The Mutation Causing the Common Apolipoprotein A-IV Polymorphism Is a Glutamine to Histidine Substitution of Amino Acid 360

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Apolipoprotein (apo) A-IV is a protein involved in the metabolism of chylomicrons and high density lipoproteins. This protein displays genetic polymorphism due to two main codominant alleles, A-IV' and A-IV2. We have identified the mutation that leads to this polymorphism. It is caused by a single-base substitution of guanine for thymine in the third base of codon 360. This substitution leads to a glutamine to histidine change. Direct sequencing of amplified DNA from eight subjects in a three-generation pedigree has demonstrated that the guanine to thymine substitution can explain the apo A-IV polymorphism. In 32 unrelated individuals, a correspondence between apo A-IV phenotype determined by isoelectric focusing and genotype determined with Fnu4HI digestion of amplified DNA could be demonstrated. The enzyme lecithin:cholesteryl acyltransferase (LCAT) is activated by apo A-IV. Under our in vitro conditions, the isoprotein apo A-IV 1-1 is a better LCAT activator than is the isoprotein apo A-IV 2-2. A knowledge of the molecular mechanism underlying the apo A-IV polymorphism will help to elucidate the mechanisms involved in LCAT activation. (Arteriosclerosis and Thrombosis 1991;11:851-856)
by centrifugation and stored at \(-20\)°C until used. Plasma used for purification of apo A-IV was collected by plasmapheresis.

**Apolipoprotein A-IV Phenotyping**

Phenotype analysis was performed by immunoblotting after IEF of the serum.\(^{18}\)

**Isolation of Apolipoproteins**

Human apo A-IV was isolated from lymph obtained from a patient with chylothorax. The lymph was centrifuged in a Beckman Model 50.2 rotor (Beckman Instruments, Palo Alto, Calif.) for 12 hours at 40,000 rpm. After washing and delipidation, the chylomicron apolipoprotein was dissolved in 10 mM tris(hydroxymethyl)aminomethane (Tris) HCl (pH 8.0) buffer containing 6 M urea,\(^7\) chromatographed on a DEAE-Sepharose column (Pharmacia–LKB Biotechnology, Uppsala, Sweden) equilibrated with the same buffer, and eluted with linear gradient 10–300 mM Tris HCl (pH 8.0) containing 6 M urea. The apo A-IV–containing fractions were pooled, dialyzed against 50 mM NH\(_4\)HCO\(_3\), lyophilized, and subjected to high-pressure liquid chromatography (HPLC).\(^{20}\)

Human apo A-IV was isolated from plasma as described by Weinberg and Scanu.\(^{27}\) The proteins absorbed to intralipid were, upon delipidation, further purified by DEAE–Sepharose chromatography and HPLC.

**Apolipoprotein A-I Preparation**

Human apo A-I was isolated from HDLs (d=1.063–1.210 g/ml) after delipidation by preparative IEF in Ultradex (Pharmacia–LKB Biotechnology) as described.\(^{28}\)

**Lecithin:Cholesterol Acyltransferase Preparation**

LCAT was isolated from fresh plasma collected by plasmapheresis by a modification\(^{28,29}\) of the method described by Albers et al.\(^{30}\) The following changes in purification steps were made: the anion exchanger Accell QMA (Millipore Corp., Bedford, Mass.) replaced DEAE–Phadex A-50 (Pharmacia–LKB Biotechnology), and the final purification step was gel filtration on Sephacryl S-200 (high resolution, Pharmacia–LKB Biotechnology). The purified enzyme was stored at 4°C in 10 mM Tris HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and 5 mM \(\beta\)-mercaptoethanol. The enzyme preparation was used within 4 weeks.


Apo A-I– and apo A-IV 1-1– or apo A-IV 2-2–egg phosphatidylcholine–cholesterol proteoliposomes were prepared by a slight modification of the sodium cholate dialysis method described by Chen and Albers.\(^{31}\) The substrates consisted of complexes of egg yolk phosphatidylcholine–cholesterol–(1,2-[\(^3\)H]cholesterol–apo A-I, –apo A-IV 1-1, or –apo A-IV 2-2) in the molar ratio of 250:12.5:0.8, respectively. Typically, egg phosphatidylcholine, cholesterol, and \([\(^3\)H]cholesterol as a tracer in organic solvents were mixed in a glass vial, and the organic solvents were evaporated to dryness under \(N_2\). The residual organic phase was removed by lyophilization. The dried lipid mixture was then solubilized by vortexing in 10 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA. Then homogeneous apo A-I or apo A-IV (isoforms 1-1 or 2-2) and sodium cholate (final concentration, 55 mM) were added. The mixture was vortexed for 1 minute and then incubated at 25°C for 20 minutes after which it was dialyzed against 10 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA for approximately 50 hours to remove sodium cholate. The molar ratio of the components in the proteoliposome was determined by cholesterol, phospholipid, and protein quantification. The substrates were stored at 4°C under \(N_2\) until used.

**Lecithin:Cholesterol Acyltransferase Assay**

LCAT transacylase activity (phosphatidylcholine cleavage followed by cholesterol esterification) was analyzed in an assay mixture typically consisting of 0.2–0.25 ml assay buffer, 10 mM Tris HCl (pH 7.4), with 150 mM NaCl and 1 mM EDTA, 0.125 ml 2% bovine serum albumin, and 0.05–0.1 ml apo A-I– or apo A-IV–containing proteoliposome substrate. The mixture was preincubated at 37°C for 15 minutes to activate the substrate, after which 0.025 ml 0.1 M \(\beta\)-mercaptoethanol and 30–50 \(\mu\)l enzyme was added. After vortexing, the reaction mixture was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 8 ml CHCl\(_3\)/CH\(_3\)OH (2:1, vol/vol), and the lipids were extracted by the method of Folch et al.\(^{32}\) and analyzed by thin-layer chromatography.\(^{28}\)

**N-Terminal Sequence Analysis**

The isolated proteins (apo A-IV 1-1 and apo A-IV 2-2) were digested with Endoprotease Lys-C (Wako Chemicals, Neuss, FRG), and the released peptides were separated by HPLC by use of a Vydac C-18 reverse-phase column equilibrated with 0.1% trifluoroacetic acid in H\(_2\)O eluted with a gradient of acetonitrile (0–70% in 30 minutes). The sequence analysis was performed by automated Edman degradation with a modified Applied Biosystems Model 477A/120A on-line pulsed liquid-phase/gas-phase sequencer in the gas-phase mode.

**DNA Preparation**

DNA was isolated from 5 ml of EDTA-augmented blood\(^{35}\) from subjects belonging to different apo A-IV phenotypes.

**Amplification of DNA**

Genomic DNA (0.5 \(\mu\)g) isolated from apo A-IV 1-1 and 2-2 subjects was amplified by the polymerase chain reaction (PCR) technique.\(^{34}\) The primers used...
Tenkanen et al APO A-IV Polymorphism 853

 were chosen according to the published apo A-IV gene structure 13: exon 1, 5'-CACTGCAGGCGAGGTAGCCT-3' and 5'-GCCTCCATCCTGCACTCT-3'; exon 2, 5'-GAGCCAGGGCTAGGGTCTGTCGTC-3' and 5'-TTAGGGCTGGGCTGGTGC-3'; exon 3, 5'-TGCCCCTGCTCCGACAAATTGCT-3' and 5'-GCTGTCCAAGGGGAGCAG-3'.

The PCR was performed in a reaction mixture containing 1 μM of primers, deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, (0.2 mM each), 20 mM Tris HCl (pH 8.8), 1.5 mM MgCl2, 0.1% Tween-20, 0.1 mg/ml gelatin, and 2.5 units Thermus aquaticus (Taq) DNA polymerase (US Biochemical Corp., Cleveland, Ohio) in a final volume of 100 μl. The cycles of denaturation (1 minute at 95°C), annealing (1 minute at 60°C), and elongation (2 minutes at 72°C) were repeated 30 times. The elongation time for exon 3 was 3 minutes. The amplified products were analyzed on 2% agarose gels and identified by staining with ethidium bromide and Southern blotting.

Cloning

For sequencing, amplified DNA from each exon was blunt-end ligated into the Smal I site in the pGem3 (Promega, Madison, Wis.).

Sequence Analysis

The cloned fragments were sequenced with the dideoxy-chain termination reaction 37 by use of a commercial kit (Sequenase.2, Cleveland, Ohio) with SP6 and T7 primers (Promega). For sequencing of the third exon, two additional primers, located about 250 nucleotides from each end of the exon, were used.

For direct sequencing of PCR fragments of exon 3, the DNA was subjected to agarose gel electrophoresis, eluted from the agarose gel with a commercial kit (Gene clean II, La Jolla, Calif.), boiled for 10 minutes, cooled on ice, and sequenced according to the Sequenase.2 kit protocol except that the annealing time used was 10 minutes. A sequencing primer, 5'-CACTTCAGGCCTTGGAG-3', located 107 nucleotides 5' of the mutation point, was used.

Synthesis of Primers

Primers for PCR amplification and sequencing were synthesized on an Applied Biosystems Model 381A DNA synthesizer.

Statistical Methods

Paired t tests (two-sided p values) were used to assess differences in LCAT activation properties of apo A-IV isoforms.

Results

Sequence analysis of amplified DNA corresponding to the three exons of the apo A-IV gene from subjects homozygous either for apo A-IV 1-1 or 2-2 only revealed one difference, a single-base substitution at position 2,387. An autoradiogram of sequencing gels of DNA from subjects with the phenotypes apo A-IV 1-1, apo A-IV 1-2, and apo A-IV 2-2 are shown in Figure 1. The substitution is a guanine to thymine change in the third base of codon 360, causing a glutamine to histidine substitution. This amino acid substitution results in an increase in the positive charge of the apo A-IV 1-1 isoprotein by one unit and explains the difference observed between the isoproteins on IEF.
To establish that this point mutation is the basis for the common apo A-IV polymorphism, the inheritance of this mutation was studied in a family and compared with the inheritance of the electrophoretic apo A-IV\(^1\) and apo A-IV\(^2\) alleles. The family tree of a kindred consisting of three generations is illustrated in Figure 2. From these subjects, the portion of the apo A-IV gene that corresponds to exon 3 was amplified by PCR as illustrated schematically in Figure 3. After amplification, the DNA from the family members was analyzed by direct DNA sequencing (data not shown). It is evident that the apo A-IV\(^1\) allele cosegregates with the -CAG- coding for glutamine, while the -CAT- coding for histidine at position 360 segregates with apo A-IV\(^2\).

The guanine to thymine substitution in the apo A-IV\(^2\) allele results in the loss of one recognition site for the restriction enzyme Fnu4HI. This enzyme can be used to demonstrate the substitution after amplification of part of exon 3 with a tailored primer. Using this method, we determined the genotypes of 32 unrelated subjects, and the results were concordant with the phenotypes obtained by IEF and immunoblotting.

The mutation was further verified by amino acid sequence analysis of apo A-IV protein purified from one subject homozygous for the apo A-IV\(^1\) allele and from two subjects homozygous for the apo A-IV\(^2\) allele. In all cases, the results were in accordance with those obtained by DNA sequencing.

As apo A-IV is an activator of LCAT, it was of interest to study whether the mutation described interferes with the activating capacity of apo A-IV. The isoproteins apo A-IV 1-1 and apo A-IV 2-2 were each isolated from two different subjects. In three separate experiments the activation of LCAT by the two isoproteins was compared with that of apo A-I. In these experiments apo A-I always was a better activator than either of the two apo A-IV isoforms (Figure 4). The mean percent activations caused by the isoproteins apo A-IV 1-1 and apo A-IV 2-2 compared with that of apo A-I were 43% and 36%, respectively. In all three experiments apo A-IV 1-1 was a somewhat better activator (p<0.01) than was apo A-IV 2-2.

**Discussion**

In the present study we determined the mutation that causes the common electrophoretic apo A-IV polymorphism. This polymorphism, which is identical with USP1,\(^1\)\(^1\) can easily be determined by IEF followed by Western blotting.\(^1\)\(^8\)\(^2\)\(^0\) It is the result of two common codominant alleles, apo A-IV\(^1\) and apo A-IV\(^2\), at a single genetic locus. PCR amplification of the apo A-IV alleles followed by cloning into a plasmid and nucleotide sequencing revealed that a guanine to thymine transition in the third position of codon 360 was the difference between the two alleles. This single-base substitution leads to a change of amino acid 360. A glutamine at this position in apo A-IV\(^1\) is substituted by histidine in apo A-IV\(^2\).

Analysis of the apo A-IV gene from a three-generation kindred by use of PCR followed by direct sequencing of the products further established that this point mutation underlies the common apo A-IV polymorphism. Also, direct sequencing of the apo A-IV protein purified from subjects homozygous for either the apo A-IV\(^1\) or apo A-IV\(^2\) allele were in agreement with the above findings.

In a previous study\(^1\)\(^4\) in which the primary structure of apo A-IV was determined by direct protein sequencing, the amino acid at position 360 was glutamine, the amino acid corresponding to the more...
common allele apo A-IV\textsuperscript{1}. However, as this is different from the amino acid histidine derived from liver cDNA analysis by Elshourbagy et al,\textsuperscript{12} those authors concluded that sequence heterogeneity at this position could not be excluded. We now demonstrate that this sequence heterogeneity is genetically determined and explains the apo A-IV polymorphism.

This mutation causes the loss of one Fnu4HI restriction site. The DNA fragments resulting after digestion are small, thus making use of this enzyme for apo A-IV typing complicated. However, these technical difficulties can be avoided by using PCR with a tailored primer.\textsuperscript{38} In all 32 subjects studied by this method, the common apo A-IV polymorphism could be explained by a guanine to thymine substitution on which the apo A-IV polymorphism is based (at residue 360) is located in the center of one such amphipathic helix repeats, one located between residues 66–87 and the other between residues 99–120 of apo A-I.\textsuperscript{41} It is conceivable that amphipathic regions of the apo A-IV molecule in a similar way take part in the activation of LCAT. The amino acid substitution on which the apo A-IV polymorphism is based (at residue 360) is located in the center of one such 22-mer amphipathic helix, amino acids 351–373. Whether the small changes in the activating capacity of different apo A-IV isoforms that we have observed during in vitro incubations have any physiological relevance in vivo remains to be established.

The role of apo A-IV in lipoprotein metabolism is present far from clear. It has been postulated to be involved in chylomicron and HDL metabolism.\textsuperscript{6} Therefore, the observation that in at least some populations\textsuperscript{20,24} the alleles at the apo A-IV locus have significant effects on plasma HDL cholesterol and triglycerides is of interest and may help to unravel the physiological role of apo A-IV. In this regard, knowledge on the variation in the apo A-IV gene, which gives rise to apo A-IV polymorphism, may be important for understanding the underlying molecular mechanisms.

The LCAT enzyme is important for normal HDL metabolism,\textsuperscript{39} and apo A-IV has been shown to be an activator of LCAT at least in vitro.\textsuperscript{7,8} Our observation, that under the assay conditions described apo A-IV is about one third as effective as apo A-I in activating LCAT, is in line with previous work.\textsuperscript{7,8} The use of different isoforms of apo A-IV for activation indicated that the isoform apo A-IV 1-1 is a somewhat better activator than apo A-IV 2-2. This observation is contradictory to data recently reported that apo A-IV 2-2 is more efficient in activating LCAT than is apo A-IV 1-1.\textsuperscript{40} As it is known that LCAT activation by apoproteins depends on the composition of the lipid substrate,\textsuperscript{7} it is possible that this apparent discrepancy is due to assay conditions.

The activating capacity of apo A-I on LCAT is thought to be due to its amphipathic helices, which are similar to those found in apo A-IV. Recently, it was shown that a major LCAT-activating effect of apo A-I is associated with two 22-mer tandem amphipathic helix repeats, one located between residues 66–87 and the other between residues 99–120 of apo A-I.\textsuperscript{41} It is conceivable that amphipathic regions of the apo A-IV molecule in a similar way take part in the activation of LCAT. The amino acid substitution on which the apo A-IV polymorphism is based (at residue 360) is located in the center of one such 22-mer amphipathic helix, amino acids 351–373. Whether the small changes in the activating capacity of different apo A-IV isoforms that we have observed during in vitro incubations have any physiological relevance in vivo remains to be established.

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References


FIGURE 4. Bar graph showing activation (nmol/ml/h at left; percent at right) of purified lecithin:cholesterol acyltransferase (LCAT) by apolipoprotein (apo) A-I and two isofoms of apo A-IV: Activating capacity of apo A-I and of the two isoforms of apo A-IV, apo A-IV 1-1 and apo A-IV 2-2, are represented by columns. Each column represents the activating capacity of apoprotein isolated from a separate subject. The assay was performed with apoprotein-containing proteoliposomes in which the molar ratio of egg phosphatidylcholine/cholesterol/apoprotein was 250:12.5:0.8. They were prepared by the sodium cholate detergent dialysis procedure. Each column represents the mean of six assays (±SD) performed in three separate experiments.


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