Dietary fat clearance in type V hyperlipoproteinaemia secondary to a rare variant of human apolipoprotein E: the apolipoprotein E3 (Arg 136 → Ser)

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(Received 25 March 1999 – Revised 20 September 1999 – Accepted 8 November 1999)

This present case report describes two siblings with severe type V hyperlipoproteinaemia, diagnosed very early in life and due to the combination of the common apolipoprotein (Apo) E2 allele and a rare mutant variant of ApoE, ApoE3 (Arg 136 → Ser). Phenotyping of ApoE falsely identified E2/E2 phenotype. The presence of mutated ApoE was suspected on an unusual restriction polymorphism of a Hha 1 restriction site and confirmed by sequence analysis of the cloned polymerase chain reaction fragment of exon 4 and familial segregation study. The severity of the hypertriacylglycerolaemia was modulated by the lipid content of the diet. A low-fat diet enriched in medium-chain triacylglycerol (TAG) decreased but did not normalize plasma TAG levels in both affected patients of the pedigree. A standardized lipid-enriched test meal showed a marked impairment of TAG-rich lipoprotein (TRL) clearance, especially the exogeneous TRL bearing ApoB-48 which still represented 79% of total TRL 7 h after the fat load. Finally, differences between the male and female siblings with the existence of a consanguine relationship in their parents suggested the involvement of other genetic factors in modulating the severity of phenotypic expression. This observation reinforces the usefulness of genotyping of ApoE for the characterization of genetic hypertriacylglycerolaemia and selection of the appropriate diet and treatment.

Apolipoprotein E: Hypertriacylglycerolaemia: Hyperlipoproteinaemia: Triacylglycerol-rich lipoprotein

Apolipoprotein (Apo) E is a component of VLDL, intermediate-density lipoprotein, HDL, chylomicrons and remnants. This protein is the ligand which promotes the recognition and catabolism of ApoE-containing lipoproteins by hepatic receptors (LDL, ApoB,E receptor and ApoE receptor) (Mahley, 1988). The ApoE gene is 3.6 kb long, contains four exons and maps on chromosome 19 (Das et al. 1985). Three common isoforms are observed (E2, E3 and E4) resulting from cysteine to arginine interchanges at residues 112 and 158. Other variants exist but they are rather rare (Rosseneu & Labeur, 1995). As affinity for the LDL-receptor depends on the isoforms of ApoE, this Apo can be considered as a modulator of lipoprotein clearance. In this respect, polymorphism of the ApoE gene currently offers an example of diet–gene interactions conditioning the lipid metabolism. Numerous reports in the literature showed that the presence of ApoE2 was associated with an elevation of plasma triacylglycerol (TAG) in obesity (Parlier et al. 1997), type 2 diabetes (Reznik et al. 1996), and combined (Sijbrands et al. 1996) or drug-induced hyperlipidaemias (Tozuka et al. 1997; Hozumi et al. 1998). In contrast, the ApoE2/E2 genotype inconstantly leads to type III hyperlipoproteinaemia (Walden & Hegele, 1994).

The opportunity to observe a pedigree with a rare mutant form of ApoE3 (Arg 136 → Ser) leading to severe familial type V hyperlipoproteinaemia when associated with the presence of ApoE2 isoform, offered the possibility of studying the interactions between this allele and nutritional factors. A point mutation in which serine replaces arginine at position 136 has already been reported under the name of

Abbreviations: Apo, apolipoprotein; TAG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein.
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‘Christchurch’ but associated with ApoE2 framework in a case of classical type III hyperlipoproteinaemia by Wardell et al. (1987). The unusual presentation of the present case led us to study the sensitivity to alimentary TAG by short-term dietary manipulations and by following exogenous and endogenous TAG-rich lipoproteins during a standardized fat-enriched test meal in comparison to ApoE3/E3 bearing controls.

Subjects and methods

Subjects

The two propositi were a boy (J.B.) and his sister (S.B.) born from related parents, both native of Spain. The diagnosis of hyperlipidaemia was made after the discovery of a splenomegaly during the first year of life in both subjects. An eruptive cutaneous xanthomatosis was also noted in some episodes. There was no other detectable lipidic deposition. Digestive, pancreatic or cardiovascular manifestations were absent during the follow-up (22 years for J.B. and 27 years for S.B.). Both subjects had a normal body weight. Diabetes, hypothyroidism and other causes of secondary hyperlipidaemia were excluded. The very high level of fasting hyperlipidaemia was made after the discovery of a splenomegaly during the first year of life in both subjects. An eruptive cutaneous xanthomatosis was also noted in some episodes. There was no other detectable lipidic deposition.

Apolipoprotein E pheno- and genotyping

Analysis of ApoE was only performed in one proband (J.B.) and his parents (his sister (S.B.) lived abroad). ApoE phenotyping was performed by immunoblotting after isoelectric focusing in an immobilized pH gradient (Martz et al. 1991). ApoE genotyping was performed by restriction fragment length polymorphism method. Genomic DNA was prepared with the Ready Amp Genomic DNA Purification System (Promega, Charbonières, France). ApoE genotypes were determined by digestion of an amplified portion of exon 4 with Hha1 as described by Hixson & Vernier (1990). Samples of the digest (5 μl) were loaded onto 8 % polyacrylamide gel and restriction fragments were detected by ethidium bromide.

Cloning experiments were performed on polymerase chain reaction products of exon 4 using a TA cloning kit (Invitrogen, San Diego, USA) and each selected clone was subsequently analysed by either genotyping or sequencing (fmol DNA sequencing kit, Promega).

Evaluation of the clinical efficacy of alimentary fat restriction

In order to find the most appropriate nutritional approach, the two patients of the family were submitted to diets differing only by the fat content. Their usual diet was of normal energy value with 35 % of energy from fat (balance saturated : monounsaturated : polyunsaturated : 25, 50 and 25 g/100 g respectively). The content of their diet was progressively decreased to 30 % and 20 % of energy from lipids. Finally, a drastic reduction of alimentary fats was obtained (15 %) in which a daily dose of 20 g of medium-chain TAG oil was used as dressing of salads. Medium-chain TAG were used as they are not included in chylomicrons and are directly conveyed to the liver by the portal route (for review see Papamandjaris et al. 1998).

Test meal and analytical determinations

After a 12 h overnight fast according to a previously used procedure (Dubois et al. 1994), patient J.B. was given a test meal containing 40 g TAG in the form of sunflower margarine (50 g) and 50 000 IU retinyl ester as well as five rusks, 125 g yoghurt, half of a boiled egg, 200 ml skimmed milk and decaffeinated coffee. Separately, a control group of eight normolipidic young males (20–29 years) with an ApoE3/3 genotype ingested a comparable test meal. Blood samples were obtained before the test meal and every hour for 7 h after the meal. Chylomicrons were isolated from 1 ml plasma layered under 2 ml NaCl (9 g/l) by ultracentrifugation at 100 000 g for 15 min at 25 000 g in a Beckman TL 100.3 rotor (Palo Alto, CA, USA). The TAG-rich lipoprotein (TRL) supernatant fraction was separated from the whole plasma at a density of 1.019 g/l and 16 °C, at 100 000 revs/min for 3.5 h in a 100.3 rotor with a Beckman TL 100 centrifuge. Subsequently, ApoB-100-containing TRL particles were separated from apoB-48-containing TRL by affinity chromatography using an immobilized 2G8 monoclonal antibody (Mona, Moscow, Russia) which does not cross-react with apoB-48 (Kosykh et al. 1991) according to a procedure described by Cohn et al. (1993). In previously published work using the same method, the presence of residual ApoB-100 was measured in the unbound ApoB-48 containing fraction by SDS-PAGE separation and quantification in plasma of normal and hypertriacylglycerolaemic subjects, i.e. 5–8 % and 7–11 % respectively (Mekki et al. 1999). Thus, ApoB-48-containing TRL were, on the whole, overestimated by about 10 %, which is consistent with results from others (Cohn et al. 1993; Björkergen et al. 1997).

TAG was measured by an enzymic procedure with kits purchased from BioMerieux (Marcy l’Etoile, France). Free fatty acids were measured in serum with a kit provided by Wako chemicals GmbH (Neuss, Germany). Retinyl palmitate was assayed in the chylomicron fraction and the non-chylomicron fraction by using a HPLC method as previously described (Dubois et al. 1994).

Post-heparin plasma samples (2–3 ml) were obtained 15 min after intravenous administration of 50 U heparin/kg. Lipoprotein lipase and hepatic lipase activities were measured according to the method described by Nilsson-Ehle & Schotz (1976). Measurement of Apo (-E, -A, -CII
and -CIII) levels was performed using immunoenzymic assays as previously described (Bard et al. 1990).

**Results**

**Identification of the genetic abnormality in this pedigree**

The fasting lipid profile of J.B. suggested the involvement of ApoE-rich lipoparticles. Indeed, the plasma ApoE level was dramatically increased (0.6 g/l; normal range 0.013–0.061 g/l). Isoelectric focusing electrophoresis phenotyping suggested the presence of ApoE2/E2 isoforms. The postheparin plasma lipoprotein lipase and hepatic lipase activities were 28.8 and 99.0 nmol free fatty acids/ml per min respectively in patient J.B. These values are comparable to those found in the young normolipidaemic males (i.e. 37.1 (SEM 6.3) nmol free fatty acids/ml per min and 104.3 (SEM 6.1) nmol free fatty acids/ml per min for lipoprotein lipase and hepatic lipase respectively).

ApoC II and -C III levels were also in the normal range. The HDL-cholesterol as well as LpA1 levels were low (0.14 g/l and 0.16 g/l respectively). Determination of the ApoE genotype by polymerase chain reaction/restriction fragment length polymorphism of the exon 4 was performed according to the method of Hixson & Vernier (1990) and showed the presence of an unusual fragment of 109 base pairs due to a restriction polymorphism of a Hha I restriction site. Restriction and sequence analysis of the cloned polymerase chain reaction fragments allowed the characterization of the two alleles: one allele was the common allele e2 (Arg 158→Cys) and the other allele was a variant e3 (Arg 136→Ser). The substitution is due to a point mutation of CGC to AGC occurring in the fourth exon of the ApoE gene. In order to confirm that this rare mutation was carried by the e3 allele, the portion of exon 4 was amplified by polymerase chain reaction and subsequently cloned. Several clones were genotyped. The presence of the unusual fragment of 109 base pairs was only found in clones expressing e3 alleles. The sequencing of these clones definitively ascribed the (Arg 136→Ser) mutation to this isoform. These analytical data were confirmed by the study of familial segregation. The parent’s genotypes were determined: the father’s geno-

**Modulation of the severity of hypertriacylglycerolaemia by fat content of the diet**

These investigations confirmed an impairment in the clearance of exogenous TRL induced by the genetic abnormality of ApoE. The effect on plasma TAG levels of four different diets respectively composed of 35 % (normal diet), 30 %, 20 % and 15 % (including 20 g medium-chain TAG) energy from fat are shown in Fig. 2. The low-fat diets only partially reduced hypertriacylglycerolaemia in both subjects, particularly in the female subject.

A standardized lipid-enriched mixed meal test was also performed in patient J.B. at the age of 20 years. Fasting serum and chylomicron-TAG (5-7 and 3.7 mmol/l respectively) were markedly elevated in patient J.B. After test meal intake, serum and chylomicron-TAG concentrations of patient J.B. rose to very high values at 4 h (9.4 and 6.4 mmol/l respectively) and plateaued until 7 h. The relative postprandial increases in serum and chylomicron-TAG (Fig. 3(a)) and chylomicron retinyl palmitate (Fig. 3(b)) were markedly elevated after 4 h (3-7, 2.7 mmol/l and 3663 nmol/l respectively) and remained elevated until 7 h. The mean postprandial pattern exhibited by eight young normolipidaemic males (Fig. 3(a) and (b)) was very different with serum and chylomicron-TAG maximal increases of 0.87 and 0.472 mmol/l respectively, at 3 h and return to baseline values after 7 h. These control subjects also showed a much reduced accumulation of chylomicron retinyl palmitate after 3–7 h.

The relative proportions of TAG in ApoB-48 and ApoB-100-containing TRL particles in the fasting and postprandial state are given in Table 1. In control subjects, a high proportion of plasma TAG was carried by ApoB-100 bearing particles in both fasting and postprandial conditions. In the fasting state, a small but measurable proportion of ApoB-48-containing TRL was found in normolipidaemic subjects as already observed by others (Cohn et al. 1993) by using comparable methodology. In contrast, in patient J.B., a high percentage of TAG was found to be associated with ApoB-48 bearing particles after the lipid load. After 2–4 h, the calculated concentrations of ApoB-48 TRL were 3.09 and 0.7 mmol/l in patient J.B. and young normolipidaemic males respectively. Even after an overnight fasting, a large proportion of TAG was in the form of ApoB-48-bearing TRL in patient J.B. (168 % of normal values). The relative proportions of TAG in ApoB-48-containing TRL particles were calculated from raw data, thus giving a 4–11 %...
overestimation due to some residual traces of ApoB-100 in this fraction, as mentioned earlier.

In patient J.B., the concentration of free fatty acids was 0.38 mmol/l in serum collected after fasting as compared with the mean concentration of 0.50 (SEM 0.06) mmol/l in the group of young normolipidaemic males. Free fatty acids in serum decreased 1±2 h postprandially in patient J.B. (0.20 mmol/l) as in control subjects and then returned to baseline level after 7 h. Fasting insulin (14.8 mU/l) and glucose (4.5 mmol/l) levels in patient J.B. were in the normal range. The postprandial insulin rise was comparable in patient J.B. and in control subjects (data not shown). These data suggest that insulin sensitivity was unaltered in patient J.B.

Discussion

The present study describes a family in which two siblings exhibited a severe type V hyperlipoproteinemia secondary to a rare mutated form of ApoE3, combined with a more common ApoE2 isoform. This point mutation in which serine replaces arginine at a position of 136 has already been reported under the name of ‘Christchurch’ but carried by the ApoE2 framework in a case of type III hyperlipoproteinemia by Wardell et al. (1987). The frequency and the penetrance of this mutation have been evaluated in a Spanish population (Civeira et al. 1996; Pocovi et al. 1996). In Spain the ‘Christchurch’ mutation seems more common, as in a group of fifteen patients with authenticated type III hyperlipoproteinemia, the anomaly was found in six subjects (Civeira et al. 1996). The pathogenic role of the ApoE3 (Arg 136→Ser) mutation can be linked to an impairment in the binding of ApoE to the ApoB/E (LDL) receptor. More generally, it has been shown that residues in the vicinity of 136–150 of ApoE molecule, especially including basic amino acids, are necessary for this function (Lalazar et al. 1988; Wilson et al. 1991). The replacement of basic residues by neutral ones in this critical area as in ApoE2 ‘Christchurch’ or other mutations reported in literature leads to a defective binding to ApoB/E (LDL) receptor. It is noteworthy that Lalazar et al. (1988) has shown by genetically engineered site directed mutagenesis using E. Coli expression system, that an Arg 136→Ser mutation on human ApoE-3, similar to that found in this present family, induces an in vitro reduction of 60% in normal receptor binding activity. The normal ApoE2 isoform, which is combined with the mutated ApoE3 isoform in our observation, has been associated with even more defective binding activity to the specific receptors (Weisgraber et al. 1982). Thus it is likely that the presence of both isoforms with a low affinity to the receptor contributes to the large impairment in TRL clearance in the two patients. The mutated ApoE3 in our observation was transmitted with normal ApoE2. In that line, it is likely that both isoforms are required for the development of severe hyperlipoproteinemia as the mother who exhibits ApoE3 (Arg 136→Ser) genotype showed a normal fasting lipid profile. But the existence of more subtle anomalies during the postprandial phase cannot be excluded. This point should be checked as this mutated ApoE has been found not to be uncommon in some Spanish populations.

Indeed, the abnormal elevated fasting plasma TAG concentration in patient J.B. can be explained by a greatly reduced ability to remove TRL from the circulation. This is especially the case for intestinally derived chylomicrons and remnants containing ApoB-48 which were shown to abnormally accumulate 7 h postprandially in the circulation and consequently, were present in exaggerated amounts in the fasting plasma of this patient too. Such an impairment of exogenous lipoprotein clearance has already been noted in patients with the ApoE2 genotype (Brenninkmeijer et al. 1987; Weintraub et al. 1987; Brown & Roberts, 1991; Boerwinkle et al. 1994).

In line with the Zilversmit (1979) hypothesis which states...
Table 1. Distribution (%) of triacylglycerol-rich lipoprotein (TRL)-triacylglycerol in apolipoprotein (Apo) B-48- and ApoB-100-containing lipoproteins, at fasting and 2–4 h postprandially in eight normal subjects and one subject with type V hyperlipidaemia associated with an ApoE3 isoform

<table>
<thead>
<tr>
<th></th>
<th>Fasting state</th>
<th>Postprandial state* (2–4 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal subjects</td>
<td>Normal subjects</td>
</tr>
<tr>
<td></td>
<td>Patient J.B.</td>
<td>Mean</td>
</tr>
<tr>
<td>TRL-triacylglycerol†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoB-48 (%) particles</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>ApoB-100 (%) particles</td>
<td>55</td>
<td>74</td>
</tr>
</tbody>
</table>

* The standardized lipid-enriched mixed test meal consisted of 40 g triacylglycerol in the form of sunflower margarine (50 g) and 50 000 IU retinyl ester, five rusk, 125 g yoghurt, half of a boiled egg, 200 ml skimmed milk and decaffeinated coffee.

† For details of the analytical procedure see p. 616.
that postprandial lipoproteins are important in atherogenesis in human subjects, several recent studies have shown exacerbated accumulations of TRL and TRL-remnants postprandially in normolipidaemic patients with coronary artery disease (Simons et al. 1987; Groot et al. 1991; Patsch et al. 1992; Karpe et al. 1994). More specifically, the accumulation of ApoB-48-containing TRL in the postprandial state has been correlated to the progression or severity of coronary artery disease (Simons et al. 1987; Karpe et al. 1994). Thus, the presence of mutated forms of ApoE, which are associated with impaired TRL clearance and TRL-remnant accumulation in the postprandial state which lasts more than overnight, might be involved in the development of atherosclerosis.

This rare mutation of human ApoE gene associated with severe hyperlipoproteinaemia extends the list of metabolic syndromes related to this type of genetic abnormality as summarized in Table 2. The recognition of this new mutated form of ApoE3 associated with severe hyperlipoproteinemia was facilitated by the presence of a new site of restriction for Hha 1 which led us to sequence the exon 4 of the ApoE gene. It is noteworthy that ApoE3 (Arg 136 → Ser) was falsely recognized as normal ApoE2 by isoelectric focusing analysis, probably as a direct result of charge alteration.

The very large heterogeneity, however, in the phenotypic expression observed in patients with ApoE genetic abnormalities (summarized in Table 2) and the fact that only 1–2% of the subjects exhibiting the ApoE2/E2 genotype suffer from type III hyperlipoproteinemia (Walden & Hegele, 1994) suggest that factors other than the structure–function relationship in ApoE receptor binding may also be involved in the pathogenesis of hyperlipoproteinemia. The absolute and relative number of ApoE molecules carried by the different TRL probably account for the affinity for these specific receptors (Havel et al. 1980). It is also likely that other factors either genetic or environmental can modulate phenotypic expression. A defect in hepatic lipase activity has been noted in some patients (Hegele et al. 1993). In our proband, both post-heparin hepatic TAG lipase and lipoprotein lipase activities were shown to be normal. However, the consanguinity noted in this family could have promoted some other possible recessive abnormality. Among the environmental factors, gender can also play a role particularly in the present family, explaining the relatively better therapeutic response obtained in the sister after puberty. Orlov et al. (1987) have already shown in a group of subjects with ApoE2 isoform that females are relatively protected from the tendency to increase total VLDL-TAG and -cholesterol noted in males in comparison with their counterparts without this specific isoform. Oestrogens have been reported to ameliorate the lipid profile in patients with type III hyperlipoproteinaemia (Kushwaha et al. 1977; Falko et al. 1979). Oestrogens also improve the uptake of normal human TRL by rat liver but this effect was significantly decreased (but not suppressed) when ApoE2 isoform was present in the particles (Havel et al. 1980). Finally, oestrogens have been shown to increase ApoE gene expression (Srivasta et al. 1997).

In conclusion, this present case report confirms that some familial hyperlipoproteinaemia characterized by an abnormal accumulation of exogenous (chylomicrons) and endogenous TRL (type V) can be related to genetic abnormality of ApoE leading to a dramatic impairment in the clearance process of TRL. This reinforces the usefulness of ApoE genotyping in the characterization of hypertriacylglycerolaemia in order to improve diet and drug prescriptions.

**Table 2. Mutations of human apolipoprotein E gene associated with hyperlipoproteinaemia**

<table>
<thead>
<tr>
<th>Mutation of apolipoprotein E</th>
<th>Classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E Deficiency</td>
<td>Type III</td>
<td>Ghielli et al. (1981)</td>
</tr>
<tr>
<td>E1 Harrisburg (Lys 146 → Glu)</td>
<td>Type III</td>
<td>Riche et al. (1989)</td>
</tr>
<tr>
<td>E1 Bethesda (Gly 127 → Asp)</td>
<td>Type III</td>
<td>Weigler et al. (1984)</td>
</tr>
<tr>
<td>E1 (Gly 127 → Asp, Arg 158 → Cys)</td>
<td>Type IV/V</td>
<td>Wardell et al. (1987)</td>
</tr>
<tr>
<td>E2 Christchurch (Arg136 → Ser)</td>
<td>Type III</td>
<td>Feussner et al. (1996)</td>
</tr>
<tr>
<td>E2 (Arg 142 → Leu)</td>
<td>Type III</td>
<td>Okubo et al. (1998)</td>
</tr>
<tr>
<td>E2 Toramonom (Glu187 → Gin)</td>
<td>Type III</td>
<td>Rall et al. (1982d)</td>
</tr>
<tr>
<td>E2 (Arg 145 → Cys)</td>
<td>Type III</td>
<td>Rall et al. (1983)</td>
</tr>
<tr>
<td>E2 (Lys 146 → Gin)</td>
<td>Type III</td>
<td>Rall et al. (1982a)</td>
</tr>
<tr>
<td>E2 (Arg 156 → Cys)</td>
<td>Type III</td>
<td>Moriyama et al. (1996)</td>
</tr>
<tr>
<td>E2 Fukuyama (Arg 224 → Gin)</td>
<td>Type IV</td>
<td>Zhao et al. (1994)</td>
</tr>
<tr>
<td>E2 (Val 236 → Glu)</td>
<td>Type IV</td>
<td>Walden et al. (1994)</td>
</tr>
<tr>
<td>E3 (Arg 136 → Cys)</td>
<td>Type III</td>
<td>Zhao et al. (1994)</td>
</tr>
<tr>
<td>E3 (Cys 112 → Arg, Arg 251 → Gly)</td>
<td>Type IV</td>
<td>Havel et al. (1986)</td>
</tr>
<tr>
<td>E3 Leyden (seven amino acids insertion at residue 121)</td>
<td>Type III</td>
<td>Havel et al. (1986)</td>
</tr>
<tr>
<td>E3 (Arg 136 → His)</td>
<td>Mild dyslipoproteinaemia</td>
<td>Minnich et al. (1995)</td>
</tr>
<tr>
<td>E4 Philadelphia (Glu 13 → Lys, Arg 145 → Cys)</td>
<td>Type III</td>
<td>Lohse et al. (1991)</td>
</tr>
</tbody>
</table>

**References**


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