In Vivo Metabolism of Apolipoprotein A-I in a Patient With Homozygous Familial Hypercholesterolemia


Familial hypercholesterolemia (FH), caused by a defect in the low density lipoprotein (LDL) receptor, results in high plasma concentrations of LDL cholesterol due to both overproduction and delayed catabolism of LDL. FH is also associated with significantly lower levels of plasma high density lipoprotein cholesterol and apolipoprotein (apo) A-I in both heterozygous and homozygous patients. However, the metabolic basis of the hypoalphalipoproteinemia in FH has not been elucidated. We investigated the kinetics of apo A-I in a homozygous FH patient and two normal control subjects by using endogenous labeling with a stable isotopically labeled amino acid. Study subjects were administered a primed constant infusion of $^{13}$C$_2$-phenylalanine for 12 hours. Apolipoproteins were isolated from plasma drawn at selected time points and analyzed for their isotopic enrichment by gas chromatography-mass spectrometry. The fractional catabolic rate of apo A-I in the FH subject was found to be substantially increased (0.38 day$^{-1}$) compared with that of the normal subjects (mean, 0.26 day$^{-1}$). In addition, the apo A-I production rate was decreased in the FH subject (6.5 mg/kg·day$^{-1}$) compared with the normal subjects (mean, 11.1 mg/kg·day$^{-1}$). In conclusion, the low levels of high density lipoprotein cholesterol and apo A-I in this homozygous FH patient are due to the combined metabolic defects of increased apo A-I catabolism and decreased apo A-I production.

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KEY WORDS • familial hypercholesterolemia • fractional catabolic rate • apolipoprotein A-I • low density lipoproteins


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reduced. The apo E phenotype of the FH subject was apo E-3/3. The two control subjects, who were 22- and 25-year-old men, had normal plasma lipid and lipoprotein levels. Their apo E phenotypes were apo E-3/4 and apo E-3/3, respectively. Clinical characteristics and laboratory values of the study subjects are summarized in Table 1.

The study protocol was approved by the Internal Review Board of the National Heart, Lung, and Blood Institute, and informed consent was given by each of the participants.

Preparation of Stable Isotopically Labeled Amino Acid

The stable isotopically labeled amino acid used in this study was a custom-synthesized \(^{13}\)C-labeled L-phenylalanine, with the six labeled carbons positioned in the aromatic ring (Cambridge Isotope Laboratories, Woburn, Mass.). Multiple labeling of the amino acid was selected because the additional mass shifts the labeled amino acid peak well out of the range of the peak due to the natural abundance of heavy isotope on mass spectrometric analysis. The use of \(^{13}\)C-labeled phenylalanine therefore significantly increases the accuracy of the mass spectrometric detection of the tracer because the background level is virtually zero. Phenylalanine was chosen as the tracer amino acid because it undergoes irreversible conversion to tyrosine by the hepatic intracellular enzyme phenylalanine hydroxylase, resulting in the generation of sixfold-labeled tyrosine in plasma. A plasma steady state of \(^{13}\)C-phenylalanine was dissolved in physiological salt solution, sterile filtered, and tested for pyrogenicity and sterility before the study.

Study Protocol

Three days before the start of the study, subjects were placed on a controlled isoweight diet containing 37% fat, 47% carbohydrate, 16% protein, 200 mg cholesterol, 1,000 kcal, and a polysaturated to saturated fat ratio of approximately 0.3. After a 12-hour fast, the \(^{13}\)C-phenylalanine was administered as a priming bolus of 550 \(\mu g\) /kg immediately followed by a constant infusion of 12 \(\mu g\) /kg\(^{-1}\) min\(^{-1}\) over a period of 12 hours. Meals were provided in small equal portions every 2 hours during the infusion. Blood samples were drawn before the priming bolus, 10 minutes after the bolus, and then at selected times during the infusion. Blood was drawn into tubes containing EDTA at a final concentration of 0.1% (wt/vol). Plasma was separated by low-speed centrifugation for 30 minutes at 4°C. Sodium azide and aprotinin were added to the plasma at a final concentration of 0.05% (wt/vol) and 200 kIU/ml, respectively.

Isolation of Free Plasma Amino Acids

Free plasma amino acids were isolated from 0.5 ml plasma by cation-exchange column chromatography as previously described. Briefly, 1 ml 30% acetic acid was added to 0.5 ml plasma and applied to pre-filled PolyPrep columns (Bio-Rad, Richmond, Calif.). The columns were washed with 8 ml distilled water and eluted with 5 ml 4N NH\(_4\)OH directly into 5-ml reaction vials (Pierce, Rockford, Ill.).

Isolation and Preparation of Apolipoproteins

Lipoprotein density subfractions were isolated by sequential ultracentrifugation of 5 ml plasma collected at selected time points. Very low density lipoproteins (VLDLs; \(d<1.006\) g/ml) and HDL (\(d=1.063-1.21\) g/ml) were used for isolation of apolipoproteins. Apo B-100 was isolated from delipidated VLDL by preparative discontinuous gradient sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis (SDS-PAGE) (5%\%/15% acrylamide) using a modification of a previously published method. Approximately 0.5 mg VLDL protein was delipidated using chloroform/methanol (2:1, vol/vol) and solubilized in 2% SDS containing 0.1 M tris(hydroxymethyl)aminomethane HCl (pH 6.8) and 1% (vol/vol) dithiothreitol. The samples were heated at 100°C for 10 minutes and applied to a 3-mm-thick vertical slab gel (12x14 cm). After electrophoresis the gels were stained with Coomassie Blue R-250 (Bio-Rad) and destained in a solution of 5% (vol/vol) methanol and 7.5% (vol/vol) acetic acid. Apo A-I was isolated from HDL by preparative isoelectric focusing. Approximately 1 mg HDL protein was solubilized in buffer containing 8.6 M urea and 1% (vol/vol) dithiothreitol. Isolelectric focusing was carried out in a 3-mm-thick vertical slab gel using 7.5% acrylamide, 8 M urea, and 2% (vol/vol) Pharmalyte, pH 4–6.5 (Pharmacia, Piscataway, N.J.). Gels were electrofocused at 250 V for 20 hours at 4°C with 0.02 M NaOH and 0.01 M H\(_2\)PO\(_4\) as the upper and lower buffers, respectively. After electrophoresis the gels were stained with Coomassie Blue G-250 (Bio-Rad). Stained apolipoprotein bands were cut from the gels, and the gel slices were dried in a 90°C oven and then hydrolyzed in 6N HCl (Pierce) for 24 hours at 110°C under nitrogen vacuum. After removal of HCl by speed-vac lyophilization the samples were dissolved in 50% (vol/vol) acetic acid and applied to cation-exchange columns as described above. Amino acids were recovered by elution with 4N NH\(_4\)OH into reaction vials.
Gas Chromatographic–Mass Spectrometric Analysis

Recovered amino acids were derivatized to the N-heptfluorobutyryl isobutyl esters as previously described. 20 Briefly, 500 μl isobutyl HCl (Alltech Associates, Inc., Deerfield, Ill.) was added to the samples, and vials were heated for 1 hour at 110°C, cooled to room temperature, and lyophilized. The residue was dissolved in 200 μl high-performance liquid chromatography-grade ethyl acetate and further treated with 150 μl heptfluorobutyric acid (Alltech Associates Inc.) at 150°C for 10 minutes to complete the derivatization. After cooling to room temperature and lyophilizing to dryness, the derivatized product was dissolved in 100 μl ethyl acetate and stored at -20°C until gas chromatographic–mass spectrometric (GC/MS) analysis.

Samples were analyzed on a Finnigan MAT 4500 GC/MS (Finnigan, San Jose, Calif.) in the chemical ionization mode with isobutane as the reagent gas. The selected positively charged ions of 418 m/z for phenylalanine, 424 m/z for 13C6-phenylalanine, 630 m/z for tyrosine, and 636 m/z for 13C6-tyrosine were monitored. At least three measurements were made of each apolipoprotein at each time point. In addition, a blank gel slice comparable in size to the protein gel slices was analyzed as a control. The amount of unlabeled phenylalanine in the blank gel slices was always less than 3% of the total phenylalanine in the study samples. The isotopic enrichment of the sample was determined as the fraction of labeled to total (labeled plus unlabeled) phenylalanine.

Analytical Methods

Apo A-I and apo B were quantified by enzyme-linked immunosorbent assay as previously described. 24–25 Apo E was quantified by radioimmunoassay. 26 Plasma cholesterol and triglycerides were quantified by using an enzymatic assay (Wako Pure Chemical Industries, Ltd., Houston, Tex.). HDL cholesterol was determined in plasma after dextran sulfate precipitation. 27

Determination of the fractional synthetic rate (FSR) of apo A-I was performed as recently reported. 28–31 The isotopic enrichment of VLDL apo B-100 at plateau was used as an estimate of the precursor pool enrichment for apo A-I synthesis. The isotopic enrichment of apo A-I was divided by the precursor enrichment to obtain a “corrected isotopic enrichment.” A “best-fit” line was fit to the data by using linear regression analysis. The apo A-I FSR was determined as the slope of the apo A-I corrected isotopic enrichment curve. At steady state, the FSR equals the FCR. The apo A-I production rate (PR) was determined by the formula

$$PR = \frac{FSR \times \text{apo A-I concentration} \times \text{plasma volume}}{\text{body weight}}$$

Plasma volume was assumed to be 4% of body weight.

Results

A plasma steady state of free 13C6-phenylalanine was achieved within 30 minutes in all three study subjects (Figure 1). The mean isotopic enrichment of 13C6-phenylalanine at steady state was 5.6±0.7% in the FH subject, 5.7±0.4% in control subject 1, and 4.9±0.3% in control subject 2. A steady state of endogenously de-
Figure 3. Line plot of corrected isotopic enrichment values for high density lipoprotein (apo A-I) in two control subjects (open symbols) and the familiar hypercholesterolemia subject (closed squares).

Control 2
Control 1
FH subject

TABLE 2. Kinetic Parameters of Apo-A-I Metabolism

<table>
<thead>
<tr>
<th>Subject</th>
<th>FSR (day⁻¹)</th>
<th>Apo A-I (mg/dl)</th>
<th>PR (mg/kg · day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH subject</td>
<td>0.376</td>
<td>44</td>
<td>6.2</td>
</tr>
<tr>
<td>Control 1</td>
<td>0.230</td>
<td>109</td>
<td>9.7</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.286</td>
<td>111</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Apo, apolipoprotein; FSR, fractional synthetic rate; PR, production rate; FH, familial hypercholesterolemia.

Discussion

Decreased plasma concentrations of HDL cholesterol and apo A-I are risk factors for coronary heart disease (CHD), independent of LDL cholesterol level. Even within the heterozygous FH population, HDL cholesterol level is inversely related to the incidence and severity of CHD. Homozygous FH patients have not only higher LDL cholesterol but also lower HDL cholesterol levels than heterozygotes and are at much greater risk for CHD. Therefore, low HDL levels may further increase CHD risk in FH patients.

Despite its clinical relevance, the metabolic basis of the low HDL cholesterol levels in FH has not been investigated. In contrast, the decreased HDL levels found in hypertriglyceridemic subjects have been the focus of several kinetics studies. The cause of the low HDL and apo A-I levels in hypertriglyceridemia has been shown to be an increased FCR of apo A-I. The apo A-I PRs in hypertriglyceridemic subjects were reported to be normal or increased. Because so little is known about the mechanisms of the hyperalphalipoproteinemia in FH, we chose to investigate the apo A-I kinetics in a homozygous FH subject.

We demonstrate that this patient has a dual metabolic defect: an increased apo A-I FCR as well as decreased apo A-I production. Similar results were recently reported by Saku et al. In the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model of FH. In that study, radiolabeled LDL turnover studies revealed that WHHL rabbits have an increased apo A-I FCR (0.874 day⁻¹) compared with control rabbits (0.502 day⁻¹). In addition, the WHHL rabbits have a decreased apo A-I PR (8.67 mg/kg · day⁻¹) compared with control rabbits (18.14 mg/kg · day⁻¹). Subsequent studies have demonstrated that hepatic apo A-I mRNA levels in WHHL rabbits are significantly lower than in normal rabbits.

FH is characterized metabolically by overproduction and delayed clearance of apo B, delayed catabolism of apo E, and delayed catabolism of Lp(a). The lack of a functional LDL receptor presumably accounts for the delayed catabolism of apo B, apo E, and Lp(a). The increased apo B production in FH subjects is less well understood and may be related to disturbances of intracellular cholesterol metabolism.

In the FH patient described in this report, apo A-I has an accelerated catabolism and a decreased rate of production. The mechanism by which the lack of functional LDL receptors results in these metabolic alterations is uncertain. The increased apo A-I catabolic rate has several potential explanations. One may be related to the expanded pool size of apo E in FH. Two reports have documented that the HDL-associated apo E mass in FH is more than twice that in normolipidemic subjects. In our study, the mass of apo E in HDL was 2.3 times higher in the FH subject (4.7 mg/dl) than in the normal subject with the apo E-3/3 phenotype (2 mg/dl). It has been established that apo E–enriched HDL is catabolized rapidly via a receptor-dependent pathway mediated by apo E. Given that the remnant receptor pathway in FH appears to be normal, it may be that the apo E–enriched HDL in FH is catabolized via this pathway, resulting in an increased apo A-I FCR. A second possibility is that the expanded pool of apo B–containing lipoproteins in FH results in a greater net mass transfer of cholesteryl ester from HDL to apo B particles via the cholesterol ester transfer protein. We have recently demonstrated that deficiency of cholesterol ester transfer protein results in delayed apo A-I catabolism, and therefore increased cholesterol ester transfer from HDL may cause more rapid apo A-I catabolism. This remains to be established.

The reduction in apo A-I production in this FH subject is consistent with the observations in WHHL rabbits and is intriguing in light of the documented increase in apo B production in FH. Monge et al. reported that incubation with LDL resulted in an increase of apo A-I mRNA in HepG2 cells. Recently, Mitchell et al. reported that treatment of rats with drugs that upregulate the LDL receptor resulted in a marked increase in hepatic apo A-I mRNA levels and a decrease in hepatic apo B mRNA levels. Thus, there...
may exist a feedback system involving the LDL receptor that regulates the hepatic expression of both apo A-I and apo B. It is well established that FH fibroblasts have markedly impaired ability to normally regulate the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene; FH hepatocytes also have abnormal intracellular cholesterol homeostasis that may affect the production of apo A-I and apo B.

In conclusion, we have established that low levels of HDL cholesterol and apo A-I in a homozygous FH subject are due to a combined metabolic defect of increased apo A-I catabolism and decreased apo A-I production compared with control subjects studied under identical conditions. These results provide new insights into the regulation of apo A-I metabolism in FH.

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References


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