Pravastatin Sodium, an Inhibitor of HMG-CoA Reductase, Decreases HDL Cholesterol by Transfer of Cholesteryl Ester from HDL to VLDL in Japanese White Rabbits

Atsuhiro Miyazaki¹, Tadashi Koieyama², Yukio Shimada³, Takashi Kikuchi⁴, Kayoko Ito⁵, Naomi Kasanuki⁶, and Teiichiro Koga⁷

¹ Clinical Development Department,
² Pharmacology and Molecular Biology Research Laboratories,
³ Research Administration Department Research Laboratories,
⁴ Intellectual Property Department,
⁵ Research Information Department,
⁶ Biomedical Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan.

In a recent paper, we reported that pravastatin sodium (pravastatin), an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, decreases the concentrations of low density lipoprotein (LDL) cholesterol through an LDL receptor pathway in Japanese White (JW) rabbits, whereas this agent lowers high density lipoprotein (HDL) cholesterol in a manner correlated with a reduction of very low density lipoprotein (VLDL) cholesterol secretion from the liver. In the present study, we administered pravastatin to JW rabbits at 30 mg/kg for 14 days and examined further the mechanisms for the reduction of HDL cholesterol. A striking finding was that the 4-day administration of pravastatin at 30 mg/kg selectively decreased the concentration of HDL cholesterol. Since 4-day administration of pravastatin to JW rabbits did not change the concentrations of hepatic LDL receptor proteins, these receptors were not likely to be involved in the reduction of HDL cholesterol. Another important finding was that pravastatin suppressed VLDL cholesteryl ester (CE) secretion from the liver, but not that of other VLDL lipids and VLDL proteins, indicating that the CE-poor VLDL particles were secreted by the consecutive administration of pravastatin. There were, however, no differences in the levels of VLDL cholesterol between the control and pravastatin-treated groups over the experimental period of 14 days. These observations raised the possibility that the reduction of HDL cholesterol in the pravastatin-treated group was due to the transfer of CE molecules from HDL particles to these CE-poor VLDL particles. Molecular species analysis supported this notion that the VLDL-CE in the pravastatin-treated group was rich in cholesteryl linoleate, indicating that the CE in this group mainly originated from HDL, whereas the VLDL-CE in the control group was rich in cholesteryl oleate, indicating that the CE in this group originated from the liver. The present study suggests that pravastatin lowers HDL cholesterol by transferring CE from these lipoproteins to VLDL in JW rabbits. J Atheroscler Thromb, 2004; 11: 22–28.

Key words: Pravastatin, Japanese White rabbit, VLDL secretion, HDL cholesterol

Address of corresponding: Teiichiro Koga, Ph.D., Biomedical Research Laboratories, Sankyo Co., Ltd., Hiromachi 1–2–58, Shinagawa-ku, Tokyo 140–8710, Japan.
E-mail: kogasa@shina.sankyo.co.jp
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Abbreviations:
Introduction

Pravastatin sodium (pravastatin) is a potent and specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (1-7), a rate-limiting enzyme in cholesterol biosynthesis (8). This drug has been shown to decrease the concentrations of low density lipoprotein (LDL) cholesterol and increase those of high density lipoprotein (HDL) cholesterol in humans (9-12), whereas in some experimental animals, the concentrations of both LDL- and HDL cholesterol were decreased (6, 13, 14).

In a recent paper (15), we demonstrated that in Japanese White (JW) rabbits, pravastatin decreased the concentration of LDL cholesterol through a well-understood LDL receptor pathway (15). Although this agent lowered HDL cholesterol of JW rabbits in a time- and dose dependent manner (15), the mechanisms for the reduction of HDL cholesterol by pravastatin were unknown (15).

We have also shown (15) that pravastatin lowered VLDL cholesterol secretion from the liver in these animals, but there were no differences in the concentrations of VLDL cholesterol between the control and pravastatin-treated groups. We have further demonstrated (15) that pravastatin similarly decreased the concentration of HDL cholesterol and the secretion rates of VLDL cholesterol from the liver. From these observations, we speculated that the transfer of CE molecules from HDL to VLDL caused the reduction of HDL cholesterol.

In the present study, to address the mechanisms by which pravastatin decreased HDL cholesterol in experimental animals, we examined the relationship between the concentration of HDL cholesterol and the reduction of VLDL cholesterol by pravastatin was unknown (15).

We have also shown (15) that pravastatin lowered VLDL cholesterol secretion from the liver in these animals, but there were no differences in the concentrations of VLDL cholesterol between the control and pravastatin-treated groups. We have further demonstrated (15) that pravastatin similarly decreased the concentration of HDL cholesterol and the secretion rates of VLDL cholesterol from the liver. From these observations, we speculated that the transfer of CE molecules from HDL to VLDL caused the reduction of HDL cholesterol.

In the present study, to address the mechanisms by which pravastatin decreased HDL cholesterol in experimental animals, we examined the relationship between the concentration of HDL cholesterol and the reduction of VLDL cholesterol by pravastatin. Molecular species analysis of the CE in VLDL particles was conducted to determine the origin of CE molecules in VLDL particles. The data obtained suggest that the consecutive administration of pravastatin caused the secretion of CE-poor VLDL particles from the liver and the HDL cholesterol-lowering action of pravastatin occurred by the transfer of CE molecules from HDL particles to these CE-poor VLDL particles.

Materials and Methods

Materials

Pravastatin was prepared as described previously (1, 2). Triton WR-1339 was purchased from Nakarai Tesque (Tokyo, Japan). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and experimental design

Animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co. (Tokyo, Japan). Male JW rabbits (SPF grade, 5 months old) were purchased from Kitayama Labs (Shiga, Japan) and were kept as described in the previous paper (15). After matching for the baseline serum total cholesterol (TC) concentrations, the rabbits were assigned to either the control (0.5% carboxymethyl cellulose: CMC) or pravastatin (30 mg/kg, 0.5% CMC solution) groups as described previously (15).

The present study consisted of two experiments. In the first experiment, we primarily determined changes in the concentrations of TC, triglycerides (TG), lipoprotein cholesterol and hepatic LDL receptor proteins after oral administration of 30 mg/kg pravastatin to rabbits for 4, 7 or 14 days. In the second experiment, we determined the VLDL secretion rates from the liver as well as changes in the levels of serum lipids after oral administration of 30 mg/kg pravastatin to rabbits for 4, 7 or 14 days.

The drug administration, blood sampling and serum preparation were performed as described in the previous paper (15).

Determination of serum lipid levels

The determination of serum lipid was performed as described in the previous paper (15).

Determination of lipoprotein lipid concentrations

Lipoproteins were isolated by ultracentrifugation according to the method of Hatch and Lees (16) at the densities of d < 1.006 g/ml (VLDL), d = 1.006–1.063 g/ml (LDL) and d > 1.063 g/ml (HDL) as described in the previous paper (15). The concentration of TC in each lipoprotein was determined using the Cholesterol C-II Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Determination of VLDL secretion rate

VLDL secretion rate was determined as described in the previous paper (15).

Preparation of liver microsomes

The preparation of liver microsomes was described in the previous paper (15).

LDL receptor activity

The concentrations of LDL receptor proteins were determined by immunoblot analysis as described in the previous paper (15).

High performance liquid chromatograph (HPLC) analysis

Lipids were extracted from VLDL fractions according to the method of Folch et al. (17) and the levels of cholesteryl oleate and cholesteryl linoleate were determined using the HITACHI Type 655 HPLC system (Hitachi Co., Tokyo, Japan). Reverse-phase HPLC columns (MILLIPORE) were used (µBondasphere C8; 3.9 mm × 15
cm, 5 µm, 300 Å). The mobile phase for HPLC was 100% acetonitrile. The column was eluted at a flow rate of 1.0 ml/min at room temperature (20–24°C) and monitored at 210 nm. Data were recorded and calculated on a HEWLETT PACKARD 3390A INTEGRATOR. Authentic cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) were dissolved in 2-propanol as external standards. Cholesteryl heptadecanoate was mixed with each sample and used as an internal standard. Lipid extracts of VLDL fractions were redissolved in 2-propanol and a portion was used for HPLC analysis. Each value was determined by two injections of the same sample.

**Statistical analysis**

Values were expressed as mean ± SE. Statistical analysis was performed by Student’s t-test and Duncan’s test.

**Results**

**Relationship between concentrations of hepatic LDL receptor proteins and changes in the concentrations of serum and lipoprotein lipids**

Pravastatin, when orally administered to JW rabbits at a dose of 30 mg/kg for 14 days, decreased the concentrations of TC in a time-dependent manner (Fig. 1). On day 4, a decrease in cholesterol by up to 20% was achieved (p < 0.05). The reduction in the concentration of TC was unexpectedly due to the significant decrease in the concentration of HDL cholesterol (p < 0.05), not to decreases in either VLDL- or LDL cholesterol (Fig. 1). The decrease in the concentration of TC reached a plateau after the 7-day administration of pravastatin at 30 mg/kg (Fig. 1). Unlike the 4-day administration of pravastatin, the reductions in TC on days 7 and 14 were due to those in both LDL- and HDL cholesterol. Over the experimental period of 14 days, the decreased concentration of HDL cholesterol was almost constant, whereas LDL cholesterol was reduced in a time-dependent manner. In the present study, the concentrations of TG and VLDL cholesterol did not change (Table 1, Fig. 1).

At the end of the experiment, the rabbits were sacrificed, the livers were excised and the concentrations of LDL receptor proteins in the liver were determined (Fig. 2). The 4-day administration of pravastatin at 30 mg/kg did not change the concentrations of hepatic LDL receptor proteins (Fig. 2), although a selective decrease in HDL cholesterol was seen on day 4 of this experiment (Fig. 1). On the other hand, on days 7 and 14, the concentrations of LDL receptor proteins were elevated by 13% and 17%, respectively, although these increases were not statistically significant (Fig. 2). On these days, corresponding reductions in the concentrations of LDL cholesterol were observed (Fig. 1).

![Fig. 1. Effects of pravastatin on serum lipids and lipoprotein cholesterol in Japanese White rabbits. Pravastatin at 30 mg/kg was orally administered to Japanese White rabbits for (A) 4, (B) 7 and (C) 14 days. Blood was withdrawn on day 0 and the indicated days after overnight fasting. Each lipoprotein was isolated by ultracentrifugation at the densities of d < 1.006 (very low density lipoprotein; VLDL) g/ml, d = 1.006–1.063 (low density lipoprotein; LDL) g/ml, and d > 1.063 (high density lipoprotein; HDL) g/ml. The concentrations of serum total cholesterol (TC) and cholesterol in each lipoprotein were determined as described in the text. Each column with bar represents mean ± SE (n = 5). Statistical analysis was performed with the Student’s t-test (*: p < 0.05, **: p < 0.01 vs day 0).](image-url)
Pravastatin Decreases HDL Cholesterol by Transfer of CE from HDL to VLDL in Rabbits

Pravastatin, when orally administered to JW rabbits at 30 mg/kg for 4 days, decreased the VLDL-TC secretion rate from the liver by about 16%, which was primarily due to the reduction of the VLDL-CE secretion rate (about 39%, p < 0.05) (Table 2). The decreases in VLDL-CE secretion rates by pravastatin at 30 mg/kg were almost constant over the experimental period. No changes in the VLDL-TG, -PL and -protein secretion rates from the liver were observed (Table 2).

Determinination of cholesteryl ester molecular species in VLDL

VLDL was isolated from rabbits which received pravastatin at 30 mg/kg for 4 days. VLDL lipids were extracted and molecular species of VLDL-CE particles were determined (Table 3). In the control group, VLDL-CE was rich in cholesteryl oleate: the ratio of cholesteryl oleate to cholesteryl palmitate was 2.3:1. In the pravastatin-treated group, the ratio of cholesteryl oleate to cholesteryl palmitate was 3.1:1. The decrease in the ratio of cholesteryl oleate to cholesteryl palmitate by pravastatin was statistically significant (p < 0.05). The decreases in the ratios of cholesteryl oleate to cholesteryl palmitate by pravastatin were almost constant over the experimental period.

Table 1. Effects of pravastatin on serum triglycerides in JW rabbits.

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>initial</td>
<td>36.5 ± 1.7</td>
<td>35.8 ± 2.3</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>treatment</td>
<td>(116.7)</td>
<td>(116.7)</td>
</tr>
<tr>
<td>Pravastatin (30 mg/kg)</td>
<td>initial</td>
<td>34.3 ± 3.2</td>
<td>46.0 ± 1.3</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>treatment</td>
<td>(103.8)</td>
<td>(101.3)</td>
</tr>
</tbody>
</table>

Pravastatin at 30 mg/kg was orally administered to JW rabbits for the indicated days. Blood was withdrawn on day 0 and the indicated days after overnight fasting. The concentrations of serum TG were determined as described in the text. Values are mean ± SE (n = 5). Each value in the parentheses expresses a mean of the percent changes from the initial values.

Table 2. Effects of pravastatin on VLDL lipid and -protein secretion rates from the liver.

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 4</td>
<td>Control (n = 5)</td>
<td>10.1 ± 0.7</td>
<td>7.8 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>111 ± 4</td>
<td>11.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Pravastatin (30 mg/kg) (n = 5)</td>
<td>8.5 ± 0.6 (84.2)</td>
<td>7.1 ± 0.3 (91.0)</td>
<td>1.6 ± 0.3* (61.5)</td>
<td>109 ± 10 (98.2)</td>
<td>11.2 ± 2.6 (92.3)</td>
</tr>
<tr>
<td>day 7</td>
<td>Control (n = 5)</td>
<td>10.2 ± 0.2</td>
<td>7.3 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>142 ± 9</td>
<td>16.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Pravastatin (30 mg/kg) (n = 5)</td>
<td>8.4 ± 0.5* (82.4)</td>
<td>6.6 ± 0.6 (90.4)</td>
<td>1.8 ± 0.2* (62.1)</td>
<td>142 ± 7 (100.0)</td>
<td>14.5 ± 2.6 (90.1)</td>
</tr>
<tr>
<td>day 14</td>
<td>Control (n = 5)</td>
<td>10.1 ± 0.4</td>
<td>7.4 ± 0.6</td>
<td>2.6 ± 0.2</td>
<td>156 ± 13</td>
<td>14.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Pravastatin (30 mg/kg) (n = 5)</td>
<td>8.3 ± 0.5* (82.2)</td>
<td>6.7 ± 0.5 (90.5)</td>
<td>1.7 ± 0.2* (65.4)</td>
<td>153 ± 0.7 (98.1)</td>
<td>14.1 ± 2.6 (95.9)</td>
</tr>
</tbody>
</table>

"Pravastatin at 30 mg/kg was administered to Japanese White rabbits for the indicated days. On day 0 and the indicated days, blood sampling was performed to confirm changes in the concentrations of serum total cholesterol, triglycerides, and VLDL-, LDL- and HDL cholesterol (data not shown). After the blood sampling for the baseline on the indicated days, Triton WR-1339 (400 mg/kg) was intravenously injected. Blood was withdrawn again 6 hours after Triton WR-1339 i.v. VLDL was fractionated by ultracentrifugation and VLDL lipids and -protein secretion rates were determined as described in the text. Values are mean ± SE. (n = 5). Significant difference: *p < 0.05 vs. control by Student’s t-test."
to cholesteryl linoleate was 3.17, whereas in the pravastatin-treated group, VLDL-CE was rich in cholesteryl linoleate: the ratio of cholesteryl oleate to cholesteryl linoleate was 0.31. These data indicate that the VLDL-CE molecules in the control and pravastatin-treated groups were from different origins.

**Discussion**

The principal conclusion in the present study was that in JW rabbits, pravastatin decreases the concentration of HDL cholesterol by the transfer of CE molecules from HDL particles to VLDL particles. The striking finding in the present study was that the 4-day administration of pravastatin to JW rabbits at 30 mg/kg selectively lowered the concentration of HDL cholesterol (Fig. 1). Although many investigators have described the lipid-lowering action of HMG-CoA reductase inhibitors in experimental animals (6, 13–15, 18–21), there have been no documents which have demonstrated selective reduction in the concentration of HDL cholesterol by HMG-CoA reductase inhibitors. We speculated that since in those studies HMG-CoA reductase inhibitors were administered to experimental animals for more than 7 days or the doses used were higher compared with those in the present study, or both, they could not find selective reduction in the concentration of HDL cholesterol.

In the present study, we demonstrated that the 4-day administration of pravastatin decreased HDL cholesterol (Fig. 1) without an increase in hepatic LDL receptor proteins (Fig. 2). In the recent paper, we also reported (15) that the 21-day administration of pravastatin at 3 mg/kg induced hepatic LDL receptor proteins and decreased LDL cholesterol, but not HDL cholesterol. These data suggest that pravastatin lowers HDL cholesterol independent of an LDL receptor pathway in JW rabbits.

Another important finding was that the concentrations of HDL cholesterol and the VLDL-CE secretion were reduced similarly in a time- (Fig. 1 and Table 2) and dose-dependent (15) manner: HDL cholesterol (Fig. 1) and the VLDL-CE secretion rate (Table 2) decreased on day 4, and these reductions were maintained similarly over the experimental period. We have also demonstrated (15) that the doses required for the reduction of VLDL-CE secretion were similar to those of HDL cholesterol. These observations suggest that the reduction of VLDL-CE secretion causes the HDL cholesterol-lowering action of pravastatin in JW rabbits.

In the present study and the previous article (15), we have demonstrated that pravastatin significantly decreased the VLDL-CE secretion rate from the liver, but not the rates of other VLDL-lipids or VLDL-proteins (Table 2), indicating that CE-poor VLDL particles were secreted from the liver in the pravastatin-treated group. There were, however, no differences in the concentrations of VLDL cholesterol between the control and pravastatin-treated group over the duration of the experiment (Fig. 1). Taking all these observations together, we have developed the hypothesis that CE molecules transferred from HDL particles made up for the CE-poor VLDL particles to maintain the concentration of VLDL cholesterol.

Analysis of CE molecular species in VLDL fractions supported this hypothesis (Table 3). VLDL-CE in the control group was rich in cholesteryl oleate (Table 3), which is originally synthesized by acyl-CoA: cholesterol acyltransferase in the liver, indicating that VLDL-CE in the control group was mainly derived from the liver. In contrast, VLDL-CE in the pravastatin-treated group was rich in cholesteryl linoleate (Table 3), which is synthesized primarily by lecithin cholesterol acyltransferase (LCAT) on HDL particles in plasma, indicating that VLDL-CE in this group was transferred from HDL particles. These data suggest that in the pravastatin-treated group, CE transfer occurred from HDL particles to VLDL particles. There are other factors which may have decreased the level of HDL cholesterol in the pravastatin-treated JW rabbits: the increased expression of scavenger receptor class B type I in the liver and ATP-binding cassette transporter A1 in the macrophages, the decrease of LCAT activity, and HDL synthesis in the liver and intestine. We can not, however, rule out the possibility that these factors were involved in the decrease of HDL cholesterol in the pravastatin-treated JW rabbits in the present study.

In humans, HDL cholesterol and apo A-I levels are increased (11, 22) when statins decrease VLDL secretion or lower TG levels: Schaefer et al. (11) demonstrated that in humans, the suppression of apoB production by

| Table 3. Molecular species in VLDL cholesteryl ester in the control and pravastatin-treated rabbits. |
|-------------------------------------------------|-------------------------------------------------|-----------------|
| Cholesteryl oleate (mg/dl)                      | Cholesteryl linoleate (mg/dl)                    | CO/CL ratio     |
| Control (n = 5)                                 | 0.45 ± 0.01                                     | 0.15 ± 0.02     | 3.17 ± 0.36 |
| Pravastatin (30 mg/kg) (n = 5)                  | 0.13 ± 0.06*                                    | 0.41 ± 0.03*    | 0.31 ± 0.06* |

VLDL was isolated from rabbits which received pravastatin at 30 mg/kg for 4 days as shown in Table 1. Cholesteryl ester was extracted and the concentrations of cholesteryl oleate (CO) and cholesteryl linoleate (CL) were determined by HPLC analysis as described in the text. Values are mean ± SE. Significant difference: *p < 0.05 vs. control by Student’s t-test.
Pravastatin is linked with an increase in apoA-I production. These studies suggest that apoA-I production might be inversely correlated with the apoB production rate. In the present study, since pravastatin did not reduce VLDL production, judging from the VLDL protein secretion rate (Table 2) and serum TG levels (Table 1), we speculated that this agent also did not change the apoA-I production. It is considered therefore that pravastatin did not change the number of HDL particles, and since the CE in HDL decreased, HDL particle size became smaller in the JW rabbits.

In some experimental animals, HMG-CoA reductase inhibitors decreased the concentration of HDL cholesterol (6, 13–15). These different responses to pravastatin in the levels of HDL cholesterol in humans and experimental animals could be attributed to changes in apoB and apoA-I production, their lipoprotein profiles (13) and CETP activities (23).

We depicted the proposed mechanisms by which pravastatin decreased the concentration of HDL cholesterol in JW rabbits in Fig. 3. The consecutive administration of pravastatin to JW rabbits caused the secretion of HDL particles from the liver. After HDL particles were secreted from the liver, CE molecules were transferred from HDL particles to these HDL particles by CETP. Because of this replenishment of CE molecules, the concentration of HDL cholesterol was lowered at the expense of a declining pool of CE in HDL particles, whereas the concentrations of VLDL cholesterol remained unchanged. The present study addressed the mechanisms by which pravastatin decreases HDL cholesterol in JW rabbits.

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