Functional Characterization of 4 Polymorphisms in Promoter Region of Hepatic Lipase Gene

Ferdinand M. van ’t Hooft, Björn Lundahl, Francesca Ragogna, Fredrik Karpe, Gunilla Olivercrona, Anders Hamsten

Abstract—Hepatic lipase (HL) is a lipolytic enzyme involved in the metabolism of plasma lipoproteins, especially high density lipoproteins. Association studies have provided strong evidence for relations of common mutations in the promoter region of the HL gene to postheparin plasma HL activity and the plasma high density lipoprotein cholesterol concentration, but the functional relevance of these polymorphisms has not been evaluated to date. We analyzed the physiological significance of 4 common polymorphisms (−250G/A, −514C/T, −710T/C, and −763A/G, all in strong linkage disequilibrium) in the promoter of the HL gene by use of electrophoretic mobility shift assays and transient transfection studies in HepG2 cells. No consistent evidence was found for a significant contribution of any of these polymorphisms to the basal rate of transcription of the HL gene. These data suggest that the 4 polymorphisms in the promoter region of the HL gene are in linkage disequilibrium with ≥1 as-yet-unknown functional polymorphisms in the HL gene locus with a significant effect on HL metabolism and/or enzymatic activity. (Arterioscler Thromb Vasc Biol. 2000;20:1335-1339.)

Key Words: DNA ■ cholesterol ■ restriction fragment length polymorphism ■ lipoproteins

Human hepatic lipase (HL; triacylglycerol lipase, EC 3.1.1.3) is a lipolytic enzyme synthesized primarily in the liver (for review, see References 1–3). After secretion from hepatocytes, the enzyme binds to the hepatic sinusoidal endothelial surface, where it hydrolyzes triglycerides and phospholipids contained in plasma lipoproteins. Several lines of evidence point to the involvement of HL in HDL and possibly LDL metabolism. However, the exact role of HL in lipoprotein metabolism has not been established.

The HL gene is localized on chromosome 15q21 and is 35 kb in size, with 9 exons that encode a cognate mRNA of 1.6 kb that is translated into a mature 476–amino acid protein.4–9 Several polymorphisms have now been described in the HL gene, including a number of mutations associated with the rare HL deficiency condition.10–13 In addition, a number of polymorphisms with relatively high frequency in the general population have also been described, but it appears that these polymorphisms are not associated with heritable variation in plasma HDL levels.14 In contrast, several recent studies demonstrated that polymorphisms in the promoter of the HL gene are related to variations in plasma HDL cholesterol concentration.14–16 Furthermore, 3 studies have reported associations between HL gene promoter variants and HL activity,16–18 1 of which also showed that the rare allele of the polymorphism in the promoter of the HL gene is linked to a buoyant LDL phenotype.18 Thus, there is considerable evidence of associations between promoter polymorphisms of the HL gene and HL activity and plasma lipoprotein concentrations. However, it is not clear whether these promoter polymorphisms are functional or merely a marker of another physiological polymorphism located elsewhere, because the promoter polymorphisms were observed to be in complete linkage disequilibrium.15

In view of the role of the promoter region in the regulation of gene expression, we screened a 1267-bp section of the proximal HL promoter in search of common genetic variants with distinct effects on the transcriptional activity of the gene. Four common promoter polymorphisms (−250G/A, −514C/T, −710T/C, and −763A/G, all in strong linkage disequilibrium) were encountered, and their physiological roles were analyzed in vitro by using electrophoretic mobility shift assays (EMSAs) and transfection studies in HepG2 cells.

Methods

Subjects
The proximal promoter of the HL gene was sequenced with the use of DNA samples from 25 healthy subjects with either low, intermediate, or high postheparin plasma HL activity.16 Genotyping for the polymorphisms encountered was subsequently performed in a total of 186 apparently healthy men, aged 35 to 45 years (mean=SD 40.3±3.5 years). They were selected at random from a registry containing all permanent residents in Stockholm County. Of those initially invited, 81% agreed to participate in the research program. All the men were interviewed to exclude individuals with a history of evidence.
of cardiovascular disease. Other exclusion criteria were the presence of severely impaired renal function, arteritis, collagenosis, diabetes mellitus, a history of alcohol abuse or other forms of addiction, and non-Swedish origin of the subject. All subjects gave informed consent to their participation in the study, the protocol of which had been approved by the ethics committee of the Karolinska Hospital.

**Gene Sequencing**

For DNA procedures, nucleated cells from frozen whole blood were prepared according to Sambrook et al., and DNA was extracted by a salting-out method. For the nucleotide sequencing of the promoter of the HL gene, a 1400-bp section of the proximal promoter, spanning from positions −1267 to 133, was amplified by polymerase chain reaction (PCR) with the forward primer (5'-CTCTGCTTTAAATCCCTACCTCTTCG) and the reverse primer (5'-ACCTGTTTTCAGGCCGTTTGT). This PCR fragment was used as a template for further amplifications as part of the Taq DyeDeoxy Terminator Cycle sequencing system (Perkin-Elmer, Applied Biosystems Division). Nested primers, designed on the basis of the published sequence of the promoter of the HL gene, were used for the analysis of overlapping sections of 200 to 300 bp in both directions.

**Genotyping**

Genotyping for the −250G/A and −514C/T polymorphisms was performed with use of a PCR fragment amplified with the forward primer (5'-GGATCACCTCTCAATGGGTC) and the reverse primer (5'-GGATCACCTCTCAATGGGTC), followed by digestion with the restriction enzymes DraI and NlaIII, respectively. The −710T/C and −763A/G polymorphisms were genotyped with use of a PCR fragment amplified with the forward primer (5'-TCTGGCCAGGAATCTCCTTC) and the reverse primer (5'-GACCCATTGTGAGGTTGATC), followed by digestion with the restriction enzymes Aval and SpII, respectively.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared according to Alksnis et al. All buffers were freshly supplemented with leupeptin (0.7 μg/ml), aprotinin (16.6 μg/ml), phenylmethylsulfonyl fluoride (0.2 mmol/L), and 2-mercaptoethanol (0.33 μL/ml). The protein concentrations in the extracts were estimated by the method of Calb and Bernlohr. Incubation for EMSA was conducted as described, and the reaction products were applied to 7% (wt/vol) polyacrylamide gel (acrylamide/methylene-bis-acrylamide weight ratio 80:1), and then electrophoresis was performed in 22.5 mmol/L Tris/22.5 mmol/L boric acid/0.5 mmol/L EDTA buffer for 2.5 hours at 200 V. Nonradioactive competitor DNAs, which were either identical, of the opposite allelic variant, or of nonspecific origin, were added in 100-fold excess of the labeled DNA.

**DNA Constructs**

Two sets of double-stranded oligonucleotides were constructed, constituting the 30-bp sequence around the −710T/C polymorphism, flanked by BamHI and BglII ends. The double-stranded oligonucleotides were ligated head to tail into a BamHI-digested HCAT vector. The correct sequence and orientation of the inserts were tested by DNA sequencing. pCAT plasmids were constructed with use of 945-bp promoter fragments, spanning from −895 to 50, ligated into a pCAT-basic vector as described by the supplier (Promega Corp). The promoter fragments were obtained by PCR amplification of DNA samples from subjects homozygous for the −250A/−514T/−710C/−763G haplotype or homozygous for the wild-type haplotype with use of the forward primer (5'-AAGTGAC-TCTGGCCAGGAATCTCCTTC) and the reverse primer (5'-GCTCTAGAGTCCATCTCCGTTCTAC). The correct sequence of the inserts was tested by DNA sequencing. Plasmids specific for the −250A, −514T, −710C, and −763G mutations were generated with the QuickChange site-directed mutagenesis kit (Stratagene Cloning Systems) with use of the wild-type pCAT plasmid described above.

**Transient Transfection Assay**

Human hepatoblastoma (HepG2) cells were cultured in 90-mm dishes in DMEM supplemented with 10% FCS. Confluent cells were transfected by the calcium phosphate DNA coprecipitation method, essentially as described. The pSV-β-galactosidase gene (Promega) was cotransfected as an internal control. In all experiments, 5 μg of the CAT construct and 5 μg of the β-galactosidase plasmid were added to the medium. CAT activity was analyzed by the method of Sambrook et al. and quantified with a PhosphorImager (Fuji Photo Film Co). β-Galactosidase activity was determined as described by the supplier (Promega). CAT levels were expressed in arbitrary units after standardization for β-galactosidase activity. All constructs were tested in triplicate in 4 to 8 independent transfection experiments.

**Statistical Methods**

Allele frequencies were estimated by gene counting.

**Results**

**Detection of 4 Common Polymorphisms in Promoter Region of HL Gene**

A 1267-bp section of the proximal promoter of the HL gene was sequenced in both directions with use of DNA samples from 25 subjects with either low, intermediate, or high postheparin plasma HL activities. The sequence analysis of the present study agreed completely with the nucleotide sequence determined by Oka et al., whereas several differences were observed with other published nucleotide sequences. In agreement with the report by Guerra et al., 4 polymorphisms were found, located at positions −250 (G-to-A substitution), −514 (C-to-T substitution), −710 (T-to-C substitution), and −763 (A-to-G substitution). No additional polymorphisms were discovered. Several homozygotes for the rare alleles were found among subjects with low postheparin HL activity. Heterozygotes were found in subjects with either low or intermediate postheparin HL activity, whereas none of the subjects with high postheparin HL activity displayed any of the 4 rare alleles.

Assays were then developed for all 4 polymorphisms in the promoter of the HL gene, and DNA samples from 186 healthy middle-aged men were analyzed. All polymorphisms were found to be in Hardy-Weinberg equilibrium. The frequencies of the rare −250A, −514T, −710C, and −763G alleles were 20.7%, 19.9%, 20.7%, and 20.7%, respectively. Complete allelic association was observed for the −250G/A, −710T/C, and −763A/G polymorphisms. The −514C/T polymorphism was also in strong linkage disequilibrium with the other polymorphisms, but in DNA samples from 3 subjects, the rare −250A/−710C/−763G haplotype was found in association with the common −514C allele.

**Allele-Specific Binding of Nuclear Protein(s) to the −710T/C Polymorphic Site**

The potential physiological roles of the −250G/A, −514C/T, −710T/C, and −763A/G polymorphisms in the promoter of the HL gene were analyzed by EMSA. No evidence was found for differences in binding patterns of nuclear protein(s) when the wild-type and the mutant alleles of the −250G/A, −514C/T, and −763A/G polymorphisms were analyzed (data not shown). However, a distinct difference in binding pattern was observed between the wild-type −710T and the mutant −710C fragments (Figure 1A). The protein-DNA complex indicated with an arrow in Figure 1A was present at considerably higher concentrations when the mutant −710C...
A fragment was compared with the wild-type 710T fragment.

Quantitative analyses of this complex demonstrated significant differences between the mutant and wild-type fragments at all nuclear extract concentrations tested (Figure 1B).

Competition studies showed that a 100-fold excess of the unlabeled 710T or 710C fragment substantially reduced the interaction of the labeled 710T fragment with the nuclear proteins (Figure 1C). Similar results were obtained in competition studies that used the 710C fragment. It is noteworthy that the mixture of labeled and unlabeled 710C and 710T fragments generated an additional protein-DNA complex in these competition experiments (Figure 1C, lanes 4 and 8). The nature of this complex is presently unknown.

Taken together, the results of the EMSA studies provided evidence of a difference between the 710T and 710C fragments regarding the binding of nuclear proteins.

2710T/C Polymorphism and Transcription of HL Gene

Transient transfection studies in HepG2 cells were conducted to explore whether the 2710T/C polymorphism influences the rate of transcription of the HL gene in vitro. CAT activities were compared between constructs harboring 1 or 2 tandemly arranged 30-bp fragments with either the 710T or the 710C site were compared in transfection studies with HepG2 cells. Constructs were tested in triplicate in 4 independent experiments. The CAT activities of the 710C-1x and 710C-2x constructs were expressed relative to the CAT activities of the 710T-1x and 710T-2x constructs, respectively, with the latter jointly indicated as wild type (WT). No significant differences in CAT activities between the different constructs were observed.

Figure 2. No difference in transcriptional activity of the 710C allele. CAT activities of constructs harboring 1 or 2 tandemly arranged 30-bp fragments with either the 710T or the 710C site were compared in transfection studies with HepG2 cells. Constructs were tested in triplicate in 4 independent experiments. The CAT activities of the 710C-1x and 710C-2x constructs were expressed relative to the CAT activities of the 710T-1x and 710T-2x constructs, respectively, with the latter jointly indicated as wild type (WT). No significant differences in CAT activities between the different constructs were observed.

-710T/C Polymorphism and Transcription of HL Gene

Transient transfection studies in HepG2 cells were conducted to explore whether the 710T/C polymorphism influences the rate of transcription of the HL gene in vitro. CAT activities were compared between constructs harboring either 1 or 2 tandemly arranged 30-bp fragments of the HL promoter containing either the 710T or the 710C sites. As shown in Figure 2, no differences in CAT activities were observed between constructs containing either of the 2 sites. These results indicate that the difference in binding pattern of nuclear protein(s) between the 710T and 710C fragments does not affect the rate of transcription of the HL gene.

Presence of Rare Alleles of All 4 HL Promoter Polymorphisms Does Not Influence Basal Rate of Transcription of HL Gene

In subsequent transfection experiments, the potential effects of the promoter polymorphisms on the basal rate of transcription of the HL gene were analyzed with the use of 945-bp fragments.
the promoter polymorphisms on the rate of transcription of the HL gene. In the present study, we evaluated the physiological roles of the 4 polymorphisms by using EMSAs and transient transfection studies in HepG2 cells to determine which 1 of the 4 polymorphisms is physiologically relevant. No evidence was found for a significant contribution of any of these polymorphisms to the basal rate of transcription of the HL gene. Therefore, it is concluded that the −250G/A, −514C/T, −710T/C, and −763A/G polymorphisms in the promoter of the HL gene cannot account for the observed relations of HL genotypes with plasma HL activity and HDL cholesterol concentration.

Initial studies that made use of the EMSA technique demonstrated a difference between the −710T and −710C alleles regarding the binding of ≥1 nuclear proteins. This difference in binding properties may form the basis for differences in transcriptional activity of the 2 alleles of this genetic variant, depending on the physiological impact of the nuclear protein(s) binding at this section of the promoter. However, extensive analysis with different transfection strategies provided no evidence for a significant functional difference between the −710T and −710C alleles. This indicates that the observed differences in the EMSAs in relation to the −710T/C polymorphism are not associated with detectable changes in the basal rate of transcription of the HL gene. Moreover, no differences were observed between the 2 alleles for the −250G/A, −514C/T, and −763A/G polymorphisms as analyzed by EMSAs and transfection studies in HepG2 cells. Taken together, these studies provide considerable evidence against a potential role of the −250G/A, −514C/T, −710T/C, and −763A/G polymorphisms in the regulation of transcription of the HL gene.

There are several possible explanations for the absence of a clear physiological role of any of the 4 polymorphisms of
the HL gene, as analyzed in the HepG2 in vitro system. First, it is possible that the basal conditions used in the in vitro studies are not comparable to the situation in vivo. Second, it cannot be excluded that HepG2 cells lack some of the components necessary for expressing the potential effects of at least 1 of the promoter polymorphisms. For example, HepG2 cells may be devoid of specific nuclear factors interacting with the polymorphic sites. Third, it is indeed possible that the $-250G/A$, $-514C/T$, $-710T/C$, and $-763A/G$ polymorphisms do not influence the basal rate of transcription of the HL gene but instead are in linkage disequilibrium with an as-yet-unknown additional polymorphism in the HL gene with a profound effect on the synthesis rate of HL. We favor this latter hypothesis because it explains the relations between the promoter polymorphisms and HL activity and plasma HDL cholesterol concentration observed in previous studies as well as the lack of physiological function of the 4 promoter polymorphisms in vitro. Unfortunately, the nature of this additional polymorphism is presently unknown. Further studies are required to identify the HL gene mutation(s) responsible for the marked effects on HL activity and HDL cholesterol concentrations.

Little is known about how the expression of the HL gene is regulated. The promoter region of the human HL gene has been characterized, and possible regulatory elements were identified by searching for consensus sequences. Transfection studies have indicated that multiple elements in the proximal promoter influence the transcription of the HL gene, but none of these elements have been identified and/or analyzed in detail. Furthermore, none of the studies reported to date provide clear evidence that any of the $-250G/A$, $-514C/T$, $-710T/C$, and $-763A/G$ polymorphisms are localized in a section of the promoter with an important regulatory function in the transcription of the HL gene. In summary, no evidence was found for a significant effect of the $-250G/A$, $-514C/T$, $-710T/C$, and $-763A/G$ polymorphisms on the basal rate of transcription of the HL gene. However, the presence of HL genotype associations with postheparin plasma HL activity and plasma HDL cholesterol concentration strongly indicates that functional mutations do exist in the HL gene. Further studies are warranted to define these mutations.

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References
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