Human Evidence That the Apolipoprotein A-II Gene Is Implicated in Visceral Fat Accumulation and Metabolism of Triglyceride-Rich Lipoproteins

Ferdinand M. van ’t Hooft, MD, PhD; Giacomo Ruotolo, MD, PhD; Susanna Boquist, MD, PhD; Ulf de Faire, MD, PhD; Gösta Eggertsen, MD, PhD; Anders Hamsten, MD, PhD

Background—Apolipoprotein (apo) A-II is a major structural protein of plasma HDLs, but little is known regarding its functions.

Methods and Results—To investigate the physiological role of apoA-II in humans, we screened the promoter region of the apoA-II gene for a functional polymorphism and used this polymorphism as a tool in association studies. A common, functional polymorphism in the promoter region of the apoA-II gene, a T to C substitution at position −265, was found. Electrophoretic mobility shift assays demonstrated that the −265T/C polymorphism influences the binding of nuclear proteins, whereas transient transfection studies in human hepatoma cells showed a reduced basal rate of transcription of the −265C allele compared with the −265T allele. The −265C allele was associated with decreased plasma apoA-II concentration and decreased waist circumference in healthy 50-year-old men. In addition, oral fat tolerance tests provided evidence that the −265C allele enhances postprandial metabolism of large VLDLs.

Conclusions—ApoA-II appears to promote visceral fat accumulation and impair metabolism of large VLDLs.

Key Words: lipoproteins • apolipoproteins • metabolism • genetics • obesity

A polipoprotein (apo) A-II is a major protein constituent of HDLs, but relatively little is known about the role of this apolipoprotein in HDL metabolism. Increased plasma apoA-II concentrations were proposed to be proatherogenic, given that HDL particles that contain both apoA-I and apoA-II are less effective than HDL particles that contain apoA-I for mobilization of cellular cholesterol from nonhepatic cells. However, additional properties of apoA-II have been reported, such as the ability to influence actions of hepatic lipase, cholesterol ester transfer protein, phospholipid transfer protein, lecithin-cholesterol acyltransferase, and class B type I scavenger receptor. Moreover, studies in either human or murine apoA-II transgenic mice, and apoA-II knockout mice indicate that apoA-II is involved in plasma clearance of triglyceride-rich lipoproteins; influences plasma levels of free fatty acids, glucose, and insulin; and affects adipose mass, which suggests a role of apoA-II in insulin sensitivity and fat homeostasis. Thus, murine data indicate that apoA-II has a complex metabolic role, but whether apoA-II has similar functions in humans is not clear.

The aim of the present study was to uncover and to characterize a physiologically relevant polymorphism in the promoter region of the apoA-II gene that influences apoA-II production and to use this polymorphism as a tool to examine the overall role of apoA-II in lipid and glucose metabolism in vivo in humans.

Methods

Subjects
A total of 624 healthy men, all 50 years of age, were included in the present study. Selection of men of identical age was intended to eliminate the confounding effect of age and sex on lipoprotein and glucose metabolism. Plasma apoA-II concentration for each subject was measured by immunoturbidometric assay (Behringwerke AG). Plasma insulin was determined by ELISA (Dako Diagnostics). Plasma lipoproteins and whole-blood glucose were measured as described. ApoB-48 and apoB-100 concentrations in triglyceride-rich lipoprotein fractions, isolated in the course of an oral fat tolerance test, were determined by SDS–PAGE. The protocol was approved by the Ethics Committee of the Karolinska Hospital, and all subjects gave informed consent to participate.

Gene Sequencing
For nucleotide sequencing of the promoter of the apoA-II gene, a 1219-bp section spanning from position −1148 to +71 was amplified by polymerase chain reaction (PCR) with forward primer 5’-GCCAGATCATGCCCATTAC and reverse primer 5’-ATGCTGCTCACACATCTTG. This PCR fragment was used as a
Genotyping
DNA was isolated by use of a genomic DNA isolation kit (Qiagen Inc). The −265T/C polymorphism in the apoA-II promoter was analyzed by use of a PCR-fragment amplified with forward primer 5′-CATGGGTGTGATATGCAGGAC and reverse primer 5′-TCAGGTGACAGGGACTATGG followed by digestion with restriction enzyme BamHI. The −932G/A, −974C/G, −978G/C, and −1099T/C polymorphisms all were analyzed by use of a PCR fragment amplified with forward primer 5′-GCCGAGATCATGCGATTAC and reverse primer 5′-CACCTGTCATTCTGATCACC followed by digestion with restriction enzymes MboI, HphI, MaelIII, and BamHI, respectively. ApoE genotype was determined as described.20

DNA Constructs
Two sets of double-stranded oligonucleotides were constructed, which constituted the 27-bp sequence around the −265T/C polymorphism flanked by BamHI and BglII ends. Double-stranded oligonucleotides were ligated head to tail into a BamHI-digested human cationic amino acid transporter vector.21 Promoter cationic amino acid transporter (pCAT) plasmids were also constructed by use of an 1186-bp promoter fragment spanning from −1148 to +38 and ligated into a pCAT-Basic vector as described by the supplier (Promega Corp). The promoter fragment was obtained by PCR amplification of DNA from a subject homozygous for the −265C allele with forward primer 5′-AAGCTGACAGGGAGACTATGG and reverse primer 5′-GGCTTAGACGTACGCAGC. Wild-type plasmid and plasmids specific for the −978G and −1099C mutations were generated with the Quick-Change site-directed mutagenesis kit (Stratagene Cloning Systems), by use of the pCAT plasmid described above.

Electrophoretic Mobility Shift Assay
Electrophoretic mobility shift assay (EMSA) was conducted as described.23 Nonradioactive competitor DNAs, either identical, of opposite allelic variant, or of nonspecific origin, were added in separate experiments. Electrophoretic mobility shift assay (EMSA) was conducted as described23 and quantified by use of a phosphorimager. β-galactosidase activity was determined as described23 and quantified by use of the pSV-β-galactosidase plasmid (Promega) were added to the medium. CAT activity was analyzed as described23 and quantified by use of a phosphorimager. 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 experiments.

Transient Transfection Assay
Human hepatoma (HepG2) cells were transfected by use of the calcium-phosphate DNA coprecipitation method.25 In all experiments, 5 μg of CAT construct and 5 μg of pSV-β-galactosidase plasmid (Promega) were added to the medium. CAT activity was analyzed as described23 and quantified by use of a phosphorimager. 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 experiments.

Statistical Methods
Allele frequencies were compared by gene counting and χ² analysis. Differences in transcriptional activity were determined by Student’s paired t test, whereas differences in continuous variables according to apoA-II −265T/C genotype were tested by 1-way ANOVA. The Tukey-Kramer test was used for post hoc analysis when differences between groups were indicated by ANOVA. Responses to the oral fat load according to apoA-II genotype were compared by repeated measures ANOVA.

Results
Polymorphisms in Proximal Promoter of ApoA-II Gene
Five different polymorphisms were discovered: −265T/C, −932G/A, −974C/G, −978G/C, and −1099T/C. The −265T/C, −932G/A, −974C/G, −978G/C, and −1099T/C polymorphisms were in almost complete negative linkage disequilibrium, which indicates the presence of 3 predominant haplotypes: −265C/−978G/−1099T, −265T/−978G/−1099T, and −265T/−978G/−1099C.

-265T/C Polymorphism Influences Basal Rate of Transcription of the ApoA-II Gene
HepG2 cells were transfected with minimal promoter constructs harboring 27-bp fragments of the −265T site or the −265C site were compared in triplicate in 6 independent transfection studies with HepG2 cells. CAT activities of the −265C construct were expressed relative to the −265T construct. B, CAT activities of 1186-bp long apoA-II promoter constructs that contain the −285G, −978C, or −1099C mutations were compared in triplicate in 4 independent transfection studies with HepG2 cells. CAT activities of constructs were expressed relative to activity of the hypothetical wild-type promoter with the −265T/−978G/−1099T haplotype.

Figure 1. Reduced transcriptional activity of the −265C allele. A, CAT activities of constructs harboring 30-bp fragments of the −265T site or the −265C site were compared in triplicate in 6 independent transfection studies with HepG2 cells. CAT activities of the −265C construct were expressed relative to the −265T construct. B, CAT activities of 1186-bp long apoA-II promoter constructs that contain the −285G, −978C, or −1099C mutations were compared in triplicate in 4 independent transfection studies with HepG2 cells. CAT activities of constructs were expressed relative to activity of the hypothetical wild-type promoter with the −265T/−978G/−1099T haplotype.
120±15%, \(P<0.01\) and \(-1099\)C (71±21% versus 108±26%, \(P=0.04\)) constructs.

**Allele-Specific Binding of Nuclear Proteins to –265T/C Polymorphic Site**

Binding characteristics of a 30-bp DNA fragment that contained either the –265T or –265C site of the apoA-II promoter were evaluated by use of nuclear extracts derived from HepG2 cells. As shown in Figure 2A, increased concentrations of 2 major DNA-protein complexes (arrows 1 and 2) were observed with increased nuclear extract concentrations. The DNA-protein complex indicated by arrow 2 in Figure 2A was present at considerably higher concentrations when the –265C fragment was compared with the –265T fragment. Quantitative analysis of this complex demonstrated significant differences between the –265C and –265T fragments at all nuclear extract concentrations tested (Figure 2B, right). In contrast, no quantitative differences were observed (Figure 2B, left) for the DNA-protein complex indicated by arrow 1 in Figure 2A. Competition studies (Figure 3) showed that a 100-fold excess of unlabeled –265T and –265C fragment substantially reduced the concentration of the radiolabeled DNA-protein complex 1, but no allele-specific differences were observed. In contrast, a 100-fold excess of unlabeled –265C fragment substantially reduced the concentration of DNA-protein complex 2 when the labeled –265C fragment was analyzed (Figure 3, lane 7), whereas a 100-fold excess of unlabeled –265T fragment had only a marginal effect on the concentration of DNA-protein complex 2 (Figure 3, lane 8).

**–265C allele Is Associated With Lower Plasma ApoA-II Concentration, Lower Waist Circumference, and Enhanced Postprandial Metabolism of Large VLDLs**

Relationships between the –265T/C polymorphism and clinical and biochemical parameters were analyzed in a group of 624 healthy, 50-year-old men (Table). Plasma apoA-II concentration was significantly lower in carriers of the –265C allele than in carriers of the –265T allele. In contrast, no relationships were found between the –265T/C polymorphism and waist circumference, whereas no associations were observed between –265T/C genotype and either blood glucose or plasma insulin concentrations. Overall response of plasma triglycerides during the entire 6-hour postprandial period was unassociated with the –265T/C polymorphism in 97 subjects with an apoE3/3 genotype (T/T, \(n=39\); T/C, \(n=42\); and C/C, \(n=16\)). However, postprandial apoB-100 concentrations in the Sf >60 of triglyceride-rich lipoproteins (a measure of large VLDL particles) were significantly lower in subjects homozygous
for the −265C allele than in subjects homozygous (Figure 4) for the −265T allele (P=0.01 for genotype, P=0.02 for interaction between genotype and time by repeated measures ANOVA). Difference in plasma concentration of postprandial large VLDL particles according to genotype remained significant (P=0.02) at 6 hours after oral fat load after controlling for waist circumference or fasting plasma insulin concentration. In contrast, postprandial responses of small VLDLs (Sf 20 to 60 of apoB-100) or chylomicron remnants of different particle size (as reflected by apoB-48 concentrations in subfractions of triglyceride-rich lipoproteins) were uninfluenced by −265T/C polymorphism.

### Discussion

Three basic observations were made regarding the physiological significance of the −265T/C polymorphism. First, in EMSA studies, a distinct difference in binding of nuclear factors was observed between 27-bp fragments that contained either the −265T or −265C site. Second, transient transfection studies provided evidence for decreased basal transcription rate of constructs that contained the −265C site compared with constructs that contained the −265T site. Third, a significant association was found between the −265T/C genotype and plasma apoA-II concentration in healthy middle-aged men. These observations suggest that −265T/C polymorphism affects binding of ≥1 hepatic nuclear factors to the promoter of the apoA-II gene, which results in decreased apoA-II expression in hepatocytes, reduced secretion of apoA-II by the liver, and decreased apoA-II concentration in plasma. The corollary is that the −265T/C polymorphism can be used to analyze the physiological role of apoA-II in humans.

The human apoA-II promoter segment between nucleotides −911 and +29 is sufficient for liver-restricted expression of a reporter gene in transgenic mice. Rate of transcription of the apoA-II gene is controlled by a complex array of regulatory elements, designated A to N on the basis of DNase I footprinting analysis. The −265T/C polymorphism is found in the middle of element D, located between nucleotides −255 and −276. Element D binds several different nuclear factors, designated AIID1, AIID2, and AIID4, as well as an activity related to CCAT/enhancer binding protein-β. Researchers have proposed that element D has a negative regulatory role in apoA-II gene transcription when occupied by AIID1 or AIID4, whereas the negative effect is reversed when element D is occupied by AIID2. The −265T/C polymorphism probably interrupts this delicate balance of binding of nuclear factors. Because transfection experiments and association studies indicate that the −265C allele is associated with a reduced rate of transcription, the nuclear proteins found in protein-DNA complex 2 are likely to act as transcriptional repressors.

Overall effect of the −265T/C polymorphism on the plasma apoA-II concentration was relatively modest. This
observation is in agreement with previous reports\textsuperscript{26,27} that show that deletion of element D has only a moderate effect on the rate of transcription of the apoA-II gene. This fact limits the application of the $9-265T/C$ polymorphism in association studies and may explain the relatively small effect of the $9-265T/C$ polymorphism on biochemical and anthropometric measurements. Moreover, some of the effects of apoA-II observed in murine models (for example, those related to HDL cholesterol concentration), may not be apparent in humans as a result of the limited effect of the $9-265T/C$ polymorphism on rate of transcription of the apoA-II gene.

An interesting observation in the present study was the relationship between $9-265T/C$ polymorphism and postprandial plasma concentrations of large VLDLs. This relationship indicates that reduced plasma apoA-II concentration associated with the $9-265C$ allele enhances the ability to remove large VLDLs from circulation during alimentary lipemia. Overexpression of human apoA-II earlier was shown to increase VLDL production and impair VLDL removal in mouse models.\textsuperscript{16,17} In addition, enhanced clearance of remnant lipoproteins was observed in the apoA-II/apoE double knockout mouse compared with the apoE knockout mouse.\textsuperscript{18} These animal studies emphasize the effect of apoA-II on remnant metabolism, whereas the human data point to a role of apoA-II in clearance of large VLDL particles. Principal differences between murine and the human studies are likely to explain this discrepancy. Also, regulation of plasma concentrations of small VLDLs is more complex than that of large VLDLs, which renders demonstration of genotype-phenotype associations for small VLDLs more difficult.

The association between the $9-265T/C$ polymorphism and waist circumference but not body mass index indicates a role of apoA-II in visceral obesity. That apoA-II is implicated in visceral obesity in transgenic mice recently was reported by Castellani et al.,\textsuperscript{17} who hypothesized that overexpression of apoA-II alters HDL composition such that the HDL interaction with skeletal muscle CD36 is impaired, which results in reduced use of free fatty acids for energy production and increased fat mass. A direct relationship of apoA-II expression to plasma free fatty acid concentrations also has been observed in studies involving common inbred strains of mice,\textsuperscript{28} apoA-II transgenic mice,\textsuperscript{17} mice lacking apoA-II,\textsuperscript{18} and human families with high prevalence of coronary artery disease.\textsuperscript{28}

Additional functional polymorphisms were not found in the proximal promoter of the apoA-II gene. Common functional mutations in the coding regions thus far have not been discovered,\textsuperscript{29} and linkage disequilibrium was not observed between the $9-265T/C$ polymorphism and $MspI$ and $BstNI$ polymorphisms in the $3^\prime$ end of the human apoA-II gene (data not shown). Therefore, that the $9-265T/C$ polymorphism is in linkage disequilibrium with another functional polymorphism in the apoA-II gene is unlikely, but it remains possible that observed associations are due to functional polymorphisms in other genes. Nevertheless, the overall similarity between our present human data and previous mouse data strongly suggests that associations between the $9-265T/C$ polymorphism and lipid and fat metabolism are directly related to the apoA-II gene, thus underlining the multifunctional metabolic roles of apoA-II.

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