The Mechanism of Comparable Serum Cholesterol Lowering Effects of Pravastatin Sodium, a 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor, between Once- and Twice-Daily Treatment Regimens in Beagle Dogs and Rabbits

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ABSTRACT—In dogs, no significant difference in the reduction of serum cholesterol was observed among three dosing regimens of pravastatin: once in the morning (3 mg/kg), once in the evening (3 mg/kg), and twice-daily (1.5 mg/kg × 2) for 21 days. In rabbits, pravastatin was administered at a dose of 50 mg/kg once-daily given in the evening or 25 mg/kg twice-daily for 14 days; the respective serum and liver cholesterol levels were decreased by 41% and 7% in the once-daily dosing and by 51% and 11% in the twice-daily dosing. The amount of low density lipoprotein (LDL) receptor protein was increased 1.2–1.3-fold (P <0.05) by both treatments, and no significant difference was noted between these treatment regimens. In addition, there was no significant difference in the extent of up-regulated LDL receptor protein between once-daily dosing in the evening and once-daily dosing in the morning. In the experiments with rabbit hepatocytes, the up-regulated LDL receptor activity induced by preincubation with pravastatin was retained even 24 hr after the removal of pravastatin. These results suggest that the comparable efficacy of pravastatin between once- and twice-daily treatment could be explained by retention of up-regulated LDL receptor activity for more than 24 hr in vitro and in vivo.

Keywords: Pravastatin, Low density lipoprotein (LDL) receptor, Hepatocyte (rabbit), Immunoblotting, Dosing regimen

3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors are very effective in lowering serum cholesterol, in particular, low density lipoprotein (LDL) cholesterol, in most animal species including humans (1–5), and are now widely used for the treatment of hypercholesterolemia (6). The mechanism of lowering serum cholesterol by HMG-CoA reductase inhibitors is now believed to be as follows: hepatic cholesterol depletion by inhibition of cholesterol synthesis may trigger the induction of hepatic LDL receptor, resulting in the stimulation of LDL removal from the blood, and may decrease the secretion of very low density lipoprotein (VLDL) cholesterol (5, 7). We (8, 9) and other investigators (10, 11) reported that pravastatin increased hepatic LDL receptor activity in normal or Watanabe heritable hyperlipidemic (WHHL) rabbits. Kovanen et al. (12) reported that when mevinolin (lovastatin), an HMG-CoA reductase inhibitor, was administered to dogs, an increase in the number of hepatic LDL receptors rather than a reduction in cholesterol synthetic rate in the liver was responsible for the enhanced clearance of LDL from blood.

Meanwhile, Hunninghake et al. (13) and Pan et al. (14) reported that once-daily pravastatin administration (40 mg) to patients with primary hypercholesterolemia was as effective as twice-daily dosing (20 mg × 2). They speculated that the efficacy of once-daily administration could be explained by its long retention of up-regulated LDL receptor in spite of the rather short half-life of pravastatin (15). However, there is no evidence that the long retention of up-regulated LDL receptor is responsible for the comparable efficacy of HMG-CoA reductase inhibitors among different dosage regimens. In order to clarify
this point, we compared the lipid lowering effects of pravastatin given by either once- or twice-daily dosing in beagle dogs as well as normal rabbits, and determined the levels of up-regulated hepatic LDL receptor in normal rabbits. In cultured rabbit hepatocytes, we examined the effects of pravastatin on the induction of LDL receptor protein. We conclude that comparable efficacy of pravastatin between once- versus twice-daily treatment seemed to be a general event in most animal species, and this effect could be explained by retention of up-regulated LDL receptor activity for more than 24 hr, in spite of the short half-life of pravastatin.

MATERIALS AND METHODS

Chemicals

Sodium[125I] was purchased from Amersham (Buckinghamshire, UK). William's E medium was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase type H was obtained from Boehringer Mannheim Co. (Indianapolis, IN, USA). All other chemicals were of the highest grade commercially available. Pravastatin was obtained by microbial hydroxylation of ML-236B as described elsewhere (16).

Animals

Beagle dogs: Male beagle dogs (5–6-months-old; Hazleton Research Products, Inc., Madison, WI, USA) were housed individually under normal lighting conditions (lights on 6 a.m. to 6 p.m.). One group consisted of 6 animals. The animals had free access to water and were fed a commercial dog food (Type DS; Oriental Yeast Co., Tokyo) at 300 g per day. Pravastatin was orally administered in zelatin capsules once or twice a day (at 9 a.m. and/or at 4 p.m. in the evening). Venous blood was obtained 24 hr after the final administration in each treatment under the fasting condition.

Rabbits: Male New Zealand White rabbits (5-months old; Japan SLC, Shizuoka) were housed individually under normal lighting conditions (lights on 7 a.m. to 7 p.m.). One group consisted of 5 animals. The animals had free access to water and were fed a commercial dog food (Type DS; Oriental Yeast Co., Tokyo) at 300 g per day. Pravastatin was orally administered in zelatin capsules once or twice a day (at 9 a.m. and/or at 4 p.m. in the evening). Venous blood was obtained 24 hr after the final administration in each treatment under the fasting condition.

Measurement of lipids and serum lipoproteins fractionation

Serum cholesterol, triglyceride and phospholipid were determined by enzymatic methods: Determiner TC555 or FC555 (Kyowa Medix Co., Tokyo) for total or free cholesterol, respectively; Triglyceride G-Test Wako or Phospholipid-Test Wako (Wako Pure Chemical Industries, Osaka) for triglyceride or phospholipid, respectively. Serum was sequentially fractionated according to the method of Hatch and Lees (17) using an Hitachi SCP-70H ultracentrifuge equipped with an RPS-56T rotor (Hitachi Co., Ltd., Ibaraki). Each lipoprotein density range was as follows: VLDL, d < 1.006; LDL, 1.006 < d < 1.063; high density lipoprotein (HDL) and very high density lipoprotein, d > 1.063.

For determination of liver cholesterol, a 200-mg portion was extracted with 2 ml of isopropyl alcohol in a Polytron (Kinematica GmbH, Steinhofalde, Switzerland), and the cholesterol content in the extract was determined as described above.

Preparation of cultured rabbit hepatocytes

Rabbit parenchymal hepatocytes were isolated from male New Zealand White rabbits (23-weeks-old) by 0.05% collagenase (type H) perfusion of the liver by the method of Selgen (18). The isolated cells (viability >90%) were suspended in William's E medium containing 5% heat-inactivated fetal calf serum, 10^{-7} M insulin, 10^{-8} M glucagon, 2 \times 10^{-5} M dexamethasone, 100 U penicillin/ml and 100 μg/ml streptomycin. Cells were inoculated at a density of 5 \times 10^5 cells/ml in a collagen-coated, 24-well plate and incubated at 37°C in a humidified 5% CO_2 / 95% air atmosphere. After 1 hr, the medium was renewed to remove the detached cells, and the cells were incubated in the same medium for 18 hr before the start of the experiments.

^{125}I-LDL preparation and determination of LDL receptor activity in cell culture

Human LDL (1.019 < d < 1.063) and lipoprotein-deficient serum (d > 1.25) were prepared by ultracentrifugation (17). LDL was radiiodinated by the iodine monochloride method (19) at a specific activity of 190–300 ng/mg protein.

Rabbit parenchymal cells were preincubated at 37°C for 48 hr with or without 2 μg/ml pravastatin in William's E medium containing 15% lipoprotein deficient serum, 10^{-7} M insulin, 10^{-8} M glucagon, 2 \times 10^{-5} M dexamethasone, 100 U penicillin/ml and 100 μg/ml streptomycin. After the preincubation, cells were incubated in the fresh medium deprived of pravastatin for a further appropriate time. For determination of LDL receptor activity, cells were incubated with 20 μg/ml of ^{125}I-LDL in the presence or absence of 400 μg/ml of non-labeled LDL for 1 hr at 37°C. After the incubation, the cells were washed 5 times with 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 2 mg/ml of bovine serum albumin, followed by two washes with the same buffer without serum albumin. The cells were dissolved in 1 ml of 2 mM sodium dodecyl
sulfate (SDS) followed by determination of LDL bound and internalized according to the method of Goldstein et al. (20).

**Monoclonal antibody**

Mouse monoclonal antipeptide antibody (designated 15D3) was kindly provided by Dr. M. Kuwano, Kyushu University School of Medicine. This antibody was directed against a synthetic peptide corresponding to the 14-amino acids of the C-terminal sequence of the bovine cortex LDL receptor. This sequence is highly conserved among many animal species including rabbit, hamster, rat and humans (21).

**Preparation of liver membrane fractions and immunoblot analysis of LDL receptors**

Rabbit liver was homogenized with a Polytron in 8 volumes of homogenizing buffer (20 mM Tris-HCl, 1 mM CaCl₂, 150 mM NaCl, 1 mM PMSF, 0.1 mM leupeptin, pH 8.0), and membrane fractions that sedimented between 8,000 and 100,000 x g were prepared. The membranes were stored at -80°C until use.

Membrane pellets prepared as above were suspended in 250 mM Tris-maleate (pH 6.0) / 2 mM CaCl₂ / 1 mM PMSF / 0.1 mM leupeptin and solubilized by adding an equal volume of 2% SDS / 10% (w/v) glycerol / 0.2% bromophenol blue and then applied without addition of β-mercaptoethanol and without heating to 6% polyacrylamide slab gels containing 0.1% SDS according to the method of Laemmli (22). Electrophoresis was carried out at a constant current of 20 mA/slab gel for 3 hr. The separated proteins were electrotransferred from the gels to polyvinylidene difluoride (PVDF) membranes according to the method of Beisiegel et al. (23). The PVDF blots were blocked in buffer A (10 mM Tris-HCl (pH 7.4) / 150 mM NaCl / 5% BSA) at 37°C for 30 min, followed by overnight incubation with mouse monoclonal anti-LDL receptor antibody. The blots were incubated for 2 hr with buffer A containing 0.74 MBq 125I-protein A, followed by washing as described above and air drying. Autoradiograms were obtained with a Bio-Imaging Analyzer (BA100; Fujix, Tokyo).

**Statistical analysis**

Data from these studies were statistically analyzed by Student’s t-test. Each reported value is a mean±S.E.M.

**RESULTS**

**Effect of pravastatin on serum lipid levels of beagle dogs by once- or twice-daily dosing for 21 days (Table 1)**

The diurnal rhythm of hepatic cholesterol synthesis, with the peak occurring around midnight, is well known (24). From this point of view, we examined the difference

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<th>Table 1. Effects of administration of pravastatin for 21 days on lipoprotein lipid levels of beagle dogs (n=6 for each group) by once- or twice-daily dosing</th>
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Pravastatin was administered at the doses of 1.5 mg/kg twice a day or 3 mg/kg once a day either in the morning or in the evening for 21 days. Blood samples were obtained 24 hr after the final dosage of pravastatin. Numbers in the parentheses express % of the corresponding control levels. Each value is expressed as an average±S.E.M. Significantly different from the corresponding control value at: *P<0.05, **P<0.01, ***P<0.001. VLDL: very low density lipoprotein, LDL: low density lipoprotein, HDL: high density lipoprotein.
between once- and twice-daily treatment; and in the once-daily administration, we compared the treatment in the morning with that in the evening.

Pravastatin was administered at doses of 1.5 mg/kg twice a day or 3 mg/kg once a day either in the morning or evening for 21 days. Blood samples were obtained 24 hr after the final dosage. As shown in Table 1, once-daily dosing in the morning or in the evening decreased serum cholesterol by 25% or 28%, respectively. Twice-daily dosing decreased serum cholesterol by 32%. There was no significant difference in the extent of reduction of serum cholesterol among the three treatment groups. Cholesterol levels were preferentially decreased in VLDL plus LDL fractions. Phospholipid levels in each treatment group also showed a similar reduction to cholesterol in each lipoprotein fraction. Triglyceride levels in two treated groups (once in the evening and twice a day) were significantly reduced in the whole serum and VLDL plus LDL fractions, but showed no reduction in the HDL fractions. In the control group, lipoprotein lipid levels did not change throughout the experiment.

Effect of pravastatin on serum and liver cholesterol levels, and LDL receptor activity of rabbits by once- or twice-daily dosing (Table 2)

Pravastatin was administered at the doses of 25 mg/kg twice a day or 50 mg/kg once a day in the evening for 14 days. In the clinical studies, blood was collected in the morning. According to this schedule, in the experiments using rabbits, blood samples were obtained on the morning of the next day after the final dosage: 24 hr after the morning dosage or 14 hr after the evening dosage.

When pravastatin was given once in the evening, serum and liver cholesterol were decreased by 41% (P<0.05) and 7%, respectively, as compared with the control levels. These reductions were almost to the same extent as the twice-daily dosage regimen (51% (P<0.01) and 11% (P<0.05), respectively). LDL receptors determined by immunoblotting analysis were significantly increased 1.2–1.3-fold in the above two treatment regimens. However, no statistical difference was observed between these two treatment regimens. We compared the extent of LDL receptor induction by the once-daily administration in the morning with that of the once-daily administration in the evening. There was no significant difference between these two groups (100±4 in the evening versus 117±8 in the morning, shown as arbitrary units).

Retention of the up-regulated LDL receptor activity after preincubation with pravastatin in rabbit hepatocytes (Fig. 1)

Cholesterol synthesis in rabbit hepatocytes was inhibited dose-dependently and almost completely inhibited by 2
µg/ml pravastatin treatment (data not shown). When rabbit hepatocytes were preincubated for 48 hr with 2 µg/ml pravastatin, the LDL receptor activity was increased 2.7-fold (Fig. 1, 0 time, 20.5 ± 2.0 ng/mg protein in untreated cells versus 55.1 ± 3.7 ng/mg protein in treated cells). After pravastatin was removed, this up-regulated LDL receptor activity decreased in a time-dependent manner, and the activity was calculated to have a half-life of about 9 hr. In addition, even 24 hr after the drug removal, the up-regulated LDL receptor activity was still higher than that in untreated cells.

DISCUSSION

Several clinical studies on the efficacy and safety of once-daily versus twice-daily HMG-CoA reductase inhibitor treatment in hypercholesterolemia have been reported (11, 12, 25, 26). In these studies, researchers observed that twice-daily dosing was slightly more effective than once-daily dosing in the evening, and once-daily dosing in the evening was slightly more effective than that in the morning, but that there was no statistically significant difference in the hypocholesterolemic effects in these three groups. Our results in beagle dogs were consistent with the former observations in individuals with primary hypercholesterolemia.

Our hypothesis is that this comparable efficacy of pravastatin among different dosage regimens seems to be due to the long retention of the up-regulated LDL receptor, in spite of the short half-life of pravastatin. The half-life of pravastatin in rabbit serum was about 3 hr when pravastatin was orally administered (Nitanai et al., personal communication). In beagle dogs, the half-life of pravastatin was reported to be less than 3 hr by the determination of changes of plasma concentration of radioactivity after oral administration of [14C]-pravastatin (27). In contrast, the up-regulated hepatic LDL receptor in rabbits by pravastatin treatment remained at a higher level than that in the control group, both at 14 hr and 24 hr after the final dosage (Table 2). In addition, the groups treated with the three different dosage regimens (once in the morning, once in the evening, or twice a day) showed similar up-regulated LDL receptor levels. This is the first report to see the retention of hepatic LDL receptor activity up-regulated by pravastatin in vivo. We also observed this long retention of up-regulated LDL receptor activity in rabbit hepatocytes. The half-life of the up-regulated LDL receptor activity after pravastatin treatment was calculated to be 9 hr, and this remained at a higher level than that of the untreated cells up to 24 hr (Fig. 1). Tam et al. (28) reported that in Hep G2 cells, the half-life of the LDL receptor protein was 9-12 hr in pulse-chase experiments. Our result was consistent with this observation. In addition, retention of LDL receptor activity up to 24 hr seemed to support the result of the in vivo experiments in rabbits, although factors concerning the metabolism of the inhibitor might be excluded in cell culture experiments.

The reason for the long retention of up-regulated LDL receptors, for more than 24 hr, might be explained by the following mechanism: When pravastatin was administered to inhibit the cholesterol synthesis, hepatic HMG-CoA reductase was induced (8), owing to the liver cholesterol homeostasis. After pravastatin was metabolized, the induced HMG-CoA reductase led to the restoration of liver cholesterol. However, in consecutive administration of pravastatin, liver cholesterol levels were maintained at a lower level than that of the untreated group 24 hr after the last dosage of pravastatin (Table 2). This phenomenon, in addition to the long half-life of LDL receptors rather than that of pravastatin, might lead to the long retention of up-regulated LDL receptors in the liver.

The increase in LDL receptor protein in rabbits was modest (1.2-1.3 fold). Kume et al. (11) reported that LDL receptor activity was increased 1.5-fold when a 50% decrease of total cholesterol was observed (52% decrease in VLDL plus LDL fraction) by pravastatin treatment in heterozygous WHHL rabbits. From these observations, a modest increase in LDL receptors by pravastatin treatment might be efficient for reducing plasma LDL, the metabolite of VLDL. Although the percent decrease of VLDL plus LDL cholesterol was larger than that of HDL cholesterol by pravastatin treatment in beagle dogs, the reduction of plasma cholesterol levels resided in the HDL fraction (Table 1). Ma et al. (29) observed the same phenomenon in hamsters and WHHL rabbits. They suggested that the HDL lowering effects of pravastatin in these animals might be explained by the relative abundance of apolipoprotein E in the HDL fractions. Such HDL might be bound and internalized through LDL receptors, resulting in the reduction of HDL cholesterol. HDL is the major cholesterol-carrying lipoprotein in beagle dogs and rabbits, and a relatively large amount of apolipoprotein E-containing HDL might be efficiently metabolized by the elevated LDL receptor.

In our experiments, we used a relatively higher dose in rabbits (50 mg/kg per day) than beagle dogs (3 mg/kg per day) for the following two reasons: First, there is a difference in sensitivity towards HMG-CoA reductase inhibitors between rabbits and beagle dogs as reported previously (2). Beagle dogs were more sensitive to HMG-CoA reductase inhibitor than rabbits. Secondly, it was necessary to clearly see the changes of some parameters concerned with lipid metabolism such as liver cholesterol levels and hepatic LDL receptor activity. Duration of pravastatin treatment is also different between the two
animals (21 days for beagle dogs versus 14 days for rabbits). We examined the time course of serum cholesterol reduction during pravastatin treatment at these dosages and observed that durations of treatment in both animals described above was sufficient to produce the maximum reduction (data not shown).

In conclusion, the comparable efficacy of pravastatin given by different dosage regimens, which seemed to be a general event in most animals, could be explained by the retention of hepatic LDL receptor activity for more than 24 hr, in spite of the short half-life of pravastatin in serum, and also by the lack of any significant differences in the levels of hepatic LDL receptor activity among these dosage regimens. From the results of these animal experiments, and also in humans (13, 14), comparable hypocholesterolemic effects of pravastatin among different dosage regimens could be explained by the long retention of hepatic LDL receptor.

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