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Effects of single administration of pravastatin sodium on hepatic cholesterol metabolism in rats

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Abstract

Pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was administered to rats at 500 mg/kg, and the changes in several parameters concerning the metabolism of cholesterol in the liver were determined over 12 h. HMG-CoA reductase activity began to be induced 6 h after pravastatin dosage and continued to increase for an additional 6 h. A significant reduction of serum and liver microsomal cholesterol was observed only at 9 h. At this time point, the protein mass and activity of cholesterol 7α-hydroxylase were significantly decreased by 31% and 34%, respectively. Hepatic low density lipoprotein (LDL) receptor expression was not affected by pravastatin throughout the experimental period. These results suggest that, in rats, the compensatory mechanism to restore the cholesterol balance after depletion of liver cholesterol produced by a single administration of pravastatin might primarily depend on the induction of HMG-CoA reductase and might be facilitated by a reduction in cholesterol 7α-hydroxylase, without the induction of hepatic LDL receptor.

Keywords: Pravastatin sodium; HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase; LDL (low-density lipoprotein) receptor; Cholesterol 7α-hydroxylase; (Rat)

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are very effective in lowering serum cholesterol, in particular low density lipoprotein (LDL) cholesterol, in most animal species including humans (Mabuchi et al., 1983; Tsujita et al., 1986; Illingworth, 1987; Saito et al., 1988; Grundy, 1988), and are now widely used in the treatment of hypercholesterolemia (Hunninghake, 1992). The mechanism by which HMG-CoA reductase inhibitors lower serum cholesterol is now believed to be as follows: hepatic cholesterol depletion by inhibition of cholesterol synthesis may trigger the induction of the hepatic LDL receptor, resulting in the stimulation of LDL removal from blood, and may decrease the secretion of very low density lipoprotein (VLDL) cholesterol (Grundy, 1988; Reihner et al., 1990). In rats and mice, however, the serum cholesterol-lowering effect of HMG-CoA reductase inhibitors is not observed (Endo et al., 1979; Fears et al., 1980; Tsujita et al., 1986; Hirano et al., 1990; Yoshino et al., 1991). We have demonstrated in a previous report (Fujioka et al., 1995) that the repeated administration of pravastatin sodium (pravastatin), a hydrophilic HMG-CoA reductase inhibitor, to rats strongly induced HMG-CoA reductase in order to compensate for cholesterol depletion in the liver. Consequently, the liver cholesterol level was increased and VLDL cholesterol secretion from the liver was stimulated, resulting in an increase in serum cholesterol. In addition, no induction of LDL receptor activity was observed. It has not been clarified whether this phenomenon is caused by the increase in liver cholesterol, or is independent of the change in liver cholesterol level.

Roach et al. (1993) reported that supplementation with dietary cholesterol of Sprague-Dawley rats did not reduce LDL receptor mass, although mRNA was down-regulated. They suggested that this might be due to post-transcriptional regulation. Ness et al. (1996) reported that inhibition of cholesterol synthesis in rats by lovastatin, another HMG-CoA reductase inhibitor, or zaragozic acid, a squalene synthase inhibitor, did not change the protein mass of the hepatic LDL receptor, although hepatic LDL receptor mRNA was increased. These observations suggest that the

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LDL receptor pathway might be resistant to changes in the cellular cholesterol content in rats (Spady and Cuthbert, 1992). In our present study, we found that administration to rats of a very high dose of pravastatin (500 mg/kg), 2-fold higher than the dose at which cholesterol synthesis in the liver is completely inhibited for at least 6 h (Fujioka et al., 1995), reduced serum and liver microsomal cholesterol 9 h after its administration. For the purpose of clarifying the mechanism by which cholesterol homeostasis is maintained in response to cholesterol depletion in the liver over short time periods, we investigated the changes in hepatic LDL receptor, cholesterol 7α-hydroxylase and HMG-CoA reductase. We observed that HMG-CoA reductase was primarily induced, followed by a significant decrease in cholesterol 7α-hydroxylase protein without the induction of hepatic LDL receptor protein.

2. Materials and methods

2.1. Materials

[3-^{14}C]HMG-CoA (2.2 Gbq/mmol) was purchased from Dupont NEN. Cholesterol oxidase (E.C. 1.1.3.6) from *Streptomyces* sp. and 7β-hydroxycholesterol were obtained from Sigma. 7α-Hydroxycholesterol was from Steraloids (Wilrton, NH, USA). All other chemicals used were of the highest grade commercially available. Pravastatin was obtained by the microbial hydroxylation of ML-236B as described elsewhere (Serizawa et al., 1983).

2.2. Animal

Male Wistar-Imamichi rats (6 weeks old; Institute of Animal Production, Japan) were housed in metal cages under normal lighting conditions (lights on, 7 a.m. to 7 p.m.) and were given a standard pellet diet. Each group consisted of four animals. Pravastatin was dissolved in distilled water and orally administered at 8 a.m. At the indicated time, the rats were killed by decapitation, blood was collected and the livers were excised. Food and water were provided ad libitum during the entire experimental period.

2.3. Polyclonal antibodies

A polyclonal antibody to LDL receptor was directed against a synthetic peptide corresponding to the 15 amino acids of the C-terminal sequence of the bovine cortex LDL receptor (Cys-Tyr-Pro-Ser-Arg-Gln-Met-Val-Ser-Leu-Glu-Asp-Asp-Val-Ala). This sequence is highly conserved among many animal species including rabbit, hamster, rat and humans (Cosgrove et al., 1993). A polyclonal antibody to cholesterol 7α-hydroxylase was directed against a synthetic peptide corresponding to the 21 amino acids of the C terminal sequence of rat cholesterol 7α-hydroxylase (Arg-Ala-Gly-Leu-Gly-Ile-Leu-Pro-Pro-Leu-His-Asp-Ile-Glu-Phe-Lys-Tyr-Lys-Leu-Lys-His) (Nishimoto et al., 1991). Conjugation of the peptide to bovine thyroglobulin as a carrier protein was achieved by synthesizing a peptide with a cysteine residue at the N-terminus. Rabbits were initially immunized subcutaneously with peptide conjugate in Freund's complete adjuvant, and then the animals were immunized subcutaneously or intraperitoneally by repeated injection of peptide conjugate in Freund's incomplete adjuvant for 11 weeks. Blood was withdrawn and whole serum was stored at −20°C. Antisera were affinity-purified before use.

2.4. Preparation of liver microsomal fractions and immunoblot analysis of LDL receptor, cholesterol 7α-hydroxylase

A solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin was used as the homogenizing buffer. Rat liver was homogenized with a Polytron (Kinematica, Steinhofhalde, Switzerland) in 8 vols. of homogenizing buffer, and a crude ultracentrifugation fraction (8000–100 000 × *g*, pellet) was collected as described elsewhere (Fujioka et al., 1995). The fraction was resuspended with the homogenizing buffer and subjected to resedimentation (100 000 × *g*, 30 min). These once-washed microsomal fractions were stored at −80°C until use.

Microsomal fractions were suspended in 20 mM Tris-HCl (pH 7.5)/50 mM NaCl/1 mM $CaCl_2$/1 mM phenylmethylsulfonyl fluoride and solubilized by adding an equal volume of 2% sodium dodecyl sulfate (SDS)/10% (w/v) glycerol/0.2% bromophenol blue and loading onto 6% SDS/polyacrylamide gels in the case of the LDL receptor, or 10% SDS/polyacrylamide gels for the cholesterol 7α-hydroxylase, according to the method of Laemmli (1970). The separated proteins were electrotransferred from the gels to polyvinylidene difluoride membranes. The membranes were then overlaid with each polyclonal antibody for 12 h followed by an anti-rabbit IgG antibody conjugated to horseradish peroxidase. Each immunoreactive protein band was then stained with diaminobenzidine. The amount of each protein was analyzed using an imaging densitometer (Bio-Rad, Model GS-670).

2.5. Enzyme assays

HMG-CoA reductase activity was measured as described elsewhere (Kuroda and Endo, 1977). Hepatic microsomes were incubated for 15 min at 37°C with 200 μM [3-^{14}C]HMG-CoA and 5 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM EDTA and 10 mM dithiothreitol. The [^{14}C]mevalonic acid formed was separated by thin-layer chromatography after lactonization.

Cholesterol 7α-hydroxylase activity was measured using a high-performance liquid chromatography (HPLC) assay that quantifies the mass of 7α-hydroxycholesterol

formed from endogenous microsomal cholesterol after enzymatic conversion to 7α-hydroxy-4-cholesten-3-one, which has a specific absorption at 240 nm, using cholesterol oxidase (Ogishima and Okuda, 1986; Chiang et al., 1990). In brief, the incubation mixture consisted of 0.2 ~ 0.4 mg microsomal protein, 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 20 mM cysteamine hydrochloride, 5 mM $MgCl_2$, 5 mM isocitrate, 0.075 units of isocitrate dehydrogenase in a total volume of 0.225 ml. The reaction was started by addition of 0.5 mM NADPH. The enzyme reaction was carried out at 37°C for 20 min. At the end of the reaction, sodium cholate, cholesterol oxidase and 7β-hydroxycholesterol as an internal recovery standard were added, and the mixture was further incubated for 10 min. Reactions were stopped by adding 0.3 ml methanol, and the mixture was extracted with 3 ml petroleum ether. The extract was evaporated to dryness under N_2, and a portion of the residue was analyzed by C_{18} reverse-phase HPLC (Senshu Pak ODS-H-1251, 4.6 × 250 mm, 5 μm).

2.6. Other analytical determinations

Serum and liver microsomal cholesterol levels were determined by an enzymatic method (Determiner TC 555, Kyowa Medix). The protein concentration was determined according to the method of Lowry et al. (1951).

2.7. Statistical analysis

Data from these studies were statistically analyzed by two-tailed Student's *t*-test. Values were expressed as means ± S.E.M.

3. Results

3.1. Effects of pravastatin on serum and liver microsomal cholesterol

In the following experiments, protein mass detected by immunoblotting and enzyme activities were measured in microsomal fractions. Therefore, we used microsomal fractions, rather than whole liver homogenates, for determining cholesterol levels in the livers. At 9 h after pravastatin dosage, serum and liver microsomal cholesterol were significantly decreased by 13% and 15%, respectively, whereas no significant differences were observed at other time points (Table 1).

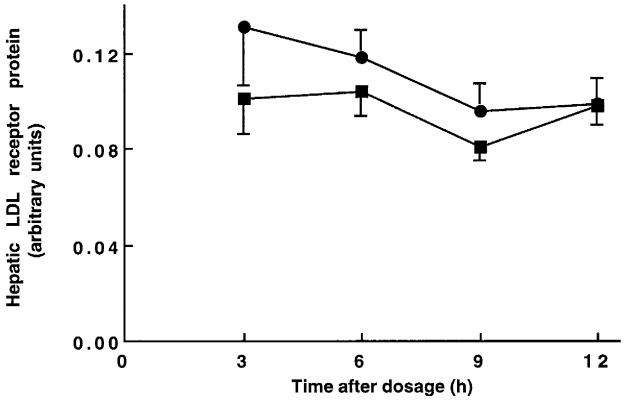

Fig. 1. Effect of pravastatin on changes in LDL receptor protein expression. Groups of four rats were killed every 3 h for 12 h after pravastatin dosage (8 a.m.). (●) control, (■) 500 mg/kg pravastatin. Liver microsomal fractions were prepared and we determined the LDL receptor protein mass as described in Section 2.

3.2. Effects of pravastatin on hepatic LDL receptor mass

In the control group, hepatic LDL receptor protein mass was slightly decreased during the experimental period. At all time points examined, no significant differences were observed between the respective control group and the pravastatin-treated group (Fig. 1).

3.3. Effects of pravastatin on cholesterol 7α-hydroxylase protein mass and activity

In the control group, both cholesterol 7α-hydroxylase protein mass and activity in the evening (at 12 h, 8 p.m.) were about 2 times higher than those in the morning (at 3

Table 1
Changes in the cholesterol concentration in serum and in the liver microsomal fraction

Time after dosage (h)	Serum cholesterol (mg/dl)		Liver microsomal cholesterol (μg/mg protein)	
	Control	Pravastatin 500 mg/kg	Control	Pravastatin 500 mg/kg
3	89.6 ± 2.1	84.6 ± 3.3	24.9 ± 1.0	24.5 ± 2.2
6	86.8 ± 3.1	81.1 ± 3.3	21.4 ± 0.6	20.5 ± 1.6
9	88.5 ± 3.8	76.8 ± 1.6 [a]	24.4 ± 0.5	20.7 ± 0.9 [b]
12	78.7 ± 1.7	84.3 ± 2.1	25.7 ± 2.0	23.6 ± 0.8

Groups of four rats were killed every 3 h for 12 h after pravastatin dosage (8 a.m.). Blood was collected and microsomal fractions were prepared from livers. Values are expressed as means ± S.E.M. Significantly different from the corresponding control value, [a] $P < 0.05$, [b] $P < 0.01$.

h after dosage, 11 a.m.) (Fig. 2A, B). This change seems to be due to the diurnal rhythm of this enzyme (Brassil et al., 1995). At 9 h after pravastatin dosage, cholesterol 7α-hydroxylase protein mass and activity were significantly decreased by 31% and 34%, respectively, as compared to those of the control group. The extent of the decrease in protein mass and activity was almost the same. In addition, pravastatin showed no inhibitory effect on cholesterol 7α-hydroxylase activity in vitro at 10 μg/ml (data not shown). Therefore, the decrease in activity might be explained by the decrease in enzyme mass, rather than by a decrease in cholesterol substrate in the microsomal fraction. At 12 h after pravastatin dosage, there were no significant differences in either enzyme mass or activity between the control group and the pravastatin-treated group.

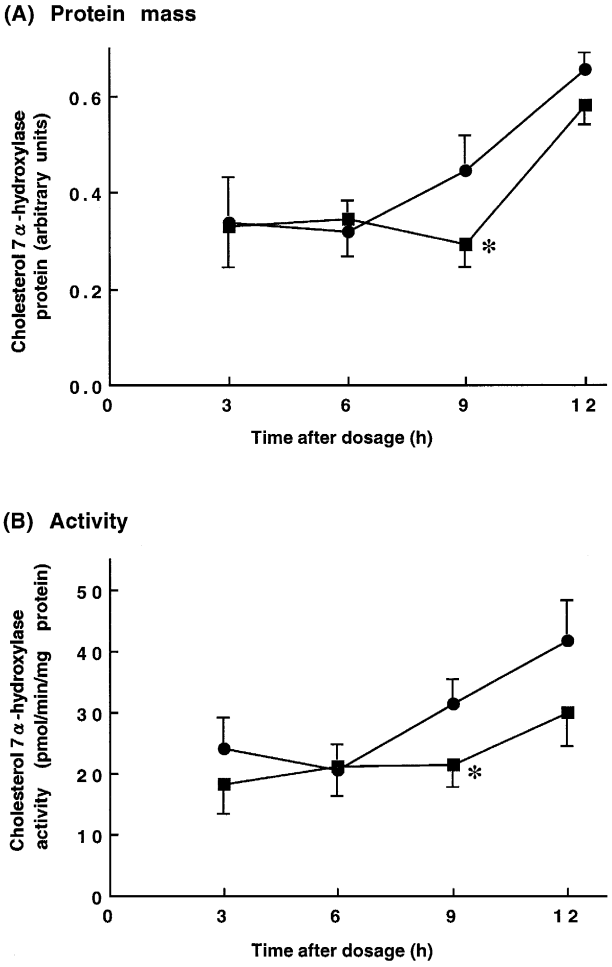

Fig. 2. Effect of pravastatin on changes in cholesterol 7α-hydroxylase protein expression and activity. Groups of four rats were killed every 3 h for 12 h after pravastatin dosage (8 a.m.). (●) control, (■) 500 mg/kg pravastatin. Liver microsomal fractions were prepared and we determined (A) cholesterol 7α-hydroxylase protein mass and (B) cholesterol 7α-hydroxylase activity as described in Section 2. Significantly different from the corresponding control value, * $P < 0.05$.

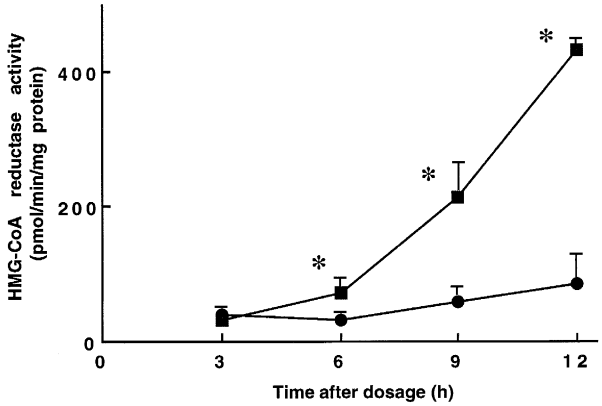

Fig. 3. Effect of pravastatin on changes in HMG-CoA reductase activity. Groups of four rats were killed every 3 h for 12 h after pravastatin dosage (8 a.m.). (●) control, (■) 500 mg/kg pravastatin. Liver microsomal fractions were prepared and we determined the HMG-CoA reductase activity. Significantly different from the corresponding control value, * $P < 0.001$.

3.4. Effects of pravastatin on HMG-CoA reductase activity in the liver

HMG-CoA reductase activities were determined in the once-washed microsomal fractions (Fig. 3). Since pravastatin is a hydrophilic HMG-CoA reductase inhibitor, large amounts of pravastatin are washed out during the preparation procedure. Therefore, HMG-CoA reductase activity determined by using once-washed microsomal fraction could reflect the content of HMG-CoA reductase protein itself (Fujioka et al., 1995).

In the control group, HMG-CoA reductase activity in the evening (at 12 h, 8 p.m.) was about 2 times higher than that in the morning (at 3 h after dosage, 11 a.m.). The difference in the activity of this enzyme between the morning and the evening was as great as the difference in cholesterol 7α-hydroxylase activity. At 6 h after pravastatin dosage, HMG-CoA reductase activity was increased 2.3-fold. The activity increased continuously and, at 12 h, the increased activity was 5-fold higher than in the respective control group.

4. Discussion

In our current study, we investigated how to restore the cholesterol balance when liver cholesterol in rats was depleted by a single administration of pravastatin, a hydrophilic HMG-CoA reductase inhibitor. The data showed that HMG-CoA reductase activity was primarily induced at 6 h after pravastatin dosage, and cholesterol 7α-hydroxylase activity was significantly reduced at 9 h after pravastatin dosage, without hepatic LDL receptor being induced at any time. HMG-CoA reductase activity continued to

increase over 12 h, while no significant difference in cholesterol 7α-hydroxylase protein mass was observed between control rats and pravastatin-treated rats at 12 h after pravastatin dosage. In order to compensate for cholesterol depletion in the liver, both facilitation of cholesterol synthesis and suppression of cholesterol excretion by bile acids might have taken place. The uptake of cholesterol from plasma LDL through hepatic LDL receptors might not act on this compensatory mechanism. We previously reported (Fujioka et al., 1995) that the repeated administration of an HMG-CoA reductase inhibitor, such as pravastatin, simvastatin or lovastatin, did not exert hypocholesterolemic effects in rats, and demonstrated that in rats HMG-CoA reductase activity was strongly induced in order to compensate for cholesterol depletion in liver by pravastatin treatment, resulting in accumulation of cholesterol in the liver, with no changes in LDL receptor and cholesterol 7α-hydroxylase activities. Together with the current results, it appears that repeated administration of pravastatin mainly leads to the induction of HMG-CoA reductase activity, and does not reduce cholesterol 7α-hydroxylase activity, possibly because there is no necessity for an additional compensatory mechanism.

Spady and Cuthbert (1992) and Horton et al. (1995) discussed that when cholesterol was given in the diet, hepatic sterol synthesis was primarily suppressed in rats as well as other animal species. As an additional compensatory mechanism, bile acid synthesis was increased and hepatic LDL uptake remained unchanged, whereas in many other species down-regulation of the LDL receptor occurs. Their results seem to be consistent with our study in relation to the regulatory mechanism of hepatic cholesterol balance in rats in which cholesterol was depleted.

Ness et al. (1996) demonstrated that when liver cholesterol was depleted by inhibition of cholesterol synthesis, hepatic LDL receptor protein expression was not increased, in spite of the induction of LDL receptor mRNA. This result suggests that changes in liver cholesterol levels might not affect LDL receptor protein expression. Whereas the mechanism of this post-transcriptional regulation has not been clarified yet, Ness et al. suggested that one possible explanation for the phenomenon was an increase in the degradation of LDL receptor through the inhibition of cholesterol synthesis in rats.

Although LDL receptor protein was not increased, a reduction in serum cholesterol was observed 9 h after the administration of 500 mg/kg pravastatin. This phenomenon might be explained by a decrease in VLDL-cholesterol secretion from the liver, in accordance with the reduction of liver microsomal cholesterol at 9 h. However, since the reduction of serum cholesterol was rather small, there still remains the possibility of some variation between individuals.

In conclusion, the compensatory mechanism to restore cholesterol balance in response to the depletion of liver cholesterol in rats produced by a single administration of HMG-CoA reductase inhibitors in a short period might be mediated through the induction of HMG-CoA reductase activity and then facilitated by a reduction in cholesterol 7α-hydroxylase activity, without the induction of hepatic LDL receptor. In the case of the repeated administration of inhibitors, this mechanism might only involve a strong induction of HMG-CoA reductase, leading to an increase in cholesterol synthesis and liver cholesterol levels.

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