Cholesterol Absorption From the Intestine Is a Major Determinant of Reverse Cholesterol Transport From Peripheral Tissue Macrophages

Ephraim Sehayek, Stanley L. Hazen

Objective—We examined the effect of ezetimibe, a cholesterol absorption (CA) inhibitor, and genetic determinants of CA on reverse cholesterol transport (RCT) from subcutaneously injected macrophages using a new dual isotope label technique.

Methods and Results—Treatment with ezetimibe decreased dietary CA by 86% and increased RCT from peripheral tissue macrophages (PTM) by 6-fold (P<0.0001). Moreover, congenic 14DKK mice with a modest 41% decrease in dietary CA displayed a 67% increase in RCT from PTM (P<0.007).

Conclusions—These findings indicate that pharmacological and genetic modifiers of cholesterol absorption are major determinants of reverse cholesterol transport from peripheral tissue macrophages. (Arterioscler Thromb Vasc Biol. 2008;27:000-000)

Key Words: atherosclerosis ■ cholesterol absorption ■ cholesterol efflux ■ ezetimibe ■ macrophage ■ reverse cholesterol transport

Reverse cholesterol transport (RCT) is a multi-step process that involves the efflux of cholesterol from peripheral tissue macrophages (PTM) for ultimate excretion in the feces. In the initial step, the high-density lipoprotein (HDL) particle promotes cholesterol efflux from macrophages for delivery to the liver. Subsequently, HDL cholesterol is excreted into bile and the intestinal lumen where biliary cholesterol is partly reabsorbed, whereas the remainder is excreted into feces. Rader and colleagues provided the first direct in vivo evidence for transport of cholesterol from PTM into feces by intraperitoneal injection of [3H]-cholesterol-labeled macrophages and detection of tracer in plasma, liver, bile, and feces.1 The presence of [3H]-cholesterol in feces suggests that the intestines may play a role in modifying RCT from PTM. Herein we used pharmacological and genetic approaches to show that cholesterol absorption (CA) from the intestine plays a major role in determining the RCT from PTM.

Methods
Detailed descriptions of all methods used as well as the generation and characterization of 14DKK congenic animals are available in the supplemental materials (available online at http://atvb.ahajournals.org).

Results
Time Course Excretion of Fecal [14C]-Cholesterol and [3H]-β-Sitostanol From PTM
Animals were injected subcutaneously with dual-labeled macrophages, and feces were collected daily for 48 hours. As shown in Figure 1, fecal excretion of the [14C]-cholesterol label significantly increased, whereas that of the [3H]-β-sitostanol label did not change from day 1 to day 2.

Treatment With Ezetimibe and the Congenic 14DKK Interval Suppress the Absorption of Dietary Sterols
Feeding for 10 days with 0.005% ezetimibe largely suppressed the absorption of dietary sterols, as indicated by a decrease of 86%, 94%, and 91% in dietary CA, plasma campesterol, and plasma sitosterol levels, respectively. When compared to ezetimibe treatment, congenic 14DKK animals were characterized by only a modest decrease in

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dietary sterol absorption with 41%, 17%, and 27% decrease in CA, plasma campesterol, and plasma sitosterol levels, respectively (Table).

**Treatment With Ezetimibe and the Congenic 14DKK Interval Increase the RCT From PTM**
As shown in Figure 2A, in chow-fed animals the 48-hour fecal excretion of [14C]-cholesterol label from PTM amounted to 3.1±0.7%, whereas in ezetimibe-treated animals the excretion was 6-fold higher reaching 19.4±6.6%. Concordant with these findings, as shown in Figure 2B, in C57BL/6J control animals the 48-hour fecal excretion of [14C]-cholesterol label from PTM amounted to 4.3±1.8% whereas in 14DKK congenics the excretion was 67% higher reaching 7.2±2.0%. In contrast, neither ezetimibe treatment nor the 14DKK interval displayed an effect on 24-hour and 48-hour plasma levels of [14C]-cholesterol and [3H]-β-sitostanol, or on liver content of the [14C]-cholesterol and [3H]-β-sitostanol labels (Table).

**Discussion**

Treatment with bile acid sequestrants and partial ileal bypass, 2 interventions that decrease the absorption of cholesterol, are associated with decreased mortality from atherosclerotic cardiovascular disease. Similarly, atheroprotective effects were reported in apolipoprotein E null mice treated with ezetimibe. Herein we show that potent pharmacological inhibition and moderate genetic suppression of CA markedly increased RCT from PTM. These findings raise the possibility that the atheroprotective effects reported for CA inhibitors may arise not only from reductions in plasma LDL cholesterol levels but also by...

**Table. Effect of Ezetimibe Treatment and Congenic 14DKK Interval on Dietary Cholesterol Absorption, Plasma Plant Sterol Levels, and Plasma Levels and Liver Content of [14C]-Cholesterol and [3H]-β-Sitostanol Labels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cholesterol Absorption</th>
<th>Campesterol (μg/mg)*</th>
<th>Sitosterol (μg/mg)*</th>
<th>[14C]-Cholesterol</th>
<th>[3H]-β-Sitostanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>76±5</td>
<td>44.8±5.0</td>
<td>11.7±1.0</td>
<td>0.037±0.021</td>
<td>0.045±0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.035±0.015</td>
<td>0.040±0.007</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>11±5†</td>
<td>2.7±0.4†</td>
<td>1.0±0.1‡</td>
<td>0.045±0.022</td>
<td>0.045±0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.035±0.012</td>
<td>0.045±0.010</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.050±0.010</td>
<td>0.045±0.010</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11±0.03</td>
<td></td>
</tr>
<tr>
<td>Control-C57BL/6J</td>
<td>85±6</td>
<td>22.6±2.0</td>
<td>5.2±0.6</td>
<td>0.041±0.025</td>
<td>0.042±0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.049±0.026</td>
<td>0.040±0.015</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06±0.01</td>
<td></td>
</tr>
<tr>
<td>14DKK</td>
<td>44±2§</td>
<td>18.7±1.4§</td>
<td>3.8±0.4†</td>
<td>0.046±0.022</td>
<td>0.048±0.016</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>0.068±0.025</td>
<td>0.048±0.011</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.31±0.11</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

*Ratio of plasma plant sterol (μg/dl) to cholesterol (mg/dl) levels.
†P<0.0001, †P<0.002, ‡P<0.002 vs chow or control-C57BL/6J.
§†% of injected dose in 10 μL plasma and 100 mg of liver tissue.
impacting on net transfer of cholesterol from peripheral cells, such as macrophages in the arterial wall, for ultimate elimination in the feces.

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Disclosures
None.

References
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Methods

**Generation and characterization of 14DKK congenic animals:** We used an intercross between two inbred mouse strains, C57BL/6J and castaneus (CASA/Rk), to map a locus on chromosome 14 in linkage with plasma plant sterol levels, a surrogate measure of cholesterol absorption from the intestine\(^1\). Next, we generated a congenic mouse strain, designated 14DKK, which consists of a 40.5cM CASA/Rk chromosome 14 interval (from 19.5-60cM) introgressed onto the C57BL/6J background. Characterization of 14DKK congenics disclosed that, when compared to control C57BL/6J mice, these animals are characterized by moderately decreased plasma plant sterol levels and cholesterol absorption rates from the intestine\(^2\).

**Animal studies:** Control and 14DKK congenic females on the C57BL/6J background were placed in metabolic cages and fed for 10 days with either a Teklad chow diet or chow supplemented with 0.005% w/w ezetimibe as indicated. Plasma plant sterol levels were determined by GC/MS following derivatization with hexamethyldisilazane\(^1\), and dietary cholesterol absorption was determined in 12 week old animals as previously described\(^3\).

**In-vitro labeling of murine RAW 264.7 macrophages with radiolabeled cholesterol and \(\beta\)-sitostanol:** RAW 264.7 macrophages were incubated for 48h with DMEM medium supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 2mM glutamine, and 0.5 mg/ml of acetylated LDL protein supplemented with 0.2 \(\mu\)Ci /ml of \(^{14}\text{C}\)-cholesterol. After 48h cells were trypsinized, washed twice and resuspended in plain DMEM
medium. Resuspended cells were supplemented with 2 µCi of \([^3H]-\beta\)-sitostanol and the ratio of \([^{14}C]/[^3H]\) in resuspended cells was recorded.

**Reverse transport of cholesterol from subcutaneously injected RAW 264.7 macrophages:** Twelve week old C57BL/6J females were placed in metabolic cages and fed for 10 days with plain powdered chow or powdered chow supplemented with 0.005% ezetimibe. After 8 days feeding animals were injected subcutaneously (in the flank) with 300 µl of dual sterol-labeled macrophages and blood was sampled after 24 and 48 hours for determination of plasma \([^{14}C]\)-cholesterol and \([^3H]-\beta\)-sitostanol labels. Feces were collected for 48h, incubated overnight at 60°C, homogenized by mortar and pestle, sterols extracted with 2:1 vol/vol chloroform:methanol, sterols in the organic phase recovered following back extraction with addition of 0.88% KCl (2:5, vol/vol), the organic phase counted for \([^{14}C]\)-cholesterol and \([^3H]-\beta\)-sitostanol labels, and percent fecal excretion of macrophages cholesterol was calculated using the formula:

\[
\% \text{ fecal excretion} = \frac{\left(\frac{[^{14}C]}[^{3}H] \text{ ratio in the feces}\right)}{\left(\frac{[^{14}C]}[^{3}H] \text{ ratio in injected macrophages}\right)} \times 100
\]

In another set of experiments twelve week old control C57BL/6J and congenic 14DKK females were placed in metabolic cages and fed for 5 days with a powdered chow diet. After 3 days animals were injected subcutaneously with sterol labeled macrophages, blood sampled after 24h and 48h, liver tissue harvested, and feces were collected and processed for RCT measurement as described above.
It should be noted that the labeling of macrophages with $^{14}$C-cholesterol and $^3$H-β-sitostanol represents a modification of the original method that used macrophages labeled with $^3$H-cholesterol only. The rational behind the addition of $^3$H-β-sitostanol and the use of $^{14}$C/ $^3$H ratio rather than the cholesterol tracer alone is that it allows for correction in: (i) animal to animal variation in the injected volume of resuspended macrophages; (ii) animal to animal variation in the injection site, which may affect accessibility of macrophages to extracellular determinants of sterol efflux and transport to the liver; and (iii) animal to animal variation in the absorption of biliary cholesterol from the intestine. Briefly, it is well established that β-sitostanol and cholesterol are both excreted into bile, but unlike cholesterol, β-sitostanol is poorly re-absorbed from the intestines. Therefore the ratio of $^{14}$C-cholesterol/ $^3$H-β-sitostanol labels allows to correct for animal to animal variation in biliary cholesterol absorption from the intestine.

**Statistical analyses:** Group differences were examined by using a two-tailed unpaired Student's $t$ test. Results displayed are mean ± SD.

References:

