Upregulating Reverse Cholesterol Transport With Cholesteryl Ester Transfer Protein Inhibition Requires Combination With the LDL-Lowering Drug Berberine in Dyslipidemic Hamsters

François Briand, Quentin Thieblemont, Elodie Muzotte, Thierry Sulpice

Objective—This study aimed to investigate whether cholesteryl ester transfer protein inhibition promotes in vivo reverse cholesterol transport in dyslipidemic hamsters.

Methods and Results—In vivo reverse cholesterol transport was measured after an intravenous injection of 3H-cholesteryl-oleate–labeled low density lipoprotein particles (3H-oxLDL), which are rapidly cleared from plasma by liver-resident macrophages for further 3H-tracer egress in plasma, high density lipoprotein (HDL), liver, and feces. A first set of hamsters made dyslipidemic with a high-fat and high-fructose diet was treated with vehicle or torcetrapib 30 mg/kg (TOR) over 2 weeks. Compared with vehicle, TOR increased apolipoprotein E–rich HDL levels and significantly increased 3H-tracer appearance in HDL by 30% over 72 hours after 3H-oxLDL injection. However, TOR did not change 3H-tracer recovery in liver and feces, suggesting that uptake and excretion of cholesterol deriving from apolipoprotein E-rich HDL is not stimulated. As apoE is a potent ligand for the LDL receptor, we next evaluated the effects of TOR in combination with the LDL-lowering drug berberine, which upregulates LDL receptor expression in dyslipidemic hamsters. Compared with TOR alone, treatment with TOR+berberine 150 mg/kg resulted in lower apolipoprotein E–rich HDL levels. After 3H-oxLDL injection, TOR+berberine significantly increased 3H-tracer appearance in fecal cholesterol by 109%.

Conclusion—Our data suggest that cholesteryl ester transfer protein inhibition alone does not stimulate reverse cholesterol transport in dyslipidemic hamsters and that additional effects mediated by the LDL-lowering drug berberine are required to upregulate this process. (Arterioscler Thromb Vasc Biol. 2013;33:13-23.)

Key Words: cholesterol-lowering drugs ■ insulin resistance ■ lipoproteins ■ oxidized lipids ■ reverse cholesterol transport

Dyslipidemia in patients with the metabolic syndrome and type 2 diabetes mellitus is a major risk factor for the development of cardiovascular diseases. Although therapies reducing low density lipoprotein-cholesterol (LDL-C) have been shown to reduce cardiovascular risk by ≈30%,1 patients attaining LDL-C reduction goals still remain at high cardiovascular risk. Indeed, diabetic dyslipidemia is characterized by hypertriglyceridemia and low levels of high density lipoprotein-cholesterol (HDL-C), which are known to be inversely correlated with cardiovascular risk.2 This inverse correlation may be related to the key role of HDL in reverse cholesterol transport (RCT), a process promoting the return of cholesterol from macrophage in the arterial wall to the liver for further biliary and fecal excretion.3 Hence, a promising alternative to further reduce cardiovascular risk is represented by therapies raising HDL-C.

Among novel HDL-raising therapies, inhibition of cholesteryl ester transfer protein (CETP), a plasma protein transferring cholesteryl esters from HDL to non-HDL particles, has been investigated extensively.4 Although the benefit of CETP inhibition in preventing cardiovascular diseases was recently challenged by the failure of both torcetrapib (TOR) and dalcetrapib,5,6 other CETP inhibitors are currently evaluated. Recent clinical trials have shown that treatment with CETP inhibitors evacetrapib and anacetrapib increased HDL-C levels by ≈130%.7,8 As initially observed in CETP-deficient patients,9 the strong increase in HDL-C is concomitant with the formation of enlarged, cholesteryl ester–rich HDL, apolipoprotein E-rich HDL (apoE–HDL) particle levels under CETP inhibition.10,11

One of the most important questions is whether CETP inhibition and apoE–HDL particles stimulate RCT. Previous studies indicated that apoE–HDL particles are efficiently uptaken by hepatic LDL receptor12,13 and in vitro experiments suggest that these particles also promote macrophage cholesterol efflux.10-12 Whether these mechanisms would occur and promote RCT under CETP inhibition in type 2 diabetic patients is currently unknown. Importantly, type 2 diabetic patients are...
prone to liver steatosis and nonalcoholic steatohepatitis (NASH), which may impair cholesterol metabolism and RCT in vivo. Liver represents a crucial protection system for peripheral tissues (eg, macrophage in the arterial wall), by mediating the immediate uptake of modified lipoproteins. Indeed, atherogenic oxidized LDL (oxLDL) particles are rapidly uptaken by liver-resident macrophages (Kupffer cells), for further resecretion of oxLDL-derived cholesterol in bile and in plasma HDL particles. Hence, the impact of liver steatosis on RCT should be also considered for evaluating the benefits of CETP inhibition in metabolic syndrome/type 2 diabetes mellitus.

Although measuring the effects of CETP inhibition on human RCT remains difficult, the use of animal models, such as the hamster, represents a valuable alternative to investigate these issues. In this line, we recently developed a hamster model of metabolic syndrome, in which high-fat feeding induced insulin resistance, dyslipidemia, increased CETP activity, reduced hepatic LDL receptor expression, and severe liver steatosis. In the present study, the effects of CETP inhibition on in vivo RCT were investigated in this hamster model. To evaluate the impact of liver steatosis on cholesterol fluxes, we used an alternative method to measure in vivo RCT, by injecting 3H-cholesteryl-oleate–labeled/oxidized LDL particles.

Methods

An expanded Methods section appears in the online-only Data Supplement.

Animals and Diets

All animal protocols were approved by the local ethical committee (Comité régional d’éthique de Midi-Pyrénées). After a 1-week acclimation period, male Golden Syrian hamsters (91–100 g, 6-week-old, Elevage Janvier, Le Genest Saint Isle, France) were fed either the control chow diet with normal drinking water or a high-fat (HF) diet estimated as 3.5% of the body weight.

After 2 weeks of diet, hamsters were randomized according to their plasma levels of HDL-C, total cholesterol, and triglycerides and were then treated (n=6/treatment group) over 2 weeks with liver X receptor (LXR) agonist GW3965 30 mg/kg twice daily, TOR 30 mg/kg and BER 150 mg/kg once daily, or respective vehicle. Individually caged hamsters had free access to food and water and were kept treated over 72 hours. Hamsters were injected intravenously with 3H-cholesteryl-oleate–labeled/oxidized LDL (≈7 million dpm) and blood (150 µL/EDTA) was collected at 5 minutes, 0.5, 1, 2, 3, 5, 6, and 24 hours after injection. Plasma and HDL were immediately isolated, before radioactivity measurement in a liquid scintillation counter. An additional liver sample was used to isolate parenchymal cells and nonparenchymal cells (Kupffer cells and endothelial cells), according to the procedure described by van Berkel and colleagues. Protein concentration was then determined on each cell fraction to determine specific activity.

In the main experiment, in vivo RCT was measured over 72 hours. Individually caged hamsters had free access to food and water and were kept treated over 72 hours. Hamsters were injected intravenously with 3H-cholesteryl-oleate–labeled/oxidized LDL (≈7 million dpm) and blood (150 µL/EDTA) was collected at 5 minutes and 24, 48, and 72 hours after injection. Plasma and HDL were immediately isolated, before radioactivity measurement in a liquid scintillation counter (50 µL plasma or HDL counted). Feces were collected over 72 hours and were stored at 4°C before cholesterol and bile acids extraction, as described previously. Remaining volume of both extracts were evaporated and resuspended in ethanol, then commercial kits were used to determine fecal total cholesterol (cholesterol RTU, Biomerieux) and total bile acids (total bile acids colorimetric assay kit, Bioquant) mass. After 72 hours, hamsters were euthanized by cervical dislocation, exsanguinated, and liver was harvested from each animal. A ≈50-ng piece of liver was homogenized using an ultrasound probe in 500 µL water, then 100 µL were counted in a liquid scintillation counter.

Results were expressed as percentage of the injected radioactivity recovered in plasma, HDL, liver, and feces. The plasma volume was estimated as 3.5% of the body weight.

In Vivo Macrophage-to-Feces RCT

Macrophage-to-feces RCT was measured over 72 hours after intraperitoneal injection of 3H-cholesterol labeled/oxidized LDL-cholesterol loaded macrophage (macrophage–RCT method), as described previously.

In Vivo 3H-Cholesterol Olate–LDL Kinetics

LDL from high-fat-fed hamsters were radiolabeled with 3H-cholesterol oleate and injected intravenously, and blood was collected continuously over 72 hours to determine plasma fractional catabolic rate (FCR). Feces were also collected over 72 hours, and liver was harvested after euthanization at time 72 hours to measure fecal and hepatic radioactivity.

In Vivo Intestinal Cholesterol Absorption

Intestinal cholesterol absorption was measured over 6 hours, after an oral gavage of 3H-cholesterol–labeled olive oil and an intraperitoneal injection of poloxamer 407, as described previously.

Biochemical Analysis

Biochemical analysis was performed using commercial kits, and standard procedures were used as described previously.
Statistical Analysis
Values are presented as mean±SEM. Statistical analysis was performed using either an unpaired, 2-tailed, Student t test or ANOVA-Dunnett’s posttest. A P<0.05 was considered significant.

Results
Both High-Fat Feeding and LXR Activation Alter In Vivo RCT as Measured by the oxLDL–RCT Method
We first aimed to validate an alternative method to measure RCT (the oxLDL–RCT method), which would better evaluate the role of the liver in this process in vivo. We investigated whether injecting 3H-cholesteryl-oleate–oxidized LDL would enable to trace cholesterol fluxes from the liver in normolipidemic hamsters. Compared with nonoxidized/normal 3H-cholesteryl-oleate–labeled LDL, 3H-oxLDL nearly disappeared from plasma within 5 minutes after intravenous injection (Figure 1A), suggesting a rapid hepatic uptake. 3H-tracer then reappeared continuously in plasma and HDL fraction over 24 hours. The major role of the liver in oxidized LDL clearance was further confirmed as >65% of the 3H-tracer was recovered in liver at 5 minutes, after injection of oxidized LDL radiolabeled with 3H-cholesteryl ether (Figure 1B). At this time point, specific activity was 16-fold higher in liver nonparenchymal cells (Kupffer cells and endothelial cells) than in parenchymal cells (1.47±0.081% versus 0.094±0.004% of injected dose/mg protein, P<0.001; data not shown).

We next evaluated whether the oxLDL–RCT method would enable to measure the effect of high-fat feeding and LXR activation on RCT. Compared with chow-fed hamsters, high-fat-fed hamsters showed a dyslipidemic profile (Figure 2A), higher fecal cholesterol, and lower fecal bile acids mass excretion (Figure 2B). After 3H-oxLDL injection, 3H-tracer appearance in plasma was significantly higher at time 24 hours, but not at 48 and 72 hours, in high-fat-fed hamster (Figure 2C). However, 3H-tracer appearance in HDL was significantly reduced at 24, 48, and 72 hours (Figure 2D), indicating that the transport of oxLDL-derived cholesterol by HDL particles was severely compromised in hamsters fed a high-fat diet. After 72 hours, 3H-tracer hepatic recovery was strongly increased (Figure 2E), suggesting a possible accumulation of oxLDL-derived cholesterol in the liver of high-fat-fed hamsters. However, oxLDL-derived 3H-cholesterol fecal excretion was increased by 37% in hamsters fed the high-fat diet (Figure 2F), whereas fecal 3H-bile acids excretion was reduced by 38% (both P<0.05 versus chow). Treatment of high-fat-fed hamsters with the LXR agonist GW3965 aggravated the dyslipidemic profile (Figure 2A), but significantly increased fecal cholesterol mass excretion by 31%, as compared with high-fat-fed hamsters (Figure 2B). After 3H-oxLDL injection, hamsters treated with LXR agonist GW3965 showed a higher 3H-tracer appearance in plasma (Figure 2E). As expected, GW3965 treatment increased oxLDL-derived 3H-cholesterol fecal excretion by 38% (P<0.05 versus high-fat), but did not improve the reduction of 3H-bile acids excretion under high-fat feeding.

To better evaluate the relevance of the oxLDL–RCT method, another set of hamster was simultaneously injected intraperitoneally with 3H-oxLDL-C–loaded macrophages (Figure 3). As seen with the oxLDL–RCT method, high-fat-fed and GW3965-treated hamsters showed an increased 3H-tracer appearance in plasma (Figure 3A). However, a different pattern was observed regarding the 3H-tracer appearance in HDL (Figure 3B), which remained unchanged with both high-fat feeding and GW3965 treatment. The strong increase in 3H-tracer hepatic recovery was again observed in high-fat-fed hamsters, 72 hours after macrophage injection (Figure 3C), whereas a further 34% increase was seen with GW3965 treatment (P<0.05 versus high-fat-fed hamsters). The raising effect of high-fat diet and GW3965 on 3H-cholesterol fecal excretion was also observed,
after \(^{3}H\)-cholesterol–labeled macrophages injection (Figure 3D). However, the reducing effect of high-fat diet on bile acids fecal excretion was not seen, when hamsters were injected with \(^{3}H\)-cholesterol–labeled macrophages.

Overall, these data indicate that the oxLDL–RCT method enables to measure alterations of RCT by high-fat diet or LXR activation. Of note, some of these alterations were not detected using the macrophage–RCT method (eg, impaired HDL-C metabolism and reduced bile acids fecal excretion).

CETP Inhibition Increases Both HDL-C and ApoE–HDL Levels but Does Not Promote In Vivo RCT

We next used the oxLDL–RCT method to investigate the effects of CETP inhibition on RCT in high-fat-fed hamsters. As expected, treatment with TOR increased the level of HDL-C and induced the appearance of apoE–HDL (Figure 4A). Despite the increase in HDL-C, TOR did not increase fecal cholesterol and bile acids mass excretion (Figure 4B). As well, TOR did not change hepatic cholesterol and triglycerides levels (data not shown). After \(^{3}H\)-oxLDL injection, TOR significantly increased \(^{3}H\)-tracer appearance in both plasma and HDL fraction (Figure 4C and 4D). However, TOR had no effect on \(^{3}H\)-tracer hepatic recovery (Figure 4E). As well, \(^{3}H\)-tracer fecal excretion in both cholesterol and bile acids fraction remained unchanged with TOR treatment (Figure 4F). These data therefore indicate that CETP inhibition increases both HDL-C and apoE–HDL levels, but does not promote in vivo RCT in high-fat-fed hamsters.
Upregulating RCT With CETP Inhibition Requires Combination With the LDL-Lowering Drug BER in Dyslipidemic Hamsters

We then hypothesized that apoE–HDL might not be functional particles in face of liver steatosis for promoting HDL-derived cholesterol fecal excretion. As apoE is a ligand for the LDL receptor, we therefore tested whether the LDL-lowering drug BER, which upregulates both the mRNA and protein expression of the LDL receptor in dyslipidemic hamsters,24–26 may promote the uptake and excretion of cholesterol derived from apoE–HDL particles.

To confirm that BER was also effective in our hamster model, a separate set of hamsters was treated over 2 weeks with BER 150 mg/kg or respective vehicle. As shown in Table 1, BER treatment in high-fat-fed hamsters improved dyslipidemia with significant reduction in LDL-C (35%) and triglycerides (47%). BER also improved liver steatosis with significant reduction in liver mass (12%), and both hepatic cholesterol (16%) and triglycerides (30%). Fecal cholesterol mass excretion was increased by 46% with BER (P<0.01 versus vehicle). The effects of BER 150 mg/kg on intestinal cholesterol absorption and RCT were also measured (Figure 5). Compared with vehicle, BER tended to decrease 3H-tracer plasma appearance by 8%, 25%, and 13% at time 3, 5, and 6 hours after 3H-cholesterol–labeled olive oil gavage, but not significantly (Figure 5A). As well, BER treatment showed no significant effect on RCT as measured with the oxLDL–RCT method (Figure 5B–5E).

The potent effect of BER on LDL particles uptake was evaluated by injecting 3H-cholesteryl-oleate–labeled LDL intravenously in high-fat-fed hamsters. As shown in Figure 6, BER significantly increased 3H-cholesteryl-oleate–LDL fractional catabolic rate. At time 72 hours after injection, 3H-tracer recovery was 15% lower in the liver (P<0.01 versus vehicle), but 102% higher in bile (P<0.05 versus vehicle). Of note, BER also increased LDL-derived 3H-cholesterol fecal excretion by 30% (P<0.05).

As BER showed the expected lipid-lowering effect in high-fat-fed hamsters, we next investigated the effects of BER under CETP inhibition on in vivo RCT. Another set of hamsters was then treated with vehicle, TOR or TOR+BER. Compared with vehicle, TOR alone and TOR+BER significantly increased both total cholesterol and HDL-C levels (Table 2). Liver mass and hepatic cholesterol levels were reduced by 18% and 26%, respectively, in hamsters treated with the combination (P<0.05 versus vehicle). The effects of drug treatment were also analyzed by fast protein liquid chromatography. As expected, TOR treatment induced the appearance of apoE–HDL (Figure 7A). However, the combination of TOR+BER substantially reduced apoE–HDL levels. Coomassie staining of fractions

Figure 3. In vivo macrophage-to-feces reverse cholesterol transport in chow-fed or high-fat-fed hamsters treated with or without the LXR agonist GW3965. A, 3H-tracer appearance in plasma after intraperitoneal injection of 3H-cholesterol–labeled/cholesterol-loaded macrophages. B, 3H-tracer appearance in HDL after injection of 3H-cholesterol–labeled/cholesterol-loaded macrophages. C, 3H-tracer recovery in liver at time 72 hours. D, 3H-tracer recovery in fecal cholesterol and fecal bile acids at time 72 hours. Results are expressed as means±SEM of 6 individual hamsters (*P<0.01 and **P<0.001 high-fat vs chow-fed hamsters; †P<0.05 GW3965 vs high-fat, Student t test).
corresponding to apoE–HDL indicated a reduction of both apoE and apoA-I levels (Figure 7A). Interestingly, TOR+BER resulted in a 97% increase in fecal cholesterol mass excretion (Figure 7B). When in vivo RCT was measured after 3H-oxLDL injection, both TOR alone and TOR+BER combination tended to increase 3H-tracer appearance in plasma and HDL (Figure 7C and 7D), with a more pronounced effect for hamsters treated with TOR alone. Although TOR showed no effect on 3H-tracer recovery in both liver and feces, TOR+BER combination significantly reduced 3H-tracer in liver by 25% (P<0.05 versus vehicle) and increased oxLDL-derived 3H-cholesterol excretion by 109% (P<0.001 versus vehicle).

These data confirm that CETP inhibition alone does not stimulate RCT in dyslipidemic hamsters and that additional effects mediated by the LDL-lowering drug BER are required to upregulate this process.

**Discussion**

Raising HDL-C levels is a potential therapy to prevent atherosclerosis and cardiovascular risk in human with the metabolic syndrome. The present study aimed to investigate whether CETP inhibition promotes in vivo RCT in a hamster model of metabolic syndrome. Overall, our data suggest that CETP inhibition itself does not promote RCT. Additional mechanisms, such as those mediated by the LDL-lowering drug BER (eg, LDL receptor upregulation, apoE–HDL levels reduction, and improvement of liver steatosis), may be necessary to stimulate this antiatherogenic process under CETP inhibition.
As liver may play a central role in RCT, we used an alternative method (the oxLDL–RCT method) to measure this process by injecting 3H-cholesteryl-oleate–labeled/oxidized LDL intravenously. Animal studies suggest that normal LDL are preferentially uptaken by hepatocytes,27 whereas oxidized LDL are rapidly uptaken by liver macrophages for further excretion of cholesterol in bile and plasma HDL.16 We observed a blunted resecretion of oxLDL-derived cholesterol in HDL particles, and reduction of fecal bile acids excretion in high-fat-fed hamsters. It is possible that the induction of liver steatosis with the high-fat/high-cholesterol diet may induce hepatic inflammation, resulting in lower cholesterol efflux, hepatic cholesterol accumulation, and bile acids metabolism impairment.28 In mice, diet-induced liver steatosis increases expression of tumor necrosis factor-α,29 which is known to inhibit ATP-binding cassette transporter A1 expression,30 and reduces expression of genes involved in bile acids synthesis.29 Although further studies would be required to investigate whether similar mechanisms occur in our hamster model, the oxLDL–RCT method enabled to highlight the adverse effect of liver steatosis on in vivo RCT. Of note, these impairments were not observed with the macrophage–RCT method, as 3H-tracer recoveries in HDL and fecal bile acids fraction were not changed in high-fat-fed hamsters. This, therefore, indicates that macrophage-derived cholesterol may follow different pathways toward fecal excretion, as compared with oxLDL-derived cholesterol. Hence, different information may be collected from the macrophage–RCT versus the oxLDL–RCT method, with advantages and limitations. The macrophage–RCT is a widely used method to measure the rate of RCT that seems well associated with the degree of atherosclerosis in animal studies.31 However, this method cannot be used

<p>| Table 1. Plasma and Liver Lipids in High-Fat-Fed Hamsters Treated With Vehicle or Berberine |
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<tr>
<th></th>
<th>Vehicle</th>
<th>Berberine</th>
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<tbody>
<tr>
<td>Total cholesterol, g/L</td>
<td>3.96±0.10</td>
<td>3.04±0.15***</td>
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<tr>
<td>LDL-cholesterol, g/L</td>
<td>0.93±0.02</td>
<td>0.61±0.06***</td>
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<tr>
<td>HDL-cholesterol, g/L</td>
<td>2.16±0.15</td>
<td>1.87±0.08</td>
</tr>
<tr>
<td>Triglycerides, g/L</td>
<td>2.95±0.32</td>
<td>1.56±0.23**</td>
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<tr>
<td>CETP activity, pmol/µL h⁻¹</td>
<td>56±3</td>
<td>50±3</td>
</tr>
<tr>
<td>Liver mass, g</td>
<td>5.9±0.2</td>
<td>5.2±0.2**</td>
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<td>Hepatic cholesterol, mg/g</td>
<td>40.3±1.2</td>
<td>34.0±1.9*</td>
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<td>Hepatic triglycerides, mg/g</td>
<td>38.1±1.6</td>
<td>26.8±3.2**</td>
</tr>
<tr>
<td>Fecal cholesterol, µg/day</td>
<td>404±24</td>
<td>589±66**</td>
</tr>
<tr>
<td>Fecal bile acids, µmol/day</td>
<td>27±2</td>
<td>23±2</td>
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CETP indicates cholesteryl ester transfer protein. Plasma samples were collected in high-fat-fed hamsters fasted overnight, after 10 days of treatment with vehicle or berberine. Liver samples were collected after 14 days of treatment.*P<0.05, **P<0.01, ***P<0.001 vehicle vs berberine.

Figure 5. In vivo intestinal cholesterol absorption and reverse cholesterol transport in high-fat-fed hamsters treated with vehicle or berberine 150 mg/kg. A, ³H-tracer appearance in plasma after an oral gavage with ³H-cholesterol-labeled olive oil. B, ³H-tracer appearance in plasma after injection of ³H-cholesteryl-oleate–labeled/oxidized LDL. C, ³H-tracer appearance in HDL after injection of ³H-cholesteryl-oleate–labeled/oxidized LDL. D, ³H-tracer recovery in liver at time 72 hours after injection of ³H-cholesteryl-oleate–labeled/oxidized LDL. E, ³H-tracer recovery in fecal cholesterol and fecal bile acids at time 72 hours. Results are expressed as mean±SEM of 6 individual hamsters (⋆P<0.05, vehicle vs berberine, Student t test).
in humans and requires the assumption that the site of radiolabeled-macrophage injection (e.g., intraperitoneal cavity) is reflecting what would happen in atherosclerotic plaques. As well, the oxLDL–RCT method is not targeting macrophages in peripheral tissues, and therefore does not provide information about cholesterol efflux in the vessel wall. Meanwhile, the oxLDL–RCT represents an alternative/novel experimental approach to look at hepatic cholesterol metabolism and its impact on RCT. The results obtained from the oxLDL–RCT method could possibly translate to human experiments, where radiolabeled particulate cholesterol is used to trace lipoprotein cholesterol metabolism. As oxLDL, radiolabeled particulate cholesterol was found to be directly taken up by liver Kupffer cells for further reappearance in plasma. However, a validated human RCT experiment remains needed to go further in this line.

We observed a higher fecal \( ^3H \)-cholesterol excretion in hamster fed with the high-fat diet (as compared with chow-fed hamsters), using both the macrophage–RCT and oxLDL–RCT method. As well, LXR activation further increased fecal cholesterol excretion in high-fat-fed hamsters. As the high-fat diet was previously found to strongly reduce biliary cholesterol excretion and increase intestinal cholesterol absorption in our hamster model, the increase in fecal cholesterol excretion would be more related to the enhancement of transintestinal cholesterol excretion (TICE), also known as the nonbiliary macrophage-to-feces RCT pathway. Although the molecular mechanisms remain unknown, a number of studies have suggested that TICE plays a significant role in RCT. TICE would contribute to \( \approx30\% \) of the total fecal cholesterol excretion in mice, and is upregulated by LXR activation and high-fat feeding. If this would also occur in our hamster model, it is interesting to note that the upregulation of the TICE pathway by high-fat feeding and LXR activation can be also measured using the oxLDL–RCT method. This would suggest that the TICE pathway could play another atheroprotective role by promoting the fecal excretion of oxLDL-derived cholesterol.

Our in vivo oxLDL–RCT experiments indicate the relevance of investigating the metabolism of oxidized LDL-derived cholesterol in the RCT process, as the rapid hepatic uptake of these atherogenic particles represents a major protection system against accumulation of cholesterol in peripheral tissues (e.g., macrophages in the arterial wall). The use of the oxLDL–RCT method to evaluate the effects of CETP inhibition showed an improvement of the resecretion of oxLDL-derived cholesterol in HDL particles in hamsters treated with TOR. This beneficial effect may be linked to the HDL apolipoprotein composition changes (increased apoA-I and apoE levels) induced by CETP inhibition. Indeed, previous in vitro studies indicated

### Table 2. Plasma and Liver Lipids in High-Fat-Fed Hamsters Treated With Vehicle, Torcetrapib, or Torcetrapib+berberine

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>Torcetrapib</th>
<th>Torcetrapib+Berberine</th>
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<tr>
<td>Total cholesterol, g/L</td>
<td>3.80±0.12</td>
<td>5.15±0.27**</td>
<td>4.43±0.20††</td>
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<td>HDL-cholesterol, g/L</td>
<td>2.04±0.13</td>
<td>2.63±0.13*</td>
<td>2.34±0.09†</td>
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<td>Triglycerides, g/L</td>
<td>3.02±0.24</td>
<td>1.20±0.09**</td>
<td>1.20±0.13†††</td>
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<tr>
<td>CETP activity, pmol/µL·h(^{-1})</td>
<td>58±7</td>
<td>40±2**</td>
<td>43±3†††</td>
</tr>
<tr>
<td>Liver mass, g</td>
<td>5.4±0.3</td>
<td>5.8±0.1</td>
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<td>Hepatic cholesterol, mg/g</td>
<td>46.0±2.6</td>
<td>47.2±4.2</td>
<td>34.8±2.5†</td>
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<tr>
<td>Hepatic triglycerides, mg/g</td>
<td>33.1±3.0</td>
<td>30.1±1.1</td>
<td>32.8±2.1</td>
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CETP indicates cholesteryl ester transfer protein. Plasma samples were collected in high-fat-fed hamsters fasted overnight, after 10 days of treatment with vehicle, torcetrapib, or torcetrapib+berberine. Liver samples were collected after 14 days of treatment. *\( P<0.01 \), **\( P<0.001 \) vehicle vs torcetrapib, †\( P<0.05 \), ††\( P<0.01 \), †††\( P<0.001 \) vehicle vs torcetrapib+berberine.
that apoE–HDL particles from homozygous CETP-deficient or TOR-treated patients have increased cholesterol efflux potential. However, CETP inhibition failed to stimulate the fecal excretion of cholesterol in our hamster model of metabolic syndrome. This result is in contrast with other studies evaluating the effects of CETP inhibitors TOR, dalcetrapib, and anacetrapib in hamsters. TOR and dalcetrapib stimulates macrophage-to-feces RCT in normolipidemic hamsters, but it is not known whether this benefit would also occur in dyslipidemic hamsters with liver steatosis. Anacetrapib also stimulated macrophage-to-feces RCT in a diet-induced dyslipidemic hamster model. However, the induction of dyslipidemia was relatively weak (eg, low levels of LDL-C levels), and the extent of liver steatosis induced by the diet was not assessed in this study. The lower cholesterol content in the diet (0.12% versus 0.5% in the present study) likely resulted in a weaker induction of dyslipidemia and liver steatosis, as compared with our hamster model. Our data therefore suggest that the extent of liver steatosis may compromise the hepatic uptake and excretion of HDL-derived cholesterol under CETP inhibition.

To test this hypothesis, we used BER, as an alternative to the lack of LDL-lowering effect by statins in hamsters, to both reduce liver steatosis and stimulate LDL receptor expression in high-fat-fed hamsters. However, the mechanism by which TOR and BER combination enhanced fecal cholesterol excretion remains unclear. Although BER alone did not affect intestinal

Figure 7. Lipoprotein profile, fecal excretion, and in vivo reverse cholesterol transport in high-fat-fed hamsters treated with vehicle, torcetrapib, or torcetrapib+berberine. A. Total cholesterol levels in fractions collected after Fast Protein Liquid Chromatography of pooled plasma samples. ApoE and apoA-I bands from pooled fractions corresponding to apoE–HDL are shown. B. Fecal cholesterol and bile acids excreted daily in feces. C. 3H-tracer appearance in plasma after injection of 3H-cholesteryl-oleate–labeled/oxidized LDL. D. 3H-tracer appearance in HDL after injection of 3H-cholesteryl-oleate–labeled/oxidized LDL. E. 3H-tracer recovery in liver at time 72 hours. F. 3H-tracer recovery in fecal cholesterol and fecal bile acids at time 72 hours. Results are expressed as mean±SEM of 6 individual hamsters (*P<0.05 vehicle vs torcetrapib; †P<0.05 and †††P<0.001 vehicle vs torcetrapib+berberine, ANOVA and Dunnett’s posttest).
cholesterol absorption and showed no effect in the oxLDL–RCT experiment, we cannot exclude that an increased LDL catabolism may play a role in the higher cholesterol fecal excretion in hamsters treated with both TOR and BER. TOR and BER combination also reduced plasma apoE–HDL and hepatic cholesterol levels, which may have also contributed to enhanced cholesterol fecal excretion for both 1H-tracer and mass. For technical reasons, we could not isolate HDL from TOR-treated or TOR+BER-treated hamsters to evaluate HDL catabolism and HDL-derived cholesterol fecal excretion (large volume of plasma would be required to radiolabel HDL particles). Such experiment would have probably confirmed that the upregulation of LDL receptor with BER stimulates the uptake of apoE–HDL for further fecal cholesterol excretion, as it did for LDL particles.

Although we could not discriminate the mechanism by which BER upregulates RCT under CETP inhibition, the present study suggests that additional lipid-lowering effects are required to potentiate the benefits of CETP inhibition in the face of metabolic syndrome. The use of the oxLDL–RCT method also highlighted under-considered aspects of in vivo RCT. Obviously, promoting cholesterol efflux from the vascular macrophages is a primary choice in developing drugs affecting RCT and HDL metabolism. However, the present study also suggests that promoting the egress of oxLDL-derived cholesterol in both HDL and fecal steroids may be an important aspect to prevent the uptake of these atherogenic particles by vascular macrophages. As TOR and BER were presently used as test compounds to evaluate the effects of CETP inhibition on oxLDL–RCT in a hamster model of metabolic syndrome, further studies would be required to evaluate other CETP inhibitors currently evaluated in the clinics.

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**Disclosures**

Dr Briand, Mr Thieblemont, Miss Muzotte, and Dr Sulpice are employees of Physiogenex.

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Methods

Animals and diets
All animal protocols were approved by the local ethical committee (Comité Régional d’Ethique de Midi-Pyrénées). Male Golden Syrian hamsters (91-100g, 6 wk old, Elevage Janvier, Le Genest Saint Isle, France) were housed in plastic cages containing wood shavings and maintained in a room with a 12-h light cycle with free access to food and tap water. Hamsters were adapted to these conditions and fed a commercial non purified diet containing 214g/kg protein, 51g/kg fat and 0.1g/kg cholesterol (Diet#A03, Safe Diets) for 1 wk. This non purified diet was defined as the control chow diet.

After 1 week of acclimation, hamsters were fed ad libitum over 4 wk either the control chow diet with normal drinking water or a high fat diet (HF) with 10% fructose in the drinking water, as described previously. The high fat diet was prepared by mixing 762.5g of control diet with coconut oil (115g), corn oil (115g), cholesterol (5g) and deoxycholate (2.5g) to obtain 1kg of HF diet (pelleted from Safe Diets).

After 2 weeks of diet, hamsters were fasted overnight and blood was collected by retro-orbital bleeding to randomize hamsters according to their HDL-cholesterol (phosphotungstate/MgCl₂ precipitation method), total cholesterol and triglycerides plasma levels. Hamsters were then
treated (n=6/treatment group) over 2 weeks with LXR agonist GW3965 30mg/kg twice daily, torcetrapib 30mg/kg once daily, berberine 150mg/kg once daily, a combination of torcetrapib 30mg/kg and berberine 150mg/kg once daily, or respective vehicle (GW3965: saline solution of 0.5% hydroxypropyl methylcellulose; torcetrapib: 1% DMSO and 99% of a saline solution of 0.5% hydroxypropyl methylcellulose; berberine: 1% Tween 80 and 99% of a saline solution of 0.5% hydroxypropyl methylcellulose).

After 10 days of treatment, hamsters were fasted overnight and blood was collected by retroorbital bleeding to measure biochemical analysis. After a 1-day recovery period, hamsters underwent the in vivo experiments described below.

**In vivo reverse cholesterol transport**

In vivo reverse cholesterol transport was measured after an intravenous injection of $^{3}$H-cholesteryl oleate labeled/oxidized LDL (oxLDL-RCT method). Human LDL (1.019<d<1.063) was isolated by ultracentrifugation, dialyzed against saline and labeled with $^{3}$H-cholesteryl oleate, as described. Briefly, $^{3}$H-cholesteryl oleate (1mCi in 1mL toluene) was evaporated under nitrogen, resuspended in 50µL ethanol and added drop wise to LDL (2.5mg protein) and lipoprotein deficient serum isolated from human plasma (200mg protein), prior a 24-hour incubation at 37°C. Radiolabeled LDL were then re-isolated by ultracentrifugation, dialyzed against saline and then oxidized with CuSO$_4$ at 37°C over 20 hours, as described previously.

The day before the in vivo experiment, a catheter was inserted into the jugular vein of each hamster under isoflurane anesthesia. Catheter was kept patent with NaCl 0.9%. To prevent blood circulation and coagulation inside the catheter, a small volume of heparin (500
IU/mL) and glycerol (1g/mL) was injected at the catheter extremity. The day of the experiment, hamsters were weighed and placed into individual cages.

In a preliminary experiment, chow-fed hamsters were injected intravenously with ≈ 7 million dpm of $^3$H-cholesteryl oleate labeled/oxidized LDL ($^3$H-oxLDL) and blood (70µL/EDTA) was collected at time 5 minutes, 0.5, 1, 2, 3, 5, 6 and 24 hours. Plasma and HDL (phosphotungstate precipitation method) were immediately isolated to measure $^3$H-radioactivity. Another group of chow-fed hamster was simultaneously injected with $^3$H-cholesteryl oleate labeled/non-oxidized LDL as control and blood was collected at time 1, 3, 6 and 24 hours after injection.

Another set of chow-fed hamsters was used to measure oxidized LDL tissue uptake. Human LDL were labeled with $^3$H-cholesteryl ether and oxidized using the same procedure as described above prior intravenous injection. At time 5 minutes after injection, blood was collected and hamsters were then sacrificed and exsanguinated. Liver and other tissues were collected and weighed. For each tissue, a 50-100mg sample was homogenized using an ultrasounds probe in 500µL distilled water then 100 µL were counted in a liquid scintillation counter. An additional liver sample was used to isolate parenchymal cells and non-parenchymal cells (Kupffer cells and endothelial cells) according to the procedure described by van Berkel and colleagues. Protein concentration was then determined on each cells fraction to determine specific activity.

In the main experiment, in vivo reverse cholesterol transport was measured over 72 hours. Individually caged hamsters had free access to food and water and were kept treated over 72 hours. Hamsters were injected intravenously with $^3$H-cholesteryl oleate labeled/oxidized LDL

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(≈ 7 million dpm) and blood (150µL/EDTA) was collected at time 5 minutes, 24, 48 and 72 hours after injection. Plasma and HDL were immediately isolated prior radioactivity measurement in a liquid scintillation counter (50µL plasma or HDL counted). Feces were collected over 72 hours and were stored at 4°C before cholesterol and bile acids extraction, as described previously.\(^1\) Remaining volume of both extracts were evaporated and resuspended in ethanol, then commercial kits were used to determine fecal total cholesterol (cholesterol RTU, Biomerieux) and total bile acids (total bile acids colorimetric assay kit, Bioquant) mass. After 72 hours, hamsters were then sacrificed by cervical dislocation, exsanguinated and liver was harvested from each animal. A ≈ 50mg-piece of liver was homogenized using an ultrasounds probe in 500µL water then 100µL were counted in a liquid scintillation counter.

Results were expressed as a % of the radioactivity injected recovered in plasma, HDL, liver and feces. The plasma volume was estimated as 3.5% of the body weight.

**In vivo macrophage-to-feces reverse cholesterol transport**

Macrophage-to-feces reverse cholesterol transport was measured over 72 hours after injection of \(^3\)H-cholesterol labeled/oxidized LDL-cholesterol loaded (macrophage-RCT method), as described previously.\(^1\) Hamsters were injected simultaneously with those receiving the \(^3\)H-oxLDL to enable the comparison of both methods. Results were expressed as a % of the radioactivity injected recovered in plasma, HDL, liver and feces. The plasma volume was estimated as 3.5% of the body weight.
In vivo $^3$H-cholesteryl oleate-LDL kinetics

A plasma pool was obtained from dyslipidemic hamsters after a 4-week high fat diet to isolate LDL by ultracentrifugation. After extensive dialysis against saline, LDL were labeled with $^3$H-cholesteryl oleate in the presence of lipoprotein deficient serum collected from human plasma, as described above. The labeled LDL particles were re-isolated by ultracentrifugation and then extensively dialyzed prior injection.

The day before the in vivo experiment, a catheter was inserted into the jugular vein as described above. The day of the experiment, hamsters were weighed and placed into individual cages. Animals had free access to food and water and were treated once daily with vehicle or berberine 100mg/kg once daily over 72h. The $^3$H-cholesteryl oleate-labeled LDL ($\approx$ 7 million dpm) were injected intravenously and blood samples (100µL) were collected at time $t=5$ minutes, 1h, 3h, 6h, 24h, 48h and 72h after injection. Plasma was immediately isolated by centrifugation and stored at 4°C prior radioactivity measurement in a liquid scintillation counter (40µL plasma counted). Feces were collected over 72 hours and were stored at 4°C before cholesterol and bile acids extraction, as described above. After 72 hours, hamsters were sacrificed by cervical dislocation and exsanguinated. Bile was collected using a Hamilton precision syringe. Collected bile volume was measured and counted in a liquid scintillation counter. Liver was then harvested from each animal. A $\approx$ 50mg-piece of liver was homogenized using an ultrasounds probe in 500µL water then 100µL were counted in a liquid scintillation counter. Bile and liver radioactivity was expressed as a % of the estimated injected dose. The estimated injected dose was calculated using the plasma counts at time 5 minutes, as follow: dpm per mL plasma at time 5 minutes x 3.5% of body weight.
Plasma decay curves were normalized to radioactivity at the initial 5-minute time point after $^3$H-cholesteryl oleate-labeled LDL injection. Plasma fractional catabolic rate (FCR) for each individual hamster was calculated using the SAAM II software.

**In vivo intestinal cholesterol absorption**

Intestinal cholesterol absorption was measured over 6 hours after an oral gavage of $^3$H-cholesterol labeled olive oil and an intraperitoneal injection of poloxamer 407, as described previously.\(^1\) Briefly, hamsters were feed-deprived overnight, weighed and were given by oral gavage 4µCi of $^3$H-cholesterol in 750µL olive oil containing 2mg/mL cholesterol. Immediately after oral gavage, hamsters were injected i.p. with poloxamer-407 (1g/kg body weight) as a lipase inhibitor. Blood (200µL) was then collected by retro-orbital bleeding under isoflurane anesthesia at time 3, 5 and 6 h to measure $^3$H-tracer appearance in plasma. Results were expressed as the % of injected dose in plasma. The plasma volume was estimated as 3.5% of the body weight.

**Biochemical analysis**

Commercial kits were used to measure total cholesterol (Cholesterol RTU, Biomerieux), triglycerides (triglyceride PAP 150, Biomerieux) and LDL-cholesterol (L-type LDL-c, Wako Chemicals). HDL-cholesterol was determined using the phosphotungstate/MgCl\(_2\) precipitation method, as described.\(^1\) Non-HDL cholesterol levels were then determined by subtracting HDL-cholesterol values from total plasma cholesterol. Plasma cholesteryl ester transfer protein (CETP) was measured by fluorescence using a commercial kit (Roarbiomedical, New York, NY, USA). Fast protein liquid chromatography (FPLC) lipoprotein analysis (total
cholesterol) using pooled plasma was performed as previously described. Fractions corresponding to apoE-HDL were used to determine apolipoproteins E and A-I by coomassie staining, as described. Hepatic cholesterol and triglycerides were determined from liver homogenate incubated with deoxycholate.

**Statistical analysis**

Values are presented as mean ± SEM. Statistical analysis was performed using either an unpaired, 2-tailed, Student t-test or ANOVA-Dunnett’s post test. A p<0.05 was considered significant.
References


