Very-Low-Density Lipoprotein Response Element in the Promoter Region of the Human Plasminogen Activator Inhibitor-1 Gene Implicated in the Impaired Fibrinolysis of Hypertriglyceridemia

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Abstract—Hypertriglyceridemia and impaired fibrinolytic function are linked to coronary heart disease and other atherothrombotic disorders. Triglyceride-rich lipoproteins may attenuate fibrinolysis by increasing the plasma levels of plasminogen activator inhibitor-1 (PAI-1). Furthermore, a common 4/5 guanosine (4G/5G) polymorphism in the promoter region of the PAI-1 gene has been indicated to influence plasma PAI-1 activity and to be involved in an allele-specific response to triglycerides. Herein we show by transfection assays that VLDLs induce transcription of the human PAI-1 promoter in endothelial cells. A VLDL response element (VLDLRE) is located to residues 2672 to 2657 in the promoter region by electromobility shift assay, methylation interference, and DNase I footprinting, and its activity is shown to be influenced by the common 4G/5G polymorphism located adjacent to and upstream of the binding site of a VLDL-inducible transcription factor. These findings may provide a molecular explanation to the link between VLDL and PAI-1 activity elevation in plasma and to the interaction between the 4G/5G polymorphism and plasma triglycerides. (Arterioscler Thromb Vasc Biol. 1998;18:20-26.)

Key Words: PAI-1 ▪ VLDL ▪ promoter ▪ genotype ▪ triglycerides

Increased plasma PAI-1 activity is a common finding in patients with CHD, particularly in young postinfarction patients with hypertriglyceridemia,1 and is associated with recurrence of cardiovascular events.2–5 The prognostic role of PAI-1 in CHD is related principally to insulin resistance.5 Since PAI-1 is a rapid inhibitor of tissue-type plasminogen activator, the major proteolytic activator of plasminogen, high plasma PAI-1 activity is thought to be linked to increased risk of coronary thrombosis. Elevated PAI-1 expression has also been demonstrated in atherosclerotic plaques,6,7 suggesting that PAI-1 may play a role in atherogenesis as well. Several common polymorphisms have been discovered in the PAI-1 locus,8–11 some of which have been reported to be associated with plasma PAI-1 activity. In particular, the 4G allele of the 4G/5G polymorphism in the promoter region has been linked to higher PAI-1 levels in healthy individuals,12,13 in patients with previous myocardial infarction10,12 or suspected CHD,14 and in patients with NIDDM.15,16 The prevalence of the 4G allele was also found to be significantly higher in patients with myocardial infarction before the age of 45 than in population-based control subjects (allele frequencies of .63 versus .53),12 and the 4G allele has recently been linked to an increased risk of coronary artery disease also in middle-aged subjects.14 However, the association between the 4G/5G polymorphism and plasma PAI-1 activity has not been observed uniformly.11,13 Furthermore, no association between 4G/5G genotype and myocardial infarction was found in the ECTIM study, examining postinfarction patients recruited from different geographical and cultural areas.13

Both environmental and genetic factors contribute to determining plasma PAI-1 activity. A striking feature of PAI-1 is its positive association with the VLDL triglyceride concentration.17 There is also some indication that the potential triglyceride regulation of PAI-1 is mainly confined to individuals with a certain PAI-1 genotype. Subjects who are homozygous for the HindIII noncutting allele (genotype 1/1) of the 3’ flanking region of the PAI-1 gene have higher plasma PAI-1 activity in the presence of a raised VLDL triglyceride concentration than individuals who are homozygous for the cutting allele (genotype 2/2), with the heterozygotes lying in between.8 This finding suggests that the HindIII polymorphism could be in linkage disequilibrium with a base change at a site of functional importance in the regulation of PAI-1 by VLDL. Furthermore, the 4G/5G polymorphic region in the PAI-1 promoter has been implicated in an allele-specific response to triglycerides in studies of patients with NIDDM, in whom the triglyceride level and its interaction with the 4G/5G genotype appeared to be strong determinants of plasma PAI-1 activity.13,16 A genotype-specific association between triglyceride level and PAI-1 antigen concentration was found also in
patients undergoing coronary angiography because of chest pain. However, such gene/environment interaction was not encountered in healthy individuals or postinfarction patients in the ECTIM study or in healthy participants of the PRIME study.

Endothelial cell synthesis and secretion of PAI-1 is indicated to contribute to the regulation of plasma PAI-1 activity. Furthermore, VLDL induces secretion of PAI-1 from cultured HUVECs. In the present study, we have characterized an element in the PAI-1 promoter that mediates VLDL induction of PAI-1 in endothelial cells.

The promoter element is located downstream of and adjacent to the 4G/5G polymorphic site. A VLDL-inducible transcription factor shows competitive binding, with the transcription factors binding to the 4G/5G polymorphic site. Competition between the 5G allele-specific transcriptional repressor protein and the VLDL-inducible factor could explain the 4G/5G allele-specific relation between VLDL triglyceride and PAI-1 activity levels in plasma.

Methods

DNA Constructs

For EMSA and footprinting analysis, double-stranded oligonucleotides were designed. The oligonucleotides used in EMSA are shown in Figs 2A and 5. In the methylation interference assay and the DNase I footprinting, a double-stranded oligonucleotide of the −676/−641 segment of the human PAI-1 promoter with flanking cytosine residues (CGGGGAGTACGCCGCTGATACATCGAGGCAGCCGGGGG) was constructed. The probes were end labeled with [γ-32P]ATP at either end using T4 polynucleotide kinase. The 2×5G-HCAT and the 2×4G-HCAT vectors, containing the 4G/5G polymorphic site coupled to a minimal and heterologous promoter, were constructed as described earlier.

The 5G-PAI-pCAT and the 4G-PAI-pCAT comprise the human PAI-1 sequences −805/−804 to +17. Polymerase chain reaction primers with flanking PstI and XbaI sites were used (upstream primer: AACTGCAGCTAGCAGCAGACGGACTCCCAGAGC; downstream primer: GCTCTAGAGCCAAACACAGCTGTGCTC). The polymerase chain reaction products from amplifying the PAI-1 promoter fragment containing the 5G allele (3.8/4.0 guanosine) from genomic DNA of individuals who were homozygous for either the 4G or 5G allele were inserted into the pCAT-Basic vector (Promega). The correct sequence of the inserts was verified by sequencing.

Methylation Interference and DNase I Footprinting

Methylation interference was conducted essentially as described previously. The methylated DNA fragments were analyzed on an 11% (wt/vol) denaturing polyacrylamide/bisacrylamide (19:1) gel. DNase I footprinting was conducted using the same DNA fragment, labeled at either end. Partial DNase I digestion was performed as described earlier, except that the reaction was terminated with 0.02 mol/L Na2EDTA and directly put on ice. The different protein-DNA complexes were directly separated by EMSA as described above. Retarded DNA fragments were electroblotted onto a Schleicher & Schuell diethylaminoethyl membrane and analyzed on an 11% (wt/vol) denaturing polyacrylamide/bisacrylamide (19:1) gel.

Statistical Methods

Differences in continuous variables between two groups were tested by paired or unpaired t tests.

Results

VLDL-Induced PAI-1 Expression and the 4G/5G Polymorphic Site

Because VLDL induction of PAI-1 in endothelial cells may at least in part be mediated by transcriptional activation, transfection studies were performed in HUVECs to further examine potential 4G/5G allele-specific mechanisms by which VLDL could enhance PAI-1 expression. Fragments (804 bp) of the PAI-1 promoter that contained either the 4G or 5G allele were coupled to a CAT gene and transiently transfected into HUVECs (Fig 1A). As shown in Fig 1B and 1C, VLDL-induced transcription of the PAI-1 promoter in HUVECs. Maximum induction was obtained at 0.075 mg VLDL per milliliter culture medium (data not shown). There was a tendency to an allele-specific response to VLDL. The promoter fragment containing the 4G allele showed a slightly higher promoter activity in response to VLDL than did the promoter fragment containing the 5G allele (3.8±1.0-fold induction, mean±SD, n=4, versus 2.3±0.9-fold induction, mean±SD, n=6; P=.06 in an unpaired t test).

We have recently demonstrated in HUVECs that both the 4G and 5G alleles bind a transcriptional activator (factor A), whereas the 5G allele also binds a repressor protein (factor B) to an overlapping binding site.

The 4G/5G allele-specific
differences in the relations between the VLDL triglyceride and PAI-1 activity levels in plasma thus suggest that the transcription factors binding to this polymorphic region could be involved in an activation of the PAI-1 promoter mediated by VLDL. We therefore performed an EMSA on the 4G/5G polymorphic region, using VLDL-induced nuclear extracts from HUVECs. However, we could detect neither any increased binding of the common factor to this region nor of the 5G allele-specific factor (data not shown). Furthermore, a transfection assay using two tandem copies of a 30-bp DNA segment containing either the 4G or 5G allele sequence inserted upstream of a minimal and heterologous promoter driving the CAT gene did not respond to VLDL when inserted upstream of a minimal and heterologous promoter. After transfection, cells were incubated for 24 hours with 0.075 mg VLDL per milliliter culture medium. C. Example of autoradiograph of thin-layer chromatography analysis of CAT assay using 1-deoxycylorhaphenicol (Amersham) as a substrate. D. Results from transfections using truncation of the human PAI-1 promoter in HUVECs when induced by VLDL. P < 0.05 denotes the result of a paired t test comparing the induction of −804-PAI–pCAT and −609-PAI–pCAT. n.s. indicates not significant.

Figure 1. VLDL-induced transcription from the PAI-1 promoter in endothelial cells. A, DNA constructs of the 4G-PAI–pCAT and the 5G-PAI–pCAT. B, VLDL induction of the PAI-1 promoter in endothelial cells, with a tendency to an allele-specific response to VLDL. Bars represent mean (SD). Results are based on four experiments with the 4G-PAI–pCAT and six experiments with the 5G-PAI–pCAT constructs. After transfection, cells were incubated for 24 hours with 0.075 mg VLDL per milliliter culture medium. C. Example of autoradiograph of thin-layer chromatography analysis of CAT assay using 1-deoxycylorhaphenicol (Amersham) as a substrate. D. Results from transfections using truncation of the human PAI-1 promoter in HUVECs when induced by VLDL. P < 0.05 denotes the result of a paired t test comparing the induction of −804-PAI–pCAT and −609-PAI–pCAT. n.s. indicates not significant.

Localization of the VLDLRE in the PAI-1 Promoter
To localize the VLDLRE within the PAI-1 promoter, we next constructed several truncations of the promoter region coupled to a CAT gene and transfected the new constructs into HUVECs. Successive 100-bp segments were deleted from the initial promoter construct, which contained the 804 bp located upstream of the start of transcription. The results obtained from induction of transiently transfected cells with 0.075 mg/mL VLDL are shown in Fig 1D. The VLDL response was lost in the construct containing 609 bp of the promoter region. This finding indicates that the VLDL response region is located within the 609 to 708 bp upstream of the transcription start site in the PAI-1 promoter. We therefore evaluated potential binding regions for any VLDL-induced transcription factor(s) that could mediate transcriptional activation. For this purpose, three overlapping EMSA probes, in addition to the 4G/5G probe, were constructed that covered the −717 to −607 region (Fig 2A). HUVECs were induced with 0.075 mg/mL VLDL for 1 to 24 hours, and nuclear extracts were prepared. Neither the −717 to −607 region of the promoter showed increased binding of one factor (Fig 2B). The specificity of the binding was analyzed by including nonlabeled EMSA probes in excess as competitors.

Figure 2. Binding of VLDL-induced factor to the human PAI-1 promoter. A, Human PAI-1 sequence from −717 to −607. Boxes delineate the four different probes: −717/−689/−665, −672/−637, and −642/−607. B, EMSA of HUVEC nuclear extract induced by VLDL for 16 hours and bound to the −672/−637 probe. The arrow denotes VLDL-induced factor. Lane 1, no lipoprotein; lane 2, 5 μg VLDL; lane 3, 10 μg VLDL; lane 4, 20 μg VLDL; lane 5, 40 μg VLDL; lane 6, 80 μg VLDL; and lane 7, 160 μg VLDL per milliliter. The figure shows a representative EMSA from five different experiments. C, EMSA on nuclear extract derived from HUVECs bound to the −672/−637 probe. Lane 1, no extract; lane 2, HUVEC extract stimulated by 80 μg VLDL per milliliter with no competitor; lane 3, 200-fold excess of −717/−684; lane 4, −672/−637; lane 5, −642/−607; and lane 6, −689/−665 as competitors. F indicates free DNA.
Fig 2C shows that the VLDL-induced factor exhibits sequence-specific binding, as the band induced by VLDL was not decreased when either the $2^717/2^684$ probe (lane 3) or the $2^642/2^607$ probe (lane 5) was added as competitor, whereas the VLDL-induced band diminished when using the same probe ($2^672/2^637$) as competitor (lane 4). Interestingly, the VLDL-induced factor was competed by both the 4G and 5G ($2^689/2^665$) probes (lane 6). This observation indicates that the VLDL-induced factor is bound within the region covering the $2^672$ to $2^637$ residues of the PAI-1 promoter adjacent to the binding site of the 5G allele–specific factor ($2^679/2^673$).12

**Identification of the Binding Site of the VLDL-Inducible Transcription Factor**

Footprinting studies using nuclear extract derived from VLDL-induced HUVECs were performed to map the specific binding site of the VLDL-induced factor. Methylation interference (Fig 3A) and DNase I footprinting (Fig 3B) showed that the VLDL-induced factor is bound to the residues $-672$ to $-637$ of the PAI-1 promoter (Fig 3C). To further study the involvement of this promoter element in the VLDL induction of promoter activity (Fig 3C). To further study the involvement of this promoter element in the VLDL induction of promoter activity, a 9-bp deletion (residues $-662$ to $-670$) of the VLDLRE was introduced into the 804-bp 4G–PAI-1 promoter construct (Fig 4A). This deletion eliminated the VLDL induction of promoter activity (Fig 4B). The wild-type 804-bp 4G–PAI-1 promoter construct was significantly induced by VLDL (2.0±0.6-fold induction, mean±SD, n=4, P<.05 in a paired t test). In contrast, the mutated promoter showed no significant response to VLDL (1.3±0.5-fold induction, mean±SD, n=4, P=.31 in a paired t test).

**VLDL-Inducible Factor Shows Competitive Binding With the Transcription Factors Binding to the 4G/5G Polymorphic Site**

Since the VLDL-inducible factor was found to bind to the region adjacent to and partly overlapping the 4G/5G polymorphic site, competition between the 5G allele–specific transcriptional repressor protein12 and the VLDL-induced factor could explain the 4G/5G allele–specific relations between VLDL triglyceride and PAI-1 activity levels in plasma. To resolve this issue, several binding sites were constructed for use in an EMSA (Fig 5). Base pair substitutions were designed on the basis of the results of footprinting and methylation interference assays (Reference 12 and Fig 3). Fig 6A shows an EMSA demonstrating the differential binding of the 4G/5G polymorphic site as previously described,12 using the 4G or 5G alleles as probe. The EMSA shows that both alleles bound a common factor (factor A), while the 5G allele bound an additional factor (factor B). Additional complexes have earlier been shown to have nonspecific interactions.12 Previous work has demonstrated that the common factor, factor A, acts as a transcriptional activator, whereas the 5G allele–specific factor, factor B, acts as a transcriptional repressor.12 As demonstrated in Fig 6A, binding of factor B to the 5G allele results in a decreased binding of factor A compared with the 4G probe. To further study the interaction between factors B and A, two mutated EMSA probes were designed, containing mutations that impair the binding of factor A and factor B, respectively (Fig 5). As shown in Fig 6B, competition of factor B resulted...
in an increased binding of factor A. Similarly, competition of factor A (Fig 6C) resulted in increased binding of factor B. Thus, the binding of the 5G allele–specific factor impaired the binding of the common transcriptional activator (factor A).

To study whether the 5G allele–specific repressor also influenced the binding of the VLDL-induced factor, EMSA probes were constructed that included the binding sites for both of these factors (Fig 5). As demonstrated in Fig 7A, factor A and the VLDL-induced factor bound to the 4G/VLDLRE construct, whereas the 5G/VLDLRE construct also bound factor B. Densitometric scanning showed a 30% decrease in binding of the VLDL-induced factor to the 5G/VLDLRE probe compared with the 4G/VLDLRE. Furthermore, an interaction between factor B and the VLDL-induced factor was demonstrated by using a probe containing the B site and the VLDLRE but with a mutated binding site for factor A. As shown in Fig 7B, addition of VLDLRE as competitor decreased the binding of the VLDL-induced factor and increased the binding of factor B. Taken together, these studies show that the 5G allele–specific repressor can compete for binding to the PAI-1 promoter with both the common transcriptional activator (factor A) and the VLDL-induced factor.

### Discussion

Increased PAI-1 expression in endothelial cells induced by VLDL may predispose to both arterial thrombosis and atherosclerosis by promoting persistence of fibrin on injured intimal surfaces and incorporation of fibrin into the artery wall. It is notable in this context that impaired fibrinolytic function secondary to elevated plasma PAI-1 activity has been shown to be of particular importance for myocardial infarction in men with hypertriglyceridemia. However, the fairly strong positive relation between triglyceride and PAI-1 levels has been a consistent observation not only in young male postinfarction patients but also among healthy normolipidemic subjects, obese individuals, and patients with angina pectoris or NIDDM. The evidence that PAI-1 plays a role in CHD now also extends beyond the rare cases of myocardial infarction before the age of 45.

The delineation in the present work of a molecular event in endothelial cells that could link hypertriglyceridemia to impaired fibrinolytic function secondary to elevated plasma PAI-1 activity has been shown to be of particular importance for myocardial infarction in men with hypertriglyceridemia. However, the fairly strong positive relation between triglyceride and PAI-1 levels has been a consistent observation not only in young male postinfarction patients but also among healthy normolipidemic subjects, obese individuals, and patients with angina pectoris or NIDDM. The evidence that PAI-1 plays a role in CHD now also extends beyond the rare cases of myocardial infarction before the age of 45.

The 5G allele–specific repressor with the binding of VLDL-induced factor. Representative autoradiograms of five different experiments are shown. A, EMSA of HUVEC nuclear extract bound to the 4G + VLDLRE and 5G + VLDLRE probes. The left lane shows the 4G + VLDLRE probe in the absence of nuclear extract. B, EMSA of HUVEC nuclear extract bound to the B + VLDLRE probe with increasing amounts of the VLDLRE as competitor.

Figure 5. DNA constructs used in the EMSAs presented in Figs 6 and 7. Boxes indicate the binding sites of the common transcriptional activator (factor A), the 5G allele–specific transcriptional repressor (factor B), and the VLDL-induced factor binding to the VLDLRE. Mutated bases are underlined and indicated with bold letters.

Figure 6. Interference by the 5G allele–specific repressor with binding of the common transcriptional activator binding to both alleles. Representative autoradiograms of five different experiments are shown. A, EMSA of HUVEC nuclear extract bound to the 4G and 5G alleles. B, EMSA of HUVEC nuclear extract bound to the 5G-allele with the B site or nonspecific DNA as competitors. C, EMSA of HUVEC nuclear extract bound to the 5G-allele with the A site or nonspecific DNA as competitors.

Figure 7. Interference by the 5G allele–specific repressor with the binding of VLDL-induced factor. Representative autoradiograms of five different experiments are shown. A, EMSA of HUVEC nuclear extract bound to the 4G + VLDLRE and 5G + VLDLRE probes. The left lane shows the 4G + VLDLRE probe in the absence of nuclear extract. B, EMSA of HUVEC nuclear extract bound to the B + VLDLRE probe with increasing amounts of the VLDLRE as competitor.
CHD14 or NIDDM.15,16 The present work disentangles the underlying molecular mechanisms. The VLDL-induced factor was found to bind to the region adjacent to and partly overlapping the binding site of the 5G allele. Competition between the 5G allele-specific transcriptional repressor protein13 (factor B) and the VLDL-induced factor could explain the 4G/5G allele-specific relations between VLDL triglyceride and PAI-1 activity levels in plasma. In addition, competitive binding between the 5G allele-specific repressor and the common transcriptional activator could explain the differences in basal transcriptional activity.

Analysis of the VLDLRE showed that there is some homology with a peroxisome proliferator activator response element. The footprint included the sequence TCAAGCCG TGT4ATC, which is similar to the peroxisome proliferator activator response element consensus sequence A/T C/G A C/A C T A/T T G/T N C C C T/T.30 The members of the PPAR family are ligand-dependent transcription factors that bind their cognate ligand with high affinity and specificity and then activate gene transcription through binding to a specific hormone response element in the promoter region of the target gene. The ligand activating PPAR is unknown. Interestingly, a variety of fatty acids can activate PPAR,30–34 and it has been proposed that fatty acids, or their acyl-CoA derivatives, are the natural ligands of PPAR and that the physiological role of PPAR is to regulate fatty acid homeostasis.35 At this stage, one could speculate that fatty acids derived from VLDL triglycerides may cooperate to regulate the PAI-1 gene. In contrast to VLDL, native LDL has no effect on PAI-1 expression in endothelial cells.35 This observation argues for a nutritional regulation of PAI-1 secretion from endothelial cells by fatty acids, as the major difference between VLDL and LDL is the high triglyceride content of the former. In fact, unsaturated fatty acids increase circulating PAI-1 levels.37–39 Differences between populations in plasma PAI-1 levels and in the relationship of the 4G/5G polymorphism to plasma PAI-1 activity might thus be accounted for by differences in dietary habits.

In conclusion, the present work has contributed a molecular explanation to the link between VLDL and PAI-1 activity elevation in plasma and a direct mechanism by which hypertriglyceridemia may increase the risk of CHD and other atherothrombotic disorders.

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References


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