Cholesterol and Triglyceride Fatty Acid Synthesis in Apolipoprotein E2–Associated Hyperlipidemia

P.J.H. Jones, S.M. Dendy, J.J. Frohlich, C.A. Leitch, and D.A. Schoeller

To investigate whether increased endogenous lipogenesis contributes to elevated plasma lipid levels in individuals with apolipoprotein (apo) E2–associated hyperlipidemia (E2-HL), plasma pool cholesterol and triglyceride fatty acid syntheses were measured in subjects with E2-HL and in those with normal lipid levels. Subjects were given a priming dose of deuterium oxide (D₂O) followed by maintenance doses over 48 hours. During the first 24 hours, subjects consumed prepared meals, whereas during the 24–48-hour interval, they consumed water only. Blood samples were drawn every 12 hours, and cholesterol and triglyceride fatty acid formation rates were determined from the change in deuterium enrichment. The free cholesterol fractional synthesis rate over 0–24 hours of E2-HL subjects (0.057±0.010 day⁻¹, mean±SEM) was not significantly different from that of normolipidemics (0.075±0.005 day⁻¹). Calculated cholesterol net synthesis was not different between the two groups (0.56±0.07 and 0.75±0.05 g/day, respectively). Mean free cholesterol synthesis for all subjects was higher in the fed (0–24-hour) compared with the fasted (24–48-hour) condition. Initial 12-hour triglyceride fatty acid fractional synthesis was significantly (p<0.01) increased in E2-HL subjects (0.143±0.012 day⁻¹) compared with controls (0.082±0.013 day⁻¹). These findings suggest that in E2-HL, elevated plasma cholesterol levels are due to factors other than increased sterol synthesis, while higher de novo fatty acid synthesis contributes to the observed hypertriglyceridemia. (Arteriosclerosis and Thrombosis 1992;12:106–113)

A polipoprotein (apo) E exhibits genetic polymorphism with its structural gene locus, yielding three common alleles designated e2, e3, and e4. These alleles code for apo E isoforms E2, E3, and E4, which are codominantly inherited to produce native apolipoprotein. The apo e3 allele is the most common, with the other isoforms exhibiting lesser population phenotypic frequency. An association between apo E phenotype and plasma total and low density lipoprotein cholesterol level has been demonstrated, with elevated plasma values in individuals carrying the e3 or e4 allele compared with the e2 allele.

Although possession of the e2 allele is usually associated with lower cholesterol levels, certain individuals with this isoform spontaneously develop hyperlipidemia such as type III hyperlipoproteinemia (HLP). Type III HLP is a fasting hyperlipidemia characterized by elevated intermediate density lipoprotein levels, xanthomatosis, and premature coronary artery disease. Type III HLP commonly develops in individuals with the apo E2/E2 phenotype and less commonly in other phenotypes or in those with apo E deficiency. The incidence of the e2 allele has also been found at a higher-than-normal frequency in individuals with hypertriglyceridemia and mixed forms of hyperlipidemia. Thus, there exists a tendency for individuals possessing the e2 allele to develop hypertriglyceridemia and mixed hyperlipidemia.
Subjects and Screening Procedures

Eight apo E2–possessing hyperlipidemic patients were recruited from the Lipid Clinic, University Hospital, Vancouver, Canada, by review of patient charts (Table 1). Four type III HLP patients had the apo E2/E2 phenotype, hyperlipoproteinemia, planar xanthomas, and very low density lipoprotein of <3-mmol/1, triglycerides ^2.26 mmol/1). The E2-HL group included four clinically obese subjects and three subjects who had elevated circulating lipid levels (cholesterol ^6.35 mmol/1 and triglycerides ^2.26 mmol/1). The E2-HL group included four clinically obese subjects and three subjects who had elevated circulating lipid levels (cholesterol ^6.35 mmol/1 and triglycerides ^2.26 mmol/1). The E2-HL group included four clinically obese subjects and three subjects who had elevated circulating lipid levels (cholesterol ^6.35 mmol/1 and triglycerides ^2.26 mmol/1). The E2-HL group included four clinically obese subjects and three subjects who had elevated circulating lipid levels (cholesterol ^6.35 mmol/1 and triglycerides ^2.26 mmol/1). The E2-HL group included four clinically obese subjects and three subjects who had elevated circulating lipid levels (cholesterol ^6.35 mmol/1 and triglycerides ^2.26 mmol/1).

Methods

Protocol

Lipogenesis was measured by using the rate of deuterium incorporation from deuterium oxide (D2O) in body water into plasma cholesterol and TGFA. Cholesterol synthesis with D2O has been previously described in plasma and erythrocytes. This approach, derived from animal studies with tritiated water (3H2O), yields a measure of the fraction of the body central pool synthesized per unit time. TGFA formation rate has been examined by using 3H2O in animals and D2O in humans.

One week before the cholesterol synthesis study, subjects recorded intakes of all food and beverages consumed over 3 days to assess habitual dietary consumption patterns. Nutrient intake analysis was performed with a computerized nutrient analysis program (Smart Engineering Ltd., Vancouver, Canada) based on the Canadian Nutrient File. Subjects were then studied in the Metabolic Research Unit over 48 hours. On the first day (0-24 hours), three isocaloric meals were provided, and on day 2, subjects drank only deuterium-labeled water. The isocaloric meals, fed to subjects at 8 AM, 12:30 PM, and 5:30 PM, contained 40% fat, 45% carbohydrate, 15% protein, and 220 mg cholesterol/1,000 kcal and had a polyunsaturated to saturated (P/S) fatty acid ratio of 0.7, as computed by food nutritional composition data. Cumulative energy content of the three meals equaled each subject’s calculated energy requirement. Just before 8:00 AM (time=0) on day 1, a blood sample was drawn for baseline plasma lipids and deuterium incorporation. Over the 24-hour period, the cumulative energy content of the three meals equaled each subject’s calculated energy requirement. Just before 8:00 AM (time=0) on day 1, a blood sample was drawn for baseline plasma lipids and deuterium incorporation. Over the 24-hour period, subjects were studied in the Metabolic Research Unit.
TABLE 2. Habitual Dietary Intake From 3-Day Food Records of Control and Apolipoprotein E2 Hyperlipidemics

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<th>Subject by group</th>
<th>Mean±SEM</th>
<th>Energy (kcal)</th>
<th>Protein (% kcal)</th>
<th>CHO (% kcal)</th>
<th>Fat (% kcal)</th>
<th>P/S ratio</th>
<th>Alcohol (% kcal)</th>
<th>Cholesterol (mg/Mcal)</th>
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<td>19.8±1.6*</td>
<td>46.6±4.2</td>
<td>30.5±3.6</td>
<td>0.6±0.1</td>
<td>3.1±1.4</td>
<td>142±18</td>
</tr>
</tbody>
</table>

CHO, carbohydrates; P/S, polyunsaturated to saturated ratio of fatty acids.

*Significantly different from controls at p<0.05.

was taken; then subjects consumed as a bolus 0.7 g D2O (Merck Sharp and Dohme Isotopes, Montreal, Canada) per kilogram of estimated body water. Maintenance doses of D2O (1.4 g and 0.7 g D2O/kg drinking water on days 1 and 2, respectively) were provided subsequently in drinking water given to subjects to maintain a plateau deuterium enrichment of body water. Blood samples were collected at 12-hour intervals over 48 hours, and plasma was immediately centrifuged and frozen.

Analytical Procedures

Total plasma lipids were extracted with hexane/chloroform (4:1, vol/vol) after addition of methanol and heating at 55°C. Water was added, and the mixture was shaken mechanically for 15 minutes and then centrifuged 10 minutes. The upper solvent phase was removed, and the remaining aqueous phase was reextracted. After centrifugation, the solvent phases were combined and the extract dried under nitrogen. Extracts were dissolved in chloroform and spotted on thin-layer chromatography plates (Whatman Inc., Clifton, N.J.). Plates were developed in petroleum ether/ethyl ether/acetic acid (135:15:1.5, vol/vol/vol). After drying, individual lipid bands were identified by developing in iodine vapor and compared with cochromatogramed cholesterol and triglyceride standards. To remove free cholesterol and triglyceride bands from the silica gel, hexane/chloroform/ether (5:2:1, vol/vol/vol) was added, shaken for 10 minutes, and centrifuged, and the solvent was removed. The extraction was repeated twice. Solvent layers were combined and thoroughly dried after transfer into 15-cm×6-mm lengths of pre-annealed quartz (Corning Glassware, Corning, N.Y.) tubing containing 0.5 g CuO and a 2.5-cm×1-mm length of sterling silver wire. The quartz tubing was evacuated to <30 mtorr and then flame-sealed.

Samples were combusted within quartz tubes at 725°C for 2 hours. The tubes were then slowly cooled.
to room temperature, the carbon dioxide was removed, and the combustion water was transferred via vacuum distillation to Vycor reduction tubes (Corning Glassware, Corning, N.Y.) containing 60±5 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, Ind.). Heating at 500°C for 30 minutes reduced the combustion water to hydrogen gas, using similar methodology as described previously.22

Analysis of plasma water deuterium enrichment was carried out after dilution of 24-hour and 48-hour postdose plasma samples (1:5 with distilled water) of known isotopic abundance to obtain deuterium enrichments within the normal analytical range of the mass spectrometer. Duplicate samples were transferred in 2-μl capillary tubes by direct addition to 15×6-mm Pyrex reduction tubes containing 60 mg zinc reagent. Reduction tubes were frozen with liquid nitrogen, evacuated, and flame-sealed before heating at 500°C for 30 minutes to generate hydrogen gas.

Deuterium enrichments were measured by direct introduction of reduction tube gas into a differential isotope ratio mass spectrometer with electrical H$_3^+$ compensation (Nuclide 3-60 H/D; MAAS, Bellefonte, Pa., and VG Isogas 903D, Cheshire, UK). Corrections were made for the solute content of plasma during dilution.

Additional aliquots of plasma were used for total cholesterol and triglyceride analyses. These determinations were carried out by enzymatic methods$^{23,24}$ with an autoanalyzer system (Technicon Instruments Corp., Tarrytown, N.Y.).

Calculations and Statistical Analyses

Assumptions of cholesterol synthesis rate calculations have been considered previously.$^{16,18}$ The proportion of central-pool free sterol derived from synthesis, expressed as the fractional synthetic rate (FSR), was calculated by using the equation

\[
\text{FSR (day}$^{-1}$\text{)} = \frac{\delta \text{cholesterol (‰)}}{\delta \text{plasma water (‰)} \times 0.475}
\]

(1)

where $\delta$ cholesterol is the change in cholesterol deuterium enrichment over 24-hour measurement intervals and $\delta$ plasma water is deuterium enrichment achieved at plateau, expressed as parts per thousand (‰), relative to the standard mean ocean water value. Isotopic enrichments are expressed in per mil using the $\delta$ notation (‰) defined as

\[
\delta (\‰) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1,000
\]

(2)

where $R$ is the ratio of the heavy to the light isotope. The denominator factor 0.475 in Equation 1 corrects for the number of hydrogen atoms on cholesterol that are predicted to be replaced by deuterium.$^{16}$

Net free cholesterol synthesis was obtained by multiplying FSR by central free cholesterol pool size. Total central-pool size was calculated using the equation of Goodman et al.$^{25}$

\[
\text{Central cholesterol pool size (g) =}
[0.287 \text{ wt (kg)} + 0.0358 \text{ chol (mg/dl)} - 2.40 \text{TGGP} - 1.72]
\]

(3)

where wt is body weight, chol is plasma total cholesterol concentration, and TGGP is a variable (1–3)
corresponding to serum triglyceride levels of <2.26 mmol (1), 2.26–3.39 mmol (2), or >3.39 mmol (3). The fraction of central-pool total cholesterol existing in free form was estimated to be 0.40. Individual net synthesis was thus calculated for each subject as

$$\text{Free cholesterol net synthesis (g/day)} = \frac{\text{FSR (day}^{-1}) \times \text{central pool (g)} \times 0.40}{(4)}$$

TGFA synthesis was determined as the change in enrichment over the initial 12-hour period of day 1. The 12-hour interval was selected because the pool into which newly synthesized hepatic triglycerides is released is small and rapidly turning over. Due to mixing of exogenous and endogenous triglyceride within the plasma pool, absolute formation rates are not obtainable. However, because diets were controlled over the study period, the contribution of exogenous, that is, dietary TGFA should be relatively constant across groups. Fractional synthesis indexes were obtained from the incorporation of deuterium into plasma TGFA over the initial 12-hour interval of study. The equation used was

$$\text{FSI (day}^{-1}) = \frac{\delta \text{ triglyceride ()}\%\text{e} \times 2}{\delta \text{ plasma water (}}\%\text{e} \times 0.477} (5)$$

where FSI is the fractional synthesis index, $\delta$ triglyceride is the change in deuterium enrichment (‰) over 12 hours, and $\delta$ plasma water is the enrichment of body water above baseline over the measurement period. The correction factor 0.477 was derived from the ratio observed by Jungas of 0.87 gram-atom tritium per gram-atom carbon incorporated into adipose fatty acids. After correcting for the carbon atoms of glycerol (Gly) in a hypothetical triglyceride containing three monounsaturated 17-carbon fatty acids (FA), the ratio was adjusted in the following manner:

$$0.477 = \frac{0.87 D}{CFA} \times \frac{51CFA}{54Cgly+FA} \times \frac{54Cgly+FA}{93HFA} (6)$$

Results were analyzed by using the SYSTAT PC software package (Systat, Inc., Evanston, Ill.). Independent Student's $t$ tests were performed to determine the significance of differences in means for anthropometry, the plasma lipid profile, usual dietary intakes, and central-pool cholesterol level. Cholesterol synthesis data were analyzed by two-way repeated-measures analysis of variance. TGFA synthesis data were analyzed using an independent Student's $t$ test. Linear regression analysis comparisons of plasma lipid levels with cholesterol or TGFA synthesis were also carried out for all subjects. A probability value <0.05 was considered to indicate a significant between-group difference.

**Results**

Demographic, anthropometric, and plasma lipid characteristics of subjects are shown in Table 1. Control subjects are numbered 1–8; type III HLPs, 9–12; and heterozygous E2/E3 hyperlipidemics, 13–16. Subject groups were similar in sex, age, height, and weight, although subjects with E2-HL showed a tendency to be heavier than controls. Significantly elevated plasma cholesterol ($p<0.05$) and triglyceride ($p<0.005$) levels were observed in E2-HL compared with control subjects.

Computerized nutrient intake analysis of control and hyperlipidemic subjects' usual dietary intakes, as reported by 3-day food records before the experimental trial, showed differences ($p<0.05$) in protein consumption between groups (Table 2). No significant between-group differences were observed for daily mean energy, carbohydrate, total fat, p/s of dietary fat, alcohol, or cholesterol consumption.

Plasma total cholesterol levels averaged over 0-, 24-, and 48-hour time points in control subjects (5.60±0.32 mmol/l, mean±SEM) were significantly lower ($p<0.005$) than in those with E2-HL (7.45±0.40 mmol/l). Day-to-day fluctuations in cholesterol level measured at 0, 24, and 48 hours were
small (percent coefficients of variation = 2.83 and 3.52 in control and E2-HL, respectively) and nonsystematic in direction.

Mean plasma water deuterium enrichments and calculated central cholesterol pool size are depicted in Figure 1. Mean postdose deuterium enrichments, calculated as the average of 12- and 36-hour plasma water time points, did not differ between control (4.473 ± 1.86 %) and E2-HL (4.854 ± 1.27 %) subjects. Calculated total central-pool sizes (grams) were 24.9 ± 0.6 and 26.1 ± 1.9 for controls and E2-HLs, respectively.

Deuterium uptake with respect to baseline into plasma free cholesterol and TGFA at each 12-hour time point is shown in Figure 2. For each lipid species, enrichment increased on day 1 (feeding) and remained constant or decreased over day 2 (fasting). Values for free cholesterol were considered meaningful over the entire 48-hour test period, as the total pool size is large relative to the daily turnover. For triglycerides this pool is smaller; thus, only the initial 12-hour interval was taken as an indicator of synthesis. Cholesterol FSR in E2-HLs (0.057 ± 0.010 day⁻¹) over 0–24 hours was not significantly different from controls (0.075 ± 0.005 day⁻¹). Comparison of free cholesterol FSR values for control and E2-HL subjects over fed and fasted days revealed significant time effects (p < 0.001) but insignificant group effects (Figure 3). Mean FSR for all subjects was higher (p < 0.001) in the fed (0–24 hours) (0.066 ± 0.006 day⁻¹) compared with the fasted (24–48 hours) (0.001 ± 0.004 day⁻¹) state.

When FSR and central free pool size determinations were combined to yield values for net synthesis, no significant differences were found between E2-HL and control groups during fed (0.56 ± 0.07 g and 0.75 ± 0.05 g, respectively) or fasted (0.03 ± 0.05 g and −0.02 ± 0.06 g, respectively) conditions (Figure 4).

Fractional synthesis indexes for TGFA, calculated from 0–12-hour enrichment data, are shown in Figure 5. A significantly elevated (p < 0.01) triglyceride 0–12-hour fractional synthesis index was observed for E2-HLs (0.143 ± 0.012 day⁻¹) compared with controls (0.082 ± 0.013 day⁻¹). No significant associations were observed for regression analysis comparisons of plasma level and synthesis for cholesterol or TGFA (data not shown).

Discussion

Although possession of the apolipoprotein e2 allele has been associated with hyperlipidemia, the underlying mechanisms have not been determined. Results from this investigation suggest that increased whole-body cholesterol synthesis does not contribute to elevated circulating cholesterol levels observed with E2-HL. In contrast, elevated triglyceride uptake of deuterium was observed with E2-HL, consistent with higher formation rates for TGFA within individuals with this disorder.

Screening of plasma cholesterol values of control subjects was appropriate for normolipidemics of the age range tested. Subjects with E2-HL exhibited elevated circulating cholesterol and triglyceride levels with or without clinical features of type III HLP. Because of limited subgroup sample sizes, a statistical comparison was not performed between type III HLP and heterozygous E2/E3 hyperlipidemic subjects. However, no obvious differences in plasma cholesterol and triglyceride levels or synthesis are apparent between these subgroups. Therefore, the significant differences between controls and E2-HLs are likely attributable to a general e2 allele-associated effect.

The present technique for measuring cholesterol synthesis has previously been used to examine natural rhythmicity in formation rate,₁⁶ the influence of dietary cholesterol level on cholesterol synthesis,₁⁷ and the effect of apo E phenotype on cholesterologenesis in healthy men.₂⁶ FSR values represent the proportion of the rapidly exchangeable pool of free body cholesterol, which is replaced by newly formed, labeled sterol per unit time. Input of unlabeled, that is, nonsynthetic cholesterol into the pool results in underestimation of true FSR. Negative FSR values indicate situations where cholesterol from unlabeled sources enters the central free pool more rapidly than that from synthesis itself.

Goodman et al²⁵ have developed a multiple-regression prediction equation that expresses the relation between the size of the cholesterol central pool and physiological variables, including weight and plasma lipid levels. This equation accounts for at least 76% of the total variation in central-pool size. As the population from which this equation was derived contained both normolipidemic and hyperlipidemic individuals, it was judged to be appropriate in both subject groups in the present investigation. Thus, we extended the deuterium uptake methodology to estimate net synthesis as the product of FSR values and the central free cholesterol pool size derived from the Goodman equation. De novo synthesized cholesterol enters and equilibrates across the central free pool before undergoing esterification or export to more slowly exchanging pools. The free portion of the central pool is thus most sensitive to changes in formation rate.

The present mean cholesterol FSR value (0.075 day⁻¹) in normolipidemic E2 subjects agrees closely with that (0.070 day⁻¹) obtained in a separate study of a similar although younger group of healthy men possessing the E2 isoform.₂⁶ The cholesterol FSR data in E2-HL subjects showed a trend toward lower fed-state sterol formation compared with the control group. Exclusion of subject 16, whose values were exceptionally high, from the E2-HL group resulted in a statistically significant reduction in FSR for E2-HL compared with control subjects, a finding consistent with the notion of feedback inhibition of sterol synthesis by higher circulating cholesterol levels in E2-HL. However, there was no valid exclusion criterion for this subject.
In normolipidemic individuals, the E2 isoform may confer changes in cholesterol metabolism. Increased and reduced synthesis as well as reduced absorption of cholesterol have been reported to accompany the lower total circulating cholesterol levels in E2-possessing subjects in comparison with E3- and E4-possessing phenotypes. Which processes are altered with E2-HL is unknown. As the present data indicate that overproduction is not the cause of the E2-HL hypercholesterolemia, it is possible that some defect in removal of cholesterol from plasma is responsible.

TGFA synthesis in hyperlipidemias as assessed by deuterium uptake from D2O has not been previously examined. Although theoretical aspects of the use of isotopic water to quantify TGFA synthesis rate have been thoroughly considered, its use in the systematic study of fat synthesis by sensitive isotope ratio mass spectrometric methods has only recently been examined. In the present work, initial 12-hour deuterium enrichment differences were taken to most accurately represent actual formation rates, as rapid attainment of an enrichment plateau would be expected in the small plasma TGFA pool relative to overall synthesis. Indeed, in subjects fed three meals per day, plasma TGFA deuterium enrichment plateaued after 36 hours at a level similar to that observed presently.

The reciprocal pattern in cholesterol and TGFA syntheses displayed by E2-HL compared with normolipidemic subjects suggests differences in regulation between hepatocyte lipoprotein uptake and formation rate. With E2-HL, high circulating cholesterol levels are perhaps a product of an apo E2-associated hepatic receptor-binding defect, resulting in inefficient removal of circulating cholesterol. Insensitivity of the feedback mechanism of hepatic de novo cholesterol synthesis has been suggested, with sterol synthesis remaining unchanged despite reduced hepatic receptor-mediated uptake. In contrast, the hepatocyte responds to the lower lipoprotein uptake rate by elevating TGFA production. Thus, controlling factors involved in regulating synthesis for cholesterol and TGFA exhibit different sensitivities.

To investigate the possibility that differing habitual dietary patterns might influence lipogenesis between the two subject groups, an analysis of previous dietary intake was performed. Limitations of dietary intake data interpretation have been previously recognized. However, because subjects were not fed test diets, nutrient intake analysis comparisons were considered to be a reasonable means of identifying significant intergroup or intersubject differences. The observed elevated protein intake of hyperlipidemics was considered to have only minor potential impact on cholesterol formation. The absence of differences in energy, fat, and cholesterol intakes between groups suggests that any influence of the preceding diet on lipid synthesis rates should be minimal.

Mechanisms responsible for the observed feeding-state modulation of cholesterol synthesis remain to be determined; however, several possibilities exist. First, endocrine alterations in response to fluctuating levels of caloric intake likely influence the rates of cholesterol formation. In in vitro systems, insulin and glucagon produce stimulation and inhibition, respectively, of hepatic cholesterol synthesis. Animals lacking insulin show reduced activities of 3-hydroxy-methylglutaryl-coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, which is reversed on treatment with insulin. Second, the macronutrient intake level may influence the level of substrate available to HMG CoA reductase, thereby modulating its activity. During feeding, the supply of the base unit for cholesterol formation, acetyl CoA, is high, thereby promoting synthesis, whereas without incoming calories, the glycolytic, lipogenic state is replaced by an environment of lypoysis and reduced acetyl CoA availability for sterol and TGFA synthesis.

In summary, these findings demonstrate differences in the mechanism of hypercholesterolemia and hypertriglyceridemia in E2-HL. Whereas cholesterol synthesis was not different between study groups, TGFA formation rate was elevated in E2-HL compared with control subjects. It is suggested that in E2-HL, elevated plasma cholesterol levels are due to factors other than increased sterol synthesis, whereas higher TGFA synthesis contributes to the observed hypertriglyceridemia.

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References


KEY WORDS: hyperlipidemia • apolipoprotein E • cholesterol synthesis • deuterium • type III hyperlipoproteinemia
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