriptional responses to different stimuli in cell culture, including glucocorticoids, transforming growth factor-β, and phorbol myristate acetate. Recently, tumor necrosis factor-α 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-α–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, S100 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, Lp(a), 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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