Effects of a Frequent Apolipoprotein E Isoform, ApoE4Freiburg (Leu28→Pro), on Lipoproteins and the Prevalence of Coronary Artery Disease in Whites

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Abstract—Different isoforms of apoE modulate the concentrations of plasma lipoproteins and the risk for atherosclerosis. A novel apoE isoform, apoE4Freiburg, was detected in plasma by isoelectric focusing because its isoelectric point is slightly more acidic than that of apoE4. ApoE4Freiburg results from a base exchange in the APOE gene that causes the replacement of a leucine by a proline at position 28. Analysis of the allelic frequencies in whites in southwestern Germany revealed that this isoform is frequent among control subjects (10:4264 alleles) and is even more frequent in patients with coronary artery disease (21:2874 alleles; \(P=0.004\); adjusted odds ratio, 3.09; 95% confidence interval, 1.20 to 7.97). ApoE4Freiburg affects serum lipoproteins by lowering cholesterol, apoB, and apoA-I compared with apoE4 (\(P<0.05\)). Our 4 apoE4Freiburg homozygotes suffered from various phenotypes of hyperlipoproteinemia (types IIa, IIb, IV, and V). In vitro binding studies excluded a binding defect of apoE4Freiburg, and in vivo studies excluded an abnormal accumulation of chylomicron remnants. ApoE4Freiburg and apoE4 accumulated to a similar extent in triglyceride-rich lipoproteins. HDLs, however, contained about 40% less apoE4Freiburg than apoE4. In conclusion, our data indicate that apoE4Freiburg exerts its possible atherogenic properties by affecting the metabolism of triglyceride-rich lipoproteins and HDL.


Key Words: apoE polymorphism ■ mutation ■ atherosclerosis ■ isoelectric focusing

Apolipoprotein E is a constituent of all human lipoproteins except LDL and occurs in 3 common isoforms (E2, E3, and E4) that are encoded by 3 codominant alleles (\(e_2\), \(e_3\), and \(e_4\)) of the APOE gene.2 ApoE plays a pivotal role in triglyceride and cholesterol metabolism by mediating the sequestration and removal of remnants of triglyceride-rich lipoproteins via the heparan sulfate proteoglycan-LDL receptor-related protein (LRP) and LDL receptor pathways.3-5 The apoE isoforms have different affinities for these pathways.3-5 The APOE alleles modulate the risk for coronary artery disease (CAD),9,10 cerebral atherosclerosis,11 and Alzheimer’s disease.12,13 In addition to the common apoE isoforms, several rare apoE variants have been identified in hyperlipidemic patients and their kindreds. Most of the variants are characterized by replacements of one or more charged amino acids by uncharged amino acids or vice versa. Some replacements in the LDL receptor-binding region (positions 136 to 150) cause defective binding to the LDL receptor and are associated with the recessive form of type III hyperlipoproteinemia, and some replacements of basic amino acids with neutral or acidic amino acids, leading to defective heparan sulfate proteoglycan (HSPG) binding, were associated with the dominant form of type III hyperlipoproteinemia.3,18 Variants with a basic amino acid at amino acid residue 3 display increased binding to LDL receptors and were associated with increased LDL cholesterol.25,26 The total number of patients with mutant APOE alleles identified by studying hyperlipidemic patients and their families, however, is very low.

The common isoforms of apoE and most of the known rare variants of apoE have been detected on the basis of different isoelectric points caused by replacements of charged amino acids by uncharged species (or vice versa). Replacements involving only uncharged amino acids should result either in no or in only small changes of the isoelectric point of the newly formed isoproteins. (The total charge of a protein is determined by the charges of the side chains of its amino acids and, to a lesser extent, by the 3-dimensional arrange-
ment of the side chains.) These small changes of the isoelectric point are difficult to detect by isoelectric focusing in polyacrylamide gels and subsequent immunoblotting. Therefore, we developed a more sensitive protocol for these small charge differences using isoelectric focusing in agarose gels and immunofixation, combining specificity with improved resolution. Using this protocol, we detected a frequent novel isoform, apoE4 Freiburg, that migrates to a slightly more acidic position than apoE4 after isoelectric focusing.

The aims of this study were to elucidate the underlying molecular basis of the apoE4 Freiburg isoform, to compare its allelic frequencies among healthy control subjects and CAD patients in southwester Germany, and to study its effects on fasting and postprandial plasma lipoprotein and apolipoprotein concentrations, receptor-binding activity, and accumulation in the various lipoprotein classes.

Methods

The protocol for all studies involving human subjects was approved by the local ethics committee, and all participants gave written informed consent for their participation in this study.

Control Subjects and Patients of the Epidemiological and the Family Studies

Subjects for the biochemical studies, the analysis of allelic frequencies, the analysis of the effects of $e_4_{\text{Freiburg}}$, and other APOE alleles on plasma lipids and apolipoproteins, and cosegregation analysis came from 3 study groups (control subject group, patient group, and family study group). All subjects were whites residing in the region surrounding Freiburg in southwestern Germany. The control group consisted of 1589 healthy blood donors (1180 men, 409 women), who were recruited in 60 different towns, and 543 healthy female employees of a large factory. All control subjects had been screened by physicians for the absence of clinically overt CAD. The patient group originated from the same geographical area and consisted of 1437 patients (548 men, 889 women) from a cardiological hospital and from the Cardiology Department of the Freiburg University Hospital. All patients had a clinical diagnosis of CAD or had survived a myocardial infarction. In addition, 991 had undergone coronary angiography, and of these, 794 patients (80%) displayed 1 vessel with >50% coronary stenosis. The family study group consisted of 7 healthy apoE4 Freiburg blood donors and 56 of their relatives. The 63 patients in this group were studied to determine heritability and cosegregation of phenotype and genotype of the new apoE isoform. These relatives were not included in the analysis of allelic frequencies.

Blood was drawn by a standard procedure. Cholesterol, apoB, apoA-I, and triglycerides were measured in serum by enzymatic and turbidimetric tests (Boehringer Mannheim), as described. Genomic DNA was isolated from blood leukocytes of subjects of the family study by the alkaline lysis method. At the time of the blood sampling, the patients, the relatives of the blood donors in the family study, and the factory workers had been fasting overnight. Blood donors were in an undefined postprandial state and were therefore excluded from analysis of triglycerides. All study subjects were included in the statistical analysis of serum cholesterol, apoB, and apoA-I because these variables are not altered postprandially.

Isoelectric Focusing of ApoE

In all subjects, phenotyping of apoE by isoelectric focusing was performed as described. Briefly, 12 μL of a delipidation solution (76 mM/μL urea [Mercr], 19.2 mM/μL DTT [Sigma], 10 mM/μL Tris [Merck], and 23% [vol/vol] Tween 20 [Sigma]) was added to 50 μL of serum. After 30 minutes, the samples were applied to horizontal 2% agarose gel containing 3 mol/L urea and 4% (vol/vol) of an ampholyte mixture (pH 3 to 10 and pH 5 to 6, 1:1; Serva) and subjected to isoelectric focusing. After focusing, the apoE isoforms underwent immunofixation by incubating the gel with goat antiserum against apoE (Greiner) and staining the immunofixed bands with Coomassie blue.

APOE Gene Sequencing

Genomic DNA was amplified by PCR for sequencing. Primers (21 to 24 bases long) were placed approximately 30 bases upstream of the 5′ end of the gene segments to be sequenced. The PCR reaction was performed in 100 μL of PCR buffer (Beckman) with 10% DMSO (Sigma), containing 0.5 to 1 μg genomic DNA, and final concentrations of 200 μmol/L dNTPs (Pharmacia) and 0.1 μmol/L of each primer. Initial denaturation at 100°C for 10 minutes was followed by the addition of 2 to 5 U Taq polymerase (Beckman) and 30 incubation cycles of 96°C (90 s), 60°C (1 minute), and 70°C (1 minute). For the sequencing of the G/C-rich exon 4 of apoE, 75% of the dGTP content in the reaction mix was replaced with 7-deaza-dGTP (Pharmacia), and temperature cycles were changed to 96°C (80 s), 55°C (1 minute), and 71°C (70 s) and repeated for 40 cycles. The product was purified by electrophoresis in 2% NuSieve agarose (FMC); DNA of the expected size was cut out of the gel and electroeluted in 0.5× Tris-acetate/EDTA for 75 minutes at 200 V. The DNA was desalted and concentrated to 70 μL by ultrafiltration in Centricron X-100 tubes (Amicon).

Single strands for sequencing were produced by the Gyllensten and Erlich method using the same scheme as above. Primer concentration was 0.1 μmol/L; a second primer was not used. Single-strand templates containing exon 4 were synthesized with 7-deaza-dGTP at the same concentration used for double-strand production. Sequencing was done from single-strand templates using T7 polymerase (Pharmacia) and 32P-dNTP (Amersham) following the supplier’s instructions. Reaction products were run on a sequencing gel, dried, and visualized by autoradiography.

Genotyping for $e_4_{\text{Freiburg}}$ by Restriction Fragment Polymorphism

Rapid genotyping was performed by restriction digest of PCR-amplified fragment of genomic DNA (primer 1, TGACCGCACT-TGAACCTTGGTCCA; primer 2, GGTATAGCCGCCCACCAG-GAGG) with MspI (Boehringer Mannheim) and subsequent fragment length determination in a gel containing 3% NuSieve agarose and 1% standard agarose. The gel was stained with ethidium bromide, and the DNA visualized under UV light.

Receptor-Binding Studies

Human apoE4 Freiburg, apoE3, and apoE4 were isolated from d<1.006 kg/L lipoproteins of homozygotes, as described previously, and checked for purity by SDS-PAGE. Human LDL were isolated from plasma of normal fasting subjects by sequential ultracentrifugation and radiolabeled by the iodine monochloride method. All of the various isoforms of apoE and dimyristoylphosphatidylcholine (DMPC, Sigma) were mixed at a ratio of 1:3 75 (wt/wt, protein:DMPC), and complexes were isolated by density gradient ultracentrifugation. All of the apoE · DMPC complexes contained apoE as the only protein moiety. Normal human fibroblasts were plated at 3.5×10^6 cells/dish 1 week before the experiment. On day 5, the cells were switched to medium (DMEM, Life Technologies) containing 10% human lipoprotein-deficient serum. On day 7, the cells were incubated at 4°C in medium containing 2.0 μg/mL 125I-LDL and increasing concentrations of apoE · DMPC complexes. The competitive binding of these complexes against human 125I-LDL was determined.

Distribution of ApoE with Different Lipoproteins

Plasma from fasting apoE4 apoE4 Freiburg, heterozygotes was separated by gel filtration (Sepharose 6B-CL, Pharmacia) as described. Gel filtration was used instead of ultracentrifugation to avoid the high centrifugal forces that strip apolipoproteins from lipoproteins during separation. Fractions containing VLDL, IDL, and HDL peaks were identified by measuring cholesterol and triglycerides with enzymatic tests (Boehringer Mannheim) and apoE with turbidimetry, using goat antiserum against apoE (Greiner). Fractions obtained from the VLDL, IDL, and HDL peaks were dialyzed to eliminate buffer...
consistently, concentrated in vacuo (if necessary), separated by isoelectric focusing, and immunofixed. The ratios of the different apoE isoforms in VLDL, IDL, and HDL were calculated after densitometry of the Coomassie-stained gels (SigmaGel, SPSS).

**Effects of ApoE4~Freiburg~ on Postprandial Metabolism**

The postprandial clearance of lipoproteins after the ingestion of a standard fatty meal containing retinyl palmitate was assessed in 3 e4~Freiburg~ homozygotes. Blood samples were obtained every other hour for 10 hours after the fatty meal. Lipoproteins were separated by ultracentrifugation and by gel filtration. Retinyl esters in chylomicrons and chylomicron remnants were measured by a fluorometric assay.

**Statistical Analysis**

Differences in the allele frequency distribution were tested with Fischer’s exact test. Statistical comparisons of the means between groups were performed by t test. Multigroup comparisons were tested by analysis of variance and were followed by the Scheffé test if the F statistic was significant. Odds ratios (ORs) as estimators of relative risk, together with their 95% approximate confidence intervals (CIs), were calculated to assess the association with CAD and relative risk, together with their 95% approximate confidence intervals (CIs), were calculated to assess the association with CAD and relative risk.

**DNA Sequencing and Segregation Analysis**

The molecular basis of this isoform was identified by sequencing all exons, 80 bases of 5’ sequence, and the consensus splice donor and acceptor sites of the APOE gene of 4 independent e4~Freiburg~ carriers. A single mutation was detected in all carriers studied. This mutation, a T-to-C mutation in position 3100 of the APOE4~Freiburg~ gene (GenBank accession M10065), changes CTG of codon 28, coding for leucine, to CCG, coding for proline (Figure 2).

The DNA mutation underlying the Leu→Pro replacement leads to the formation of an MspI restriction site (C CCG). In the absence of the mutation, the 271-bp fragment of genomic PCR-amplified DNA is cleaved into 2 fragments of 191 and 80 bp. In the presence of the mutation, the 191-bp fragment is cleaved into fragments of 136 and 55 bp (Figure 2).

Heritability and cosegregation of genotype and phenotype were studied in 7 study participants (blood donors) and 56 of their relatives. Genotype (ie, presence of a specific MspI restriction site of PCR-amplified genomic DNA) and phenotype (ie, apoE4~Freiburg~ band in isoelectric focusing) were congruent in all families studied indicating cosegregation of phenotype and genotype. The CCG mutation was only observed on e4 chromosomes and not on e2 or e3 chromosomes. Screening of the 56 relatives (30 men, 26 women) identified 33 clinically healthy heterozygous and homozygous apoE4~Freiburg~/apoE4~Freiburg~, apoE4~Freiburg~/apoE4~Freiburg~, and apoE4~Freiburg~/apoE4~Freiburg~. In lanes marked with an asterisk, the use of VLDL that has undergone delipidation instead of serum resulted in decreased nonspecific background staining.

**Results**

**Mobility of ApoE4~Freiburg~ by Isoelectric Focusing**

By isoelectric focusing of serum, the apoE4~Freiburg~ isoform was discovered in combination with the common apoE isoforms E3, E4, and E2 (Figure 1A) and as the only apoE isoform (Figure 1B). It is evident from these gels that apoE4~Freiburg~ is easily detected in serum of apoE4/apoE4~Freiburg~ heterozygotes because it focuses in a slightly more acidic position than apoE4, and a pronounced double band of similar intensity is easily discernible (Figure 1A). To detect apoE4~Freiburg~ in apoE2/apoE4~Freiburg~ heterozygotes, apoE3/apoE4~Freiburg~ heterozygotes, or homozygotes, samples containing apoE4 were run in an adjacent lane to detect the slight acidic shift of apoE4~Freiburg~ versus apoE4.
TABLE 1. Lipids and Apolipoprotein Concentrations and Risk Factors for CAD in 4 Homozygous apoE4 Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>8.368</td>
<td>5.217</td>
<td>6.475</td>
<td>18.828</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>2.914</td>
<td>3.060</td>
<td>0.764</td>
<td>53.257</td>
</tr>
<tr>
<td>VLDL cholesterol, mmol/L</td>
<td>0.439</td>
<td>1.524</td>
<td>0.285</td>
<td>16.986</td>
</tr>
<tr>
<td>VLDL triglycerides, mmol/L</td>
<td>1.912</td>
<td>2.981</td>
<td>0.342</td>
<td>52.818</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>6.224</td>
<td>2.763</td>
<td>2.464</td>
<td>10.059</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.705</td>
<td>0.930</td>
<td>1.554</td>
<td>0.801</td>
</tr>
<tr>
<td>ApoA-I, mg/dL</td>
<td>158</td>
<td>126</td>
<td>164</td>
<td>112</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>181</td>
<td>105</td>
<td>99</td>
<td>162</td>
</tr>
<tr>
<td>Age, y/sex</td>
<td>55/M</td>
<td>51/F</td>
<td>52/M</td>
<td>57/M</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>31</td>
<td>28</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>150/100</td>
<td>135/90</td>
<td>130/85</td>
<td>165/105</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

*This patient was identified among the patients attending the lipid clinics of Magdeburg and not included in the evaluation of allelic frequencies or the effects of apoE4 on lipoproteins.

Allele Frequencies of ApoE4

The allelic frequencies of apoE4 were studied by phenotyping 2132 clinically healthy subjects (control group) and 1437 CAD patients (patient group). The absolute and relative frequencies of the common and apoE4 phenotypes are given in Table 2. A total of 30 carriers of apoE4 were detected in these 3569 subjects. One CAD patient was homozygous and 29 subjects were heterozygous for apoE4.

The frequencies of the apoE4 allele were 1:426 (10:426 apoE4) and 1:400 (10:400 apoE4) for the control and patient groups, respectively. The frequency of apoE4 was significantly lower in CAD patients (1:7) than in healthy subjects (1:4), p < 0.001.

Effects of ApoE4 on Lipids and Apolipoproteins

Three homozygotes from 2 unrelated families were identified in this study, and a fourth homozygote was identified among the patients of our lipid clinics. All homozygotes had various types of hyperlipoproteinemia (type IIa, type IIb, type IV, and type V) and 2, both with hypertriglyceridemia, had CAD (Table 1).

The phenotypes of the homozygotes led us to study the effects of the common APOE alleles (e2, e3, and e4) and of apoE4 on lipoprotein concentrations in all subjects. The effects of the different common APOE alleles were analyzed by comparing the wild-type apoE3/E3 with apoE2/E2, apoE4/E4, and apoE3/E4 (Figure 3) because these phenotypes modulate concentrations of lipids and apolipoproteins.

The effects of apoE4 on lipids and apolipoproteins were studied in 2 steps. First, all carriers with 1 or 2 alleles of apoE4 were compared with all noncarriers of apoE4 (n=63) by using the aim of including as many apoE4 individuals as possible (Figure 4). The caveat of this analysis is that the results are easily confounded by known (opposite) effects of the other apoE isoforms. Second, only the most frequent combinations of apoE4 were studied (ie, the combinations apoE3/E4 and apoE4/E4) to be compared with apoE3/E4 and apoE4/E4, respectively. Although fewer subjects were analyzed than in the first analysis, this analysis allows the effects of apoE4 to be clearly differentiated from those of apoE (Figure 3 and Table 3).

Analysis of cholesterol and apoB concentrations revealed, as expected, that e2 and e4 had opposite effects on lipid and apolipoprotein concentrations. Cholesterol and apoB were lower in apoE2/E2 and higher in apoE4/E4 and in apoE3/E4 than in apoE3/E3 (Figure 3A and 3B). No
effects of the $\epsilon_4_{\text{Freiburg}}$ allele on cholesterol and apoB were detected when all subjects were analyzed (Figure 4A and 4B). When the opposite effects of the $\epsilon_2$ and $\epsilon_4$ alleles were excluded by the direct comparison of apoE3/E4$_{\text{Freiburg}}$ with apoE3/E4, however, this analysis revealed that apoE3/E4$_{\text{Freiburg}}$ subjects had a 0.524 mmol/L lower cholesterol (Figure 3A), and the comparison of apoE4/E4$_{\text{Freiburg}}$ with apoE4/E4 revealed that apoE4/apoE4$_{\text{Freiburg}}$ heterozygotes had 20.7 mg/dL lower apoB (Figure 3B). ApoB was not significantly different between apoE3/E4$_{\text{Freiburg}}$ and apoE3/E4 (Figure 3B), and cholesterol was not significantly different between apoE4/E4$_{\text{Freiburg}}$ and apoE4/E4 (Figure 3A; for CIs, see Table 3).

ApoA-I concentrations were lower in apoE4/E4 and in apoE3/E4 than in apoE3/E3, but no differences were observed in apoE2/E2 (Figure 3C). The $\epsilon_4_{\text{Freiburg}}$ allele had a profound effect on apoA-I concentration even when all subjects were analyzed (Figure 4C). Mean apoA-I concentrations were 17.2 mg/dL lower in carriers of $\epsilon_4_{\text{Freiburg}}$ than in noncarriers (95% CI, 10.2 to 24.0 mg/dL; $P=0.000$; mean in noncarriers, 153.4 mg/dL); this relationship also held true after adjustment for age and sex (21.1 mg/dL lower in subjects with the apoE4$_{\text{Freiburg}}$ isoform; $P=0.003$).

ApoA-I was 15.9 mg/dL lower in apoE3/E4$_{\text{Freiburg}}$ compared with apoE3/E4 but did not reach statistical significance between apoE4/E4$_{\text{Freiburg}}$ and apoE4/E4 (Figure 3C and Table 3).

### Table 2. Observed and Calculated Phenotype Frequencies of the Common ApoE Isoforms and of ApoE4$_{\text{Freiburg}}$

<table>
<thead>
<tr>
<th>E2/E2</th>
<th>E2/E3</th>
<th>E2/E4</th>
<th>E2/E4$_{\text{Freiburg}}$</th>
<th>E3/E3</th>
<th>E3/E4</th>
<th>E3/E4$_{\text{Freiburg}}$</th>
<th>E4/E4</th>
<th>E4/E4$_{\text{Freiburg}}$</th>
<th>E4$<em>{\text{Freiburg}}$/E4$</em>{\text{Freiburg}}$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>21(1.0%)</td>
<td>241(11.3%)</td>
<td>52(2.4%)</td>
<td>3(0.1%)</td>
<td>1356(63.6%)</td>
<td>419(19.7%)</td>
<td>4(0.2%)</td>
<td>33(1.6%)</td>
<td>3(0.1%)</td>
<td>0(0%)</td>
<td>2132</td>
</tr>
<tr>
<td>16(1.1%)</td>
<td>149(10.4%)</td>
<td>21(1.5%)</td>
<td>2(0.1%)</td>
<td>890(61.9%)</td>
<td>323(22.5%)</td>
<td>16(1.1%)</td>
<td>18(1.3%)</td>
<td>1(0.1%)</td>
<td>0(0%)</td>
<td>1437</td>
</tr>
<tr>
<td>37(1.0%)</td>
<td>390(10.9%)</td>
<td>73(2.4%)</td>
<td>5(0.1%)</td>
<td>2246(62.5%)</td>
<td>742(20.8%)</td>
<td>20(0.6%)</td>
<td>51(1.4%)</td>
<td>4(0.1%)</td>
<td>0(0%)</td>
<td>3569</td>
</tr>
<tr>
<td>21(0.6%)</td>
<td>429(12.0%)</td>
<td>70(2.0%)</td>
<td>2(0.1%)</td>
<td>2231(62.5%)</td>
<td>728(20.4%)</td>
<td>25(0.7%)</td>
<td>59(1.7%)</td>
<td>4(0.1%)</td>
<td>0(0%)</td>
<td>3569</td>
</tr>
</tbody>
</table>

$^*$Allelic frequencies for $\epsilon_2$, $\epsilon_3$, $\epsilon_4$, and $\epsilon_4_{\text{Freiburg}}$ were 0.0759, 0.7907, 0.1290, and 0.0043, respectively. The estimated number of all subjects was calculated from these allelic frequencies. Note that the 56 participants of the family study were not included in this analysis of the apoE phenotype frequencies.

ApoE4$_{\text{Freiburg}}$ Effect on Lipoproteins

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**Figure 3.** ApoE phenotype-specific box-and-whisker plots for cholesterol (A), apoB (B), apoA-I (C), and triglycerides (D). The box represents the interquartile range, which contains 50% of the values. The whiskers extend from the box to the highest and lowest values, excluding outliers. The line across the box indicates the median. Differences from the values of apoE3/E3 (wild-type) were calculated to identify the effects of the common apoE phenotypes and between apoE4/E4 and apoE4/E4$_{\text{Freiburg}}$ as well as between apoE3/E4 and apoE3/E4$_{\text{Freiburg}}$ to identify the effects of E4$_{\text{Freiburg}}$. For confidence intervals, see Table 3. Fewer subjects were included in the analysis of triglycerides because it was restricted to subjects with fasting triglycerides <7.874 mmol/L. Subjects with apoE2/E3, apoE2/E4, apoE2/E4$_{\text{Freiburg}}$, and apoE4$_{\text{Freiburg}}$/E4$_{\text{Freiburg}}$ are not shown.
The analysis of the effects of different APOE alleles on fasting triglycerides was restricted to the study subjects in whom the blood samples were obtained under fasting conditions (ie, all patients, all control subjects from the occupational health clinics, and all participants of the family study, n = 2036). Fasting triglycerides were <7.874 mmol/L in 99.4% of all subjects, but the variability of triglyceride concentrations (as indicated by standard deviation and skewness) in all different apoE phenotypes studied exceeded by far the variability of cholesterol, apoB, and apoA-I concentrations (Figures 3 and 4). This higher variability was an obstacle for the statistical analysis. Nevertheless, triglycerides were higher in apoE2/E2, apoE4/E4, and apoE3/E4 than in apoE3/E3. Although the number of subjects examined in our study was quite large, the only difference that reached statistical significance was between apoE3/E3 and apoE2/E2 (Figure 3D). When the effects of the apoE4Freiburg isoform on triglycerides were studied in all carriers of apoE4 Freiburg, triglyceride concentrations were higher by 0.402 mmol/L in subjects with apoE4Freiburg (n = 56) than in noncarriers (n = 1968) (95% CI, 0.032 to 0.837 mmol/L; P = 0.035; mean in apoE4Freiburg, 2.359 mmol/L) (Figure 4D), and this difference was still present after adjustment for sex (P = 0.006). Differences between apoE4 and apoE4Freiburg did not reach statistical significance in the comparison of certain phenotypes. Triglycerides were 0.187 mmol/L higher in apoE3/E4Freiburg (n = 39) than in apoE3/E4 (n = 419) and 0.462 mmol/L higher in apoE4/E4Freiburg (n = 8) than in apoE4/E4 (n = 31) (Figure 3D; for CIs, see Table 3).

In summary, the subjects with apoE4Freiburg had significantly lower cholesterol (versus E4/E4), lower apoB (versus E3/E4), and lower apoA-I, and, possibly, higher fasting triglycerides than those with apoE4.

**Distribution of ApoE4 Freiburg and ApoE4 with Different Lipoproteins**

Analysis by gel filtration of lipoproteins in 2 apoE4/apoE4 Freiburg subjects on 2 independent occasions (n = 4) revealed that most of the plasma apoE is present in VLDL and IDL and only very little is in HDL (Figure 5). To test whether the apoE isoforms differ in their accumulation in VLDL and HDL, as has been observed for apoE3 and apoE4, the amounts of apoE4 and apoE4Freiburg present in VLDL, IDL, and HDL were measured in these apoE4/apoE4Freiburg heterozygotes. Both isoforms showed a very similar accumulation in VLDL (ratio apoE4Freiburg to apoE4, 0.99) and in IDL (ratio, 0.95), but much less apoE4Freiburg than apoE4 (ratio, 0.67) accumulated in HDL (Figure 5).

**Effects of ApoE4 Freiburg on Postprandial Lipoproteins**

We also analyzed the effects of the apoE4Freiburg isoform on postprandial lipoproteins because the epidemiological data

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Effects of E4Freiburg on cholesterol (A), apoB (B), apoA-I (C), and triglyceride (D) concentrations. Carriers include the apoE phenotypes E2/E4Freiburg, E3/E4Freiburg, E4/E4Freiburg, and E4Freiburg/E4Freiburg. Noncarriers included the apoE phenotypes E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4. Cumulative frequency distribution analysis of all subjects for cholesterol, apoB, apoA-I, and triglyceride concentrations. Carriers include the apoE phenotypes E2/E4Freiburg, E3/E4Freiburg, E4/E4Freiburg, and E4Freiburg/E4Freiburg. Noncarriers included the apoE phenotypes E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4. Cumulative frequency distribution analysis of all subjects for cholesterol, apoB, and apoA-I (n = 3625) and of all fasting subjects for triglycerides (n = 2036) showed no differences for cholesterol and apoB between carriers of the E4Freiburg allele and noncarriers, but apoA-I was lower (P = 0.001) and triglycerides higher (P = 0.048) in carriers of E4Freiburg than in noncarriers. Subjects with triglycerides >7.874 mmol/L were excluded from statistical analysis (n = 12).

**TABLE 3. Effects of ApoE4 Freiburg on Lipid and Apolipoprotein Concentrations**

<table>
<thead>
<tr>
<th>Lipid or Apolipoprotein</th>
<th>ApoE3/E4 (n = 41) vs ApoE3/E4 (n = 747)</th>
<th>ApoE4/apoE4Freiburg (n = 11) vs ApoE4 (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Difference</td>
<td>95% CI</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>-0.524</td>
<td>-0.945 to -0.108</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>-4.26</td>
<td>-16.9 to 8.4</td>
</tr>
<tr>
<td>ApoA-I, mg/dL</td>
<td>-15.9</td>
<td>-24.3 to -7.4</td>
</tr>
<tr>
<td>Triglycerides, mmol/L*</td>
<td>0.187</td>
<td>-0.325 to 0.533</td>
</tr>
</tbody>
</table>

*Analysis of triglyceride concentrations was restricted to fasting subjects with triglycerides <7.874 mmol/L (apoE3/E4Freiburg, n = 39; apoE3/E4, n = 419; apoE4/E4Freiburg, n = 8; apoE4/E4, n = 31).
revealed several effects of the apoE4 Freiburg isoform on fasting lipoproteins and because carriers of many rare apoE variants display a delayed clearance of postprandial lipoproteins. A standard fatty meal containing retinyl palmitate was given to 3 E4 Freiburg homozygotes (for clinical characteristics, see subjects 1, 2, and 3 in Table 1), and the different lipoprotein classes were monitored for 10 hours. These data were compared with those from 2 control groups of normolipemic subjects, 1 group of 29 subjects (with the apoE phenotypes E2/E3, n = 8; E3/E3, n = 8; E3/E4, n = 7; E4/4, n = 6) designated non-apoE2/E2, and 8 subjects with the phenotype E2/E2. Although all 3 E4 Freiburg homozygotes displayed different forms of hyperlipoproteinemia in the fasting state, their postprandial increases of chylomicrons, chylomicrons remnants, and VLDL were moderate and did not differ from those obtained in non-apoE2/E2 normolipemic control subjects (Figure 6A through 6C).

We next examined in detail the composition of HDL after a fatty meal. Postprandial concentrations of HDL triglycerides, an indicator for both high cholesteryl ester transfer protein activity and the presence of high concentrations of triglyceride-rich lipoproteins, increased in response to the postprandial lipoprotein surge in all 3 groups (Figure 6D). There were, however, differences between the groups. The total triglyceride enrichment, as measured by the area under the curve of HDL triglycerides, tended to be lowest in (hyperlipidemic) E4 Freiburg homozygotes, followed by normolipemic non-apoE2/E2 subjects, and was significantly higher in normolipemic e2 homozygotes (0.834, 1.129, 1.390 mmol·h⁻¹·L⁻¹, respectively; P = 0.038 for E4 Freiburg/E4 Freiburg versus e2/e2). The ratio of apoA-I to HDL cholesterol (Figure 6F) and the molar ratio of HDL cholesterol to phospholipids (E4 Freiburg/E4 Freiburg, 0.86±0.05; non-apoE2/E2, 0.94±0.02; e2/e2, 0.92±0.04) were similar among these 3 groups. Both ratios did not change postprandially in homozygous E4 Freiburg patients or in normolipemic control subjects. In summary, these studies revealed a normal clearance of postprandial lipoproteins and a slightly lower enrichment of HDL with
triglycerides in e4Freiburg homozygotes. The postprandial changes of HDL composition (phospholipids, cholesterol) were otherwise unremarkable.

Discussion

The molecular basis of the novel apoE isofrom apoE4Freiburg is a thymidine-to-cytidine substitution at position 3100 of APOE4, which changes leucine to proline at position 28 of the polypeptide chain. ApoE4Freiburg differs from most other known apoE variants in that the amino acid replacement involves 2 neutral amino acids and therefore does not change the net charge of the isoprotein. Denaturing conditions present at isoelectric focusing allow the detection of a slightly more acidic net charge of the isoform, especially in apoE4/ apoE4Freiburg heterozygotes (Figure 1A). In this case, isoelectric focusing with subsequent immunofixation leads to a doublet at the apoE4 position with the additional band migrating to a position about a fifth net charge more acidic than apoE4. The leucine-to-proline substitution in codon 28 is not located within the receptor-binding region (residues 136 to 150), the HSPG-binding sites (142 to 147), or the lipid-binding domain in the carboxyl terminus (residues 244 to 272), and the predicted normal functions of these regions could be demonstrated. In fact, normal receptor-binding function could be demonstrated by comparing the binding properties of apoE4Freiburg · DMPC complexes with those of DMPC complexed with apoE3 or apoE4. Defective HSPG-binding is very unlikely because of the phenotype of heterozygotes, which in cases of defective HSPG-binding were otherwise unremarkable.

Screening of 3569 subjects for this isoform revealed that e4Freiburg heterozygotes were more likely to survive a myocardial infarction or if the e4Freiburg allele was associated with longevity.53 survivors of a myocardial infarction and CAD patients who had not yet suffered a myocardial infarction were included in the patient group. Adjustment for age and sex did not change the higher allelic frequency among CAD patients. Therefore, selection bias is not likely and the higher allelic frequency in CAD patients may in fact reflect the atherogenic properties of this isoform.

All 4 e4Freiburg homozygotes identified suffered from various forms of hyperlipoproteinemia and 3 had different types of hypertriglyceridemia. The analysis of 60 heterozygotes did not link e4Freiburg with a specific hyperlipoproteinemia phenotype. We analyzed the effects of the common APOE alleles and of e4Freiburg on the modulation of serum lipids and apolipoproteins. Because e4Freiburg is probably derived from e4, special emphasis was placed on the comparisons of apoE3/E4Freiburg with apoE3/E4 and of apoE4/E4Freiburg with apoE4/E4 to identify effects specific to apoE4Freiburg. Our study confirmed the modulation of plasma lipoproteins and apolipoproteins by the common APOE alleles observed in previous studies.52–54 Specifically, the e2 allele decreased cholesterol and apoB and increased triglycerides, and the e4 allele increased cholesterol and apoB and decreased apoA-I. The e4 allele also increased triglycerides, but because of the higher variability of triglyceride concentrations,55 this effect did not reach significance. These and other data52,53 indicate that the effects of different APOE alleles on triglycerides are opposite to the effects on HDL. Our interpretation is that the effects of APOE on apoA-I concentrations are secondary to the effects of modulating triglyceride concentrations. The much higher intraindividual variability of triglycerides (compared with apoA-I),56 however, makes it more difficult to detect these modulating effects on triglycerides.

The effects of e4Freiburg on the concentration of fasting lipids and apolipoproteins were significantly different from those of e4. e4Freiburg lowered cholesterol (e3/e3 versus e3/e4), apoB (e4/e4 versus e4/e4), and apoA-I. The mechanism of the effect of apoE4Freiburg on lipoproteins is unknown, but the epidemiological data, together with data from the postprandial studies, indicate a connection of e4Freiburg with triglyceride-rich lipoproteins, apoA-I, and HDL triglycerides. The effects of apoE4Freiburg on cholesterol and apoB concentrations can be explained by the known metabolic link between high triglycerides and low LDL cholesterol.57 To get some insight into the molecular mechanism of apoE4Freiburg in lipoprotein metabolism, we characterized the receptor-mediated clearance of lipoproteins containing apoE4Freiburg with 2 approaches.

First, the in vitro clearance by LDL receptors was analyzed by receptor-binding studies of apoE4Freiburg and other apoE isofroms complexed with small DMPC disks. These receptor-binding studies revealed no binding defect of apoE4Freiburg. This is consistent with the observation that none of the e4Freiburg homozygotes presented with type III hyperlipoproteinemia, which is associated with LDL receptor-binding-defective isofroms of apoE.7

Second, the in vivo clearance of postprandial lipoproteins from 3 e4Freiburg homozygotes was unremarkable and did not differ from the clearance of triglyceride-rich lipoproteins in
normalipemic control subjects\textsuperscript{28} despite higher fasting triglycerides in the $\epsilon_{4}$\textsubscript{Freiburg} homozygotes. Surprisingly, the postprandial accumulation of triglycerides in HDL was even less pronounced than in the normalipemic control subjects. Higher fasting and postprandial triglycerides have been shown to increase triglycerides in HDL.\textsuperscript{28} A possible clue for the molecular mechanism comes from the studies of the association of apoE4 and apoE4\textsubscript{Freiburg} with different lipoproteins. Although the majority of each isoform accumulated in triglyceride-rich lipoproteins (Figure 5), marked differences were observed in their accumulation in HDL. Unequivocally less apoE4\textsubscript{Freiburg} than apoE4 was associated with HDL in the patients studied. These data do not exclude a lower HDL preference of apoE4\textsubscript{Freiburg} caused by certain lipid-binding characteristics.\textsuperscript{58} However, certain lipoprotein particles (Lp) in HDL containing apoE (ie, triglyceride-rich LpE:A-I) may be removed more rapidly in carriers of apoE4\textsubscript{Freiburg} than in carriers of the common isoforms. Rapid removal of LpE4\textsubscript{Freiburg}:A-I also reduces apoA-I concentrations. The rapid removal of LpE4\textsubscript{Freiburg}:A-I might be partly responsible for the lower apoA-I concentrations in carriers of $\epsilon_{4}$\textsubscript{Freiburg}, the lower postprandial triglyceride-enrichment of HDL in $\epsilon_{4}$\textsubscript{Freiburg}, homozgyotes observed after a fatty meal, and the higher allelic frequency of $\epsilon_{4}$\textsubscript{Freiburg} in CAD patients. Because of the low number of homozgyotes available and the multifactorial modulation of HDL concentrations, we cannot calculate the quantity of HDL cholesterol and HDL triglycerides removed after a fatty meal. However, the preferential uptake of LpE4\textsubscript{Freiburg}:A-I might take place at all times and not only after a fatty meal and might decrease HDL long-term. We further speculate that these LpE4\textsubscript{Freiburg}:A-I might interfere with the catabolism of triglyceride-rich lipoproteins and subsequently decrease HDL. Elevated concentrations of triglyceride-rich lipoproteins decrease HDL, presumably by the activation of cholesteryl ester transfer protein–mediated lipid transfer.\textsuperscript{45} High concentrations of triglyceride-rich lipoproteins and low concentrations of HDL promote CAD.\textsuperscript{55,56} In this context, it is intriguing to see a 3-fold higher allelic frequency of $\epsilon_{4}$\textsubscript{Freiburg} among CAD patients than among control subjects. The receptor-binding studies with apoE4\textsubscript{Freiburg}–DMPC cannot affect the effects of different apoE isoforms on the clearance of LpE:A-I. Because of their high homogeneity and good reproducibility between different preparations, these DMPC complexes have been successfully used for numerous receptor-binding studies. In alternative methods (eg, use of native lipoproteins obtained from different subjects), differences in protein and lipid composition can confound the results. An alternative hypothesis for the differences in the accumulation of apoE4\textsubscript{Freiburg} in HDL is that the effects result from differences in its lipoprotein preference, either directly by stabilization of the long helices at the expense of short helices, which might cause a low affinity for HDL\textsuperscript{59} or indirectly by its effects on the arginine-61 side chain.\textsuperscript{58}

In summary, apoE4\textsubscript{Freiburg} is a frequent apoE mutation and is significantly more common among CAD patients. The observed effects of this isoform on plasma lipids and apolipoproteins under fasting and postprandial conditions, the phenotype of homozygous and heterozygous carriers, receptor-binding studies, and the distribution of apoE4\textsubscript{Freiburg} among different lipoproteins imply that this isoform exerts its atherogenic properties by modulating the metabolism of triglyceride-rich lipoproteins and HDL.

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Effects of a Frequent Apolipoprotein E Isoform, ApoE4Freiburg (Leu28→Pro), on Lipoproteins and the Prevalence of Coronary Artery Disease in Whites
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