Effects of Six APOA5 Variants, Identified in Patients With Severe Hypertriglyceridemia, on In Vitro Lipoprotein Lipase Activity and Receptor Binding


Objective—The purpose of this study was to identify rare APOA5 variants in 130 severe hypertriglyceridemic patients by sequencing, and test their functionality, because no patient recall was possible.

Methods and Results—We studied the impact in vitro on LPL activity and receptor binding of 3 novel heterozygous variants, apoAV-E255G, -G271C, and -H321L, together with the previously reported -G185C, -Q139X, -Q148X, and a novel construct -Δ139 to 147. Using VLDL as a TG-source, compared to wild type, apoAV-G255 and -L321 and -C185 showed reduced LPL activation (~25% [P=0.005, ~36% [P<0.0001], and ~23% [P=0.02], respectively). ApoAV-C127, -X139, -X148, and Δ139 to 147 had little affect on LPL activity, but apoAV-X139, -X148, and -C271 showed no binding to LDL-family receptors, LR8 or LRP1. Although the G271C proband carried no LPL and APOC2 mutations, the H321L carrier was heterozygous for LPL P207L. The E255G carrier was homozygous for LPL W86G, yet only experienced severe hypertriglyceridemia when pregnant.

Conclusion—The in vitro determined function of these apoAV variants only partly explains the high TG levels seen in carriers. Their occurrence in the homozygous state, coinheritance of LPL variants or common APOA5 TG-raising variant in trans, appears to be essential for their phenotypic expression.

Key Words: apolipoprotein AV ■ LDL-R family ■ LR8 ■ LRP1 ■ HSPG-bound LPL

Premature truncations of APOA5, Q139X, Q148X, and the 1V3S+3g>c are associated with severe Hypertriglyceridemia (HyperTG),1,2 behaving as phenocopies of lipoprotein lipase (LPL) deficiency. These rare APOA5 variants do not always lead to a deficiency in circulating plasma apoAV, and carriers present with a range of apoAV levels (reviewed in ref 3). Kao et al4 identified a common polymorphism G185C in a Taiwanese study which occurs at a minor allele frequencies of 0.04 in controls but at a 6.3-fold higher frequency of 0.27 in hypertriglyceridemic patients (P<0.001).

ApoAV is present on chylomicrons, VLDL, and HDL, but not on IDL or LDL, suggesting that VLDL-containing apoAV is cleared before the lipolytic cascade.6 One function of apoAV is to activate LPL, and ApoAV knockout mice have 4-fold higher TG levels than wild type.7,9 Whereas LPL transgenic mice can rescue ApoA5 knockout mice from hypertriglyceridemia (HyperTG), this is not entirely reciprocal, suggesting that the effect of apoAV on plasma TG is dependent on heparin-sulfate proteoglycan (HSPGs)-bound LPL.7,8 In addition, apoAV-dependent TG catabolism acts by enhancing receptor-mediated endocytosis via members of the LDL-receptor (LDLR) family.10,11

We have identified 3 novel APOA5 missense variants (E255G, G271C, H321L) in patients with TG levels >10 mmol/L. Although LPL and APOC2 variants had been excluded for the G271C carrier, the coding exons of LPL and APOC2 were sequenced in the 2 other probands. Because family studies were not possible, the APOA5 variants were expressed in vitro together with G185C, Q139X, Q148X.12,5 In addition we designed a deletion construct Δ139 to 147, to

Original received April 18, 2008; final version accepted July 8, 2008.

From the Division of Cardiovascular Genetics, Department of Medicine (B.D., W.W.Z., J.A.C., W.P., R.W., S.E.H., P.J.T.), UCL, London, UK; the Department of Medical Biochemistry (A.D., W.J.S.), Max F. Perutz Laboratories, Medical University Vienna, Austria; the Department of Medical Biosciences/Physiological Chemistry (S.K.N., A.L., G.O.), Umeå University, Sweden; AMC Liver Center (F.G.S., M.N., J.A.K., J.J.P.K.), Amsterdam, The Netherlands; the Institute for Clinical and Experimental Medicine (J.A.H.), Prague, Czech Republic; the Department of Internal Medicine I (M.M.), University Hospital Hamburg-Eppendorf, Hamburg, Germany; the National Hospital for Neurology & Neurosurgery (P.J.L.), London, UK; the Centre de Biophysique Moléculaire Numérique (L.L.), Gembloux, Belgium; BIOSIRIS (N.d.), Crealys Park, Gembloux, Belgium; the 3rd Department of Medicine, 1st Faculty of Medicine (M.V.), Charles University, Prague, Czech Republic; the Institute for Biochemistry and Molecular Biology II (J.H.), University Hospital Hamburg-Eppendorf, Hamburg, Germany; and the Department of Chemistry (A.L.), Tallinn University of Technology, Estonia.

B.D. and W.W.Z. contributed equally to this study.

Correspondence to Professor Philippa Talmud, Division of Cardiovascular Genetics, Department of Medicine, British Heart Foundation Laboratories, Rayne Building, Royal Free and University College Medical School, 5 University Street, London WC1E 6JF, UK. E-mail p.talmud@ucl.ac.uk

Arterioscler Thromb Vase Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.108.172866
study whether the deleted region could explain the difference in phenotypes between Q139X and Q148X. To fully characterize these mutant proteins, their effects on LPL activation and receptor binding, were undertaken.

**Methods**

See supplemental methods (available online at http://atvb.ahajournals.org) for full details.

**Patients**

In total 130 patients with TG levels $>$10 mmol/L were recruited into the study; seven patients from the UK, 28 patients from the Netherlands with LPL and APOC2 mutations excluded, and 95 patients from the Czech Republic.

**Resequencing of APOA5**

Sequence the coding exons of APOA5 was performed in 4 fragments.

**Expression and Purification of Recombinant ApoAV**

For expression WT apoAV and all mutant proteins in pET20b vector were transformed into BL21(DES) cells (Novagen, UK) and cultured following standard conditions. Recombinant apoAV was purified under denaturing conditions on a Ni-NTA-His Bind resin column (Novagen, UK).

**Ligand Binding Experiments**

Details using either LR8 or LRP1 are described in detail elsewhere.$^{10,11}$

**Results**

**Identification of Novel APOA5 Variants in Patients With Severe HyperTG**

Our aim was to identify novel variants in APOA5 and assess their frequency in patients with severe HyperTG. Sequencing the APOA5 coding region in 130 individuals with TG levels $>$10 mmol/L identified 4 nonsynonymous variants: E255G (c.764 A $>$ G), G271C (c.821 G $>$ T), H321L (c.962 A $>$ T), and A315V (c.824 C $>$ T), all in exon 3. A315V was present in 3 HyperTG Czech individuals and occurs at polymorphic frequencies in a Czech cohort and was not studied further. Two intronic point variants (IVS1 $>$ IVS3) were predicted by splice recognition algorithms (http://www.fruitfly.org/seq_tools/splice.html) to not affect splicing and were not investigated further. The frequency of these 3 novel variants was 2.3%, and that of the common SNPs S19W (rs1131 T $>$ C) was identified in the heterozygous state in a 43-year-old Dutch female with persistent HyperTG and bouts of pancreatitis. She abstained from alcohol and had a stable BMI of 28 kg/m$^2$. Her pretreatment plasma TG was $\approx$ 20 mmol/L and when treated and adhered to a fat-restricted diet, her TG levels dropped to 6.30 mmol/L (Table). She had no family history of cardiovascular disease, pancreatitis, or HypertG. The patient was homozygous for $\approx$ 1131C. Plasma apoAV levels, as determined by ELISA, were 3762 ng/mL, 15-fold higher than mean levels in normolipidemic volunteers (257 ng/mL). On a nonreducing Western blot (supplemental Figure I) her plasma apoAV appeared mainly in a 120 kDa form, 3-fold larger than wild type (WT) apoAV (around 40 kDa). Her postheparin LPL activity and mass was 121% and 8.2, respectively, in the Czech and Dutch HyperTG patients compared to reported normolipidemic controls from those countries.$^{13,14}$

E255G (c.764 A $>$ G) was identified in a 32-year-old female Asian UK patient with gestational Type I hyperlipoproteinemimia. She was also heterozygous for APOA5 S19W. Coding exons for both the LPL and APOC2 genes were sequenced and revealed that the proband was homozygous for LPL W86G (with a normal APOC2 sequence). This variant had been previously reported in a pediatric Type I hyperlipoproteinemic patient,$^{15}$ so it was surprising that it was only under the stress of her 2 pregnancies that her plasma TGs rose to $\approx$ 75 mmol/L and she developed cutaneous xanthomas. Before and after her pregnancies she was able to maintain TGs levels between 2 and 10 mmol/L with a low-fat diet alone (Table). APOA5-G255 was not detected in 260 healthy white controls and in the absence of Asian controls, we tested for its presence in an Asian cohort (n = 508) with type 2 diabetes (T2D).$^{16}$ Ten patients were heterozygous for E255G (carrier frequency 1.7%), one of whom also carried the APOA5 $\approx$ 1131T $>$ C rare allele. Fasting lipid levels were available on 9 E255G carriers and their mean TG levels were 1.64±0.48 mmol/L, which did not differ significantly from the sample mean (2.44±1.7 mmol/L, $P=0.17$ (supplemental Figure I, available online at http://atvb.ahajournals.org).

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Gender</th>
<th>BMI, kg/m$^2$</th>
<th>Treatment</th>
<th>Lipid levels, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low fat diet</td>
<td>Acipimox &amp; atorvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fenofibrate &amp; atorvastatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>anti-diabetic drugs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S19W genotype</th>
<th>E255G</th>
<th>G271C</th>
<th>H321L</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW</td>
<td>32</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>TT</td>
<td>28</td>
<td>4.5</td>
<td>8.3</td>
</tr>
<tr>
<td>SW</td>
<td>1.29</td>
<td>1.14</td>
<td>8.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LPL variant</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>W86G (HMZ)</td>
<td>P207L (HTZ)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table. Baseline and Posttreatment Characteristics of the 3 Patients With Identified APOA5 Variants From the Current Study**
detected in 282 healthy Czech controls. The sequencing of coding exons of \textit{LPL} and \textit{APOC2} identified that he was a carrier of the French Canadian \textit{LPL} P270L mutation.

Using Protein Prediction Methods to Understand the Potential Functional Domains of ApoAV

Molecular modeling was used to characterize the effect of these novel and previously reported variants on apoAV function. Results from mutation prediction algorithms, SIFT and PolyPhen, are shown in supplemental Table II. ApoAV is a 366-aa apoprotein which is highly \(\alpha\)-helical and free of beta structure with a calculated molecular weight of 41.2 kDa. The 5 coiled-coil elements and 9 predicted Receptor Binding Domains (RBD)\(^{19}\) represent protein–protein, protein–DNA, or protein–ion interaction domains (supplemental Table II). E255 is in an alpha helix in a putative RBD. Glycine rotates easily and adds flexibility to the protein chain. Thus E\(\rightarrow\)G is likely to create a conformational change with glycine inside the protein molecule, reducing the ability of apoAV to interact with HSPG-bound LPL.

Although WT apoAV has only a single cysteine residue at position 227, a G\(\rightarrow\)C at either site would allow the formation of a disulfide bond. Western blotting confirmed that these variant proteins form dimers and multimers, changing the tertiary structure (Figure 1). In addition, A171-R245 is a potential lipid-binding domain, including leucine residues at positions 162, 163, 172, and 173, and a tryptophan residue at position 170, which facilitate lipid interactions. Furthermore, residue 185 occurs within a RBD and may therefore have an effect on protein–protein interactions.

H321 lies outside any of the predicted coiled-coil or RBDs (supplemental Table II), but within a disordered domain (G201 to K335). Although hydrophilic histidine can stabilize the folded structures of proteins, hydrophobic leucine tends to be located inside the protein molecule. This change is likely to affect the interaction with LPL.

X139, X148, and \(\Delta\)139 to 147 all are predicted to lie in a coiled-coil domain with a putative RBD at position 146 to 152 (supplemental Table II). The X148 and \(\Delta\)139 to 147 maintain most of the coiled-coil domain, whereas X139 only retains 30%.

Expression of Recombinant APOAV Proteins

Recombinant WT apoAV, the 6 variants and the novel \(\Delta\)139 to 147 variant were expressed in vitro with and without the C-terminal His\(_{6}\)-tag sequence. This enabled us to establish that the C-terminal His\(_{6}\)-tag sequence was not interfering in the LPL activity assay. The 2 truncated variants could not be detected with the apoAV polyclonal antibody directed against the C terminus of the protein. Expression of all proteins was confirmed by Western blot analysis under nonreducing conditions, using either the apoAV polyclonal antibody or a His\(_{6}\)-tag antibody (Figure 1).

Effect of Recombinant ApoAV on Human VLDL Hydrolysis by HSPG-Bound LPL In Vitro

To mimic the activation of LPL at the vascular endothelium by the different apoAV proteins, the previously described assay which incorporates HSPG was used,\(^{8}\) and a concentration titration using WT apoAV confirmed that 5 \(\mu\)g/mL was within the assay linear range. The results are shown in Figure 2. Compared to the negative control (LPL-only) WT stimulated LPL activity by 35% ([\(P=0.003\)], confirming apoAV as an activator/stabilizer of LPL. Compared to WT, apoAV-L321 had the largest impact on LPL activation (\(-36\%\) [\(P<0.0001\]), next apoAV-G255 showed a \(-25\%\) effect ([\(P=0.005\]). The two G\(\rightarrow\)C substitutions showed different effects, with apoAV-C271 having a nonsignificant effect of \(-11\%\) ([\(P=0.24\]), whereas apoAV-C185 displayed a \(-23\%\) on LPL activation ([\(P=0.02\]). Of the two truncated proteins X139 showed 18% ([\(P=0.04\]) lower LPL activity whereas X148 and \(\Delta\)139 to 147 showed only borderline effects on LPL activity of \(-15\%\) ([\(P=0.09\]) and \(-16\%\) ([\(P=0.08\]), respectively.

Effect of Recombinant ApoAV on Lipid Emulsion Hydrolysis by HSPG-Bound LPL In Vitro

To test whether the presence of apoAV in human plasma influenced the LPL hydrolysis of VLDL, we repeated the
assay using a lipid emulsion as a TG source (Figure 3). Essentially the results were the same, however the different effects seen for the 2 G>C mutants in the VLDL assay were reversed.

**Binding of Recombinant ApoAV to LDLR Relatives (LRs) by Ligand Blotting**

Direct ligand blotting was carried out to compare mutant and WT protein binding to the chicken LDLR relative, LR8 based on Dichlbenger et al. Binding to the 95-kDa LR8 from chicken follicle extract is shown in Figure 4. Binding of apoAV-L321, -E255, -C271, -C185 as well as apoAV-X139 to 147 was comparable to WT binding, suggesting that the 9 deleted amino acids are not involved in receptor binding, which must be C-terminal of apoAV-Q148 because neither apoAV-X139 nor -X148 bound to LR8. ApoAV-C271 showed no binding be C-terminal of apoAV-Q148 because neither apoAV-X139 nor -X148 bound to LR8. ApoAV-C271 showed no binding to the LR8.

**Binding of Recombinant ApoAV to LR1 Using Surface Plasmon Resonance**

Binding studies using surface plasmon resonance (SPR) to LR1 confirmed these results, identifying apoAV -X139, -X148, and -C271 as nonbinders. Dissociation constants (K_d) were calculated to determine overall affinity for this interaction. Compared to WT, apoAV-C185 displayed close to unchanged affinity for LR1, K_d WT = 134×10^{-9} mol/L and K_d G255 = 107×10^{-9} mol/L, respectively. apoAV-L321, -L321 and -X148, and -C271 as nonbinders. Dissociation constants (K_d) could not be calculated for these 3 mutants also displayed a significant loss of number of binding sites compared to WT as shown by the lower response in Figure 5 and could represent conformational changes in the mutant proteins. K_d could not be calculated for nonbinders.

**Discussion**

We have identified 3 novel APOA5 missense variants, E255G, G271C, and H321L, in patients with severe HyperTG (>10 mmol/L at a frequency of ~2.3%). However, in addition to these variants, the E255G proband was homozygous for the previously reported LPL W86G15 and the H321L carrier was heterozygous for LPL P207L suggesting that, at best, variants in APOA5 are making a rather minor contribution to the disorder. We expressed these APOA5 variants together with those previously reported1-5 and carried out functional studies in vitro obtaining novel insights into the structure: function relationship of apoAV.

Wang et al resequenced coding exons in the APOA5, LPL, and APOC2 in 110 HyperTG patients. They identified no rare APOA5 variants, but 10% had LPL or APOC2 variants compared to 0.2% in normolipidemic controls.20 The carrier frequency of the S19W was 4.7-fold higher in Hyper TG patients than controls.20 In our study the carrier frequency of rare APOA5 and LPL variants was 2.3% and 1.5%, respectively. Rare allele frequency differences for S19W and 1131T>C in HyperTG patients compared to same nationality controls were ~3.5- and ~2.4-fold higher, respectively.

**ApoAV-G255**

The impact of E255G on TG levels in vivo is modest at best. The proband was also homozygous for LPL W86G, a variant

---

**Figure 3.** Lipid emulsion hydrolysis associated with different recombinant apoAV proteins (L321, G255, C271, C185, X139, X148, and Δ139 to 147) by HSPG-bound LPL. The percentage increase/decrease relative to LPL was: WT, +46% (P<0.0001), G255+15% (P<0.05), X139+23%, C185+31%, 321L+19%, X139+8, X148+12%, Δ139 to 147+35%, WT apoCIII−74%. Probability values compared to WT.

**Figure 4.** LR8 ligand blot under nonreducing conditions. Human ApoAV WT (lane 1), Δ139 to 147 (lane 2), Q139X (lane 3), Q148X (lane 4), G165C (lane 5), G271C (lane 6), E255G (lane 7), and H321L (lane 8), incubated with anti-His antibodies, anti-LR8 (lane 9) incubated with anti-LR8.

**Figure 5.** LR1-apoAV WT and mutant interaction using surface plasmon resonance.
known to cause Type I hyperlipoproteinemia in the homozygous state.\textsuperscript{15} Interestingly, the E255G proband only expressed severe HyperTG during her 2 pregnancies. Gestational HyperTG has been reported in patients homozygous or compound heterozygotes for LPL variants\textsuperscript{21,22} and is suggested to be related to increased VLDL secretion from the liver, especially in the third trimester of pregnancy, overwhelming the hydrolytic capability of the mutant LPL.\textsuperscript{22} ApoAV-G255 does show some reduced functionality and is less effective in activating LPL (as seen when VLDL or lipid emulsion were the TG-source), and this could exacerbate the HyperTG. However this variant occurred at a carrier frequency of 1.7\% in a study of T2D Indian Asians and was not associated with TG levels that differed significantly from noncarriers. The change from a negatively charged glutamic acid to small neutral glycine could affect protein tertiary structure, alpha helices formation or the putative RBD predicted at 251 to 257.

**ApoAV-C271 and -C185**

The patient heterozygous for G271C had a 15-fold higher plasma apoAV level compared to the normal plasma pool. The estimated molecular weight of her plasma apoAV-G271C was 3-fold higher (120 kDa) than WT, and the unpredecnted apoAV levels suggest that this is a function of polymerization of the apoAV. Western blot show that the majority of this protein exists in the multimeric form. In vitro LPL activation by apoAV-C271 was not significantly different from the WT but apoAV-C271 did not bind to the receptors, and this is likely to represent the basis for the HyperTG. Another contributing factor to the HyperTG is likely to be the homozygosity for the rare allele of the APOA5 -1131T>C SNP (Table) which itself is associated with 40\% TG-raising effect in the homozygous state.\textsuperscript{23}

G185C was identified at a polymorphic frequency of 4.2\% in healthy Taiwanese controls, but at significantly higher frequency (\textasciitilde27\%) in Taiwanese HyperTG patients (\textasciitilde0.001).\textsuperscript{5} but is absent in whites.\textsuperscript{13} Compared to G185 homozygotes (mean TG 1.06mmol/L), heterozygous G185C individuals had a modest 15\% higher TG (1.22 mmol/L), whereas the CC185 individuals had TG levels of 21.0 mmol/L, with >10 fold higher risk of HyperTG.\textsuperscript{5} Thus C185 appears only to present with severe TG-raising effects in the homozygous state. In vitro, apoAV-C185 forms multimers, but the majority of this protein, in contrast to -C271, appears to be in the monomeric form (illustrated in Figures 1 and 4a). However, sequence analysis predicts that, in contrast to apoAV-C271, LPL activation would be significantly impaired by apoAV-C185, because of the relative position of the mutated glycine in the protein. G185C showed normal binding to receptors LR8 and LR1, confirming that residue 185 is more likely to be important in LPL activation than receptor binding. The differences between G185C and G271C in binding to LRs could be explained by the quaternary structure of the protein. We speculate that apoAV-C185 favors binding to the receptor over multimerization to itself or to C227, the only cysteine residue present in WT apoAV, whereas apoAV-C271 multimerizes more effectively, which could sterically blocks accessing LR8.

**ApoAV-L321**

The Czech patient, heterozygous for both H312L and APOA5 S19W, was also heterozygous for the LPL variant P207L. He presented with severe HyperTG and T2D. Of all the recombinant apoAV variants tested in vitro, L321 showed the most reduced LPL activation in the VLDL and lipid emulsion assays, and clearly showed an effect on LR1 binding in the more sensitive SPR system. LPL P207L has been well characterized in vitro\textsuperscript{24} and in vivo.\textsuperscript{18} In a study of 34 P207L carriers, in the presence of a APOC3 TG-raising SNP, mean TG were 10.31 mmol/L compared to 5.58 mmol/L in non-carriers of this SNP.\textsuperscript{18} We propose that the presence of APOA5 H321L and S19W compound the effect of P207L, resulting in pretreatment TG levels reaching 63mmol/L, considerably higher than that reported by Garenc et al.\textsuperscript{18}

**ApoAV-X139, -X148, and Δ139 to 147**

The structure–function studies of these 2 reported truncation variants reflect the disparity in their observed LPL activity in vivo. The Q139X proband had only 21\% residual LPL activity (comparable to homozygous LPL deficiency; 1) whereas the Q148X homozygous proband had 60\% residual LPL activity (2). The deletion Δ139 to 147 construct was created to test whether these 9 amino acids were of particular importance in LPL activation. In the VLDL assay X139 showed 18\% lower LPL activity than WT, whereas X148 and Δ139 to 147 had little effect. In the lipid emulsion assay, both X139 and X148 showed significantly reduced LPL activation (10\% and 9\%, respectively). However, in vivo, in the heterozygous state, apoAV from the WT allele is likely to be present in plasma and thus the VLDL experiment should represent a more realistic assay. This supports the in vivo observation that the penetrance of these variants is low\textsuperscript{1,2} and common APOA5 SNPs, as well as obesity and age, are required for expression of HyperTG. It seems unlikely that residues 139 to 147 are crucial for LPL activation. When tested against LR1 and LR8, neither truncation showed binding, suggesting that the receptor-binding domain is located C-terminal to residue 148.

Of the naturally-occurring variants only X148,\textsuperscript{2} C185,\textsuperscript{5} and the recently identified Q97X\textsuperscript{25} have been found in the homozygous state, associated with severe HyperTG. Whereas E255G and H321L probands coinherited LPL variants in the HMZ or HTZ state, respectively, all the other rare APOA5 variants were coinherited with S19W in the heterozygous state or, in the case of G271C, with -1131T>C in the homozygous state. The family studies of X139 and X148 carriers\textsuperscript{1,2} clearly show that these rare APOA5 variants are only of clinical importance when co-inherited with an additional APOA5 TG-raising SNP, primarily S19W. The case of Q97X adds strength to this hypothesis, because the obligate heterozygous parents and heterozygous sibling are all normalTG and homozygous for the common alleles of both S19W and -1131T>C.

A major limitation of this study was that because of the inability to recall these patients and their families, we could not perform cosegregation analysis nor measure postheparin LPL activity. Only in the case of G271C carrier did we have plasma apoAV measures. However, these are the first in vitro
studies of apoAV variants. It now seems clear that the impact of rare variants on TG levels is only evident in the homozygous state, or with coinheritance of LPL variants or in the presence of common APOA5 variants. Thus, missense or truncation variants in APOA5 might occur more commonly, but without these compounding effects they are unlikely to be associated with HyperTG.

Acknowledgments

We thank Prof Jorgen Gliemann, Aarhus University, Aarhus, Denmark for LRPI and receptor-associated protein (RAP).

Sources of Funding

B.D., J.A.C., W.P., R.W., S.E.H., and P.J.T. are supported by the BHF (RG2005/014, and PG 04/110/17827). A.D. and W.J.S. were supported by the “Deutsche Forschungsgemeinschaft and LiDia (Lipids and Diabetes).”

Disclosures

None.

References


Effects of Six APOA5 Variants, Identified in Patients With Severe Hypertriglyceridemia, on In Vitro Lipoprotein Lipase Activity and Receptor Binding


Arterioscler Thromb Vasc Biol. published online July 17, 2008;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2008/07/17/ATVBAHA.108.172866.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/07/17/ATVBAHA.108.172866.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplementary Figures

Figure I: Boxplot of triglyceride levels of Indian Asians with T2D \(^1\). E255E homozygotes (n=508) versus carriers of the E255G variant (n=9), \(p=0.17\). Bar: Median (50\(^{th}\) percentile), top of box = 75\(^{th}\) percentile, bottom of box = 25\(^{th}\) percentile, 0 = outlier values, * = extreme values.
**Figure II:** Western blot analysis of plasma apoAV from the Dutch subject heterozygous for G271C [under reducing (lane 1) and non-reducing conditions (lane 2)] analysed with apoAV polyclonal antibody. The positions of migration (KDa) of marker proteins are indicated.
Reference

Supplementary Figure legends

**Figure 2**: VLDL hydrolysis associated with different recombinant apoAV proteins (L321, G255, C271, C185, X139, X148 and Δ139-147) by HSPG-bound LPL. VLDL (1mM) was isolated from human plasma by ultracentrifugation and incubated with wildtype apoAV and different apoAV expressed variants (1µg/ml) for 30 min at 37 °C. Following this, the VLDL/expressed protein complexes were incubated with 0.5 µg/ml LPL immobilised on HSPG. Free fatty acid release was measured colorimetrically and presented as mean (95 % CI). Experiments were repeated nine times in quadruplicate. P values shown are compared to wildtype (ie. LPL + apoAV WT). The percentage increase/decrease relative to LPL (represented by the dotted line) was: WT, +44%, G255 +2%, C271 +26%, C185 +5%, 321L -17%, X139 +14, X148 +19%, Δ139-147 +17%.

**Figure 3**: Lipid emulsion hydrolysis associated with different recombinant apoAV proteins (L321, G255, C271, C185, X139, X148 and Δ139-147) by HSPG-bound LPL. The lipid emulsion (1mM) was incubated with wildtype apoAV and different apoAV expressed variants (2.5µg/ml) for 30 min at 37 °C. Following this, the liposome/expressed protein complexes were incubated with 1.5 µg/ml LPL immobilised on HSPG. Free fatty acid release was measured colorimetrically and presented as mean (95 % CI). Experiments were repeated four times in triplicate. P values shown are compared to wildtype. The percentage increase/decrease relative to LPL (represented by the dotted line) was: WT, +46%, G255 +15%, C271 +23%, C185 +31%, 321L +19%, X139 +8, X148 +12%, Δ139-147 +35%, WT + apoCIII -74%. Bovine apoCIII (10µM) acted as a positive control, for the inhibition of LPL activation by WT apoAV.

**Figure 4**: Ligand blot. Nitrocellulose strips containing SDS-PAGE-separated proteins under non-reducing (lane 1 - 9) conditions from chicken ovarian follicle membrane extracts were incubated with crude protein extracts (100 µg/ml) obtained from BL21 cells expressing His-tagged human ApoAV WT (lane 1), Δ139-147 (lane 2), Q139X (lane 3), Q148X (lane 4), G185C (lane 5),
G271C (lane 6), E255G (lane 7) and H321L (lane 8), anti-LR8 (lane 9). The strips were washed and incubated with anti-His antibodies (lane 1 - 8). An antibody against LR8 (lane 9) was used to visualize LR8 in the ovarian follicle membrane extract.

**Figure 5:** LRP1-apoAV WT and mutant interaction using surface plasmon resonance.

Binding studies were performed under conditions given in the methods. LRP were immobilized (1.7 fmol/mm²) and an equivalent mass of BSA were used in the reference flow cell. Figure shows plotted RU values from 10 consecutive sample injections for each apoAV variant.
Supplementary methods

Patients. In total 130 patients with TG levels >10 mmol/l were recruited into the study; seven patients from the UK, diagnosed with Type 1 hyperlipoproteinemia, twenty-eight patients from the Netherlands with Type I and Type V hyperlipoproteinemia in whom mutations in LPL and APOC2 in the coding region had been excluded, and ninety-five patients from the Czech Republic, in whom no information existed concerning LPL or APOC2 mutations.

Resequencing of APOA5. Four fragments which cover the coding region of APOA5 gene (exons 1-3) were amplified using the following four pairs of primers (for primer sequences see supplementary table): AV5 (524bp, cover exon 1 and exon 2); AV3 (486bp, cover 5’ half of exon 3), AV2 (546bp, cover middle part of exon 3); and AV1 (493bp, cover 3’ half of exon 3). Overlapping fragments of AV3, AV2 and AV1 span the whole sequence of exon 3. The amplification conditions were as follows: hot start at 95°C for 5 min followed by 30 cycles of 95°C/30 sec, 55°C (except 65°C for AV3)/30 sec, 72°C/45 sec, ending by 72°C/3 min. The PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK) or AMPure PCR Purification System (Agencourt Bioscience, UK).

Sequencing reaction was proceeded by DYEEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE™ DNA Analysis Systems (Amersham Biosciences, UK) under the condition of 95°C/20 sec, 50°C/15 sec and 60°C/60 sec, 25 cycles. Purified by CleanSEQ Dye-Terminator Removal (Agencourt Bioscience, UK), the products of sequencing reaction were run on MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences, UK).

Genotyping for the APOA5 -1131T>C (rs662799) was carried out using the protocol reported previously in 1. The S19W SNP (rs3135506) was ascertained from the resequencing.

ApoAV/pETplasmid vector. The coding sequence of human APOA5 lacking the signal peptide was amplified using synthetic oligonucleotide primers and inserted into pET20b+ expression vector using BamHI restriction enzyme (Novagen, UK). Site-directed mutagenesis was used to introduce the different mutations using the Stratagene QuickChange site-directed mutagenesis kit (Stratagene, UK) and sequencing was performed to verify that no unwanted
mutations had occurred. Primers used for site-directed mutagenesis are shown in the supplementary table. Maxipreps were prepared using Plasmid Midi kit (Quiagen) and sequenced with Applera BigDye Terminator v3.1 sequencing kit on an ABI 377 automated sequencer.

Expression and purification of recombinant apoAV. For protein expression, WT apoAV as well as -G255, -C271, -C185, -L321, -X139 and -X148 in pET20b+ vector were transformed into BL21(DES) cells (Novagen, UK) and cultured in 250ml M9-Medium at 30 °C (supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 1/20 of LB Medium, 20 mM Glucose, 0.1 mg/ml Carbenicillin). The deletion construct and ∆139-147, designed to test the importance of residues 139-147, was also expressed in a similar fashion. When the culture reached OD600 0.5-1.0, apoAV synthesis was induced by the addition of isopropyl thiogalactopyranoside to a final concentration of 0.8 mM. After 4h, the bacteria were pelleted by centrifugation at 2500 rpm for 10 minutes. The pellet was washed with 0.9 % NaCl solution. Recombinant apoAV was purified under denaturing conditions. The pellet was resuspended in solubilisation buffer (containing 6 M guanidine HCl, 10 mM Tris, 100 mM NaH₂PO₄·H₂O, 5 mM imidazole, 5 mM mercaptoethanol, pH 8.0) and disrupted by sonication. The cell lysate was centrifuged for 15 minutes at 10000 rpm and the supernatant fraction was then mixed with a 3 ml bed volume of Ni-NTA-His Bind resin (Novagen, UK). After 1 h incubation on a wheel rotor, the suspension was applied to a column. The column was washed three times with solubilisation buffer and proteins were eluted in solubilisation buffer containing 0.5 M imidazole. Fractions containing the apoAV protein were pooled and dialysed against 1x PBS. Protein concentrations were determined using the bicinchoninic acid method (Pierce, UK).

Protein sequence modeling. ApoAV sequence was analyzed by Protein Investigator™ (Bioiris, http://www.biosiris.com/02prod_serv/ProteinInvestigator/ProteinInvestigator_Info.php). Protein Investigator™ is a rapid, accurate and in-depth study of a protein based on the protein sequence only. This analysis includes prediction of structural and functional properties of the protein using Biosiris’ technology platform and a selection of the most relevant and up-to-
date techniques available on the market. Secondary structure prediction was assessed by two
methods, Psipred \(^2\) and NPSA consensus prediction \(^3\). To obtain a better understanding of the
impact of the missense and truncated proteins on LPL activation, we used an amino acid
substitution (AAS) prediction method (SIFT http://blocks.fhcrc.org/sift/SIFT.html) as well as
a tool which predicts the possible impact of an amino acid substitution on the structure and
function of a human protein using physical and comparative considerations, called PolyPhen
(http://genetics.bwh.harvard.edu/pph/).

Western blotting. 500 ng of recombinant ApoAV were subjected to 4-12 % gradient gels
(Invitrogen). WT apoAV protein and -G255, -C271, -C185, -L321, -X139, -X148 as well as
Δ139–147 proteins were transferred to a nitrocellulose membrane (Hybond C-extra,
Amersham, UK) and proteins were detected with apoAV polyclonal antibody \(^4\) or His\(_6\)-tag
antibody for WT, Q139X and Q148X (Novagen, UK) and corresponding horse-radish
peroxidase-conjugated goat-anti rabbit antibody from Jackson ImmunoResearch (Stratech,
UK). Blots were developed with the enhanced chemiluminescence protocol (Pierce, UK). The
sizes of proteins were estimated with a broad-range molecular mass standard from Invitrogen,
UK.

In vitro hydrolysis of lipoproteins. To investigate the hydrolysis rate of lipoproteins by HSPG-
bound LPL, a previously reported assay was used \(^5\). A 96-well microtiter plate was incubated
for 18 h at 4 °C with 100 µl/well of 5 µg/ml of HSPG (from the basement membrane of
mouse sarcoma, Sigma H4777) in PBS. After washing three times with PBS, the wells were
blocked with PBS containing 1% free fatty acid (FFA)-free bovine serum albumin (Merck,
Germany) for one hour at 37 °C. Bovine LPL in 0.1 M Tris, 20 % glycerol, pH 8.5, were
added to the wells (100 µl/well at 5 µg/ml) and incubated for 1 h at 4 °C. Then the wells were
washed again three times with 0.1 M Tris, pH 8.5. Human VLDL was isolated by density
gradient and adjusted to 1 mM TG concentration (measured by a commercial TG Kit (α-
Laboratories, UK) in 0.1 M Tris, pH 8.5, containing 1 % FFA-free bovine serum albumin. To
prepare the lipid emulsion, 28 mg triolein, 4 mg cholesterol-oleate, 4 mg cholesterol and 8 mg
phosphatidylcholine were mixed in chloroform. The solvent was removed and the lipid
emulsion was formed by vigorous sonication in PBS. Aggregates were removed by filtration using a 450 nm filter (Millipore). The hydrodynamic diameter of nanosomes was approximately 250 nm as determined by dynamic light scattering (data not shown). The lipid emulsion was adjusted to 1 mM triglyceride concentration as described before for human VLDL. Human VLDL as well as lipid emulsion were associated for 30 minutes at 37 °C with the different recombinant apoAV mutants (1 µg/ml for VLDL and 2.5µg/ml for the lipid emulsion with 1µM bovine apoCII for activation of LPL) and lipolysis was started by the addition of VLDL or liposome/apoAV complexes to the HSPG-LPL-containing plates. Lipolysis was stopped after 10 minutes by addition of Triton X-100 (1 % final concentration). FFA concentration was measured by using a commercial NEFA-C Kit (WAKO Chemicals) adapted to 96 well microtiter plates. Experiments were repeated nine times, each time in quadruplicate.

Ligand binding experiments:

Animals: Mature Derco-Brown hens (30–40 weeks old) were purchased from Heindl Co. (Vienna, Austria). All animals were maintained on layer's mash with free access to water and feed and with a daily light period of 14 h.

Antibodies: Antiserum against recombinant ggapoaV was raised in adult female New Zealand White rabbits by injections of 250 µg each of antigen as described previously 6. Murine monoclonal His5-tag antibody (Qiagen; used at 1:500) and mouse anti-glutathione S-transferase (GST) antibody (BD Pharmingen; used at 1:2000) were purchased from the indicated sources. Rabbit anti-LR8 IgG was prepared as described previously 7. Recombinant GST-receptor-associated protein (RAP) fusion protein was produced in DH5 bacteria using a PGEX 2T-derived (Pharmacia) expression plasmid 8.

Preparation of tissue and membrane protein extracts. All operations were performed at 4 °C. For total protein extracts, fresh chicken tissues were homogenized (5 ml/g wet tissue) with an Ultra-Turrax T25 homogenizer in buffer containing 20 mM HEPES (pH 7.4), 300 mM sucrose, 150 mM NaCl, and complete protease inhibitor cocktail (Roche, Austria). Homogenates were spun at 620 g for 10 min, and Triton X-100 was added to the supernatant
to a final concentration of 1%. After incubation for 30 min, a supernatant (designated the total tissue extract) was obtained by centrifugation at 300000 g for 1h, quickly frozen in liquid N2, and stored at −70 °C until use. Membrane fractions and extracts were prepared from fresh chicken tissues as described 9, except that the extraction buffer contained 1% Triton X-100.

The clear supernatant, designated the membrane extract, was treated as described above. LRP1 and RAP was prepared as described in 10.

Western and ligand blotting of apoAV or LR8 were performed as described previously 11.

Surface plasmon resonance experiments

Binding studies were performed on a Biacore 2000 (Biacore, Uppsala, Sweden) using CM5 sensor chips. Low density related protein 1 (LRP1) and bovine serum albumin were immobilized using the Biacore amine coupling kit. Running buffer used contained 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.05 % v/v P20 and 2.5 mM CaCl. All experiments were performed at 25 ºC with 30µl/min flow. Sample proteins were dialysed against running buffer. The receptor associated protein (RAP) was used as a positive control of the system and apoCIII was used as negative control. The reference flow cell containing an equivalent mass as the active flow cell were subtracted from the raw data. Sensorgrams were analyzed using the BIAevaluation software version 3.2 (Biacore, Uppsala, Sweden). Calculations were performed in Sigma Plot.

Statistical analysis. Free fatty acid release (from the LPL activity assays) was log-transformed before analysis to give a normal distribution. The comparison of groups was made using two-way analysis of variance to take into account variation between experiments performed on nine different occasions (in quadruplicate) for human VLDL and data for the lipid emulsion were performed on three different occasions (in triplicates). Regression estimates from this model were used to obtain the ratio of each group relative to the WT, and 95% confidence intervals were constructed. Statistical analysis was performed using SPSS version 12.0.1 (SPSS, Chicago, IL).
References


Supplementary Tables

Table I: Computational analysis of apoAV by the amino acid substitution (AAS) prediction method SIFT and POLYPHEN, a tool which predicts the possible impact of an amino acid substitution on the structure and function of a protein.

<table>
<thead>
<tr>
<th>Amino acid prediction method (SIFT)</th>
<th>Predicted to be</th>
<th>Score</th>
<th>Mean sequence conservation</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>G255</td>
<td>tolerated</td>
<td>0.17</td>
<td>2.34</td>
<td>not affected</td>
</tr>
<tr>
<td>C271</td>
<td>deleterious</td>
<td>0.04</td>
<td>2.65</td>
<td>affected</td>
</tr>
<tr>
<td>C185</td>
<td>deleterious</td>
<td>0.03</td>
<td>2.20</td>
<td>affected</td>
</tr>
<tr>
<td>L321</td>
<td>tolerated</td>
<td>0.78</td>
<td>2.93</td>
<td>not affected</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PolyPhen</th>
<th>Predicted to be</th>
<th>PSIC score difference</th>
<th>PSIC score 1</th>
<th>PSIC score 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G255</td>
<td>possibly damaging</td>
<td>1.58</td>
<td>1.08</td>
<td>-0.50</td>
</tr>
<tr>
<td>C271</td>
<td>possibly damaging</td>
<td>1.75</td>
<td>0.72</td>
<td>-1.04</td>
</tr>
<tr>
<td>C185</td>
<td>possibly damaging</td>
<td>2.00</td>
<td>1.11</td>
<td>-0.88</td>
</tr>
<tr>
<td>L321</td>
<td>probably damaging</td>
<td>2.96</td>
<td>2.21</td>
<td>-0.75</td>
</tr>
</tbody>
</table>
Table II: Predicted Receptor binding domains [RBD, {Gallet, 2000 /id}] of apoAV – representing protein-protein, protein-DNA and protein-ion interactions.

<table>
<thead>
<tr>
<th>RBD</th>
<th>Location</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21 - 26</td>
<td>TKARKG</td>
</tr>
<tr>
<td>2</td>
<td>36 - 43</td>
<td>GDKGRVEQ</td>
</tr>
<tr>
<td>3</td>
<td>48 - 54</td>
<td>KMAREPA</td>
</tr>
<tr>
<td>4</td>
<td>90 - 98</td>
<td>VGMRRQLQE</td>
</tr>
<tr>
<td>5</td>
<td>146 - 152</td>
<td>ELQEQLR</td>
</tr>
<tr>
<td>6</td>
<td>182 - 190</td>
<td>HHTGRFKEL</td>
</tr>
<tr>
<td>7</td>
<td>232 - 237</td>
<td>SRKLTL</td>
</tr>
<tr>
<td>8</td>
<td>251 - 257</td>
<td>DQLREEL</td>
</tr>
<tr>
<td>9</td>
<td>281 - 292</td>
<td>VRQRLQAFRQDT</td>
</tr>
</tbody>
</table>