Effect of Ezetimibe on the In Vivo Kinetics of ApoB-48 and ApoB-100 in Men With Primary Hypercholesterolemia

André J. Tremblay, Benoît Lamarche, Jeffrey S. Cohn, Jean-Charles Hogue, Patrick Couture

Objective—To examine the impact of ezetimibe, a selective inhibitor of intestinal cholesterol absorption, on the in vivo kinetics of apolipoproteins (apo) B-48 and B-100 in humans.

Methods and Results—Kinetics of triglyceride-rich lipoprotein (TRL) apoB-48 and very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL) apoB-100 labeled with a stable isotope were assessed at baseline and at the end of 8 weeks of treatment with 10 mg/d of ezetimibe in 8 men with moderate primary hypercholesterolemia. Data were fit to a multicompartmental model using SAAMII to calculate fractional catabolic rate (FCR) and production rate (PR). Ezetimibe significantly decreased total and LDL cholesterol concentrations by −14.5% and −22.0% (P=0.004), respectively, with no significant change in plasma triglyceride and high-density lipoprotein (HDL) cholesterol levels. Ezetimibe had no significant effect on TRL apoB-48 kinetics and pool size (PS). However, VLDL and IDL apoB-100 FCRs were significantly increased (+31.2%, P=0.02 and +20.8%, P=0.04, respectively) with a concomitant elevation of VLDL apoB-100 PR (+20.9%, P=0.04). Furthermore, LDL apoB-100 PS was significantly reduced by −23.2% (P=0.004), caused by a significant increase in FCR of this lipoprotein fraction (+24.0%, P=0.04).

Conclusions—These results indicate that reduction of plasma LDL cholesterol concentration after treatment with ezetimibe is associated with an increase in FCR of apoB-100–containing lipoproteins. (Arterioscler Thromb Vasc Biol. 2006;26: 1101-1106.)

Key Words: apolipoprotein B-48 ■ apolipoprotein B-100 ■ cholesterol absorption ■ ezetimibe ■ gas chromatography/mass spectrometry ■ intestine ■ kinetic

Elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) constitute a major risk factor for the development of atherosclerosis and reduction of LDL-C has been well-established as a primary strategy for lowering the risk of coronary heart disease. Blood cholesterol levels are regulated by various mechanisms, including de novo synthesis, cholesterol absorption, and biliary excretion. Dietary and biliary cholesterol is absorbed by the proximal jejunum of the small intestine. Ezetimibe is a member of a new class of lipid-altering agents that inhibits the absorption of dietary and biliary cholesterol at the brush border of the intestine without affecting the absorption of triglycerides or fat-soluble vitamins. It has been shown that glucuronidated ezetimibe undergoes enterohepatic recirculation, thereby repeatedly delivering drug to its site of action. Results from a number of studies in various animal models have demonstrated the lipid-lowering properties of ezetimibe as a single agent and the additive cholesterol-lowering effects of ezetimibe when combined with statin. In human, ezetimibe at a dose of 10 mg per day has been shown to reduce LDL-C levels by 12% to 14%. Recent studies have suggested that Niemann-Pick C1 Like 1 protein plays a critical role in ezetimibe-sensitive intestinal cholesterol absorption. However, the effect of ezetimibe on lipoprotein metabolism is not fully understood. The aim of the present study was therefore to investigate the effects of 8 weeks of treatment with ezetimibe 10 mg/d on the kinetics of apolipoproteins (apo) B-48 and apoB-100 labeled with a stable isotope [L-(5,5,5-D3) leucine] in 8 men with moderate primary hypercholesterolemia. ApoB-48 is the principal structural protein of intestinally derived lipoprotein particles and remains associated with them throughout the lipolytic cascade. However, apoB-100 is a large glycoprotein of hepatic origin that plays an indispensable role in the assembly, secretion, and intravascular transport of distinct classes of lipoproteins.

Methods

Subjects

Eight men with plasma LDL-C levels >50th percentile for their age were recruited from a pool of patients currently followed at the lipid
clinic of Laval University Medical Center. Subjects were 42.8 years old (range, 33 to 55) and had a body mass index of 28.2 kg/m² (range, 24.4 to 30.3). Subjects were excluded if they had acute liver disease, hepatic dysfunction, persistent elevations of serum transaminases, familial hypercholesterolemia, or plasma triglyceride levels >4.5 mmol/L. They were also excluded if they had a recent history of alcohol or drug abuse, diabetes mellitus, or a history of cancer. Furthermore, all participants were unrelated at the first and second degree. All eligible subjects had to be withdrawn from lipid-lowering medications for at least 6 weeks before the kinetic study in the basal state. A second kinetic study was then performed after 8 weeks of therapy with ezetimibe 10 mg/d. Patients were instructed to take 1 capsule at the time of their evening meal. Compliance was assessed by pill counting. The research protocol was approved by the Laval University Medical Center ethical review committee and written informed consent was obtained from each subject.

Experimental Protocol for In Vivo Stable Isotope Kinetics
To determine kinetics of triglyceride-rich lipoprotein (TRL) apoB-48, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL apoB-100, subjects underwent a primed-constant infusion of L-[5,5,5-D$_3$]-leucine while they were in a constantly fed state. Starting at 7:00 AM, the subjects received 30 identical small cookies every half hour for 15 hours, each equivalent to one-third of their estimated daily food intake based on the Harris-Benedict equation, with 15% of calories as protein, 45% carbohydrate, 40% fat (7% saturated, 26% monounsaturated, 7% polyunsaturated), and 85 mg of cholesterol/100 kcal. At 10:00 AM, with 2 intravenous lines in place, 1 for the infusate and 1 for blood sampling, L-[5,5,5-D$_3$]-leucine (10 μmol/kg body weight) was injected as a bolus intravenously and then by continuous infusion (10 μmol/kg body weight $^{1\text{h}^{-1}}$) over a 12-hour period. Blood samples (20 mL) were collected at hours 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12.

Characterization of Plasma Lipids and Lipoproteins
Twelve-hour fasting venous blood samples were obtained from an antecubital vein into Vacutainer tubes containing EDTA (0.1% final concentration) before the beginning of the kinetic study. Plasma was separated from blood cells by centrifugation at 3000 rpm for 10 minutes at 4°C. Plasma cholesterol and triglyceride concentrations were determined with an Analyzer RA-1000 (Technicon Instruments Corporation, Tarrytown, NY), as previously described. VLDL (d<1.006 g/mL), IDL (d=1.006-1.019 g/mL), and LDL (d=1.019 to 1.063 g/mL) fractions were isolated from fresh plasma by sequential ultracentrifugation, and HDL cholesterol was measured as previously described. One-dimensional non-denaturing 2% to 16% polyacrylamide gradient gel electrophoresis (1D-PAGGE) was used to determine the LDL particle size.

Quantification and Isolation of ApoB-48 and ApoB-100
ApoB concentration in TRL, IDL, and LDL were determined by noncompetitive enzyme-linked immunosorbent assay (ELISA) using immunopurified polyclonal antibodies (Alerchek Inc, Portland, Maine) to calculate their respective pool size (PS). The coefficient of variation for the apoB assay was between 6% and 10% depending on the region of the standard curve. ApoB-100 and apoB-48 were then separated by SDS polyacrylamide gel electrophoresis according to standardized procedures. Briefly, 50 μL of TRL, IDL, or LDL were mixed with 50 μL of 3% SDS sample buffer and subjected to electrophoresis in 3% to 10% linear gradient polyacrylamide slab gels. Gels were stained overnight in 0.25% Coomassie Blue R-250, destained for 7 to 8 hours. Based on the assumption that both apoB-100 and apoB-48 have the same chromogenicity, the relative proportion of apoB-100 and apoB-48 was assessed by scanning each gel with laser densitometry. We scanned lipoprotein fractions from 3 different time points to calculate ratios and to estimate the average concentrations of apoB-100 and apoB-48 using the total apoB concentration.

Isotopic Enrichment Determinations
ApoB-48 and apoB-100 bands were excised from polyacrylamide gels, and bands were hydrolyzed in 6N HCl at 110°C for 24 hours. Trifluoroacetic acid and trifluoroacetic anhydride (1:1) were used as derivatization reagents for the amino acids before analysis on a Hewlett-Packard 6890/5973 gas chromatograph/mass spectrometer. Isotope enrichment (%) and tracer/tracee ratio (%) were calculated from the observed ion current ratios. The isotopic enrichment of leucine in the apolipoproteins was expressed as tracer/tracee ratio (%) using standardized formulas.

Kinetic Analysis
Kinetics of TRL apoB-48 were derived by a multicompartmental model as previously described. We assumed a constant enrichment of the precursor pool and used the TRL apoB-48 plateau tracer/tracee ratio data as the forcing function to drive the appearance of tracer into apoB-48. Kinetics of apoB-100 in VLDL, IDL, and LDL fractions were derived by a multicompartmental model as previously described, with each compartment representing a group of kinetically homogenous particles. This simplified model has been compared with more complex multicompartmental models with either ≥2 VLDL compartments or direct input from the liver into VLDL, IDL, and LDL compartments and was selected based on a statistically better fit of tracer/tracee ratio data. Briefly, compartment 1 represents the plasma amino acid pool. Compartment 2 is an intracellular delay compartment representing the synthesis of apoB in the liver. Compartment 3 represents plasma VLDL; compartment 4, IDL; and compartment 5, LDL. It is assumed that plasma leucine (compartment 1) is the source of the leucine that is incorporated into apoB and that all apoB enters plasma via compartment 3. Therefore, transport rates into compartment 3 correspond to total apoB-100 production. We also assumed a constant enrichment of the precursor pool and used the VLDL apoB-100 plateau tracer/tracee ratio data as the forcing function to drive the appearance of tracer into apoB-100 as previously described. To model LDL data, the LDL enrichment curve has been constrained to a value equivalent to the VLDL apoB-100-tracee enrichment at 2 distant time points (1900 and 2000 hours). Given linearity of the LDL apoB-100 tracee ratio curve, this constraint was necessary to avoid an infinite number of model solutions. Under steady-state condition, the fractional catabolic rate (FCR) is equivalent to the fractional synthetic rate. ApoB production rates (PRs) were determined by the formula PR = [FCR (pools/d) x apoB concentration (mg/ DL) x plasma volume (L)/body wt (kg)]. Plasma volume was estimated as 4.5% of body weight. The SAAM II program (SAAM Institute, Seattle, Wash) was used to fit the model to the observed tracer data.

Statistical Analysis
A matched pairs t-test was used to compare the effects of ezetimibe on the lipid–lipoprotein profile and kinetic parameters. All analyses were performed using JMP Statistical Software (version 5.1; SAS Institute, Cary, NC).

Results
Characteristics of Subjects
Mean age and body mass index of participants were 42.8±8.2 years and 28.2±2.4 kg/m², respectively. Subjects maintained their weight throughout the study. Table 1 shows the lipid–lipoprotein profile of subjects in the basal state and after 8 weeks of treatment with ezetimibe (10 mg/d). Ezetimibe significantly reduced total, LDL-C, and apoB levels by −14.5%, −22.0%, and −17.1%, respectively. However, ezetimibe had no significant effect on plasma triglyceride, HDL-C, apoA-I levels, and LDL particle size.
Analyses of deuterated plasma amino acids and lipid/lipoprotein measurements indicated that plasma leucine enrichments as well as plasma triglyceride and TRL apoB-48 levels remained constant during the course of the infusion (data not shown). Mean TRL apoB-48 tracer/tracee ratios at baseline and after treatment with ezetimibe are shown in Figure 1A. Table 2 shows PS, FCR, and PR of TRL apoB-48 at baseline and after ezetimibe treatment. Ezetimibe had no significant effect on TRL apoB-48 kinetics and PS. No significant correlation was found between ezetimibe-induced changes in LDL-C concentration and changes in TRL apoB-48 PS ($r = -0.29; P = 0.49$) or between changes in LDL-C concentration and changes in TRL apoB-48 PR ($r = -0.31; P = 0.46$).

Ezetimibe has been shown to decrease plasma LDL-C levels by $\approx 15\%$ in patients with primary hypercholesterolemia\(^{29,30}\) and coadministration of ezetimibe with a statin can provide as much as an additional 12% to 14% reduction in LDL-C concentrations as compared with a monotherapy with statin.\(^{31–34}\) In the present study, ezetimibe as monotherapy at a dose of 10 mg/d for 8 weeks resulted in a $\approx 22.0\%$ reduction in LDL-C concentrations and a significant increase in the catabolic rate of VLDL, IDL, and LDL apoB-100. Furthermore, increase in the catabolic rate of VLDL apoB-100 was partly compensated by a $\approx 20.9\%$ increase in VLDL apoB-100 PR. Finally, ezetimibe has no significant impact on TRL apo-B48 kinetics or PS.

Ezetimibe is known to selectively inhibit the absorption of biliary and dietary cholesterol and phytosterols at the intestinal brush border.\(^{35}\) Recent studies have shown that Niemann-Pick C1 Like 1 plays a critical role in the absorption of intestinal cholesterol and has been established as the direct target of ezetimibe action.\(^{11,36}\)

### Table 1. Lipid/Lipoprotein Profile After an 8-Week Treatment With Ezetimibe (10 mg/d)

<table>
<thead>
<tr>
<th></th>
<th>Total-C, mmol/L</th>
<th>TG, mmol/L</th>
<th>LDL-C, mmol/L</th>
<th>HDL-C, mmol/L</th>
<th>ApoB, g/L</th>
<th>ApoA-I, g/L</th>
<th>LDL Particle Size, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>6.0 (5.4–6.9)</td>
<td>1.5 (0.8–2.9)</td>
<td>4.2 (3.2–5.0)</td>
<td>1.1 (0.8–1.3)</td>
<td>1.23 (1.11–1.47)</td>
<td>1.26 (1.03–1.49)</td>
<td>255.2 (251.3–258.11)</td>
</tr>
<tr>
<td><strong>Ezetimibe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (range)</td>
<td>5.1 (4.3–5.7)</td>
<td>1.5 (0.9–3.4)</td>
<td>3.3 (2.3–3.9)</td>
<td>1.2 (0.9–1.5)</td>
<td>1.02 (0.89–1.11)</td>
<td>1.30 (1.06–1.57)</td>
<td>255.3 (251.6–258.9)</td>
</tr>
<tr>
<td>%Δ</td>
<td>$-14.5$</td>
<td>$-2.6$</td>
<td>$-22.0$</td>
<td>$+6.7$</td>
<td>$-17.1$</td>
<td>$+3.2$</td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>0.004</td>
<td>0.5</td>
<td>0.004</td>
<td>0.1</td>
<td>0.004</td>
<td>0.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; C, cholesterol; TG, triglyceride. $P$ represents the $P$ value for the difference between baseline and treatment values. %Δ represents the percentage change after the treatment with ezetimibe.
Ezetimibe at 10 mg/d has been shown to decrease cholesterol absorption by 54% in mildly to moderately hypercholesterolemic subjects. In animal models, ezetimibe also appears to decrease delivery of cholesterol from the intestine to the liver, to reduce hepatic cholesterol stores, to upregulate LDL-C receptors on liver cell membranes, and to increase clearance of cholesterol from blood. More specifically, lipid content of postprandial lipoproteins and apoB-48 concentrations in chylomicrons have been examined in cynomolgus and rhesus monkeys after treatment with ezetimibe. In this study, ezetimibe was shown to reduce cholesterol content of chylomicrons and LDL-C levels but did not significantly affect apoB-48 concentrations. In agreement with these findings, our study shows that ezetimibe has no significant impact on TRL apoB-48 kinetics or PS, suggesting that ezetimibe reduces LDL-C by decreasing delivery of cholesterol from the intestine to the liver rather than by reducing chylomicron particle number.

The present study has also shown an increased FCR of VLDL, IDL, and LDL apoB-100 after ezetimibe treatment. Increased clearance of apoB-100-containing lipoproteins with ezetimibe is likely caused by an increase in the expression of the LDL receptor in the liver. Thus, by blocking cholesterol absorption, cellular cholesterol content of the liver decreases resulting in upregulation of LDL receptor activity and decrease in LDL production. A recent animal study reported an increase in the LDL receptor mRNA levels in mice after ezetimibe treatment and earlier studies in hamsters fed various lipid-enriched diets showed that changes in hepatic LDL-R mRNA levels faithfully reflected changes in LDL receptor protein as well as LDL clearance rates. Furthermore, this same study in LDLR−/− mice showed that the cascade of changes in intrahepatic cholesterol metabolism and the resultant increase in plasma LDL-C levels that ensue from the absorption of excess cholesterol are preventable by imposing a pharmacological blockade on the uptake of cholesterol at the intestine level. The ezetimibe-induced LDL-C response in the absence of any hepatic LDL receptor activity suggests a major effect of diminished chylomicron cholesterol delivery to the liver on the rate of cholesterol secretion in VLDL and, hence, on LDL-C production. The results of the present study, however, show no change in LDL apoB100 PR. This is likely caused by an increase in VLDL apoB-100 production in response to decreased cholesterol content in the liver and a compensatory increase in hepatic cholesterol synthesis. In fact, this elevation in VLDL apoB-100 synthesis could reduce the cholesterol-lowering effect of ezetimibe by maintaining the LDL PR relatively stable as compared with pre-treatment levels. These data are in agreement with the study of Sudhop et al that reported a compensatory increase of cholesterol synthesis associated with significant reductions in LDL (20%) and total.

### Table 2

<table>
<thead>
<tr>
<th>TRL ApoB-48</th>
<th>ApoB-48, mg/dL</th>
<th>PS, mg</th>
<th>FCR, pools/d</th>
<th>PR, mg · kg⁻¹ · d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.2 (0.6–2.2)</td>
<td>42.6</td>
<td>7.7 (5.8–11.3)</td>
<td>3.9 (1.9–6.8)</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>1.0 (0.4–1.9)</td>
<td>37.5</td>
<td>7.9 (4.4–12.5)</td>
<td>3.2 (1.9–5.6)</td>
</tr>
<tr>
<td>%Δ</td>
<td>−16.7</td>
<td>−12.0</td>
<td>+2.6</td>
<td>−17.9</td>
</tr>
<tr>
<td>P</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*P* represents the *P* value for the difference between baseline and treatment values. %Δ represents the percentage change after the treatment with ezetimibe.

### Table 3

<table>
<thead>
<tr>
<th>VLDL ApoB-100</th>
<th>IDL ApoB-100</th>
<th>LDL ApoB-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoB-100, mg/dL</td>
<td>PS, mg</td>
<td>FCR, pools/d</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
<td>--------------</td>
</tr>
<tr>
<td>Baseline</td>
<td>10.4</td>
<td>387.7</td>
</tr>
<tr>
<td></td>
<td>(7.4–15.6)</td>
<td>(253.8–618.5)</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>9.6</td>
<td>350.2</td>
</tr>
<tr>
<td></td>
<td>(6.6–14.3)</td>
<td>(225.7–462.0)</td>
</tr>
<tr>
<td>%Δ</td>
<td>−7.7</td>
<td>−9.7</td>
</tr>
<tr>
<td>P</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

FCR indicates fractional catabolic rate; PS, pool size; PR, production rates. *P* represents the *P* value for the difference between.

%Δ represents the percentage change after treatment with ezetimibe.
cholesterol in a 2-week, double-blind, placebo-controlled, crossover study in 18 hypercholesterolemic patients. Interestingly, bile acid sequestration with resins has been shown to increase the hepatic production of triglyceride-rich VLDL,44,45 most probably in response to concomitant elevation in hepatic cholesterol and triglyceride synthesis.44,45 The observed increase in hepatic cholesterol synthesis might explain the additive lipid-lowering effects of the coadministration of ezetimibe and an 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor.9 It should be pointed out, however, that because LDL composition is heterogeneous and the relationship of LDL-C to apoB is variable for different populations, it may be difficult to translate the apoB data into cholesterol data, so this study can only provide a qualitative estimate of cholesterol metabolism.

In conclusion, the present study indicates that the LDL-C lowering effect of ezetimibe is mainly caused by an increase in the catabolism of apoB-100–containing lipoproteins. Our study also shows that ezetimibe has no significant effect on TRL apoB-48 kinetics although variability in apoB-48 measurements could have reduced the power to detect a true effect of ezetimibe. Finally, our results suggest that the ezetimibe-induced reduction in cholesterol delivery to the liver is associated with a compensatory increase in hepatic VLDL apoB-100 production, which may limit the lipid-lowering effect of ezetimibe.

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