Rosuvastatin Activates ATP-Binding Cassette Transporter A1–Dependent Efflux Ex Vivo and Promotes Reverse Cholesterol Transport in Macrophage Cells in Mice Fed a High-Fat Diet

Tomohiko Shimizu, Shin-ichiro Miura, Hiroyuki Tanigawa, Takashi Kuwano, Bo Zhang, Yoshinari Uehara, Keijiro Saku

Objective—It is controversial whether statins improve high-density lipoprotein (HDL) function, which plays an important role in reverse cholesterol transport in vivo. The aim of the present study was to clarify the effects of rosuvastatin and atorvastatin on reverse cholesterol transport in macrophage cells in vivo and their underlying mechanisms.

Approach and Results—Male C57BL mice were divided into 3 groups (rosuvastatin, atorvastatin, and control groups) and orally administered rosuvastatin, atorvastatin, or placebo for 6 weeks under feeding with a 0.5% cholesterol+10% coconut oil diet. After administration, although there were no changes in plasma HDL cholesterol levels among the groups, plasma from the rosuvastatin group showed an increased ability to promote ATP-binding cassette transporter A1–mediated cholesterol efflux ex vivo. In addition, capillary electrophoresis revealed a shift in HDL toward the pre-β HDL fraction only in the rosuvastatin group. Mice in all 3 groups were intraperitoneally injected with 3H-cholesterol–labeled and cholesterol-loaded macrophages and then were monitored for the appearance of 3H-tracer in plasma and feces. The amount of 3H-tracer excreted into feces during 48 hours in the rosuvastatin group was greater than that in the control group. Finally, 3H-cholesteryl oleate-HDL was intravenously injected into all groups, blood samples were taken, and the count of 3H-cholesterol was analyzed. Plasma 3H-cholesteryl oleate-HDL changed similarly, and no differences in fractional catabolic rates were observed.

Conclusions—Rosuvastatin enhanced the ATP-binding cassette transporter A1–dependent HDL efflux function of reverse cholesterol transport, and this finding highlights the potential of rosuvastatin for the regression of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:2246-2253.)

Key Words: ABC transporters ▪ lipoproteins, HDL ▪ macrophages ▪ statins

A higher level of serum low-density lipoprotein cholesterol (LDL-C) is a major risk factor for atherosclerosis, which is a cause of cardiovascular disease. Several clinical trials have demonstrated that cholesterol-lowering therapy with statin reduces the risk of cardiovascular events.1,2 On the contrary, a lower level of serum high-density lipoprotein cholesterol (HDL-C) is considered to be independently and inversely associated with the risk of cardiovascular disease,3 and in addition to a lower LDL-C level, an increase in HDL-C has been suggested to be a secondary lipid target for reducing the risk of cardiovascular disease.4 Even in patients who are treated aggressively with statins to reduce LDL-C levels <70 mg/dL, low levels of HDL-C remain a significant predictor of major cardiovascular events.5 Statins are becoming increasingly recognized for their pleiotropic effects, which include anti-inflammation, antioxidant, vasodilation, improved endothelial function, and stabilization of atherosclerotic plaque. Statins promote the regression of atherosclerotic plaque in the carotid artery, coronary artery, and thoracic aorta through their ability to reduce LDL-C in addition to pleiotropic effects. Little is known about their effects on reverse cholesterol transport (RCT) in macrophage cells, which is the main function of HDL. Thus, it is controversial whether they improve HDL function, which plays an important role in macrophage RCT, whereas statins significantly decrease LDL-C.

RCT is the physiological process by which excess cholesterol in peripheral tissues is excreted to outside of the body.8 The process of RCT can be divided into 3 stages. First, cellular cholesterol is effluxed from peripheral cells to HDL, and macrophage ATP-binding cassette transporter (ABC) A1, ABCG1, and scavenger receptor class B type I (SR-BI) play

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Important roles. Second, HDL-C is transported in blood to the liver, and the cholesterol in HDL is converted to cholesteryl ester (CE) by the enzyme lecithin-cholesterol acyltransferase (LCAT) and carried as CE in the core of the HDL particle to the liver. Finally, CE is excreted from the liver into bile, either directly or after conversion to bile acids.

Atorvastatin and rosuvastatin are well known to be the most effective statins for lowering LDL-C levels. Several clinical trials have reported that aggressive lipid-lowering therapy not only suppresses the progression of atherosclerosis, but also contributes to its regression. Although the administration of rosuvastatin and atorvastatin resulted in a significant regression of coronary atherosclerosis and significantly increased HDL-C levels, the effects of statins on the 3 stages of RCT in vivo have not yet been reported. We hypothesized that these strong statins might promote the expression of several factors involved in RCT independent of lowering LDL-C, and there might be differences in the effects of rosuvastatin and atorvastatin on RCT. Therefore, the aim of the present study was to clarify the effects of rosuvastatin and atorvastatin in an in vivo macrophage RCT system and their underlying mechanisms.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Rosuvastatin and Atorvastatin Prevented the Increase in Triglyceride Without Changes in HDL-C, LDL-C, or non–HDL-C
Changes in the lipid profile before and after 6 weeks of statin administration are shown in the Table. The control group did not show significant changes in plasma total cholesterol, LDL-C, or non–HDL-C levels before and after 6 weeks. Both the rosuvastatin and atorvastatin groups also did not show changes of the levels before and after treatment. In addition, there were no significant changes in the levels of HDL-C in all 3 groups before and after 6 weeks. However, there were significant changes in plasma triglyceride levels after treatment. There was a significant increase in the triglyceride level at 6 weeks in the control group, whereas neither rosuvastatin nor atorvastatin changed the triglyceride level, indicating that statins prevented the increase in the triglyceride level at 6 weeks.

Rosuvastatin, but Not Atorvastatin, Increased Pre-β HDL Without Decreasing LCAT Activity
Next, we analyzed HDL subfractions as assessed by capillary isotachophoresis on a Beckman P/ACE MDQ system (Beckman-Coulter, Tokyo, Japan) as described previously, as shown in Figure 1A and 1B. Capillary isotachophoresis is a technique for analyzing charge-based lipoprotein subfractions directly in plasma. Capillary isotachophoresis can separate plasma lipoproteins into 3 HDL subfractions (fast-, intermediate-, and slow-migrating HDL) according to their electrophoretic mobilities. The rosuvastatin group, but not the atorvastatin and control groups, showed a significant increase in pre-β HDL, which indicates peaks in slow-migrating HDL, after administration. The absolute amounts of slow-migrating HDL in each group were analyzed at weeks 0 and 6. There were no significant differences among the 3 groups at week 0. At 6 weeks, the rosuvastatin group showed a significant increase in the percentage of the slow-migrating HDL fraction compared with the atorvastatin and control groups. Because plasma LCAT is critical for HDL maturation, we analyzed whether these changes were influenced by LCAT activity (Figure 1C). There were no differences in the activity of LCAT between the groups, indicating that rosuvastatin increased pre-β HDL without decreasing LCAT activity.

Rosuvastatin, but Not Atorvastatin, Decreased mRNA Levels of Apolipoprotein A-I in the Liver
We isolated the livers from mice in the rosuvastatin, atorvastatin, and control groups at 6 weeks and analyzed mRNA expression levels of various factors (Figure 2). Rosuvastatin and atorvastatin had no significant effects on mRNA levels of SR-BI.

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**Table 1. Lipid Profile in Rosuvastatin, Atorvastatin, and Control Groups at 0 and 6 Weeks**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks</th>
<th>TC, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>LDL-C, mg/dL</th>
<th>Triglyceride, mg/dL</th>
<th>Non–HDL-C, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin</td>
<td>0</td>
<td>95±12</td>
<td>51±16</td>
<td>41±10</td>
<td>21±8</td>
<td>45±9</td>
</tr>
<tr>
<td>(n=8)</td>
<td>6</td>
<td>86±20</td>
<td>44±10</td>
<td>46±14</td>
<td>21±10*</td>
<td>42±22</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0</td>
<td>95±12</td>
<td>51±14</td>
<td>40±11</td>
<td>23±7</td>
<td>45±12</td>
</tr>
<tr>
<td>(n=8)</td>
<td>6</td>
<td>93±11</td>
<td>43±9</td>
<td>45±7</td>
<td>30±8*</td>
<td>50±8</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>99±14</td>
<td>51±15</td>
<td>43±11</td>
<td>24±11</td>
<td>48±8</td>
</tr>
<tr>
<td>(n=7)</td>
<td>6</td>
<td>101±14</td>
<td>50±15</td>
<td>42±10</td>
<td>45±11†</td>
<td>51±10</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and TC, total cholesterol.

*P<0.05 vs control group at weeks 6.
†P<0.05 vs control group at weeks 0.
and ABCA1 (Figure 2A and 2B). The mRNA levels of liver X receptor, farnesoid X receptor, apolipoprotein A-I (ApoA-I), and ABCG8 in the liver in the rosuvastatin group were significantly lower than those in the control group (Figure 2C–2F). The atorvastatin group also showed a significant reduction in liver X receptor mRNA expression compared with the control group (Figure 2C). Furthermore, ApoA-I mRNA expression in the rosuvastatin group was significantly less than that in the atorvastatin group (Figure 2E). On the contrary, there were no significant differences in ABCG5 or sterol regulatory element-binding protein 2 among the 3 groups (Figure 2G and 2H).

**Rosuvastatin, but Not Atorvastatin, Increased Cholesterol Efflux via ABCA1 Ex Vivo**

We examined whether cholesterol efflux via the ABCA1 pathway was enhanced by rosuvastatin, because rosuvastatin increased pre-β HDL after treatment. Pooled plasma from mice that had been treated with rosuvastatin, atorvastatin, or placebo for 6 weeks was used for ex vivo cholesterol efflux studies with bone marrow macrophage from mice and J774 macrophages. Before the experiments, apolipoprotein B–containing protein was excluded from pooled plasma. For the preparation of apolipoprotein B–depleted plasma, plasma was mixed with phosphotungstic acid and magnesium chloride. As shown in Figure 3A to 3C, we measured plasma HDL-C–mediated efflux in bone marrow macrophage with or without probucol, which specifically inhibits ABCA1. Plasma from the rosuvastatin group had significantly greater cellular cholesterol efflux capacity than plasma from the atorvastatin and control groups (Figure 3A). However, no significant difference was seen with the use of probucol (Figure 3B). In addition, plasma from the rosuvastatin group had a significantly greater capacity to promote ABCA1-specific efflux than plasma from the atorvastatin and control groups (Figure 3C). Similar results were observed regarding cholesterol efflux using J774 cells. The rosuvastatin group had significantly greater total efflux than the atorvastatin and control groups (Figure 3D). In addition, with J774 cells in which ABCA1 was stimulated by 3′,5′-cAMP, total efflux in the rosuvastatin group was significantly greater than those in the atorvastatin and control groups (Figure 3E).

**Rosuvastatin, but Not Atorvastatin, Promoted Macrophage RCT In Vivo**

We investigated the differences in the effects of rosuvastatin and atorvastatin on RCT in macrophage cells. As shown in Figure 4A, after 3H-cholesterol–labeled J774 cells were injected into the peritoneal cavity of mice, the 3H-cholesterol counts in plasma (3H-plasma) were determined and expressed as a percentage of the total label injected (percentage of injection). There was no difference in the percentage of injection at each time point between the atorvastatin and control groups, whereas 3H-plasma in the rosuvastatin group was significantly greater at all time points compared with those in the atorvastatin and control groups. However, there was no difference in 3H-Liver between the rosuvastatin and atorvastatin groups (Figure 4B). Although there was no difference in 3H-Liver between the rosuvastatin and control groups, 3H-Liver in the atorvastatin group was significantly lower than that in the control group. Furthermore, the rosuvastatin group had significantly higher 3H-cholesterol levels in 3H-Feces than the atorvastatin and control groups (Figure 4C).

**Rosuvastatin, but Not Atorvastatin, Increased Cholesterol Excretion From the Liver Without Increasing Turnover**

Finally, we investigated the effects of statins on the turnover and fecal excretion of HDL-derived cholesterol. Plasma

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** A, Lipoprotein subfractions as assessed by capillary isotachophoresis (cITP) in plasma taken from wild-type (WT) mice before and after the administration of statins. The cITP technique separates plasma lipoproteins into 3 high-density lipoprotein (HDL) subfractions (fast-, intermediate-, and slow-migrating HDL [fHDL, iHDL, and sHDL]) according to their electrophoretic mobilities. Dotted squares indicate pre-β HDL which shows peak in sHDL. B, Absolute amount of sHDL as pre-β HDL in plasma taken from WT mice before (week 0) and after (week 6) the administration of statins. The levels of cITP HDL subfractions can be determined as peak areas relative to that of an internal marker. *P<0.05 vs atorvastatin and control groups at 6 weeks. C, Activity of lecithin-cholesterol acyltransferase (LCAT) in plasma at week 6.
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3H-cholesteryl oleate-loaded HDL was decreased equally in all 3 groups (Figure 5A), consistent with the results regarding fractional catabolic rate (Figure 5B). On the contrary, there were differences in fecal excretion. The rosuvastatin group showed a significant increase in 3H-cholesterol in feces compared with the atorvastatin and control groups (Figure 5C). Interestingly, the rosuvastatin group showed a significant increase in the fecal excretion of 3H-free-cholesterol, but not 3H-bile acids (Figure 5D and 5E). These results indicate that rosuvastatin, but not atorvastatin, enhanced the excretion of HDL-derived cholesterol into feces and support the finding that rosuvastatin promoted RCT in macrophage cells. This is consistent with the notion that rosuvastatin increases the excretion of cholesterol from the liver.

Discussion

In the present study, rosuvastatin, but not atorvastatin, increased macrophage-to-HDL RCT by activating cholesterol efflux via ABCA1. Furthermore, rosuvastatin increased liver-to-feces RCT by promoting the fecal excretion of HDL-derived cholesterol. This difference in the effects of rosuvastatin and atorvastatin may promote the regression of atherosclerosis.

We clarified the differences in RCT in vivo between treatment with rosuvastatin and atorvastatin in mice. There are 3 key steps in RCT: cholesterol efflux from macrophage cells to plasma HDL acceptors, uptake from plasma to the liver by HDL-C, and HDL-derived cholesterol excretion from the liver to bile. In the overall mechanism of RCT, rosuvastatin activates both the extraction of cholesterol from peripheral macrophage cells and the excretion of cholesterol from the liver into feces or bile. Rosuvastatin increased both cholesterol efflux via ABCA1 ex vivo and cholesterol excretion from the liver without increasing turnover. These results suggest a mechanism for the regression of atherosclerosis independent of lowering LDL-C and have implications for our understanding of the effects of rosuvastatin on RCT.

With regard to the mechanism by which rosuvastatin activates RCT, not only it can activate cholesterol efflux from peripheral macrophage cells, but it can also increase the excretion of cholesterol into feces. As the same conditions as the occasion where cholesterol efflux from a peripheral macrophage is equivalent (turnover study; Figure 5), we observed cholesterol excretion into feces after the intravenous injection of HDL containing an equivalent amount of cholesterol. As a result, cholesterol excretion into feces was increased as in the RCT study (Figure 4). If cholesterol transfer to the liver by HDL is increased, cholesterol excretion into feces would also be increased. Unexpectedly, there were no differences in cholesterol transfer to the liver by HDL.

Figure 2. Effects of statins on mRNA expression levels of various factors in mouse hepatocytes (n=7 in each group). Scavenger receptor class B type I (SR-B1; A), ATP-binding cassette transporter A1 (ABCA1; B), liver X receptor (LXR; C), farnesoid X receptor (FXR; D), apolipoprotein A-I (ApoA-I; E) ABCG8 (F), ABCG5 (G), and sterol regulatory element-binding protein 2 (SREBP-2; H). *P<0.05. AU indicates arbitrary units. All values represent the mean±SD of 3 replicates from each liver.
between the groups after the intravenous injection of $^3$H-labeled cholesteryl-loaded HDL, which indicated that rosuvastatin did not influence the transfer of HDL from peripheral tissue to the liver (Figure 5). Although cholesterol in feces was increased (Figure 4), there were no differences in the attenuation of cholesterol in blood after intravenous injection. This observation was important. Specifically, rosuvastatin did not alter blood cholesterol levels. Cholesterol that is transported to the liver might be excreted into bile as free cholesterol, without being metabolized to bile acid. Thus, it is not possible to obtain a final conclusion from these results.

Recently, Le May et al.\textsuperscript{20} reported that transintestinal cholesterol excretion is an important pathway for hepatobiliary secretion by statin. Little is known about the effects of statins on the intestine. Transporters, ABCG5/8 and ABCB1a, in addition to Niemann-Pick C1-Like 1 and LDL receptor, significantly contribute to transintestinal cholesterol excretion. Although we analyzed mRNA levels of ABCG5, ABCB1a, Niemann-Pick C1-Like 1, and LDL receptor in the intestine after statin treatment (Figure I in the online-only Data Supplement), there were no significant changes in the levels of these mRNAs. In addition, we also examined whether statins effect an intestinal absorption of cholesterol. Although we performed an additional experiment, there was no difference in the intestinal absorption of cholesterol between rosuvastatin and atorvastatin (Figure II in the online-only Data Supplement). We could not explain transintestinal cholesterol excretion with respect to the mechanism of the differential effects of RCT between rosuvastatin and atorvastatin.

There are several clinical reports that the administration of statins increases HDL-C.\textsuperscript{13–16} Unlike these clinical results, a significant increase in plasma HDL-C was not seen after the intravenous injection of $^3$H-labeled plasma (Figure 3). $^3$H-cholesterol in plasma after macrophage injection (A), $^3$H-cholesterol in the liver (B), and $^3$H-cholesterol in feces (C). CPM indicates counts per minute. *$P<0.05$ atorvastatin and control groups in each time point (A). *$P<0.05$ (B and C).
administration of statins. A lack of cholesterol ester transfer protein in mice might be one of the reasons for this result. In addition, Tamehiro et al.\textsuperscript{21} reported that the dual promoter system driven by sterol regulatory element-binding protein 2 and liver X receptor, respectively, regulates hepatic and extrahepatic ABCA1 expression. The increase in HDL-C levels by statins in humans may be partly attributable to increased liver-specific transcripts as a result of the activation of a liver-type promoter. In the present study, statins did not increase the mRNA levels of sterol regulatory element-binding protein 2 or liver X receptor in the liver. Fortunately, there were no significant differences in HDL-C concentrations among the 3 groups, and we could perform further experiments to analyze the mechanisms by which rosuvastatin enhanced RCT independent of plasma HDL-C levels. Although rosuvastatin did not change HDL-C levels compared with those in the other groups, cholesterol efflux was enhanced via ABCA1. We considered that rosuvastatin altered the HDL composition and analyzed this composition by capillary isotachophoresis. Rosuvastatin significantly increased the pre-\(\beta\) HDL fraction, which has higher cholesterol efflux capacity. Recently, an exciting new therapeutic strategy that uses cholesterol ester transfer protein inhibitors has gained attention.\textsuperscript{22,24} Cholesterol ester transfer protein inhibitors markedly increase HDL-C and decrease LDL-C when administered as monotherapy or when administered in combination with statins. Based on the results of the present study, the combination of cholesterol ester transfer protein inhibitors with rosuvastatin may promote RCT via the induction of pre-\(\beta\) HDL.

LCAT is critical for the maturation and maintenance of normal HDL metabolism. Hydrophobic CE by LCAT moves to the core of HDL particles, where it contributes to the generation of mature HDL particles and their progressive enlargement. Glomset\textsuperscript{25} proposed that LCAT plays a central role in RCT by removing free cholesterol from the surface of HDL, thus helping to maintain a gradient of free cholesterol from cells to HDL. We considered that rosuvastatin reduces LCAT activity and increases the nascent HDL component. The effects of statins on LCAT activity are controversial. For example, the administration of rosvustatin in rats did not change LCAT activity.\textsuperscript{26} Conversely, when mice were administered simvastatin or when humans were administered atorvastatin or pravastatin, LCAT activities increased.\textsuperscript{14,27,28} In our experiment, neither rosuvastatin nor atorvastatin altered LCAT activity. In this study, rosuvastatin shifted the composition of HDL from mature HDL to pre-\(\beta\) HDL without any changes in LCAT activity. In addition to LCAT, both hepatic lipase and endothelial lipase affect the composition of HDL particles. Endothelial lipase expression resulted in the generation of small pre-\(\beta\) HDL particles in wild-type mice.\textsuperscript{29} Hepatic lipase induced the formation of pre-\(\beta\) HDL from triacylglycerol-rich HDL.\textsuperscript{30} If rosuvastatin activates hepatic lipase and endothelial lipase, this activation may change the composition of HDL and increase pre-\(\beta\) HDL.

With regard to the lipid profile, such as the plasma levels of LDL-C, HDL-C, and triglyceride, both rosuvastatin and atorvastatin suppressed the increase in triglyceride levels after a high-cholesterol diet, whereas a control group showed a significant increase in triglyceride levels. In clinical studies, statins have been shown to reduce not only LDL-C levels but also triglyceride levels.\textsuperscript{13} Although we did not analyze lipoprotein lipase activity, atorvastatin improved diabetic dyslipidemia and increased lipoprotein lipase activity in vivo while reducing LDL-C, triglyceride, and very-low-density lipoprotein cholesterol.\textsuperscript{31}

We also determined the mRNA expression of various factors in the liver. SR-BI receptor expressing a hepatocyte
cell surface is important for cholesterol transfer from HDL to the liver. However, there was no significant difference in SR-BI mRNA expression in this study. Statins did not change macrophage SR-BI expression in mice or hepatic SR-BI expression in dogs. Based on our HDL turnover study, rosuvastatin may activate RCT without increasing transport from HDL to the liver. Unexpectedly, rosuvastatin decreased ApoA-I mRNA, although clinical studies have shown an increase in ApoA-I levels. Marchesi et al. reported that rosuvastatin did not increase ApoA-I transcription or hepatic secretion. The unexpected result obtained in our study, that is, rosuvastatin reduced hepatic ApoA-I mRNA, could be a consequence of hepatic cholesterol depletion attributable to the inhibition of statin, which may reduce hepatic HDL production.

Study Limitations
There were several study limitations in this study. First, there was a problem about optimal doses of rosuvastatin and atorvastatin. We analyzed the mRNA levels of Cyp7A1, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and LDL receptor (Figure III in the online-only Data Supplement). The mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the atorvastatin group, but not the rosuvastatin group, were significantly higher than those in the control group. Although the dose of rosuvastatin in the present study may not be optimal, rosuvastatin enhanced RCT in vivo. On the contrary, although the dose of atorvastatin was optimal, atorvastatin did not enhance RCT. We think that rosuvastatin could enhance RCT, even though the dose was low. Second, we did not analyze the hepatic levels of HDL-derived \(^{3} \text{H}-\text{cholesterol in Figure 5 because we used } {^{3} \text{H}-\text{cholesterol oleate in the experiment, which is excreted into the feces through bile without accumulating in the liver. Hence, we used } {^{3} \text{H}-\text{cholesterol ester which accumulates at a higher rate in the liver than the other liver. Further studies will be needed to resolve this issue.}}

Conclusions
Our results indicate that rosuvastatin, but not atorvastatin, promotes RCT in macrophage cells in vivo by activating ABCA1-dependent efflux from peripheral macrophage cells and increasing the excretion of cholesterol from the liver to bile. The regression of atherosclerosis by rosuvastatin may be more powerful than the effect of atorvastatin, and rosuvastatin might be able to further reduce the incidence of cardiovascular events.

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Supplement

Materials and Methods

Materials

The following antibodies and reagents were purchased or provided: Dulbecco modified Eagle medium (DMEM) and phosphate buffer saline (PBS) (Wako Pure Chemical Industries Ltd., Osaka, Japan); [1,2-3H]cholesterol, [1,2-3H]cholesteryl oleate (Perkin Elmer Life & Analytical Sciences Inc.); rosuvastatin and atorvastatin (Toronto Research Chemicals, Inc., Canada); 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8-CTP-cAMP) (Enzo Life Sciences, Inc., New York, NY); human HDL (Calbiochem, Darmstadt, Germany); and the liver X receptor (LXR) agonist T0-901317 and the retinoid X receptor (RXR) agonist 9-cis-retinoic acid (Sigma-Aldrich, St. Louis, MO).

Mice, Diets and Experimental Design

Wild-type (WT) C57BL/6J mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were housed in specific pathogen-free barrier facilities at Fukuoka University. Mice were maintained under a 12-h light/dark cycle and fed a 0.5% high-cholesterol diet + 10% coconut oil (purchased from Oriental Yeast Co. Ltd., Tokyo, Japan) (Content of diet were shown in Supplementary Table I), which was
provided ad libitum for 2 weeks before and during the study. In all cases, fasting plasma was obtained from the retro-orbital plexus while the mice were under isoflurane anesthesia. For each experiment, female 3- to 4-month-old C57BL/6 mice were divided into 3 groups; 4 mg/kg/day rosuvastatin (rosuvastatin group), 8 mg/kg/day atorvastatin (atorvastatin group) and 0.5 % methylcellulose (control group) dissolved in freely available drinking water for 6 weeks. In a study by de Haan et al., mice received diet with or without 0.01% (w/w) atorvastatin for 6 weeks (i.e., approximately 10 mg/kg/day, which corresponds to a dose of 70 mg/day for an average 70 kg person) [1]. Since the maximum doses of rosuvastatin and atorvastatin in humans are 40 mg/day and 80 mg/day, respectively, the doses of rosuvastatin and atorvastatin in mice were determined to be 4 mg/kg/day and 8 mg/kg/day, respectively. Animal experiments were approved by the Fukuoka University Animal Center Committee.

Plasma Lipid, Lipoprotein and lecithin-cholesterol acyltransferase (LCAT) Activity Analyses

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and LCAT activities were measured by a commercial kit (Sekisui Medical Co. Ltd., Japan). Lipoprotein subfractions in
serum and serum fractions were analyzed by capillary isotachophoresis (cITP) on a Beckman P/ACE MDQ system (Beckman-Coulter, Tokyo, Japan) as described previously [2]. cITP is a technique for analyzing charge-based lipoprotein subfractions directly in plasma [3, 4]. cITP can separate plasma lipoproteins into three HDL subfractions [fast- (f), intermediate- (i), and slow- (s) migrating HDL] according to their electrophoretic mobilities. The levels of cITP HDL subfractions can be determined as peak areas relative to that of an internal marker.

**RNA Quantification**

Total RNA was isolated from mouse primary hepatocytes with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was produced from total RNA via reverse transcription with a SuperScript First-Stand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY). mRNA expression with Taqman assay systems was quantified by an ABI 7500 Real Time PCR System according to the manufacturer’s instructions (Life Technologies, Grand Island, NY) for scavenger receptor class B type I (SR-BI), ATP-binding cassette transporter (ABC) A1, liver X receptor (LXR), farnesoid X receptor (FXR), apolipoprotein (Apo)A-1, ABCG8, ABCG5, sterol regulatory element-binding protein 2 (SREBP-2) and β-actin RNA. The expression data were
normalized for β-actin levels.

**Ex Vivo Cholesterol Efflux from Bone Marrow Macrophage and J774 Macrophage Cells**

Plasma samples were collected from mice after 6 weeks of treatment. Plasma HDL (apoB-depleted plasma)-mediated cholesterol efflux was measured in cAMP-treated J774 macrophage cells as described above. For the preparation of apoB-depleted plasma, plasma was mixed in a tube containing the precipitation reagent (0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride). After 10 min at room temperature, this mixture was centrifuged for 10 min at 3,000g. The clear supernatant was separated and stored in capped glass tubes for a maximum of 2 days at 4°C before the cholesterol content was determined by the cholesterol oxidase/p-aminophenazone method. We measured plasma HDL-C-mediated efflux in bone marrow macrophage (BMM) with or without probucol, which specifically inhibits ABCA1. J774 macrophages were plated in 12-well plates (0.25×10^6 /mL) in DMEM with 10 % fetal bovine serum (FBS) and 0.5 % penicillin G and streptomycin at 37 °C. After 1 day, the cells were labeled for 24 h at 37 °C with 3 μCi/mL [1,2-^3H]cholesterol and 50 μg/mL acetylated low-density cholesterol (ac-LDL) in the presence of 1.0 % FBS and
0.5 % penicillin G and streptomycin in DMEM. Cells were washed extensively with
PBS with 1.0 % bovine serum albumin (BSA), and J774 macrophages were then
equilibrated with or without 0.3 mmol/L cAMP, which activates mainly ABCA1, in 0.2
% BSA-containing medium for 20 h at 37 °C [5]. At the end of the equilibration
period, cell monolayers were washed twice with PBS containing 1 % BSA.

Subsequently, cholesterol efflux was induced by incubation for 4 h at 37°C with each
pooled plasma (excluding apo-B-containing protein) diluted to 2 % in DMEM. Free
cholesterol efflux was obtained by measuring the release of radiolabeled cholesterol into
the medium as described previously [6].

Next, BMM were isolated from mice by standard procedures [7]. Isolated
macrophages were suspended and cultured in 12-well plates (0.25×10⁶/mL) in DMEM
with 10 % FBS plus 0.5 % penicillin G and streptomycin at 37°C. After 4 days,
macrophages were labeled for 24 h at 37°C with 2 μCi/mL [1, 2-3H]cholesterol and 25
μg/mL ac-LDL in the presence of 1.0 % FBS and 0.5 % penicillin G and streptomycin
in DMEM. BMM were washed extensively with PBS with 1 % BSA and equilibrated
overnight in the presence of the LXR agonist T0-901317 and the RXR agonist 9-cis
(each 5 μM) in serum-free medium. For cholesterol efflux, BMM were treated with or
without 20 μM probucol for 2 h at 37°C [8]. After 4 h, aliquots of the medium were
removed, and the $^3$H-cholesterol released was measured by liquid scintillation counting.

The $^3$H-cholesterol present in the cells was determined by extracting cell lipids in 0.2 M NaOH and 0.15 M NaCl.

**Macrophage reverse cholesterol transport (RCT) Study**

The RCT study was performed as described previously [9-11]. J774 macrophages were grown in DMEM supplemented with 10 % FBS. The cells were radiolabeled with 5 μCi/mL $^3$H-cholesterol and cholesterol enriched with 25 μg/mL ac-LDL for 40 h. The labeled foam cells were washed, equilibrated in medium with 0.2 % bovine serum albumin for 4 hours, spun down and resuspended in DMEM immediately before use.

All mice were fed a 0.5 % high-cholesterol diet for 2 weeks before experiments as prefeeding. For this experiment, 21 female 3- to 4-month-old mice (n=7/each group) were divided into 3 groups (rosuvastatin, atorvastatin and control groups). After 6 weeks, $^3$H-cholesterol-labeled and acetylated LDL-C-loaded J774 cells were injected intraperitoneally. Blood was collected at 6, 24 and 48 h, and plasma samples were used for liquid scintillation counting. Feces were collected continuously from 0 to 48 h and stored at 4 °C before the extraction of cholesterol and bile acid.
**HDL Turnover Study**

Human HDL was labeled with $^3$H-cholesteryl oleate as described previously with slight modifications [11, 12]. For the experiment, 15 female 3- to 4-month-old mice (n=5/each group) were divided into 3 groups (rosuvastatin, atorvastatin and control groups). After 6 weeks, labeled HDL was injected intravenously. Blood was collected at 2 min and 1, 3, 6, 9, 24 and 48 h, and liver and feces were collected at 48 h. The fractional catabolic rate (FCR) was calculated using the SAS (Statistical Analysis System) software package (Ver. 9.2; SAS Institute Inc., Cary, NC) as described previously [12, 13].

**Statistical Analysis**

The results before and after drug administration were compared with the Student $t$ test (2-tailed) and analysis of variance (ANOVA) using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA). For the macrophage RCT studies, the appearance of tracer in plasma was analyzed by repeated measures ANOVA, and a Newman-Keuls multiple comparison test was applied to correct for multiple comparisons. One-way ANOVA with a Newman-Keuls multiple comparison test was used to compare the differences among the 3 groups. All data are presented as the
mean ± standard deviation (SD). A probability value <0.05 was considered statistically significant.

References


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Supplementary Figs and Tables.

Supplementary Table I. Content of diet.
Crude protein 20.7g/100g diet
Crude fat 15.1g/100g
Crude ash 5.2g/100g
Crude fiber 2.5g/100g
Nitrogen free extract 49.5g/100g
Water 7.1g/100g

Supplemental Figure I. mRNA levels of ABCG5, ABCB1a, NPC1L1 and LDL receptor in the intestine in the rosuvastatin, atorvastatin and control groups.

Supplemental Figure II. Intestinal absorption of cholesterol in the rosuvastatin, atorvastatin and control groups.

Methods for measurement of the intestinal absorption of cholesterol
Wild-type mice were divided into 3 groups; 4 mg/kg/day rosuvastatin (rosuvastatin
group, n=7), 8 mg/kg/day atorvastatin (atorvastatin group, n=8) and 0.5 % methylcellulose dissolved in water (control group, n=8) for 2 weeks. We measured intestinal cholesterol absorption function by a fecal dual-isotope ratio method, as described previously [1, 2].

Briefly, mice were gavaged with 150 μl of safflower oil (195-15372, Wako Pure Chemical Industries, Ltd. Japan) that contained a mixture of 2 μCi [5,6-3H] sitostanol (ART 0361, American Radiolabeled Chemicals Inc., St. Louis, MO, USA) and 1 μCi [4-14C] cholesterol (NEC018250UC, PerkinElmer Life Sciences, USA), and then returned to fresh cages. Feces were collected from individually housed mice in wire-bottom cages after the administration of label and processed as described previously following homogenization and neutral sterol extraction of fecal samples. The ratio of 14C and 3H sterol in each feces sample and the dosing mixture were determined by liquid scintillation counting and the cholesterol absorption percentage was calculated as described previously [2, 3].

References:

Supplemental Figure III. mRNA levels of Cyp7A1, HMG-CoA reductase and LDL-R in the liver in the rosvuastatin, atorvastatin and control groups.