Apolipoprotein B100 Metabolism in Autosomal-Dominant Hypercholesterolemia Related to Mutations in PCSK9

Khadija Ouguerram, Maud Chetiveaux, Yassine Zair, Philippe Costet, Marianne Abifadel, Mathilde Varret, Catherine Boileau, Thierry Magot, Michel Krempf

Objective—We have reported further heterogeneity in familial autosomal-dominant hypercholesterolemia (FH) related to mutation in proprotein convertase subtilisin/kexin type 9 (PCSK9) gene previously named neural apoptosis regulated convertase 1 (Narc-1). Our aim was to define the metabolic bases of this new form of hypercholesterolemia.

Methods and Results—in vivo kinetics of apolipoprotein B100-containing lipoproteins using a 14-hour primed constant infusion of [2H3] leucine was conducted in 2 subjects carrying the mutation S127R in PCSK9, controls subjects, and FH subjects with known mutations on the low-density lipoprotein (LDL) receptor gene (LDL-R). Apo B100 production, catabolism, and transfer rates were estimated from very LDL (VLDL), intermediate-density lipoprotein (IDL), and LDL tracer enrichments by compartmental analysis. PCSK9 mutation dramatically increased the production rate of apolipoprotein B100 (3-fold) compared with controls or LDL-R mutated subjects, related to direct overproduction of VLDL (3-fold), IDL (3-fold), and LDL (5-fold). The 2 subjects also showed a decrease in VLDL and IDL conversion (10% to 30% of the controls). LDL fractional catabolic rate was slightly decreased (by 30%) compared with controls but still higher than LDL-R–mutated subjects.

Conclusion—These results showed that the effect of the S127R mutation of PCSK9 on plasma cholesterol homeostasis is mainly related to an overproduction of apolipoprotein B100. (Arterioscler Thromb Vasc Biol. 2004;24:1448-1453.)

Key Words: PCSK9 ■ hypercholesterolemia ■ apolipoprotein B100 ■ kinetic analysis ■ modeling

Apolipoprotein B100 (apoB100) plays a central role in intracellular assembly and secretion of triglyceride (TG)-rich lipoproteins and is a ligand for low-density lipoprotein receptor (LDL-R). Many mutations in LDL-R and apoB genes characterize, respectively, familial hypercholesterolemia (FH) and familial deficiency apolipoprotein B. In FH with defective LDL-R, LDL cholesterol is higher than in familial deficiency apolipoprotein B with more severe clinical consequences. Several other genes may be candidate for causing hypercholesterolemia. For example, it has been described recently an inherited recessive form of hypercholesterolemia related to a mutation in the phosphotyrosine-binding domain protein ARH leading to a tissue-specific defect in the removal of LDLS from the circulation. We also recently described a family with autosomal-dominant hypercholesterolemia carrying a mutation in proprotein convertase subtilisin/kexine type 9 (PCSK9) in which serine was substituted by arginine at position 127 (S127R mutation). This enzyme, also called NARC-1, is a member of the secretory subtilase family. It is a novel proprotein convertase synthesized as a soluble zymogen that undergoes an autocatalytic intramolecular processing in the endoplasmic reticulum. NARC-1 mRNA is mainly expressed in the liver and small intestine, and both organs playing a key role in cholesterol homeostasis. Patients with this mutation exhibit hypercholesterolemia, a normal LDL-R activity as measured in skin fibroblasts. They also do not carry the most frequent hypercholesterolemic apoB100 gene mutation (R3500Q). It was suggested that PCSK9, by acting on apoB100 maturation, could decrease lipoprotein release, and its deficiency or dysfunction could lead to an overproduction of apoB100-containing lipoproteins.

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The aim of this study was to identify the mechanisms involved in the lipid disorders related to this novel mutation. For this purpose, we have performed in vivo kinetic studies of apoB100-containing lipoproteins in 2 patients carrying the S127R mutation and compared our results to heterozygous FH related to LDL-R mutations and controls.

Methods

Subjects

The 2 subjects carrying the S127R on PCSK9 were recruited through the French Hypercholesterolemic Family Network (Réseau National de Recherche sur les Hypercholestérolémies Familiales). The inclu-
Kinetic Studies
The endogenous labeling of apoB100 was performed by constant infusion of \([\text{H}_3]\)-leucine. Subjects fasted overnight before the study and remained fasting during the entire procedure.\(^{17}\) Each patient received intravenously a prime of 10 \(\mu\text{mol} \cdot \text{kg}^{-1}\) of tracer, immediately followed by a constant tracer infusion (10 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}\)) for 14 hours. Venous blood samples were drawn into EDTA tubes (Venoject, Paris, France) at baseline and at 15, 30, and 45 minutes, and 1, 1.5, 2, and 2.5 hours, and then hourly until 14 hours had passed. Sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montluçon, France), a protease inhibitor, were added to blood samples at a final concentration of 1.5 and 0.5 \(\text{mmol/L}\), respectively.

Analytical Procedures
Isolation by ultracentrifugation and measurement of enrichment of lipoprotein containing apoB100. VLDL1 (Sf 400 to 60) and VLDL2 (Sf 60 to 20) were separated by density gradient ultracentrifugation\(^{18}\) using a swaying bucket rotor at 40,000 revolutions/min for 24 hours at 10°C (RPS 40T, Hitachi), IDL (1.006<d<1.020 g/mL) and LDL (1.020<d<1.063 g/mL) were separated by standard sequential ultracentrifugation methods\(^{19}\) using a fixed-angle rotor at 40,000 revolutions/min for 22 hours at 10°C (CP70, Hitachi).

Isolation and measurement of leucine enrichment in apoB100 have been described previously.\(^{20}\) Briefly, apoB100 in lipoproteins was isolated by sodium dodecylsulfate polyacrylamide gel electrophoresis. Apolipoprotein bands were dried under a vacuum and then hydrolyzed. The amino acids were purified by cation exchange chromatography, then esterified and derivatized. Electron-impact gas chromatography mass spectrometry was performed on a 5891A gas chromatograph connected with a 5971A quadrupole mass spectrometer. The isotopic ratio was determined by selected ion monitoring at m/z of 282 and 285. Calculations of apoB100 kinetic parameters were based on the tracer-to-tracee mass ratio.\(^{20}\)

Isolation of Lipoproteins by Fast Protein Liquid Chromatography
Lipoprotein isolation was performed as described by Chetiveaux et al.\(^{21}\) Briefly, 200 \(\mu\text{L}\) of plasma were injected into a MV-7 multijet injection loop and separation was performed on 2 supereose 6 HR 10/30 columns in series at flow rate of elution at 0.35 mL/min; 0.35 mL was collected for each fraction and the entire profiles was completed within 105 minutes. The system was controlled by FPLC DIRECTOR software (Amersham Pharmacia Biotech Inc).

Measurements of Lipids and ApoB100
All measurements were realized at 4 different sampling times on plasma and once on fast protein liquid chromatography (FPLC) fractions. Cholesterol and TG levels were measured using commercially available enzymatic kits (Biomérieux, Marcy l’Etoile, France). ApoB100 concentrations were obtained in VLDL, IDL, and LDL by combining selective precipitation and mass spectrometry\(^{22}\) and by immunonephelometry (Biomérieux) on FPLC fractions. The percentage recovery of cholesterol, TGs, and apoB100 after centrifugation was higher than 85%.

Modeling
Kinetic analysis of tracer-to-tracee ratios was achieved using computer software for simulation, analysis, and modeling (SAAMII). This model (Figure 1) has previously been used and published for controls and hypertriglyceridemic subjects.\(^{16}\) Briefly, in this model, heterogeneity in VLDL is represented by large VLDL (VLDL1), small VLDL (VLDL2), and a shunt between VLDL and LDL. Data are obtained by chemical analysis for VLDL1 and VLDL2. For VLDL1 fraction, supplementary heterogeneity was introduced in the model by 2 compartments: the first one, named VLDL1a and linked to VLDL2 by delipidation cascade, and the second, named VLDL1 remnants (VLDL1R), related to a direct removal of VLDL. This was performed to get a better fit of the data. ApoB-100 enters into plasma through VLDL secretion and direct production of IDL and LDL. ApoB-100 direct removal occurs from VLDL1a (k (40,5)), VLDL2 (k (30,20)), VLDL1R (k (8,11)), IDL (k (10,8)), and LDL (k (10,6)). ApoB-100 transfer to higher density lipoproteins occurs by delipidation for VLDL1a (k (10,10), k (11,10)), VLDL2 (k (20,20), k (20,20)), and IDL (k (10,8)). The use of more complex models did not provide significant improvement in the fit from F test and Akaike information criterion.\(^{23}\) A forcing function determined with SAAMII program through interoperation between experimental data and corresponding to the time course of plasma leucine enrichment was used to drive the appearance of leucine tracer into apoB100 of the different lipoprotein fractions.\(^{24}\)

For comparison between the 3 groups (PCSK9-mutated patients, heterozygous FH, and controls) the VLDL1a, VLDL2, and VLDL1R data were presented as VLDL delipidation rate and VLDL fractional catabolic rate (FCR), which represents the sum of delipidation and direct removal rate. The VLDL conversion rate was calculated as VLDL2 delipidation flux divided by total VLDL mass. The VLDL direct removal was calculated as a sum of VLDL1a, VLDL2, and VLDL1R direct removal divided by total VLDL mass. The apoB100 production rate (PR) in mg/kg per day represents the product of FCR and pool size of apoB100 in lipoprotein fractions assuming plasma volume equal to 4.5% of body weight.

Results
Patients mutated for PCSK9 had higher plasma TC and apoB100 than controls (Table 1). Plasma lipids (cholesterol, TGs) profiles from FPLC lipoprotein are shown in Figure 2 for S1 and S2 and for 1 representative control and 1 representative FH. Compared with control, apoB100-rich lipoproteins from S1 and S2 exhibited higher cholesterol.
concentrations, although they remained lower than in heterozygous FH subjects. VLDL and IDL profiles were also different compared with controls (Table 2). They were more enriched in cholesterol than in TG. The TC/TG ratio was high in S1 and S2 in VLDL (0.33 for S1, 0.30 for S2) compared with controls (0.21). This ratio was, in contrast, lower for HDL in S1 (1.8) and S2 (1.0) compared with controls (6.0).

To assess LDL size, we calculated TC/apoB100 ratio for LDL and found it higher in S1 (2.1) and S2 (2.8) than controls (1.8).

For the 2 patients and control subjects, tracer/tracee curves are shown in Figure 3. Tracer appears in the controls and subjects in VLDL 1, VLDL2, IDL, and then in LDL. Model-fitted lines and experimental points showed close agreement and the masses of apoB100 calculated in each compartment and were not different from chemically measured values (<15%, data not shown). The individual and mean fractional rate constant for S1, S2, controls, and heterozygous FH are shown in Table 3. The fractional rates of VLDL apoB100 as a direct catabolism for the 2 subjects were higher than for controls (6-fold for S1 and 3-fold for S2) or in FH patients (3.5-fold for S1 and 2-fold for S2).

The fractional rate of apoB100 transfer from VLDL to IDL was decreased in S1 and S2 compared with controls (respectively, 17% and 4% of controls) and FH (respectively, 34% and 7% of FH). The VLDL direct removal is higher in S1 (6-fold) and S2 compared with controls subjects. A decrease was also observed for the fractional rate of apoB100 transfer from IDL to LDL (31% and 11% of controls for S1 and S2, respectively). This was accompanied by a lower direct removal than controls (20% of controls for S1 and 4% for S2). Compared with controls, FCR of LDL was slightly decreased (by 30% and 35%). S1 and S2 showed a higher direct production of VLDL (2.9-fold for S1 and 3-fold for S2), IDL (2.7-fold for S1 and 3-fold for S2), and LDL (6-fold for S1 and 5-fold for S2) compared with controls. The contribution of direct production of LDL was higher (73% and 72% of total production) than controls (25%) and FH (48%). The contribution of apoB100 production from IDL in LDL pool was in the same range (5.8 and 5.0 mg/kg per day) compared with controls (7.6 mg/kg per day) but lower than in FH patients (11.05 mg/kg per day).

**Discussion**

Kinetics of VLDL, IDL, and LDL apoB100 were performed in 2 hypercholesterolemic subjects carrying the mutation S127R in PCSK9 and were compared with control and heterozygous FH subjects with identified mutation on LDL-R.17 Hypercholesterolemia in these 2 subjects was the result of a dramatically apoB100-containing lipoprotein overproduction accompanied by a slight decrease in their removal. The delayed clearance observed for VLDL and IDL was a consequence of a decreased delipidation rate.

Although only 2 subjects from the same family carrying the S127R mutation were studied, the results are similar and consistent enough to be reported and used for the design of research plans on NARC-1 and hypercholesterolemia. Both subjects showed a strong increase of the direct production of VLDL, IDL, and LDL. The high VLDL release was mainly related to VLDL2 production (data not shown) and did not affect TGs pool size. These finding are different from those reported in patients with LDL receptor or apolipoprotein B100 defect. In the former, a strong reduction of catabolism and a slight overproduction of apoB100-containing lipoproteins were reported. A delayed conversion of VLDL to IDL and IDL to LDL was also reported in these subjects. Familial-deficiency apolipoprotein B100 heterozygous patients were characterized by a lower LDL catabolism and direct production rate and a low conversion rate from IDL to LDL compared with controls.18,25,26

**TABLE 1. Characteristics of Controls, Patients With PCSK9 Mutation (S1 and S2), and Heterozygous Familial Hypercholesterolemic Patients**

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Sex</th>
<th>BMI</th>
<th>TC</th>
<th>TG</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>ApoB</th>
</tr>
</thead>
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<tr>
<td>S1</td>
<td>43</td>
<td>F</td>
<td>19</td>
<td>292</td>
<td>75</td>
<td>241</td>
<td>36</td>
<td>144</td>
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<tr>
<td>S2</td>
<td>23</td>
<td>M</td>
<td>22</td>
<td>372</td>
<td>180</td>
<td>301</td>
<td>40</td>
<td>182</td>
</tr>
<tr>
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<td>53</td>
<td>2F/3M</td>
<td>24.7</td>
<td>440</td>
<td>150</td>
<td>345</td>
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</tr>
<tr>
<td>SD</td>
<td>9</td>
<td>1F/11M</td>
<td>4.3</td>
<td>28</td>
<td>21</td>
<td>40</td>
<td>6.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Controls</td>
<td>34</td>
<td>1F/11M</td>
<td>26.3</td>
<td>170</td>
<td>80</td>
<td>82</td>
<td>49.8</td>
<td>62</td>
</tr>
<tr>
<td>SD</td>
<td>12</td>
<td></td>
<td>4.4</td>
<td>38</td>
<td>20</td>
<td>16</td>
<td>14.7</td>
<td>9</td>
</tr>
</tbody>
</table>

TC, TG, LDL-C, HDL-C, and ApoB are in mg/dl.

FH indicates familial hypercholesterolemia; TC, total cholesterol; TG, Triglycerides; ApoB, Apolipoprotein B100.

**Figure 2.** Cholesterol (A) and triglycerides (B) profile on plasma fraction eluted by FPLC in control (×), S1 (−), S2 (Δ), and heterozygous familial hypercholesterolemia (○).
The overproduction of VLDL, IDL, and LDL observed in the present study was already reported in other familial hypercholesterolemia, but the magnitude of this phenomena is dramatically higher in the patients with the PSCK9 mutation. It was already shown that substrate availability, especially cholesterol ester mass, modulates hepatic secretion of lipoproteins. However, there are still controversies and in cultured hepatoma cells starved of lipoproteins, an enhancement of apoB mRNA was observed and was reversed by an addition of LDL to the culture medium, as we have observed in FH patients after LDL apheresis. A strong relationship was also recently reported between LDL-R receptor activity and apoB100 degradation in mice hepatocytes. Some studies using statins have shown that the inhibition on apoB100 synthesis is dependent on the magnitude of the inhibition of endogenous cholesterol synthesis. In this complex regulation, we can speculate that PCSK9 could increase the degradation of newly synthesized apoB100. It is well known that a large amount of novel synthesized apoB100 is not secreted but is degraded within the cell and that apoB100 concentration is mainly regulated after transcription. Numerous studies have shown that several situations like hormonal and nutritional states or apoB100 gene mutations can alter apoB100 rate of secretion by changing its intracellular degradation. The activity of PCSK9 could be critical for this pathway and its regulation, but the mechanisms related to this mutation on cholesterol homeostasis and apoB100 secretion need further investigation. A lower conversion rate of VLDL and IDL compared with controls and heterozygous FH patients was also observed in the 2 subjects. It is demonstrated that in normal subjects, most apoB100 LDL comes from VLDL lipolysis. The contribution of VLDL and IDL to LDL formation accounted for almost 28% in these subjects versus 75% in the controls, and the direct synthesis was the principal pathway for LDL production. In FH heterozygous patients, LDL direct secretion was equal to the formation from IDL, whereas in the familial deficiency apolipoprotein B the synthesis from IDL was the major source (80%). The composition of nascent VLDL, mainly TG content, may govern their subsequent metabolic behavior in plasma. TG-rich particles undergo rapid lipolysis via lipoprotein lipase. The VLDL composition of the 2 subjects showed a higher cholesterol to TGs ratio. As demonstrated by Schreier et al., this ratio is negatively correlated with the affinity of lipoprotein lipase. Thus, these enriched cholesterol particles are probably not well-recognized by lipoprotein lipase and then make little contribution to LDL production. Our kinetic data and the

### Table 2. Lipoprotein Composition in Different Subjects

<table>
<thead>
<tr>
<th></th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>TG</td>
<td>ApoB</td>
<td>TC</td>
</tr>
<tr>
<td>S1</td>
<td>9.1</td>
<td>27.4</td>
<td>7.7</td>
<td>5.9</td>
</tr>
<tr>
<td>S2</td>
<td>23.3</td>
<td>77.5</td>
<td>17.3</td>
<td>6.6</td>
</tr>
<tr>
<td>FH</td>
<td>80</td>
<td>74</td>
<td>5.9</td>
<td>46</td>
</tr>
<tr>
<td>SD</td>
<td>9</td>
<td>10</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Controls</td>
<td>4.5</td>
<td>23</td>
<td>2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>SD</td>
<td>2.6</td>
<td>10</td>
<td>1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Data are in mg/dl.
TC indicates total cholesterol; TG, Triglycerides; ApoB, Apolipoprotein B100.

### Figure 3. Time course of enrichment of VLDL1 (□), VLDL2 (★), IDL (O), and LDL (+) apolipoprotein B100 in a representative control subject (A) and in S1 (B) and S2 (C) subjects during [2H3]-leucine perfusion.
composition of lipoproteins found in the 2 subjects are in good agreement with this hypothesis. The mechanisms involved in the elevated cholesteryl ester-to-TGs ratio in the case of PCSK9 mutation are not known.

The 2 subjects also showed a decreased LDL fractional catabolic rate. Functional tests showed normal binding, internalization, and degradation of LDL particles in fibroblasts from the probands (HC2-II-9). This test was performed with LDL isolated from control subjects and showed a normal receptor activity but does not exclude an uptake deficiency caused by LDL abnormalities. As already shown, small and dense LDL enriched in cholesterol bind weakly to fibroblasts and could explain this result.

Thus, our kinetic study suggests that PCSK9 is a key enzyme in cholesterol homeostasis by inducing a dramatic increase of apoB100 production of hepatic lipoproteins enriched in cholesterol esters. The other observed abnormalities may be secondary to this overproduction. Further studies on the effect of PCSK9 on hepatic or intestinal lipoprotein synthesis are now required.

Acknowledgments

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References


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TABLE 3. ApoB Kinetic Parameters of Controls, Patients With PCSK9 Mutation (S1 and S2), and Heterozygous Familial Hypercholesterolemic Patients

<table>
<thead>
<tr>
<th>VLDL</th>
<th>Conversion</th>
<th>IDL</th>
<th>Conversion</th>
<th>LDL</th>
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<tbody>
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<td>Rate</td>
<td></td>
<td>Rate</td>
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<tr>
<td>S1</td>
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<tr>
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<td>4.21</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>3.62</td>
<td>3.38</td>
<td>1.01</td>
</tr>
</tbody>
</table>

FCR indicates fractional catabolic rate (day⁻¹); TPR, total production rate (mg/kg/day); PRd, direct production rate (mg/kg/day).


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