Combined Effects of Pravastatin and Probucol on High-Density Lipoprotein Apolipoprotein A-I Kinetics in Cholesterol-fed Rabbits

Keijiro Saku, M.D. Ph.D., Rui Liu, M.S., Shiro Jimi, Ph.D.*
Kunihiro Matsuo, M.D., Kyosuke Yamamoto, M.D. Ph.D.**
Teruyoshi Yanagita, Ph.D.*** and Kikuo Arakawa, M.D. Ph.D.

The combined effects of pravastatin and probucol on high-density lipoprotein (HDL) apolipoprotein (apo) A-I kinetics in cholesterol (Ch)-fed rabbits were investigated. Japanese White rabbits were treated with 0.15% pravastatin and 0.5% Ch (group 1) or 0.15% pravastatin plus 1% probucol and 0.5% Ch (group 2) for 2 months. After treatment, the serum total cholesterol levels in groups 1 and 2 had significantly (p<0.01) increased (37.4±6.7 mg/dl vs 117.1±46.4 mg/dl, and 31.4±4.9 mg/dl vs 143.0±84.5 mg/dl, respectively). The serum HDL-cholesterol levels in both groups decreased (18.2±2.8 mg/dl vs 16.2±3.7 mg/dl, p<0.01 for group 1; 18.2±1.9 mg/dl vs 15.5±4.5 mg/dl, ns for group 2). Apo A-I kinetics were assessed by injecting 125I-labeled HDL intravenously into both groups of rabbits, and taking blood samples periodically for 6 days. Kinetic parameters calculated from apo A-I specific radioactivity decay curves showed that the apo A-I fractional catabolic rates in rabbits fed pravastatin and Ch (group 1) were significantly less than those in rabbits fed pravastatin plus probucol and Ch (group 2) (0.546±0.017 /day vs 0.730±0.126 /day, p<0.05), while the synthetic rate of apo A-I was lower in group 2 than in group 1 (14.76±1.71 mg/kg per day vs 11.21±2.38 mg/kg per day, respectively, p<0.1). These data indicate that pravastatin and probucol have different effects on HDL-apo A-I kinetics in a diet which includes cholesterol.

(Ipn Cire J 1995; 59: 292—298)

PRAVASTATIN is a potent competitive inhibitor of HMG-CoA reductase and has a cholesterol-lowering effect in both animals1,2 and humans3—8 Pravastatin was initially found as a minor metabolite of compactin (ML-236 B), and was later produced by microbial transformation of compactin using Nocardi astratrophica. It is superior to compactin in two ways: (1) the inhibitory activity of pravastatin in sterol synthesis is more potent than that of compactin, and (2) pravastatin has an organ selectivity, in that it inhibits sterol synthesis markedly in the liver and intestine, but only slightly in other organs, including those which produce hormones.

Pravastatin at 10—40 mg/day has been shown to reduce total cholesterol by 15—25%3—9 and to decrease low-density lipoprotein (LDL)-cholesterol by 16—39%3,7—9 It
is now available world-wide, as is the widely used hypolipidemic agent probucol, which has similar cholesterol-lowering effects. Increased levels of high-density lipoprotein (HDL) and its protein apolipoprotein (apo) A-I are also strongly linked to preventing the development of coronary atherosclerosis. While some researchers have reported that pravastatin has a greater effect than probucol on HDL and apo A-I levels,\(^{10}\) the mechanism by which probucol reduces HDL has been shown to be safe.

Under steady-state conditions, the rate of apo A-I synthesis is equal to that of its turnover. Therefore, stimulation of HDL-apo A-I synthesis may be a better method for enhancing reverse cholesterol transport than would be blocking HDL catabolism to raise serum HDL levels. In contrast, a decreased synthesis would result in a decreased transport of cholesterol from peripheral cells. Therefore, a drug which affected the synthesis of HDL apolipoprotein A-I may play an important role in the regression and progression of atherosclerosis. However, no previous study has evaluated the effect of pravastatin on HDL-apo A-I synthesis and catabolism in vivo.

In our previous investigation, we reported the effect of probucol on HDL-apo A-I kinetics in rabbits\(^{11}\) and found that probucol reduced HDL by two mechanisms; ie, it increased the fractional catabolic rate of apo A-I and reduced the synthesis of apo A-I. Clinically, the combined use of pravastatin and probucol is uncommon, but it would be interesting to know whether or not HDL levels reduced by probucol might be returned to pre-probucol levels with the addition of pravastatin. In this report, we investigated the effects of pravastatin and pravastatin plus probucol on apo A-I kinetics in vivo in the cholesterol-fed state.

**MATERIALS AND METHODS**

**Rabbits**

Male Japanese White normolipidemic adult rabbits (ages approximatley 6–8 months and weighing 2.3–2.6 kg) were obtained from Kyudo Co, Ltd, Fukuoka, Japan. All of the animals were housed individually with a 12-h light/dark cycle.

**Studied Groups**

Group 1 [Ch+Pra] (n=7), 0.15% pravastatin with 0.5% cholesterol diet for 2 months; group 2 [Ch+Pra+Pro] (n=7), 0.15% pravastatin plus 1% probucol with 0.5% cholesterol for 2 months. The treatment of the experimental animals was assessed and approved by the Ethics Committee of Fukuoka University.

**Preparation of Diet**

Pure pravastatin and probucol powder were added to basal rabbit chow (RC-4 with 0.5% cholesterol diet, Oriental Yeast, Tokyo) at the rate of 0.15% and 1%, respectively. No organic solvent was used. Crude fat and cholesterol contents of RC-4 were 3.0 g and 2 mg per 100 g of dry food, respectively. A cholesterol diet was prepared by adding 0.5% cholesterol to RC-4.

**Serum Lipids and Lipoprotein Analysis**

Changes in total cholesterol (TC) and triacylglycerol (TG) concentrations in serum and high-density lipoprotein fractions (d> 1.063 g/ml, isolated by ultracentrifugation using an RPL-42T rotor) were determined by enzymatic methods.\(^{12,13}\) Protein was determined by the method of Lowry et al.\(^{14}\)

**Isolation of High-Density Lipoproteins**

Fasting (12 h) venous blood samples were obtained from each group of rabbits. HDL (1.063<d<1.21 g/ml) was isolated and purified twice by preparative ultracentrifugation\(^{15}\) using a Hitachi SRP-50AT rotor in a Hitachi 70P-72 (Hitachi, Koki), and then dialyzed against saline buffer and stored at 4 °C.

**Iodination of High-Density Lipoproteins**

Isolated HDL was labeled with \(^{125}\)I-Na according to McFarlane’s method\(^{16}\) as modified by Bilheimer et al.\(^{17}\) and as previously described.\(^{18–20}\)

**Kinetic Study of HDL-apo A-I**

Four rabbits in each group were randomly selected for the kinetic study of HDL-apo A-I. To prevent sequestration of radioiodide resulting from apolipoprotein catabolism, 3 mg of NaI was injected into each rabbit at 12 h prior to, and again just prior to, the injection of labeled HDL. Each rabbit was given
TABLE I  CHANGES IN SERUM TC, TG, HDL-C AND HDL-TG LEVELS (mg/dl) BEFORE AND AFTER TREATMENT (Mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th></th>
<th>TG</th>
<th></th>
<th>HDL-C</th>
<th></th>
<th>HDL-TG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Group 1</td>
<td>37.4 ± 6.7</td>
<td>111.7 ± 46.4 **</td>
<td>37.3 ± 7.6</td>
<td>85.0 ± 24.7 **</td>
<td>18.2 ± 2.8</td>
<td>16.2 ± 3.7 **</td>
<td>17.9 ± 5.1</td>
<td>13.4 ± 4.6</td>
</tr>
<tr>
<td>Group 2</td>
<td>31.4 ± 4.9</td>
<td>143.0 ± 84.5 **</td>
<td>28.7 ± 4.7</td>
<td>62.6 ± 13.8 **</td>
<td>18.2 ± 1.9</td>
<td>15.5 ± 4.5</td>
<td>16.3 ± 2.0</td>
<td>13.6 ± 7.0</td>
</tr>
<tr>
<td>Ch only</td>
<td>34.6 ± 13.0</td>
<td>333.1 ± 1381.5 **</td>
<td>34.8 ± 9.8</td>
<td>128.2 ± 55.5 **</td>
<td>17.2 ± 2.7</td>
<td>24.5 ± 9.6</td>
<td>19.3 ± 1.7</td>
<td>24.4 ± 5.7</td>
</tr>
<tr>
<td>Ch + Pro</td>
<td>33.4 ± 15.8</td>
<td>843.6 ± 358.7 *</td>
<td>38.2 ± 20.0</td>
<td>66.8 ± 43.9</td>
<td>17.0 ± 3.9</td>
<td>10.0 ± 1.6</td>
<td>21.5 ± 4.2</td>
<td>21.8 ± 5.7</td>
</tr>
</tbody>
</table>

*p<0.1, *p<0.05, **p<0.01 (before vs after).
B, before treatment; A, after treatment; Ch, cholesterol; Pra, pravastatin; Pro, probucol. Group 1: 0.15% Pra with 0.5% Ch diet, n=7; Group 2: 0.15% Pra and 1% Pro with 0.5% Ch diet, n=7.
Ch only: 0.5% Ch diet (n=5), Ch+Pro: 1% Pro with 0.5% Ch diet (n=4): The data presented here were published in Biochimica et Biophysica Acta 1990 [Ref no.11]

50–60 μCi of 125I-labeled HDL via the marginal auricular vein. Blood samples were taken from the marginal auricular vein at 4 min, 6 h, 12 h, 24 h and then daily for 6 days. Blood was collected in tubes containing EDTA and the plasma was obtained by low-speed centrifugation. Two hundred microliters of plasma per tube was initially adjusted to a density (d) of 1.063 g/ml using solid KBr, placed in an ultracentrifuge tube (Ultra-Clear, Beckman Instrument, Palo Alto, CA), and spun at 40,000 rpm15 for 5 h using a Hitachi RPL-42T rotor. The bottom fraction was then collected and the density was raised to 1.21 g/ml. The tube was spun again and the top HDL fraction was collected. Two tubes were usually analyzed for each data point. Isolated HDL (1.063<d<1.21 g/ml) was then dialysed, delipidated with acetone/ethanol (1:1, v/v), and apo A-I was separated by 12% polyacrylamide gel disc electrophoresis (containing 1% sodium dodecyl sulfate)21. After 3–4 h of electrophoresis, a translucent line through the gel column at the position of apo A-I was visible. The distance of these lines from the bottom of the gels was measured and the gels were removed using a syringe needle (23G, 10 cm) with air pressure on a plastic wrap film to prevent contamination. The protein bands were then sliced into 2–3 mm sections, crushed in 0.60 ml of 50 mmol/L NH4HCO3 (pH 8.0), and incubated for 2 days at 4°C. Apo A-I was eluted in NH4HCO3 solution. The extracted protein was dialysed extensively against 50 mmol/L NH4HCO3 buffer. Protein concentration and radioactivity were measured in each sample and specific radioactivities were obtained19,20. These measurements were performed in duplicate or triplicate for each sample. Deviation of replicate specific activity from the mean was less than 2.5%.

Calculation of Kinetic Parameters
The specific radioactivity decay curves of HDL-apo A-I required two exponentials for adequate fitting. The residence time (reciprocal of fractional catabolic rate (FCR)) was calculated from the area under the decay curves, as previously described19,20. Total mass (TM) of apo A-I was calculated by the radioisotope dilution technique. Under steady-state conditions, the synthetic rate (SR) was calculated using the formula19,20: SR = (TM×FCR)/kg body weight.

Statistical Analyses
Statistical analyses were performed using the SAS software package at the Fukuoka University Computer Center.

We have previously published the results of an HDL-apo A-I kinetic study in rabbits fed either a 0.5% cholesterol diet (n=5) or a 0.5% cholesterol plus 1% probucol diet (n=4) for 2 months11. These data are shown in Tables I and II for comparison with the present apo A-I kinetic data in groups 1 and 2.

RESULTS
The initial mean serum concentrations of TC, TG, HDL-cholesterol (HDL-C) and HDL-TG, and those 2 months after treatment with pravastatin and probucol, are
shown in Table I. A significant (p<0.01) increase in serum TC was observed in both groups. At 2 months after the treatment, mean serum TC in group 2 [Ch+Pra+Pro] tended to be higher than that in group 1 [Ch+Pra], but this difference was not significant. Mean serum TG in both groups was also significantly (p<0.01) increased. Mean serum TG in group 1 [Ch+Pra] tended to be higher than that in group 2 [Ch+Pra+Pro] at 2 months, but this difference was not significant. Reductions in HDL-C were observed in both groups. The mean serum HDL-C in group 2 [Ch+Pra+Pro] was lower than that in group 1 [Ch+Pra], although this difference was not significant. Although reductions in HDL-TG were noted in both groups, the differences were not statistically significant.

Kinetic parameters of HDL-apo A-I in both groups are shown in Table II and Fig.1. The total mass (TM) in group 2 [Ch+Pra+Pro] was significantly (p<0.05) decreased, compared to that in group 1 [Ch+Pra]. The FCR in group 2 [Ch+Pra+Pro] was significantly (p<0.05) greater than that in group 1 [Ch+Pra]. The SR of apo A-I in group 2 [Ch+Pra+Pro] was smaller than that in group 1 [Ch+Pra].

Concentrations of pravastatin, pravastatin metabolic product and probucol were measured in sera taken just prior to the kinetic study. The levels of pravastatin and pravastatin metabolite in group 1 [Ch+Pra] were 521.07±269.04 ng/ml and 327.50±130.23 ng/ml, respectively. Unfortunately,
serum concentrations of pravastatin in group 2 [Ch + Pra + Pro] could not be obtained. The level of probucol in group 2 [Ch + Pra + Pro] was 4.26 $\pm$ 2.83 $\mu$g/ml.

**DISCUSSION**

In our previous investigation,$^{11}$ probucol had no effect on HDL kinetics (TM, FCR, SR) in rabbits fed a normal diet, but did produce remarkable changes in the kinetics of HDL-apo A-I in cholesterol-fed rabbits (Tables I and II). Therefore, in the current investigation, we studied the effects of pravastatin and probucol on HDL-apo A-I kinetics in rabbits which had been fed 0.5% cholesterol.

We previously reported that Ch feeding resulted in extremely high levels of serum TC, approximately 1,300 to 3,300 mg/dl, after 2 months. However, serum total cholesterol levels were reduced in pravastatin-treated groups (groups 1 and 2), even with cholesterol feeding (ie, total cholesterol levels after 2 months were 117.1 $\pm$ 46.4 mg/dl in rabbits fed [Ch + Pra] (group 1) and 143.0 $\pm$ 84.5 mg/dl in rabbits fed [Ch + Pra + Pro] (group 2) (Table I)), although these cholesterol levels were significantly greater than the pre-treatment levels. Such a drastic reduction in the level of serum TC, as compared to simple cholesterol feeding, has also been reported in cholesterol-fed rabbits after treatment with either simvastatin (10 mg/kg BW),$^{22,23}$ another HMG-CoA reductase inhibitor, or pravastatin (50 mg/kg BW) in WHHL rabbits, an animal model of familial hypercholesterolemia.$^{1,2}$ Since the dosage used in these other studies is not very different from that in our pravastatin experiments (47–49 mg/kg BW), these results taken together indicate that HMG-CoA reductase inhibitors significantly inhibit the increase in serum cholesterol in cholesterol-fed rabbits.

Many reports have shown that pravastatin (10–40 mg/day), in addition to reducing serum total cholesterol, increases serum HDL-C levels from 0 to 20% both in patients with familial hypercholesterolemia and in those with non-familial hypercholesterolemia.$^{5,8,9}$ The amounts of the major protein components of HDL, apo A-I and apo A-II, have been shown to increase by 12 to 16% with pravastatin therapy,$^{5,8,9,24}$ in rabbits from groups 1 and 2, HDL-C levels decreased by 11% ($p < 0.05$) and 15%, respectively (Table I), while in our previous study serum HDL-C levels in rabbits fed only 0.5% cholesterol increased by 42% ($p < 0.1$)$^{11}$ It is likely that the blunting of the rise in serum TC by pravastatin leads to a reduced need for reverse cholesterol transport, and hence lower HDL. The drastic reductions in serum TC and smaller reductions in HDL-C also suggest that pravastatin inhibits intestinal cholesterol absorption, as well as inhibiting HMG-CoA reductase. This hypothesis is supported by a report that another HMG-CoA reductase inhibitor, simvastatin, inhibits cholesterol absorption from the gastrointestinal wall in cholesterol-fed rabbits,$^{2}$ although blocked cholesterol absorption is not a feature of statin therapy in humans. However, pravastatin did not affect HDL-C levels in WHHL rabbits, in which hypercholesterolemia is endogenous.$^{1,2}$ It seems that the action of pravastatin in rabbits, especially with regard to HDL synthesis, may differ from that in man. Since apo A-I synthesis in rabbits occurs only in the intestine, and not in the liver,$^{25}$ the substantial inhibition of cholesterol absorption by pravastatin may explain the reduced HDL-C levels.

The major finding in our study is that the apo A-I FCR in rabbits fed [Ch + Pra + Pro] (group 2) is significantly higher than that in group 1 [Ch + Pra] (Fig. 1, Table II), but tends ($p < 0.1$) to be smaller than that in rabbits fed Ch plus Pro (0.928 $\pm$ 0.109/day in our previous investigation) (Table II)$^{11}$ No statistically significant difference in FCR was observed between group 1 [Ch + Pra] and rabbits fed only Ch (0.604 $\pm$ 0.067/day) (Table II)$^{11}$ The SR of apo A-I in rabbits fed [Ch + Pra] (group 1) tended to increase, compared to that in rabbits fed [Ch + Pra + Pro] (group 2). The SRs of apo A-I in groups 1 and 2 were significantly lower than those in Ch-fed rabbits, while they were greater than those in rabbits fed [Ch + Pro] (Table II). Since pravastatin drastically reduced serum TC levels (in both groups 1 and 2), compared to those in rabbits fed cholesterol alone or cholesterol plus probucol, relatively normal apo A-I FCRs (reported previously) might be maintained in group 1. This explains the slight reduction in
the apo A-I SR in group 1, compared to the apo A-I SR in normolipidemic rabbits fed normal chow or that in Ch-fed rabbits since SR was calculated as \( \text{TM} \times \text{FCR} \) of apo A-I\(^\text{19,20} \). The serum total cholesterol was also drastically reduced in group 2 (Ch + Pra + Pro), compared to that in rabbits fed cholesterol alone, but probucol reduced the total mass of apo A-I drastically, and increased the fractional catabolic rate of apo A-I, as in our previous report. This may explain why [Ch + Pra + Pro] (group 2) caused a lower (\( p < 0.1 \)) apo A-I SR than that in rabbits fed [Ch + Pra] (group 1). The serum concentration of probucol in group 2 was sufficient to affect HDL-apo A-I kinetics, based on our previous report\(^\text{11} \). These kinetic data showed that 1) serum cholesterol levels may play an important role in determining the apo A-I FCR, 2) the effect of pravastatin on HDL-apo A-I kinetics was different from that of probucol, and 3) the addition of pravastatin to probucol had no significant effects on serum HDL-C or apo A-I levels, which is similar to the recent findings observed in man after combined therapy with pravastatin and probucol\(^\text{26} \), although they produce different apo A-I kinetics. In addition, no significant relationship was observed between the kinetic parameters mentioned above and serum concentrations of pravastatin, pravastatin metabolic product or probucol in our study.

We previously reported that cholesterol-fed rabbits showed slight (+7.9\%) increased apo A-I synthesis (19.57±3.37 vs 18.14±3.75 mg/kg daily by normal chow) (Table II), which closely corresponded to increased intestinal apo A-I mRNA levels in cholesterol-fed rabbits\(^\text{27} \). Mitchell et al\(^\text{28} \) have reported that the HMG-CoA reductase inhibitor simvastatin decreased plasma apo A-I levels and increased hepatic mRNA levels in rats, and suggested that post-transcriptional events may regulate apo A-I synthesis. Serum HDL levels were regulated not only by apo A-I kinetic parameters, but also by cholesterol ester transfer protein (CETP)\(^\text{29} \). In the current investigation, we did not measure the changes in CETP mass or activity before and after drug treatment. Further investigation will be needed to clarify the effects of CETP activity on apo A-I kinetics.

In summary, the present results indicate that pravastatin and probucol have different effects on HDL-apo A-I kinetics in a diet which includes cholesterol. In particular, the increased FCR of apo A-I and reductions in apo A-I mass and synthetic rate were more prominent in the group treated with pravastatin and probucol than in the group treated with pravastatin alone.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan (No. 02671114, No.04671503), and from the Fukuoka University Research Fund (1992, 1993). We wish to thank Dr. Noboru Takami for his continuing interest in this project.

REFERENCES


insulin dependent diabetics (NIDDM) with pravastatin (CS-514). *Atherosclerosis* 1989; 75: 67–72


