Mode of Action of Probucol in Reducing Serum Cholesterol in Mice

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Accepted September 20, 1985

Abstract—The mode of action of probucol in reducing serum cholesterol was studied in normal and cholesterol-fed mice. Probucol did not affect intestinal absorption of radioactive cholesterol in normal and cholesterol-fed mice. In normal mice, probucol treatment resulted in inhibition of incorporation of [14C]-acetate into cholesterol in the liver, while it stimulated the incorporation in the small intestines. Incorporation of [14C]-mevalonate into cholesterol was not affected by the treatment. These results were consistent with the finding that the HMG-CoA reductase activity was decreased in the liver, but increased in the intestinal tissues of the treated mice. In cholesterol-fed mice, probucol treatment had no effect on cholesterol synthesis in the liver, while it increased the intestinal cholesterol synthesis. The over-all effect of this drug on cholesterol synthesis was not significant, although it tended to be inhibitory in normal mice and stimulatory in cholesterol-fed mice. On the other hand, probucol treatment resulted in acceleration of the clearance of [14C]-cholesterol-derived radioactivity from the circulation and resulted also in a significant increase in fecal excretion of the radioactivity, cholesterol and bile acids without changes in lipid composition of the bile. Cholesterol content in and radioactivity distribution among the tissues were not affected by probucol. Hepatic cholesterol 7α-hydroxylase activity was increased by probucol. These findings indicate that probucol lowers serum cholesterol mainly by increasing catabolic excretion of cholesterol into bile.

Probucol is a potent hypocholesterolemic agent in many animal species including man. Although there are a number of papers describing the cholesterol lowering action of this agent in pharmacological and clinical studies, the mechanism of its action has not been well defined (1–8). The results of some studies with animal and human subjects suggested a possible involvement of stimulation of catabolic excretion of cholesterol (9–11) and inhibition of hepatic cholesterol synthesis at early stages (9, 10, 12), although with some variation (4, 10, 12), in the cholesterol-lowering action of probucol. However, other studies indicated a significant reduction of fecal excretion of neutral and acid sterols together with decrease in hepatic cholesterol 7α-hydroxylase activity by treatment (12). Impaired intestinal absorption of cholesterol may contribute, to some extent, to reduction of plasma cholesterol by this agent (9, 10).

More studies are required for clarifying these conflicting findings and for better understanding the mode of action of probucol. The present paper describes the effect of probucol on intestinal absorption of cholesterol, hepatic and intestinal cholesterol synthesis and catabolic excretion of cholesterol in normal and cholesterol-fed mice, under the conditions where their serum cholesterol was significantly reduced by probucol treatment.

Materials and Methods

Animals: Animals used were age-matched male STD:ddY mice (4 weeks old at the start of experiment, Shizuoka Agricultural Co-
operative Association for Laboratory Animals, Shizuoka). These mice were fed on a standard laboratory mouse chow (Funabashi Farm, Ltd., Funabashi). Hypercholesterolemia was induced by feeding a high cholesterol diet composed of 1% cholesterol, 0.5% cholic acid, 5% olive oil and 93% standard mouse chow (Funabashi Farm, Ltd.). Probucol (Lot no. 634N, Dow-Lepetit-Japan Co., Ltd., Tokyo) was dissolved in olive oil at 40 mg/ml and administered by a stomach tube at a dose of 400 or 800 mg/kg-day. Control animals received olive oil alone (10 or 20 ml/kg).

**Chemicals:** Sodium [1-14C]-acetate (1.9 mCi/mmol), DL-[2-14C]-mevalonate (DBEB salt, 40.8 mCi/mmol), [4-14C]-cholesterol (54.0 and 52.5 mCi/mmol) and DL-[glutaryl-3-14C]-3-hydroxy-3-methylglutaryl CoA (57.6 mCi/mmol) were obtained from New England Nuclear, Boston. Other chemicals used were of reagent grade.

**Intestinal absorption of cholesterol:** Intestinal absorption of exogenous cholesterol was studied according to Iritani and Nogi (13) by measuring radioactivity of serum samples taken consecutively after oral administration of [14C]-cholesterol. The labeled cholesterol was suspended in 0.2% Tween 80 at 1 µCi (25 mg)/ml and was given to mice at a dose of 0.4 µCi/mouse after overnight fasting. In the experiments with cholesterol-fed mice, the animals were not fasted before the test. Blood samples were taken consecutively from the tail vein for determination of radioactivity and cholesterol content in serum.

**Biosynthesis of cholesterol:** This was studied by measuring incorporation of radioactive precursors into cholesterol in the liver and small intestines. [14C]-Acetate (100 µCi/kg) or [14C]-mevalonate (50 µCi/kg) was intravenously injected to mice as a saline solution (10 ml/kg). The animals were sacrificed 1 hr after the injection of the labeled precursors to obtain serum, the liver and the whole small intestines. Lipids were extracted from serum and the tissues by mixing or homogenization with 20 volumes of acetone-ethanol (1:1. v/v) according to Schoenheimer (14). Aliquots of the extracts were spotted on TLC plates (Kiesel gel 60F254: Merck, Darmstadt), and major lipid classes were separated using a solvent of n-hexane-diethyl ether-acetic acid (73:25:2. v/v/v). The lipid spots were visualized by spraying with 0.03% rhodamine 6G, and those corresponding to free cholesterol and cholesterol ester were separately scrapped into scintillation vials. One ml of methanol and 15 ml of liquid scintillation mixture (dioxane scintillator) were added, and the radioactivity was measured by a liquid scintillation spectrometer. Total cholesterol was also determined by the method of Allain et al. (15).

**Fecal excretion and tissue distribution of cholesterol:** Mice were intravenously injected with [14C]-cholesterol (100 µCi/kg) which was dissolved in 20% ethanol in saline at 100 µCi/10 ml. Blood samples were taken from the tail vein of each mouse before and consecutively after the injection of [14C]-cholesterol for determination of radioactivity and cholesterol concentration in serum. All feces were collected separately from each animal for 6 days (normal mice) or 5 days (cholesterol-fed mice) after the cholesterol injection for determination of fecal excretion of radioactivity, cholesterol and bile acids. Then the mice were sacrificed for determination of radioactivity and cholesterol content in the tissues including the liver, kidneys, whole small intestines, lungs, spleen, heart and aortas. Fecal bile acids and neutral sterols were extracted according to Grundy (16) and determined enzymatically (17) and colorimetrically (18), respectively. Fecal radioactivity was measured by the combustion method. Total lipids in tissues were extracted according to Schoenheimer et al. (14). Cholesterol and radioactivity were determined by colorimetry (18) and liquid scintillation spectrometry, respectively.

**Assay of HMG-CoA reductase and cholesterol 7α-hydroxylase:** The liver and the small intestine were homogenized with 10 volumes of 0.25 M sucrose-0.01 M Tris buffer, pH 7.4. The homogenates were subjected to ultracentrifugation to obtain microsomes according to Shefer et al. (19). The microsomal fraction was resuspended in the buffer solution. HMG-CoA reductase activity of hepatic and intestinal microsomes was assayed by the method of Shapiro et al. (20) using the radioactive substrate, R,S-
[3-14C]-HMG-CoA. Cholesterol 7α-hydroxylase activity in the liver microsomes was assayed according to Lakshmanan and Veech (21) with the radioactive substrate [4-14C]-cholesterol (specific activity 52.5 mCi/mmole). The radioactive products were separated on TLC plates (Silica gel G, Merck, Darmstadt). The activity was expressed as pmoles of a product formed per min by 1 mg microsomal protein. Protein was determined by the method of Lowry et al. (22).

Analysis of data: Statistical significance of data was analyzed by Student's t-test. Data are presented as the mean±S.E.

Table 1. Effect of probucol on intestinal absorption of [14C]-cholesterol in normal mice

| Time (hr) after [14C]-cholesterol ingestion | 3     | 6     | 24    |
|-------------------------------------------|-------|-------|-------|
| Serum radioactivity (10^5 dpm/ml)         |       |       |       |
| Control                                   | 1.70±0.31 | 4.52±0.33 | 11.40±0.80 |
| Probucol (400 mg/kg)                      | 1.92±0.32 | 4.69±1.10 | 8.37±0.56** |
| Serum cholesterol (mg/dl)                 |       |       |       |
| Control                                   | 203.2±12.5 | 229.0±11.3 | 201.5±7.9 |
| Probucol (400 mg/kg)                      | 117.9±11.3** | 143.6±15.2** | 104.4±6.4** |

The values are the mean±S.E. of 8 mice. **P<0.01 versus control. The mice received daily oral doses of probucol or vehicle for 7 days. [14C]-Cholesterol was given orally 1 hr after the last dose.
of probucol, and their incorporation into cholesterol was measured in the liver and the small intestines. As shown in Table 3, probucol treatment resulted in a significant reduction of incorporation of \([^{14}C]\)-acetate into cholesterol in the liver, whereas it contrastively stimulated the incorporation of the labeled acetate into cholesterol in the intestines. Probucol did not affect the incorporation of \([^{14}C]\)-mevalonate into cholesterol in both tissues. In these experiments, there was no difference in specific radioactivity of cholesterol in serum between the treated and the control groups.

In the cholesterol-fed mice, endogenous cholesterogenesis was suppressed by about 90% in the liver and by about 50% in the small intestines (Table 4). Under these conditions, as shown in Table 4, probucol treatment at a daily dose of 800 mg/kg for 7 days no longer affected the incorporation of \([^{14}C]\)-acetate into cholesterol in the liver, but it still stimulated cholesterol synthesis in the small intestines. In this batch of experiments, probucol (800 mg/kg) was found again to inhibit cholesterol synthesis in the liver and to stimulate it in the intestines in normal mice (Table 4).

In all these experiments, tissue cholesterol concentration was not affected by probucol, except for serum cholesterol levels which were significantly lower in the treated mice than in the control mice.

Since probucol exerted dural effects on cholesterol synthesis in mice, inhibition in the liver and stimulation in the small intestines.
Table 4. Effect of probucol on incorporation of $[^14\text{C}]$-acetate into cholesterol in cholesterol-fed and normal mice

|                      | Cholesterol specific activity$^a$ | Serum cholesterol$^b$ |
|----------------------|-----------------------------------|-----------------------|
|                      | Liver | Intestine | Serum  |                  |                      |
| Cholesterol-fed mice  |       |           |        |                  |                      |
| Control              | 0.49±0.11 | 3.62±0.25 | N.D.$^c$ | 317.5±20.5      |
| Probenecid           | 0.85±0.14 | 6.76±0.91* | N.D.   | 212.8±22.8**    |
| Normal mice          |       |           |        |                  |                      |
| Control              | 6.05±0.89 | 6.97±0.52 | N.D.   | 185.7±5.2       |
| Probenecid           | 1.79±0.30** | 16.23±0.99** | N.D.   | 75.6±6.9**      |

The values are the mean±S.E. of 10 mice. *P<0.05, **P<0.01 versus respective control. The mice received daily oral doses of probucol (800 mg/kg) or vehicle for 7 days. $[^14\text{C}]$-Acetate was intravenously injected 5 hr after the last dose. Incorporation of the radioactivity into cholesterol was determined 1 hr after the $[^14\text{C}]$-acetate injection. $^a$ 10$^{-2}$ dpm/mg, $^b$ mg/dl, $^c$ not determined.

Table 5. Over-all effect of probucol on incorporation of $[^14\text{C}]$-acetate into cholesterol in whole liver and intestine in mice

|                      | Normal mice | Cholesterol-fed mice |
|----------------------|-------------|----------------------|
|                      | Control     | Probenecid (400 mg/kg) | Control     | Probenecid (800 mg/kg) | Control     | Probenecid (800 mg/kg) |
|                      |             | Control (800 mg/kg) |             |                         |             |                      |
| Cholesterol labeled by $[^14\text{C}]$-acetate (10$^{-2}$ dpm/organ)$^a$ |             |                        |             |                         |             |                      |
| Liver                | 49.44±10.63 | 27.95±3.56            | 49.16±7.83  | 17.76±3.07**            | 14.22±3.33  | 14.59±2.25            |
| Intestine            | 25.10±2.38  | 34.19±3.61*           | 8.81±1.43   | 17.89±1.24**            | 3.80±0.20   | 6.28±1.07             |
| Sum                  | 74.54±12.05 | 62.13±5.39            | 57.98±8.49  | 35.65±3.76*             | 18.02±3.21  | 20.80±2.50            |
| % of control         | 83.35       | 61.49                 |             |                         |             | 115.43                |

*P<0.05, **P<0.01 versus respective control. $^a$ Figures were calculated from the data in Tables 3 and 4 and each organ weight.
The over-all effect of this drug was calculated in each mouse. As shown in Table 5, there was no significant difference in the sum of the incorporation of \([^{14}C]\)-acetate into cholesterol in the whole liver and intestines between the probucol-treated (400 mg/kg in normal and 800 mg/kg in cholesterol-fed) and the control groups, although the over-all effect tended to be inhibitory in normal mice and stimulatory in cholesterol-fed mice. At a high dose of probucol (800 mg/kg), the over-all effect was found to be significantly inhibitory in normal mice.

Effect of probucol on catabolic excretion of cholesterol: Probucol was administered to normal mice at a daily oral dose of 400 mg/kg for 9 days. \([^{14}C]\)-Cholesterol was intravenously injected on the third day. As shown in Fig. 1a, serum cholesterol levels were significantly decreased to reach a bottom level (87.4±5.0 mg/dl) on day 5, which remained low thereafter. Serum radioactivity derived from the injected \([^{14}C]\)-cholesterol was significantly lower at every point of measurement (1, 3, 5, 20, 28, 48, 72 and 120 hr after \([^{14}C]\)-cholesterol injection) in the treated mice than in the controls (Fig. 1b). There was no significant difference in serum radioactivity within 50 min (3, 10, 20, 30, 40 and 50 min) after \([^{14}C]\)-cholesterol injection between the 2 groups (data not shown). Half life times of radioactivity decay in serum were calculated to be 31.9±1.3 hr for the treated group and 37.8±1.2 hr for the control group (P<0.01).

Table 6 shows fecal excretion of radioactivity, cholesterol and bile acids for 6 days after \([^{14}C]\)-cholesterol injection. Fecal cholesterol was significantly increased in the treated group, but there was no significant difference in fecal bile acids and radioactivity between the 2 groups, although a slight increase in fecal radioactivity was observed in the treated group. At sacrifice of animals, there was no significant difference in cholesterol content in and radioactivity distribution among various tissues including the liver, kidneys, small intestines, lungs, spleen, heart and aortas between the 2 groups (data not shown).

Another experiment was carried out with cholesterol-fed mice. Mice were maintained on the high cholesterol diet for 3 weeks. They were then divided into 2 groups of 8 mice each and fed on normal laboratory chow for 5 days thereafter. One group of mice was orally given probucol at a daily dose of 800 mg/kg, and the other was given vehicle (olive oil, 20 ml/kg). At the time of diet exchange, \([^{14}C]\)-cholesterol was intravenously injected. As shown in Fig. 2, serum

![Fig. 1](image-url)
cholesterol levels were maintained high during feeding the high cholesterol diet and were gradually decreased after the diet was exchanged to normal chow. The decrease in serum cholesterol levels was significantly greater in the probucol-treated group than in the control. Serum radioactivity derived from the injected [14C]-cholesterol was decreased significantly faster in the treated mice with a half life time of 26.9±1.2 hr than in the control mice with a half life time of 38.0±1.7 hr (n=8, P<0.01) (Fig. 3). Fractional removal rate of serum cholesterol was calculated to be 0.015±0.002 hr⁻¹ and 0.012±0.002 hr⁻¹ (P<0.05) for the treated and the control mice, respectively.

Table 7 shows fecal excretion of radioactivity, total cholesterol and total bile acids which were all significantly increased in the treated mice. At sacrifice of the animals, there was no significant difference in cholesterol content in various tissues including the liver, kidneys, small intestines, lungs, spleen, heart and aortas between the 2 groups.

Effect of probucol on HMG-CoA reductase and cholesterol 7α-hydroxylase activities: Probucol was orally administered to normal mice at a daily dose of 400 mg/kg for 7 days. About 20 hr after the last dose, blood samples

**Table 6. Effect of probucol on fecal excretion of the radioactivity, cholesterol and bile acids in normal mice**

|                          | Control                  | Producol (400 mg/kg) |
|--------------------------|--------------------------|----------------------|
| Radioactivity (10⁻⁶ dpm/6 days) | 12.10±0.66               | 13.98±0.74           |
| Cholesterol (mg/6 days)   | 21.72±1.30               | 29.14±1.62*          |
| Bile acids (mg/6 days)    | 19.64±2.11               | 18.00±0.49           |
| Fecal mass (g/6 days)     | 4.80±0.21                | 4.66±0.22            |

The values are the mean±S.E. of 8 (control) and 7 mice (producol). *P<0.05 versus control. Probucol was orally given at 400 mg/kg-day for 9 days. [14C]-Cholesterol was intravenously injected after the 3rd dose of probucol or vehicle. Feces were collected for 6 days after [14C]-cholesterol injection.
were taken for determination of serum cholesterol, and the animals were sacrificed to obtain the liver and small intestines for assay of microsomal HMG-CoA reductase and cholesterol 7α-hydroxylase activities. As shown in Table 8, HMG-CoA reductase activity was decreased by 30% in the liver, but increased by 53% in the intestines from

![Graph showing changes in serum radioactivity](image)

**Fig. 3.** Changes in serum radioactivity in cholesterol-fed mice after [14C]-cholesterol injection during daily treatment with probucol at 800 mg/kg (C) or vehicle (20 ml/kg) (A) for 5 days. [14C]-Cholesterol (100 μCi/kg) was intravenously injected immediately after the 1st dose of probucol or vehicle. Each point represents the mean value from 8 mice.

| Table 7. Effect of probucol on fecal excretion of radioactivity, cholesterol and bile acids in cholesterol-fed mice |
|---------------------------------------------------------------|
| Control | Pro布cul (800 mg/kg) |
| Radioactivity (10^-6 dpm/5 days) | 10.06±0.79 | 13.95±1.15* |
| Cholesterol (mg/5 days) | 44.01±2.63 | 68.84±8.19* |
| Bile acids (mg/5 days) | 33.30±2.40 | 51.47±2.36** |
| Fecal mass (g/5 days) | 3.32±0.24 | 3.61±0.18 |

The values are the mean±S.E. of 8 mice. *P<0.05, **P<0.01 versus control. Probucol was orally given at 800 mg/kg/day for 5 days. [14C]-Cholesterol was intravenously injected immediately after the 1st dose of probucol or vehicle. Feces were collected for 5 days after [14C]-cholesterol injection.

| Table 8. Effect of probucol on hepatic and intestinal HMG-CoA reductase and cholesterol 7α-hydroxylase activities in normal mice |
|---------------------------------------------------------------|
| HMG-CoA reductase activity* | Hepatic cholesterol 7α-hydroxylase activity* | Serum Cholesterolb |
| Liver | Intestine | Liver | Intestine | |
| Control | 25.3±4.4 | 33.6±6.3 | 23.0±3.3 | 155.6±11.3 |
| Probucol (400 mg/kg) | 17.7±3.9 | 51.4±9.3 | 42.7±4.8* | 55.9±6.5** |

The values are the mean±S.E. of 8 mice. *P<0.05, **P<0.01 versus control. The mice received daily oral doses of probucol (400 mg/kg) or vehicle for 7 days. *pmoles/min/mg protein, bmg/dl.
the treated mice, although the effects were not statistically significant. Cholesterol 7α-hydroxylase activity was significantly increased by 86% in the treated mice. Serum cholesterol levels were also significantly decreased by the treatment.

Discussion

Cholesterol concentration in the circulating blood is influenced by several factors including intestinal absorption of exogenous cholesterol, biosynthesis of cholesterol in tissues, mainly in the liver and the small intestines, and catabolic excretion of cholesterol into bile as well as reabsorption of excreted cholesterol through the enterohepatic circulation. Hypocholesterolemic agents are thought to reduce serum cholesterol levels in animals by affecting some of these factors.

Clofibrate, a widely used hypocholesterolemic agent, did not lower serum cholesterol in mice with a 2 week administration as reported before (3). On the other hand, probucol reduced serum cholesterol levels effectively in clofibrate-resistant mice. These findings suggest that the mode of the hypocholesterolemic effect of probucol may be different from clofibrate. So we have studied the mode of action of probucol in reducing serum cholesterol in mice. In this sort of a study, it seems important to examine some metabolic and biochemical changes in test animals whose serum cholesterol is actually reduced by the drug tested. In the present study, therefore, probucol was orally administered at a daily dose of 400 mg/kg to normal and at 800 mg/kg to cholesterol-fed mice on the basis of the finding reported previously (3).

The present results (Tables 1 and 2) demonstrated no difference in serum radioactivity after oral administration of radioactive cholesterol between the probucol-treated and the control mice. This indicates no effect of probucol on intestinal absorption of exogenous cholesterol. Some difference in serum radioactivity which was observed 24–72 hr after [14C]-cholesterol ingestion was thought to be due to some effect of the drug to stimulate catabolism of cholesterol absorbed (see later). The procedure of this experiment may be too simple to lead to this conclusion. Effect of a drug on cholesterol absorption is usually studied by the combined use of 2 differently radiolabeled cholesterols as reported by Barnhart et al. with probucol (10). However, the above conclusion was supported by the finding that probucol had no effect on serum cholesterol in hepatectomized mice with reduced liver functions but with intact gastrointestinal functions.

The effect of probucol on cholesterol synthesis was rather complicated. In normal mice, probucol was an inhibitor of cholesterol synthesis in the liver but was a stimulator in the small intestines when [14C]-acetate was used as a precursor of cholesterol synthesis (Tables 3 and 4). These effects were, however, not observed with [14C]-mevalonate as a precursor, indicating the site of action of probucol was on HMG-CoA reductase (Table 3). This was supported by the finding that the probucol-treated mice showed a lower HMG-CoA reductase activity in the liver and a higher activity in the intestines (Table 8). Also, in the cholesterol-fed mice whose cholesterogenesis was suppressed by the feed-back inhibition of cholesterol, probucol still stimulated cholesterol synthesis from acetate in the intestines (Table 4). As the liver and the small intestines are the main organs that produce cholesterol in the body, these results indicate that the over-all effect of probucol on cholesterol synthesis is not involved in lowering serum cholesterol, although the effect of a very high dose (800 mg/kg) could be partially responsible for the reduction of serum cholesterol in normal mice (Table 5). These findings give rise to some questions about the previous conclusion that inhibition of cholesterol synthesis is involved in the hypocholesterolemic action of this drug (9, 10, 12). The previous conclusion was drawn only from the study on hepatic cholesterol synthesis. The precise mechanism of this dural action of probucol is not clear yet.

The effect of probucol on catabolic excretion of cholesterol was the most reliable one in the present study to explain the mode of the hypocholesterolemic action of this drug. Probucol treatment significantly accelerated the decay of the injected radio-
active cholesterol in blood (Figs. 1b and 4) and increased the fecal excretion of cholesterol and bile acids as well as the radioactivity in cholesterol-fed mice (Table 7). In the study with normal mice (Table 6), the effect on fecal excretion was not so clear as that observed with the cholesterol-fed mice, although hepatic cholesterol 7α-hydroxylase activity was significantly elevated by the probucol treatment (Table 8).

Acceleration of catabolic excretion of cholesterol was also reported as a main mode of action of probucol by several workers with cholesterol-fed monkeys (10) and hypercholesterolemic patients (9, 11). However, Li et al. (12) reported the decrease in fecal sterols and hepatic cholesterol 7α-hydroxylase activity in the probucol-treated rats, the opposite findings to other workers (9-11) and our present finding. It may be possible to speculate that the effect of probucol varies depending on the stage of drug treatment: probucol strongly stimulates the fecal excretion of bile acids and neutral sterols during the early stage of drug treatment where serum cholesterol is decreasing, while it does not do so in the steady state of drug action. This concept is not inconsistent with the finding of Miettinen (9) who reported an initial, transient increase in fecal bile acids and neutral sterols followed by a lower fecal steroid values at the end of a 4 week treatment of patients with the drug. In the present study with normal mice (Table 6), the effect of probucol was not so clear. This may be due to an intermediary stage of drug action in this experiment.

From these findings presented here, probucol was found to reduce serum cholesterol mainly by accelerating catabolic excretion of cholesterol. However, further detailed studies are required to elucidate the precise mechanism of action of this drug in the steady state.

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