Effects of Pravastatin on Cholesterol Metabolism in Watanabe Heritable Hyperlipidemic Rabbits

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Summary

Pravastatin, a competitive inhibitor of hydroxymethylglutaryl CoA reductase (HMG CoA reductase) is a potent hypocholesterolemic agent in humans as well as experimental animals, including the Watanabe heritable hyperlipidemic (WHHL) rabbit, lacking low density lipoprotein (LDL) receptor activity. We studied the effect of pravastatin on several aspects of cholesterol metabolism in WHHL rabbits. Cholesterol synthesis was measured by intraperitoneal injection of radioacetate and determination of its incorporation into the nonsaponifiable lipid fraction of liver, plasma, adrenal glands and gonads. A single dose of pravastatin (25 mg/kg) caused statistically significant inhibition of hepatic cholesterol synthesis at 2, 6, 12, and 24 hours following oral administration. By 48 hours, the inhibitory effect of the drug was no longer demonstrable. The pattern of radioactivity in the plasma was similar to that in the liver. The drug had no statistically significant effect on cholesterol synthesis in adrenal glands and gonads, suggesting a selective effect on the liver. Cholesterol absorption was studied after simultaneous oral administration of [3H] cholesterol and [14C] $\beta$-sitosterol. Pravastatin, 50 mg/kg for 10 days had no effect on fecal excretion of the radiolabelled steroids over 4 days. At 24 hours the plasma level of [14C] cholesterol was 1/3 that of control in pravastatin treated animals (p<0.05) but did not undergo an accelerated decline over 6 days. The activity of acyl CoA: cholesterol acyltransferase (ACAT) in intestinal mucosa and the concentration of hepatic cholesterol were similar in animals treated over one year with pravastatin 50 mg/kg/day or with placebo.

Our data do not allow us to make definitive conclusions about the effect of pravastatin on cholesterol absorption but are compatible with the hypothesis that the drug inhibits the hepatic synthesis as well as the
assembly of cholesterol into lipoproteins.

**Key Words:**
- Pravastatin
- Cholesterol synthesis and absorption
- Acyl CoA:acyltransferase
- Watanabe heritable hyperlipidemic rabbits
- (WHHL)

PRAVASTATIN (SQ 31,000) is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and reduces serum cholesterol levels in humans and animal models. In studies with Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of human familial hypercholesterolemia associated with low density lipoprotein (LDL) receptor deficiency and accelerated atherosclerosis, pravastatin 50 mg/kg/day caused a sharp drop in serum cholesterol in one month which persisted for 12 months. The final serum cholesterol values were 458±43 versus 309±24 mg/dl, *p*<0.005, in placebo and drug treatment groups, respectively. In humans and other animal models, the mechanism of action of pravastatin is thought to result primarily from increased LDL receptor mediated cholesterol clearance. In homozygous LDL receptor deficient WHHL rabbits such a mechanism is unlikely and other mechanisms accounting for pravastatin's effects should be considered. Traditionally, inhibitors of HMG-CoA reductase have been tested by assay of the enzyme exposed to the inhibitor, by *in vitro* studies where cultured cells or tissue slices are incubated with the inhibitor, or by *ex vivo* experiments where animals are given the drug and cholesterol synthesis is determined in incubated tissue slices or cells. These procedures have shortcomings inherent to the methods employed: enzyme assays are performed under optimum assay conditions that may not reflect the *in vivo* situation; *in vitro* incubation of cells or tissue slices do not account for variations in entrance of the inhibitor into cells or transformation of the inhibitors *in vivo*, and *ex vivo* experiments may overlook *in vitro* dilution of the inhibitor.

In *ex vivo* studies with pravastatin, Tsujita et al reported that inhibition of cholesterol synthesis in intact cells and in rat tissue slices was selective for tissues with high cholesterol turnover, showing over 90% inhibition in liver and intestine, 30% in kidney and only slight inhibition in the other tissues studied. More recently Mosley et al showed that pravastatin was more specific for rat liver in relation to lens than were lovastatin and simvastatin. Selectivity of HMG-CoA reductase inhibitors in the liver is considered to be a desirable feature since inhibition of cholesterol metabolism in other tissues could interfere with normal functions, such as steroid hormone production.
Therefore, we studied the in vivo effects of pravastatin in WHHL rabbits by measuring the incorporation of intraperitoneal radioacetate into cholesterol in various tissues, the fecal excretion and plasma decay curve of oral $[^{14}C]$ cholesterol, the activity of ACAT in intestinal mucosa and the hepatic concentration of cholesterol.

**Materials and Methods**

**Animals:**

Six to nine month old homozygous WHHL rabbits with mean serum cholesterol $\geq 410 \text{ mg/dl}$ (10.6 mM) and weighing between 2.7 and 3.4 kg maintained on reverse light cycle (1900 to 0700 hours light) were used in all experiments. Rabbits were fed a commercial rabbit chow (Lab Rabbit Chow HF 5326, Purina Ralston, Chicago, IL) and given water ad libitum in all studies.

**Time course study of in vivo cholesterol synthesis in various tissues:**

WHHL rabbits were divided into drug treated and control groups. For each time point 3 rabbits from each group were used. Pravastatin was administered orally by gavage at a single dose of 25 mg/kg suspended in 1 ml of 50% ethanol and care was taken to avoid regurgitation. Control animals were given 50% ethanol only. At 2, 6, 12, 24 and 48 hours after pravastatin or ethanol administration, animals were given an intraperitoneal injection of an aqueous solution of 400 $\mu$Ci $[^{14}C]$ sodium acetate (S.A. 12.6 mCi/mmol, Amersham, Arlington Heights, IL). Exactly 2 hours later animals were anesthetized with pentobarbital intraperitoneally. Blood samples were collected in EDTA from the abdominal aorta or the inferior vena cava. Immediately afterwards, the liver, adrenals and gonads were excised, rinsed, weighed, and quickly frozen in a dry-ice acetone bath and stored at $-70^\circ\text{C}$.

Cholesterol synthesis was determined by measuring the incorporation of the injected $2[^{14}C]$ sodium acetate into the non-saponifiable fraction (NSF). Adrenals (0.1 g—0.2 g), gonads (0.1 g—0.5 g) and liver (0.5 g) were finely minced and transferred to 50 ml glass stoppered tubes. Plasma (1 ml) was similarly prepared. Saponification and extraction of cholesterol were done by slight modifications of standard methods. Thin layer chromatography showed that between 70% and 85% of the radioactivity was associated with the cholesterol fraction. This fraction was similar in control and treated animals.
**Cholesterol absorption:**

Five WHHL rabbits were given a daily oral dose of 50 mg/kg body weight of pravastatin in saline by gavage for ten days between 8:30 and 9:30 a.m. (experimental). Control animals were given saline only. On day 10, after an overnight fast, rabbits were given pravastatin or saline and immediately after they were given orally 1 ml of an emulsion containing 5 µCi of 1, 2[3H]cholesterol and 1 µCi of 4[14C]β-sitosterol (S.A. 46 mCi/mmol and 56 mCi/mmol, respectively, Amersham). This emulsion was prepared by dissolving the radiolabelled material in corn oil and mixing it with a 6.8% solution of powdered skim milk in a ratio of 1:4. Emulsification was done by sonication for 15 min at a maximum setting (Branson Instruments, Inc., Stamford, CT). Pravastatin/saline treatment was continued until day 17. Feces collection was initiated on day 10, right after feeding the radiolabelled cholesterol and continued for 7 days.

Six weeks after the end of the study, the rabbits that were used as controls in the first experiment were given the drug and vice versa and the experiment was repeated. Experimental procedures were identical in both experiments. For the quantification of [3H] cholesterol and [14C] β-sitosterol excreted in feces, 1 g of dried powdered feces from days 1 to 4 was saponified as described above. The petroleum ether extract was then evaporated to dryness under nitrogen and redissolved in 1.0 ml of chloroform: methanol (2:1). Aliquots of this solution (0.2 ml) in duplicate were placed in combustion paper, air dried overnight and combusted in a sample oxidizer (Packard Instruments Co., Downers Grove, IL). Radioactivity was determined in a Packard model 4640 liquid scintillation counter. Counting of dual labelled samples was carried out using a program which allowed separate measurements of [3H] and [14C] radioactivity. For each sample the total amount of [3H] excreted in feces was corrected based on the amount of [14C] β-sitosterol recovered in feces.

**Plasma specific activity:**

After oral administration of radioactivity in the second experiment, blood samples were collected from the ear vein at 1, 2, 3 and 6 days to determine plasma [3H] cholesterol and total cholesterol. Plasma (0.1 ml) was mixed with 0.5 ml of Biosolve (Beckman Instruments, Inc.) in a liquid scintillation vial and then heated at 60°C for 1 hour. Solubilized samples were then cooled to room temperature, 15.0 ml of Hydrofluor added to each vial and [3H] radioactivity determined in the liquid scintillation counter. Cholesterol was determined by Ciba Corning Kit, Gilford Systems, Oberlin, Ohio. After correction for background radioactivity on the day of drug
administration, the specific activity was calculated from plasma [³H] cpm/plasma cholesterol.

**ACAT activity in intestine and liver:**

These studies were done on animals that were given a single daily dose of pravastatin (50 mg/kg) or saline for 1 year. Rabbits were fasted overnight and sacrificed between 0800 and 1000 hours by pentobarbital overdose through the ear vein. Liver and intestine were excised and used for measuring ACAT activity.

**Preparation of microsomes:** After excision the liver was weighed and placed in cold buffer I [0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), and 1.0 mM EDTA]. The small intestine from the jejunum to the proximal ileum was immediately removed and washed several times with cold saline. The intestine was then cut along the mesenteric side and the intestinal mucosa was scraped into a tube containing buffer I. Mucosal scrapings and a portion of the liver were homogenized in a glass pestle homogenizer and the homogenate was initially spun for 20 min at 10,000 g at 4°C and then spun for 60 min at 105,000 g at 4°C in a Beckman L8-55 ultracentrifuge. The resulting microsomal pellet was immediately collected and stored at −70°C until needed.

**Determination of ACAT activity:** Microsomal ACAT activity was determined by a modification of the method described by Heider et al. Measuring the incorporation of [¹⁴C]-oleyl CoA into cholesteryl oleate ester. The assay mixture consisted of 70 μl of 0.1 M phosphate buffer (pH 7.4), 0.2 μmoles DTT, 0.1 μmoles ATP, 0.5 μmoles MgCl₂, 33 μmoles fatty acid free bovine serum albumin (BSA) and 0.1 mg of microsomal protein in a final volume of 0.2 ml. The assay mixture was pre-incubated for 30 min in a shaking water bath at 37°C. The reaction was then initiated by the addition of 3.0 nmol [¹⁴C]-oleyl CoA (S.A. 52 mCi/mM, Amersham) and was allowed to continue for 60 min. The reaction was terminated by the addition of 1 ml of chloroform: methanol (2:1, v/v). The lipid layer was extracted and fractionated on precoated silica gel 60 thin-layer chromatography sheets (layer thickness 0.2 mm, Alltech Associates, Inc., Deerfield, IL) and developed in heptane: diethyl ether: acetic acid (85:15:2). After visualization by iodine vapors, the labelled cholesteryl oleate bands were cut out and counted in 10.0 ml of Hydrofluor in a liquid scintillation counter. ACAT activity was expressed in pmol [¹⁴C]-cholesteryl oleate formed/mg protein/min.
Fig. 1. Effect of pravastatin on the \textit{in vivo} incorporation of $2^{[14C]}$ sodium acetate into the non-saponifiable fraction (NSF) in plasma and liver. Rabbits were given pravastatin or carrier by gavage at zero time and given an intraperitoneal injection of labelled acetate at the specified times and sacrificed 2 hours later. Bars represent mean±SEM of triplicate assays. Number in parentheses represents the number of animals. Values are expressed as percent of counts obtained from control animals (baseline), which were determined by grouping values from the animals given carrier only. Baseline values were: liver $16,500±2,100$ cpm/g wet weight ($\bar{x}$±SEM), $n=11$, and plasma $8,500±1,500$, $n=11$. Data were analyzed by a two-way ANOVA. *P<0.05.

**Determination of liver cholesterol:**
A half gram of liver tissue obtained from rabbits treated with saline/pravastatin for 1 year was saponified according to the method described above for \textit{in vivo} cholesterol synthesis. The final residue from the non-saponifiable fraction was dissolved in $900\,\mu l$ of heptane containing $5\alpha$ cholestane ($1\,mg/ml$) for further quantification using gas chromatography (model 3400, Varian Instrument Group, Walnut Creek, CA). The samples were run on a nickel column ($1.8\,m\times2\,mm$) packed with $3\%$ ov-17 on a 100–200 mesh gas chrom Q. The column temperature was set to $260^\circ C$, injection temperature at $290^\circ C$ and detector temperature at $300^\circ C$.

**Statistical analyses:**
Data were compared between groups by analysis of variance. Where appropriate, data were compared by independent t test. All analyses were performed using a SAS statistical software package (SAS Institute, Gary, IN).\textsuperscript{20}

**RESULTS**

**Time course of inhibition of cholesterol synthesis:**
\textit{In vivo} incorporation of $[14C]$ acetate into the NSF was significantly suppressed in liver and plasma of treated rabbits when compared to control
A significant suppression varying from 76 to 50% was observed in liver from 2 hours to at least 24 hours following drug administration. At 48 hours the radioactivity had reached values slightly above the baseline. However, the rebound was not statistically significant. The changes in radioactivity in plasma cholesterol were similar to that in liver. The highest rate of cholesterol synthesis in WHHL rabbits was found in the adrenal \[36,400\pm6,500 \text{ cpm/g (n=9)}\] wet weight compared to the liver \[16,500\pm2,100 \text{ (n=11)}\]. There was no suppression in acetate incorporation in the adrenals at 2 hours. Some suppression was observed 6 hours after administration of pravastatin and continued for up to 24 hours. These effects were not statistically significant. Pooling the adrenal data over 24 h showed a 31\% suppression. The changes in gonadal incorporation at various times were not statistically different from controls. Due to technical difficulties we could not perform determinations in gonads at 2 hours. Pooling of the data for 6, 12 and 24 hours showed a suppression of 2\% compared to 64\% and 70\% for liver and plasma.

**Cholesterol absorption:**

\[^{14}\text{C}\] label was not detected in plasma following oral administration of \[^{14}\text{C}\] \(\beta\)-sitosterol, indicating that \[^{14}\text{C}\] \(\beta\)-sitosterol was not degraded or absorbed. Based on fecal recovery of \[^{14}\text{C}\] \(\beta\)-sitosterol, the absorption of \[^{3}\text{H}\] cholesterol was \(68.2\pm3.7\%\) and \(59.6\pm4.4\%\) in control and pravastatin treated animals, respectively (n=10, NS). When rabbits were crossed over from the control group to the experimental and vice versa, they behaved
Fig. 3. Specific activity of [3H] cholesterol in plasma following oral administration of emulsion containing 5 μCi 1, 2[3H] cholesterol and 1 μCi 4[C14] β-sitosterol. Rabbits were receiving pravastatin (50 mg/kg) for the previous 10 days and continued to receive the drug throughout the study. Blood samples were taken on days 1, 2, 3 and 6 following administration of the emulsion. Each data point represents ±SEM, n=5. *P<0.05.

Table I. Effects of Pravastatin on Hepatic Cholesterol, and Microsomal ACAT Activity in Mucosa and Liver*

<table>
<thead>
<tr>
<th></th>
<th>Control (N)</th>
<th>Pravastatin (50 mg/kg for 1 yr)</th>
<th>P</th>
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<tbody>
<tr>
<td>Mucosal ACAT (pmoles/mg protein/min)</td>
<td>5.25±1.46 (7)</td>
<td>3.28±0.7 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Hepatic ACAT (pmoles/mg protein/min)</td>
<td>1.50±0.41 (8)</td>
<td>1.05±0.21 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Hepatic cholesterol (mg/g)</td>
<td>2.75±0.53 (6)</td>
<td>2.40±0.46 (10)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Results are expressed as mean±SEM for (N) rabbits in each group. All assays were done in triplicate.

WHHL rabbits were given pravastatin (50 mg/kg/body weight) or saline in a single daily dose for 1 year. Tissues were obtained as described in Materials and Methods.

N=number of animals; NS=statistically non-significant.

especially in the same manner.

Twenty-four hours after oral administration of [3H] cholesterol, plasma specific activity was 3 fold higher in control animals than in the pravastatin treated rabbits [p<0.05] (Fig. 3). Specific activity remained essentially unchanged in treated WHHL rabbits from days 1 to 6 in contrast to the gradual decline seen in untreated controls.

Pravastatin decreased mean intestinal and liver ACAT activity in WHHL rabbits given the drug for 1 year by 38% and 30%, respectively, compared to control animals but these changes did not reach statistical significance (Table I). Drug treatment for 1 year did not alter hepatic cholesterol content (Table I).
DISCUSSION

Large interanimal variations were observed in these studies, similar to the experience of other investigators with WHHL rabbits. The rates of incorporation of $^{14}$C acetate into NSF in the adrenals and liver are consistent with studies by Dietschy et al using intravenously administered radio-labelled $\text{H}_2\text{O}_2$ to assess cholesterol synthesis and indicate a similar tissue distribution of these tracers. Our data show that oral administration of pravastatin significantly reduced in vivo cholesterol synthesis in the liver of WHHL rabbits by 76% in 2 hours following drug administration. Tsujita et al obtained similar results studying the conversion of $^{14}$C acetate to sterols in freshly isolated rat hepatocytes under in vitro conditions and 2 hours after oral administration of 25 mg/kg. Pravastatin action was consistent in both experiments indicating that the effect of this dosage in vivo was not due to expansion of the acetate pool which would lower the specific activity of $^{14}$C acetyl CoA converted to cholesterol. Recently Koga et al using in vivo methods similar to ours showed that pravastatin dosages of 5 and 20 mg/kg in mice caused a 75 to 91% suppression of $^{14}$C acetate incorporation into sterol in liver in 1 hour, which is comparable to our data in WHHL rabbits.

Dietschy et al showed that in WHHL rabbits the liver is the major contributor to whole body sterol synthesis. Thus, the levels of radioactive sterols we observed in plasma reflect primarily the changes in hepatic synthesis. The plasma data are consistent with less hepatic cholesterol available for incorporation into lipoproteins but do not preclude the possibility that lipoprotein assembly was also impaired. The effect of pravastatin lasted for 24 hours with a slight, non-significant rebound in incorporation observed by 48 hours (18 and 39% above baseline levels for liver and plasma, respectively). The possibility that a greater rebound occurred between 24 and 48 hours cannot be ruled out. This compensatory effect is probably due to an increase in enzyme protein as reported by others.

Pravastatin’s inhibitory action over 24 h is remarkable since the drug has been shown to have a $T_{1/2}$ in plasma of 1.5–2.0 hours in all species studied. If the action of the drug were that short-lived we could expect an increase in cholesterol synthesis once the drug is cleared, since Ma et al have shown that mevinolin, another HMG-CoA reductase inhibitor, produces a 10-fold increase in mRNA levels for the enzyme. The reason for this prolonged action is unknown. It may be due to binding of the drug to the cell membrane or to the enzyme. Mosley et al showed that two washes of liver obtained from rats 2 hours after an oral dose of the drug had no ef-
fect on the inhibitory action of pravastatin, supporting the concept that the
drug attaches to the cell surface.

The effects of pravastatin in the adrenals are not readily understood. The 31±5% suppression over 24 hours can be due to direct inhibitory effects of pravastatin on cholesterol synthesis or may reflect the decreased radioactivity in plasma lipoproteins taken up by the adrenal during this period. Significant reduction in hepatic cholesterol synthesis and output of labelled lipoproteins into plasma were noted 2 hours after pravastatin treatment while no changes were noted in the adrenals. These observations infer that pravastatin, at least initially, has a preferential effect on cholesterol synthesis in the liver rather than on the higher rate of cholesterol synthesis observed in the adrenals of WHHL rabbits. Our data also suggest that pravastatin does not have a significant effect in the gonads.

Our results agree with those of Tsujita et al\textsuperscript{1} who showed greater specificity of the drug for hepatocytes. Mosley et al\textsuperscript{17} recently reported studies which showed that pravastatin was 100 fold less potent in suppressing cholesterol synthesis in lens than in liver. Both of these studies were performed \textit{ex vivo} which subjected them to nonphysiological conditions.

To determine if pravastatin interferes with cholesterol absorption, we employed the same methods used by Ishida et al in Japanese white rabbits.\textsuperscript{24} These investigators found that repetitive doses of simvastatin, a derivative of lovastatin, were effective in inhibiting cholesterol absorption in rabbits fed a 1% cholesterol diet and that this effect was related to a reduction in ACAT activity in intestinal microsomes.\textsuperscript{25} However, cholesterol absorption and ACAT activity were not changed when the same dose was given to rabbits fed a regular chow diet. In our study, pravastatin caused a slight (13%), non-significant reduction in cholesterol absorption in WHHL rabbits, based on fecal recovery of $[^3]$H-cholesterol.

We also measured the appearance of radioactivity in blood following the oral administration of $[^3]$H cholesterol and $[^14]$C $\beta$-sitosterol. Though the mean plasma cholesterol between control and treated animals did not differ over the study period, we expressed the appearance of radioactivity as specific activity to account for differences in plasma cholesterol in individual animals. Rabbits treated with pravastatin had significantly lower plasma specific activity of $[^3]$H cholesterol 24 hours after its oral administration (Fig. 3). We had anticipated no difference, based on our recovery of radio-cholesterol in feces and the results of Ishida et al with simvastatin in Japanese white rabbits.\textsuperscript{24}

To clarify further pravastatin's effects on cholesterol absorption, we turned to our recently published concurrent experiment in which WHHL
rabbits received the drug for 1 year\(^{13}\) and determined pravastatin's action on intestinal and hepatic ACAT activity and liver cholesterol concentration. ACAT catalyzes the esterification of cholesterol with fatty acids and is thought to have an important regulatory role in cholesterol absorption in the intestinal cell.\(^{26}\) Heider et al have shown that acyl amides which inhibit ACAT activity also inhibit cholesterol absorption in rabbits.\(^{19}\) Field et al showed a direct correlation between intestinal ACAT activity and cholesterol absorption.\(^{27}\) Moreover, hepatic ACAT activity and cholesterol content are also increased by cholesterol feeding.\(^{28}\) The reductions in intestinal and hepatic ACAT activity and hepatic cholesterol content which we observed were not statistically significant and were consistent with the findings of our fecal study that pravastatin has no significant effect on cholesterol absorption. Thus, under similar dietary conditions our results are consistent with the studies of Ishida et al\(^{25}\) of ACAT activity.

Since our data suggest that cholesterol absorption is not significantly affected by pravastatin, the lower specific activity of \(^{3}\)H cholesterol at 24 hours in Fig. 3 might be explained by rapid tissue clearance of lipoproteins containing the absorbed label. In a review Tsujita and Watanabe\(^{29}\) have published the preliminary data of Shiomi et al\(^{30}\) which show that pravastatin alone or in combination with cholestyramine treatment increases hepatic LDL receptor activity in WHHL rabbits. If this mechanism were functioning in our rabbits one would expect an accelerated decline of \(^{3}\)H radioactivity in the pravastatin animals over days 1 to 6. This is not shown in Fig. 3, suggesting that clearance of cholesterol containing lipoproteins might not be accelerated by pravastatin in our animal model. Thus, alternative mechanisms such as resecretion of the label in lipoproteins or decreased assembly of cholesterol into lipoproteins need be considered. Arad et al\(^{31}\) and Wyne et al\(^{32}\) have shown that inhibition of HMG CoA reductase alters synthesis of apolipoprotein B and E. These results support prior studies associating HMG-CoA reductase inhibitors with reduced production of apolipoprotein B.\(^{33},34\) Reduced availability of apolipoproteins could reduce the incorporation of cholesterol into lipoproteins secreted into plasma and explain the decrease in plasma cholesterol label we observed with pravastatin treatment. Clearly, more studies are needed to determine the possible effect of HMG-CoA reductase inhibitors on cholesterol absorption.

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