Differential Distribution of Apolipoprotein E Isoforms in Human Plasma Lipoproteins

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The polymorphism of apolipoprotein E (apo E) accounts for a substantial amount of the genetic variance of cholesterol levels in man. The ε-2 allele lowers and the ε-4 allele raises plasma and low density lipoprotein cholesterol levels as compared to the ε-3 allele. Whereas the lower cholesterol levels in carriers of the ε-2 allele can, at least in part, be attributed to the grossly deficient binding of apo E-2 to the apo B,E receptor, apo E-3 and E-4 bind to the same degree. We used gel filtration and ultracentrifugation to separate lipoproteins and subsequent immunoblotting analysis to study the apo E isoform distribution. We always found lipoproteins of lower density relatively enriched in apo E-4 and high density lipoproteins relatively depleted of apo E-4 as compared to apo E-3. This was also seen in plasma of heterozygous subjects that simultaneously express two apo E isoforms. Also, the apo E-A-II complex was directly shown by immunoblotting. Furthermore, when purified iodinated apo E was incubated with plasma in vitro, apo E-4 also reassocia
ted more with lipoproteins of lower density than apo E-3. We conclude that apo E-3 and apo E-4 have a different lipoprotein particle distribution in vivo. This differential lipoprotein distribution may account for differences in the metabolism between apo E-3 and E-4. (Atherosclerosis 8:405–411, May/June, 1989)

A polipoprotein E (apo E) is a polymorphic apolipoprotein exhibiting mainly three isoforms (apo E-2, E-3, and E-4) in the population. It is coded for by three alleles at a single gene locus. This single gene locus has a profound influence on the plasma lipid levels of the entire population. Compared to the common apo E-3 peptide (wild type), carriers of the mutant allele coding for apo E-2 exhibit lower, and carriers of the mutant allele for apo E-4 express higher, plasma and low density lipoprotein (LDL) cholesterol levels. Furthermore, the ε-2 allele has been found more frequently among hypertensive patients with hypercholesterolemic subjects, whereas the ε-4 has been associated with high density lipoprotein (HDL) levels. Furthermore, apo E isoforms differ in their cysteine-arginine content at positions 112 and 158 in the amino acid sequence. The cholesterol-lowering mechanism of apo E-2, which leads to primary dysbeta
toproteinemias in the homozygotic state, may, to a large extent, be explained by its well-documented, grossly diminished, receptor-binding activity to the apo B,E receptor as compared to apo E-3 and apo E-4. The importance of apo E-4-associated hypercholesterolemia is documented by the fact that carriers of the ε-4 allele (coding for apo E-4) were identified more frequently among hypercholesterolemic populations than in control groups and were found to suffer from myocardial infarction earlier in life than carriers of the ε-2 allele. Furthermore, recent data from comparative studies in different ethnic groups suggest that at least a part of the higher serum cholesterol levels found in the Finnish population may be due to higher frequencies of the ε-4 allele. The mechanism of apo E-4-associated hypercholesterolemia is not yet fully understood. Gregg and coworkers showed an abnormal low catabolism of apo E-4 in humans as compared to apo E-3. In their studies using radiolabeled apo E-3 and E-4, Gregg et al. showed that apo E-3 and E-4 redistribute differently among lipoproteins, with a higher percentage of apo E-4 being bound to low density lipoprotein (VLDL) and less to high density lipoprotein (HDL) when compared to apo E-3. Using immunoblotting techniques, we showed directly that apo E-3 and apo E-4 are also differentially distributed among plasma lipoproteins in the heterozygotic states.

Methods

Lipoprotein Separation

Blood was drawn from normolipemic volunteers into tubes containing 1.5 g/l Na2 EDTA and 10 μM of the thrombin inhibitor α-phenylalanyl-γ,γ-dimethyl glycylamide. Plasma was obtained by immediate low-speed centrifugation and was kept at 4°C. Lipoproteins were separated by gel permeation chromatography or ultracentrifugation. Chromatographic separation of lipoproteins was carried out by applying 2 ml of plasma to a (90 × 1.6)-cm column of Biogel A-5 M (BioRad Laboratories, Richmond, CA) and eluting at a flow rate of 8 ml/hour in a 10 mM Tris buffer (pH 7.4) containing 300 mM NaCl, 1 mM EDTA, and 0.02% NaN3. When only 150 mM NaCl was used in the

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column buffer, the lipoproteins were not recovered fully. With 300 mM NaCl, we obtained a full recovery as judged from the elution of lipoproteins retained when using only 150 mM NaCl. Cholesterol was determined enzymatically (Boehringer, Mannheim, FRG) to identify lipoprotein fractions. Lipoproteins in 2.5-ml aliquots of the column fractions were precipitated by the phosphotungstate procedure,17 and apo E was analyzed by immunoblotting in the redisolved pellet as described below.

Density gradient ultracentrifugation was carried out as described by Redgrave et al.18 After the run, the tubes were punctured from the bottom, and 1-ml fractions were collected. Lipoproteins were localized by cholesterol determination throughout the gradient fractions and precipitated before apo E analysis by immunoblotting.

For precipitation of lipoproteins in 2.5-ml aliquots of fractions after gel filtration or in 0.3-ml aliquots of fractions after density gradient ultracentrifugation (diluted to 1 ml with distilled water), two stock solutions were prepared. Solution A contained 4 g of tungstophosphoric acid, pH 7.6, (NaOH) per 100 ml. Solution B contained 2 M MgCl₂. For each milliliter of lipoprotein containing aliquots, 1 ml of Solution A and 100 μl of Solution B were added. The mixture was vortexed and incubated for 2 hours at room temperature. Then the precipitate was obtained by centrifugation (3500 g for 20 minutes). The supernate was discarded, and the pellet was resuspended in 80 μl of 150 mM NaCl and 20 μl of 10% (wt/vol) Na₂CO₃. This concentrated lipoprotein fraction was then used for immunoblotting.

Lipoproteins were further separated by ultracentrifugation in a 50.3 type rotor (Beckman Instruments, Munich, FRG) using adaptors for 240-μl tubes (Kontron Instruments, Eching, FRG). Specifically, plasma was adjusted to a density of d=1.063 g/ml by adding a KRBr stock solution (d=1.33 g/ml). Then, 200 μl of the density-adjusted plasma was pipetted into a 240-μl motor tube, and ultracentrifugation was carried out for 20 hours at 5°C. After the run, the d=1.063 g/ml super- and infranates were obtained and analyzed by immunoblotting for apo E isoforms.

**Apolipoprotein E Quantification**

Apo E was measured by electroimmunoassay using the commercially available sheep anti-human antibody and the protocol proposed by the distributor (Immuno Diagnostics, Heidelberg, FRG).

**Immunoblotting Analysis of Apolipoprotein E Isoforms**

Twenty microtiter aliquots of plasma, of the 1.063 g/ml super- or infranate, or of the redissolved pellet of precipitated lipoproteins, were incubated for 1 hour at 4°C in the presence of excess cetazide/methylammonium bromide (CTAB) and Triton X-100, 25 mM dithiothreitol (DTT).19 This was electrophoresed into detergent containing agarose gels modifying the procedures described for membrane proteins20 as outlined in detail for Group A apolipoproteins.21 After agarose gel electrophoresis, isoelectric focusing was performed essentially as outlined before,21 modifying the pH gradient by using a different ampholyte mixture (1/3 pH 5 to 7, 2/3 pH 4 to 6.5) (Pharmacia Fine Chemicals, Uppsala, Sweden).

After isoelectrofocusing, the proteins were electrophoretically blotted onto nitrocellulose sheets in a Transblott apparatus (LKB, Bromma, Sweden) essentially as outlined before22 with some modification. Blotting was carried out for a total of 2 hours: 30 minutes at 250 mA and then 90 minutes at 400 mA at 4°C. The blotting buffer consisted of 25 mM Tris and 192 mM glycine containing 20% (vol/vol) methanol. The nitrocellulose sheets were first soaked for 30 minutes at room temperature in 50 mM Tris buffer (pH 8.0) containing 90 mM NaCl and 1 mg/ml of polyvinylpyrrolidone (PVP) ( Buffer A) to saturate the nonspecific binding sites. Then the sheets were incubated and gently agitated for 1 hour at room temperature in Buffer A containing 1 μg/ml of rabbit antihuman apo E antibody. The sheets were next washed (with three changes) in 50 mM Tris (pH 8) containing 90 mM NaCl ( Buffer B). Incubation was continued in Buffer A containing 10 μl/mg of goat antirabbit IgG (peroxidase conjugated) for 90 minutes at room temperature under gentle agitation. Sheets were then washed with three changes of Buffer B. Apo E bands were visualized at 37°C in 50 mM Tris buffer (pH 6.4) containing 90 mM NaCl and 0.5 μl/ml of H₂O₂ in addition to 10 μl/ml of 1% 4-chloro-1-naphthol (Sigma Chemie, Taufkirchen, FRG) dissolved in ethanol. The sheets were dried and kept dark.

**Radiolodination of Apolipoprotein E**

Apolipoprotein E was isolated from the VLDL of subjects homozygous for apo E-3 or E-4 by preparative SDS polyacrylamide gel electrophoresis as described.23 Apo E-3 was also isolated from high density lipoprotein-2 (HDL₂) Therefore, plasma was centrifuged in the density range of d=1.063 to 1.125 g/ml. The apo E containing fraction was further fractionated by heparin Sepharose affinity chromatography as described by Weisgraber and Mahley.24 The apo E containing fraction was delipidated and subsequently subjected to preparative SDS polyacrylamide gel electrophoresis as described above. Isopeptides were radioiodinated with 125I (New England Nuclear, Dreieich, FRG) by the method of McFarlane25 or later by a procedure known to yield higher labeling efficiencies and less extreme pH.26 In both cases, lysylated apo E was redissolved in 0.1 M sodium phosphate buffer (pH 8) containing 0.1% sodium decyl sulfate (Eastman Kodak Company, Rochester, NY). Labeling efficiency was 25% to 35% with the method of McFarlane25 and 75% to 85% with the procedure of Sinn et al.26 Much less NaCl27 was needed with the second procedure to yield a specific activity of 200 dpm/ng than by the McFarlane procedure. We did not aim for higher specific activities, as only in vitro incubations were carried out.

Free iodine was removed by passing the labeling mixture over a 2 cm×10 cm Sephadex G25 column and by subsequent dialysis of the void volume fraction. Apo E labeled in parallel by the two procedures showed no difference in in vitro incubations with plasma.

**In Vitro Incubation Experiments with Iodinated Apolipoprotein E**

Aliquots (2 ml) of apo E-3/4 heterozygote plasma were incubated separately with 1.5 μg of radiolabeled apo
Lipoproteins were separated by gel permeation chromatography or ultracentrifugation, and apolipoprotein E isopeptides were analyzed within the various lipoprotein fractions by immunc blotting. Figure 1A shows the elution profile of plasma separated on a Biogel A-5-M column. Figure 1B, the separation of lipoproteins by density gradient ultracentrifugation. Lipoproteins were identified by cholesterol determination.

Apo E isoforms were determined within the lipoprotein fractions. Figure 2 demonstrates a typical apo E isof orm distribution pattern within the lipoprotein spectrum of an apo E-2/4 heterozygotic subject after gel permeation chromatography. Apo E-4 was almost completely associated with lipoproteins of larger size (of lower densities), whereas HDL exhibited predominantly apo E-2. Identical results were obtained when density gradient ultracentrifugation was used to separate lipoproteins. Figure 3 shows the apo E peptides of the same apo E-2/4 subject after density gradient ultracentrifugation.

The differential distribution of the apo E isoforms within the lipoprotein particles was also seen when aliquots of the fraction after lipoprotein separation by gel permeation chromatography or ultracentrifugation were directly subjected to isofocusing without prior precipitation of lipoproteins and going through agarose gel charge shift electrophoresis. Therefore, an aliquot of the sample was incubated with a sodium decyl sulfate-containing buffer and directly subjected to isofocusing with the procedure of Manzel and colleagues. After isofocusing, proteins were blotted onto nitrocellulose, and apo E bands were developed as outlined above (data not shown). As this procedure required dialysis of the density gradient fractions prior to isofocusing and was limited by the applicable sample volume of the column fractions, we routinely concentrated lipoproteins by precipitation and subjected them to charge shift electrophoresis, which is fairly insensitive to ionic strength.

To further analyze the relative distribution of the different apo E peptides within individual lipoproteins, we investigated apo E-3/4 heterozygotic subjects. These individuals exhibited relatively more apo E-4 associated with lipoproteins of lower density and a relative enrichment of HDL in apo E-3. The isopeptide distribution throughout the lipoprotein spectrum after density gradient ultracentrifugation of plasma from a typical apo E-3/4 subject is shown in Figure 4. Gel permeation chromatography gave identical results (data not shown). Thus, apo E-4 showed a higher association with (apo B-containing) lower density lipoproteins than did apo E-3 and E-2.

To rule out the possibility that the differential distribution of apo E isopeptides within the lipoprotein spectrum simply reflected a metabolic state rather than different affinities of the isopeptides for various lipoproteins, apo E-3 and E-4 were isolated, radiolabeled, and incubated in vitro with whole plasma. The addition of iodinated apo E-3 to the E-3 homozygotic plasma resulted in a redistribution reflecting the apo E mass in the sample. Incubation of the same plasma with iodinated apo E-4 showed less radioactivity associated with HDL and more with lipoproteins of lower density as compared to apo E-3 (data not shown). This is similar to the results obtained by Gregg et al. by in vivo redistribution of apo E-3 and apo E-4 in apo E-3 homozygotic subjects. Reducing apo E before incubation did not abolish the difference between apo E-3 and E-4.

When labeled apo E-3 and E-4 were incubated in vitro with apo E-3/4 plasma, apo E-4 again redistributed to a higher degree with VLDL as compared to HDL. The results of these experiments are shown in Table 1. Apo E-3 isolated from HDL did not differ from VLDL-derived apo E-3 in redistribution behavior.

To investigate whether the differential distribution of the various apo E isopeptides could be demonstrated in all heterozygotic subjects, we screened plasma samples by ultracentrifugation. Plasma was adjusted to a density of d=1.063 g/ml and was separated in a 240-μl tube in a 50.3 rotor, and apo E isopeptides were analyzed in the...
d = 1.063 g/ml supra- and infranate fraction. In all samples analyzed so far (n=120), we showed the differential distribution of the apo E isopeptides between lipoprotein classes. We always found apo E-4 dominating in the d = 1.063 g/ml buoyant lipoprotein fraction as compared to the infranate fraction of the same density (data not shown).

One mechanism of the differential distribution of the apo E peptides between lipoprotein fractions could be the ability of apo E-2 and E-3 to form heterodimers with apo A-II, a complex characterized earlier. Charge shift electrophoresis was therefore carried out in the absence of the reducing agent DTT. As shown in Figure 5, the apo E-A-II complex can be demonstrated directly from plasma in the unreduced state.

**Discussion**

The development of immunoblotting of apo E isoforms from plasma and subsequently within different lipoprotein fractions, especially HDL as described in the present research, provides the tools to study apo E isopeptide distribution in plasma. In the past, an individual’s apo E isopeptides were mainly only analyzed in VLDL, although large portions of apo E may be associated with other lipoproteins.

Indirect evidence that apo E-4 has an altered lipoprotein particle distribution compared to apo E-3 stems from the studies of Gregg and coworkers. They showed a marked difference in the redistribution between lipoprotein subfractions of apo E-3 and E-4 when injected as iodinated apoproteins into apo E-3 or E-4 individuals. Apo E-4 reassociated to a higher degree with VLDL and to a lesser degree with HDL than did apo E-3. Our in vitro incubation experiments showed a similar redistribution of labeled apo E-3 and apo E-4 when added to apo E-3/3 plasma.

**Figure 2.** Identification of apolipoprotein (apo) E isoforms through the elution range of plasma lipoproteins after gel permeation chromatography. Plasma of an apo E-4 heterozygotic subject was passed through a BioGel A-5-M-column, and apo E was analyzed in the fractions by immunoblotting. The predominance of apo E-4 in the lipoproteins of larger size is obvious. The numbers indicate the eluted fractions.

**Figure 3.** Identification of apolipoprotein (apo) E isoforms in plasma lipoproteins after density gradient ultracentrifugation. Plasma of the same apo E-2/4 heterozygotic individual as in Figure 2 was subjected to density gradient ultracentrifugation. After the run, the tubes were punctured from the bottom, and apo E isoforms were analyzed in representative fractions by immunoblotting. Again, apo E-4 was predominately associated with lipoproteins of lower density (Fractions 9 to 11). An immunoblot of apo E from the subject’s whole plasma is shown in Lane P.
Figure 4. Analysis of apolipoprotein (apo) E isoforms in lipoproteins of an apo E-3/4 heterozygotic plasma sample after density gradient ultracentrifugation. After centrifugation, the tubes were punctured from the bottom, and apo E isoforms were identified by immunoblotting. Again, apo E-4 shows a relative enrichment in the lipoproteins of lower density (Fractions 9 to 11). Panel P represents an immunoblot of whole plasma for comparison.

**Table 1. Redistribution of Radiolabeled Apo E-3 or E-4 after Incubation with Plasma of Apo E-3/4 Heterozygotic Subjects**

| Parameter            | VLDL   | HDL    |
|----------------------|--------|--------|
| Apo E (μg)           | 15±4.9 | 61.5±22|
| Apo E-3 radioactivity* | 19.1±5.02 | 173.3±32.8 |
| Apo E-4 radioactivity* | 34.6±6.1 | 133.8±27.4 |

*Apo E-3 and E-4 radioactivity values are dpm×10⁻⁶.

Furthermore, incubation of both apoproteins E-3 and E-4 with the plasma of apo E-3/4 subjects led again to a higher portion of apo E-4 in VLDL. The fact that reduction of apo E-3 did not abolish the differential distribution of apo E-3 and E-4 is consistent with the report of Gregg et al.35 Using apo E-2, they noted that reducing apo E-2 was not sufficient to mimic the behavior of apo E-4, but that the addition of a positive charge to the cysteine residues was necessary. Apo E-3 isolated from HDL did not differ in its redistribution behavior from VLDL-derived apo E-3, indicating that post-translational modification might not be a major determinant for particle distribution.

Measuring apo E mass between lipoprotein fractions allows conclusions with respect to isopeptide distribution only in homozygote individuals. Our results show for the first time that there exists within a heterozygote apo E individual a differential distribution of the two expressed apo E peptides. Our work furthermore directly demonstrates for the first time the distribution of apo E isoforms in all plasma lipoprotein fractions. The presence of the variant peptide E-4 in a single dose (apo E-3/4) in a normal individual was recently shown to significantly affect postprandial fat clearance.32 This was due in part to the fact that apo E-3 and E-4 in one individual distribute differently among the individual's lipoproteins. The apo E isopeptide distribution within the lipoprotein spectrum of heterozygotic individuals in our study was always compatible with the concept of a relatively higher presence of apo E-4 in lipoproteins of lower density than apo E-3. No exception was found during the screening of 120 plasma samples after ultracentrifugation at a density of d=1.063 g/ml, despite the fact that, in normallipidemic subjects, a large variability in apo E lipoprotein distribution has been reported earlier.31 The amount of VLDL in the plasma of the individuals studied here already seemed to influence the distribution of apo E between lipoproteins, although in the normal range of total plasma lipids, individuals with triglycerides in the upper normal range had more of the apo E associated with VLDL than those in the lower ranges. This is in agreement with the observation that, in plasma of patients with type IV hyperlipidemia, the majority of apo E in plasma is found in the VLDL density range.30

Apo E-4, E-3, and E-2 differ in their amino acid sequence at positions 112 and 158. Apo E-2 shows cysteine residues at these two positions, while apo E-4 contains arginines, and apo E-3 has cysteine at position 112 and arginine at amino acid position 158.11,12 These compositional differences in the amino acid sequence and charge give rise to variations in the proteins' functions. It has also been documented13 that the defective binding of the common apo E-2 to the apo B,E receptor is due to the cysteine-for-arginine interchange at amino acid position 158.11,12 Apo E-3 and apo E-4 both carry arginine at
position 158 and exhibit no difference in their ability to bind to the apo B,E receptor in vitro when complexed with dimyristoyl-phosphatidylcholine. In vivo, apo E-2 shows a diminished clearance from plasma as compared to apo E-3. Thus, the difference in lipoprotein distribution between apo E-2, on one hand, and apo E-4 and E-3, on the other hand, could to some extent be accounted for by the divergent binding of these apoproteins to lipoprotein receptors.

Nevertheless, this would not explain the difference in apo E-3 and apo E-4 isopeptide distribution between lipoprotein particles as documented here directly in plasma of heterozygotic apo E-3/4 individuals. There seem to be several reasons for the different lipoprotein particle distribution of apo E-3 and E-4. It has been shown that cysteine-containing apo E proteins may form mixed disulfides with apo A-II. By omitting the reducing agent during electrophoresis in our system, we were also able to document the existence of apo E-A-II complexes in plasma. On the one hand, these mixed disulfides inhibit the interaction of apo E containing lipoproteins with the apo B,E receptor.

Apo E-4 contains no cysteine and is thus not capable of forming complexes with apo A-II and might, therefore, not be altered in its receptor interaction. On the other hand, the apo E-A-II complex may be an important mechanism that "binds" apo E-3 and E-2 to HDL, whereas apo E-4 would not be bound via disulfide bonds.

But this would only partly explain the differential redistribution phenomena in vitro, as reduction of apo E before the in vitro incubations did not abolish the difference between apo E-3 and E-4. Recent results from Gregg et al. suggest that, in addition to the ability to form disulfides (cysteine residue), the electrical charge independently affects the apo E peptides' metabolism. The addition of positive charges by means of aminoethylation of the free sulphydryl groups of apo E-2 lead to a change in metabolic behavior of modified apo E-2 comparable to that of apo E-4. The clustering of apo E-4 in lipoproteins of lower density would then be a synergistic effect of less binding to HDL via mixed disulfides and increased affinity for VLDL via the additional positive charge. The increased metabolism of apo E-4 could, therefore, be explained by the high portion of this apoprotein in the faster turning over VLDL pool and the absence from the slower turning over HDL pool.

A higher efficiency of enteral cholesterol absorption in subjects with the e-4 allele as compared to e-3 or e-2, together with the high affinity of apo E-4 for lipoproteins of lower density and the faster postprandial fat clearance of E-4 individuals, are possible explanations for the elevated plasma and LDL cholesterol levels seen in carriers of the e-4 allele.

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