Effects of Pravastatin Sodium Alone and in Combination with Cholestyramine on Hepatic, Intestinal and Adrenal Low Density Lipoprotein Receptors in Homozygous Watanabe Heritable Hyperlipidemic Rabbits

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ABSTRACT — Pravastatin sodium (pravastatin), a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, was administered alone (50 mg/kg) or in combination with cholestyramine, a bile acid sequestrant resin, at the level of 2% in the diet to homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits for 4 weeks. The low density lipoprotein (LDL)-cholesterol levels were reduced by 29% and 56% with pravastatin alone and the combination treatment, respectively. Hepatic LDL receptor activity was increased by 11.2- and 13.9-fold with pravastatin alone and the combination treatment, respectively. The LDL receptor activity in the untreated homozygous WHHL rabbits was only 2.5% of that in the normal rabbits. mRNA for the LDL receptor in the liver was also increased by 2.1- and 3.4-fold with pravastatin alone and the combination treatment, respectively. On the other hand, mRNA for the LDL receptor in the adrenal gland was not affected by pravastatin and the combination treatment, whereas the mRNA in the intestine was increased in both groups. These results suggest the following: 1) the induction of hepatic LDL receptor activity by the treatment of pravastatin alone or in combination with cholestyramine is the main cause of the reduction of serum cholesterol levels by these treatments even in LDL receptor-deficient animals. 2) The induction of the mRNA for the LDL receptor in the liver and intestine, but not that in the adrenal gland, might be a reflection of the tissue-selective inhibition of cholesterol synthesis by pravastatin.

Keywords: WHHL rabbits, LDL receptor, Pravastatin, HMG-CoA reductase inhibitor, Cholestyramine

In homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, the amount of low-density lipoprotein (LDL) bound to LDL receptor is less than 5% of that in normal rabbits (1–4). Because of their LDL receptor deficiency, WHHL rabbits have serum cholesterol levels up to 1000 mg/dl (mainly LDL cholesterol) from birth, and consequently suffer from severe atherosclerosis as they become mature (5). Although heterozygous WHHL rabbits possess both normal and mutant genes for the LDL receptor, they are not severely hyperlipidemic, unlike the case in human familial hypercholesterolemia (FH) (2, 6). Therefore, homozygous WHHL rabbits are an excellent animal model for human FH.

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis, were reported to lower the plasma lipoprotein cholesterol levels in various kinds of animals (7–9). Cholestyramine, a bile acid sequestrant resin, has long been used as a hypocholesterolemic drug (10). The combination drug treatment of an HMG-CoA reductase inhibitor and cholestyramine was reported to be more effective for lowering plasma cholesterol (6, 11, 12).

It was reported that the main mechanism for both types of drugs to lower plasma LDL is the induction of

hepatic LDL receptor caused by hepatic cholesterol depletion (11, 13). Kume et al. recently reported (6) that pravastatin sodium (pravastatin), an HMG-CoA reductase inhibitor, and cholestyramine additively induced hepatic LDL receptor protein as well as mRNA for the receptor and consequently lowered plasma LDL cholesterol levels in heterozygous WHHL rabbits.

In addition, the induction of LDL receptor mRNA and the reduction of LDL cholesterol in homozygous WHHL rabbits were also reported using mevinolin as an HMG-CoA reductase inhibitor (14). However, the function of the mutant LDL receptor protein has not been clarified well. The question is whether mutant LDL receptors in homozygous WHHL rabbits, in which all the LDL receptors are mutant, really function to bind and internalize LDL when the receptor is highly induced.

In this study, we examined whether the LDL receptor activity was induced and consequently the plasma cholesterol levels were reduced in homozygous WHHL rabbits by pravastatin alone and in combination with cholestyramine.

MATERIALS AND METHODS

Iodine-125 (IMS.30) was purchased from Amersham (England). [3-^{14}C]DL-3-hydroxy-3-methylglutaryl CoA and [γ-^{32}P]ATP were obtained from DuPont-NEN.

Male WHHL rabbits (10–13-month-old) were divided into three groups (3 rabbits each). The rabbits were housed individually and fed a commercial rabbit chow, 120 g/day (Type GC-4, Oriental Yeast Co., Tokyo, Japan). Pravastatin solution was orally administered at the dose of 50 mg/kg per day and cholestyramine was given at the level of 2% in the diet for 4 weeks. Blood samples were withdrawn from the ear vein in the morning after overnight fasting before the drug treatment and at the day of dissection. The rabbits were sacrificed by exsanguination from the carotid blood vessel under pentobarbital sodium anesthesia (20 mg/kg, i.v.). Immediately after bleeding, liver, intestinal and adrenal tissues were removed.

Serum lipoproteins were fractionated according to the previously described method (9). Cholesterol was measured enzymatically.

The liver tissues were homogenized with a Polytron homogenizer, and membranes sedimenting between 8,000 and 100,000 $\times$ g were prepared (3). The membranes were frozen in liquid nitrogen and stored at -80°C for 4 days. LDL from WHHL rabbits was iodinated by the iodine monochloride method as described (15, 16). Membrane pellets were suspended in buffer and subjected to the LDL binding experiment (3, 6) and the HMG-CoA reductase assay (17). For the binding assay, 10 μg/ml ^{125}I-labeled LDL (specific activity 278 cpm/ng) was incubated with 100 μg of the membrane protein. EDTA-sensitive binding was calculated by subtracting the amount of ^{125}I-labeled LDL bound in the presence of 5 mM EDTA from that bound in the absence of EDTA and was considered to be specific for the LDL receptor (3).

Other portions of the liver, the small intestine (ileum) and the adrenal gland were immediately flash-frozen in liquid nitrogen. The tissue total RNAs were isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (18). Fifteen micrograms of RNA was electrophoresed through a 1% agarose gel containing 7% formaldehyde (19) and transferred to a nylon membrane. Prehybridization was performed at 42°C in 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhart's solution, 10% dextran sulfate, 1% SDS and 100 μg/ml of heat-denatured salmon sperm DNA. Hybridization was performed for 16 hr at 42°C in the same buffer containing 10^6 cpm/ml of ^{32}P-labeled cDNA probe (1×10^9 cpm/μg). The LDL receptor probe, pLDLR-2HH1, was a kind gift from Dr. D.W. Russell and consists of a 1.9-kb fragment (base pairs 1573-3486) of the human LDL receptor cDNA clone (20), which showed more than 75% homology to the rabbit cDNA (21). Filters were washed twice at room temperature with 2 $\times$ SSC/0.1% SDS, then washed twice at 50°C with 0.1 $\times$ SSC/0.1% SDS, and then exposed to X-ray film (Fuji New RX) at -80°C with a intensifying screen for 24 hr.

Protein was measured by a Bio-Rad protein assay kit using histone as the standard. All other reagents and materials used were of analytical grade.

RESULTS

Table 1 shows the hypocholesterolemic effect of pravastatin alone and in combination with cholestyramine in male homozygous WHHL rabbits. Initial plasma cholesterol levels were 438 $\pm$ 65 mg/dl ($n = 9$), and about 80% of the cholesterol was in the LDL fraction. In group 1 (control), cholesterol levels in whole plasma and any lipoprotein fraction were not changed during the experimental period. In group 2 (pravastatin), cholesterol levels were reduced by 31% and 29% in whole plasma and LDL, respectively, after 4 weeks of treatment. In group 3 (pravastatin + cholestyramine), cholesterol levels were further reduced by 57% and 56% in whole plasma and LDL, respectively. HDL cholesterol levels were not changed with these drug treatments.

Hepatic HMG-CoA reductase activity was increased 6.6-fold in group 2 (61 vs. 9.2 pmol/min/mg protein)

and 27-fold in group 3 as compared with that in group 1 (Fig. 1).

EDTA-sensitive binding of ^{125}I-labeled LDL to the membranes from WHHL rabbits showed saturation kinetics and was saturated at 10 μg protein/ml (data not shown). The ^{125}I-labeled LDL binding to hepatic membranes is shown in Fig. 1. Control membranes showed very low activities (0.92 and 0.59, average of 0.76 ng LDL/mg protein). Under the same experimental conditions, liver membranes from normal rabbits (Japanese white) showed ^{125}I-labeled LDL binding of 29.8 ± 2.8 ng/mg (n = 3). This means that liver membranes of WHHL rabbits possess only 2.5% of LDL binding activity compared with those of normal ones. Pravastatin alone and the combination drug treatment caused 11.2-fold and 13.9-fold stimulation in the LDL binding activity, respectively.

The amount of mRNA for the LDL receptor was determined by densitometry using α-actin as an internal standard for intensity. As shown in Fig. 2, hepatic

Table 1. Plasma and lipoprotein cholesterol levels in WHHL rabbits treated with pravastatin alone and in combination with cholestyramine

		Cholesterol concentration, mg/dl				
	Weeks	Plasma	VLDL	IDL	LDL	HDL
Group 1	0	454 ± 64	36 ± 21	51 ± 29	354 ± 29	10 ± 4
	4	490 ± 37	27 ± 29	35 ± 34	415 ± 65	12 ± 6
(% of initial)		(108)	(75)	(69)	(117)	(120)
Group 2	0	419 ± 71	34 ± 16	49 ± 32	329 ± 25	7 ± 2
	4	290 ± 50*	23 ± 7	26 ± 16	234 ± 51*	8 ± 1
(% of initial)		(69)	(68)	(53)	(71)	(114)
Group 3	0	443 ± 82	31 ± 17	33 ± 13	369 ± 87	10 ± 1
	4	192 ± 16**	8 ± 3	11 ± 2	164 ± 16**	9 ± 5
(% of initial)		(43)	(26)	(33)	(44)	(90)

Group 1, control; Group 2, pravastatin treatment; Group 3, pravastatin ± cholestyramine treatment. Each value represents the mean ± S.D. for 3 animals. Significantly different from the control value at $P < 0.01$ (*) or $P < 0.001$ (**) by Student's t-test.

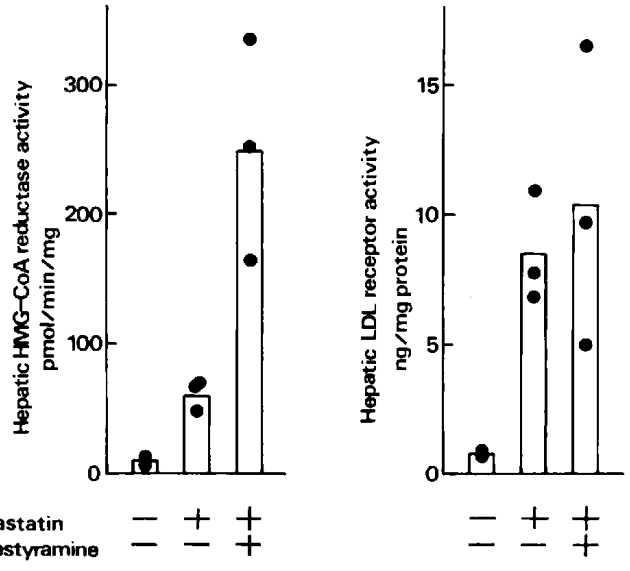

Fig. 1. Hepatic HMG-CoA reductase activity and LDL receptor activity in WHHL rabbits treated with pravastatin alone and in combination with cholestyramine. Hepatic microsomal membranes were prepared and assayed as described in Materials and Methods. Each point represents an individual value and the bars represent the average. Statistical analysis was not done because only two samples were tested in the control group (one sample lost).

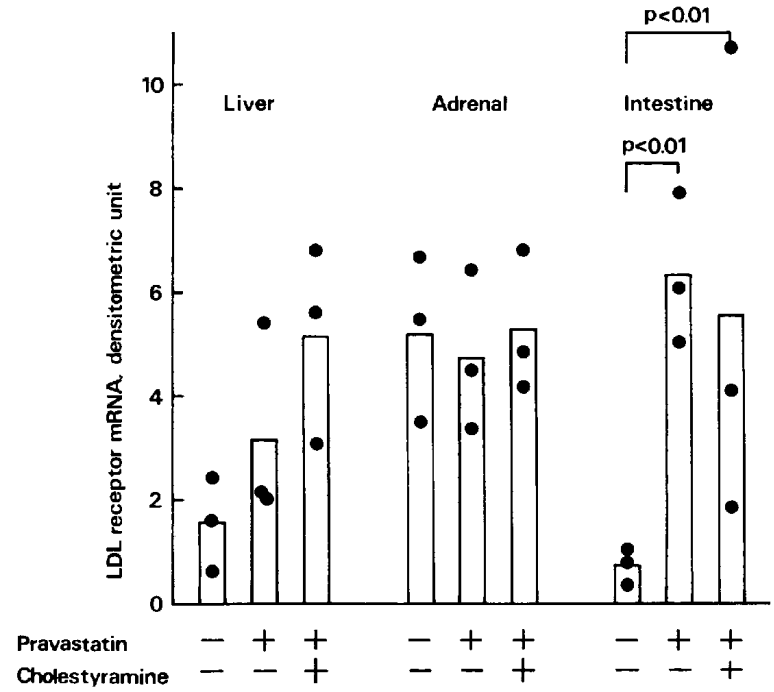

Fig. 2. Hepatic, adrenal and intestinal mRNA for LDL receptor in WHHL rabbits treated with pravastatin and in combination with cholestyramine. Each tissue was subjected to the isolation of total RNA and analyzed for the mRNA for the LDL receptor as described in Materials and Methods. Densitometric intensity was compared with α-actin as a standard. A statistically significant difference was seen only in the intestine by Student's t-test.

mRNA levels were increased to 2.1-fold and 3.4-fold by pravastatin alone and in combination with cholestyramine, respectively. The intestinal mRNA levels were increased to 8.8-fold and 7.7-fold by the single treatment and the combination treatment, respectively. The adrenal mRNA levels, however, were not altered by these treatments. In all experiments (Fig. 2), the intensity of α-actin was almost the same.

Body weights did not change during the experiment among the three groups.

DISCUSSION

The LDL binding activity of membranes from homozygous WHHL rabbits was only one-fortieth that of membranes from normal rabbits. This value is comparable to the previous data (2, 4). Ma et al. (14) first reported that an HMG-CoA reductase inhibitor induced LDL receptor mRNA and reduced plasma LDL cholesterol in homozygous WHHL rabbits, but they did not show the LDL receptor activity after the drug treatment. The present study demonstrated that the increase in hepatic mRNA for the LDL receptor in WHHL rabbits induced by pravastatin, an HMG-CoA reductase inhibitor, alone or in combination with cholestyramine led to the increment of LDL receptor binding activity, and subsequently reduced plasma LDL cholesterol.

Kume et al. (6) reported results similar to the present ones using heterozygous WHHL rabbits, where the normal and mutant mRNAs for the LDL receptor were induced comparably, and the ratios of increment in mRNA and the receptor binding activities were almost the same. Their finding was not proof that the mutant receptor can function, because induction of the normal receptor alone could explain such hypocholesterolemic effects (13). The homozygous WHHL rabbits used in this experiment, however, possess only mutant genes for the LDL receptor and can produce only mutant proteins (22). The present data showed that the induced mutant receptor functioned at least in part and contributed to lowering plasma LDL levels (Table 1).

The mechanism of reduction of plasma cholesterol levels by HMG-CoA reductase inhibitors is thought to be the induction of the hepatic LDL receptor (11). However, we can not rule out the possibility that mechanisms other than LDL receptor induction may contribute to lowering the plasma LDL levels. Vega et al. (23) reported that HMG-CoA reductase inhibitor reduced VLDL secretion as the result of inhibition of hepatic cholesterol synthesis in patients with hyperlipidemia. On the other hand, the plasma LDL cholesterol level in a homozygous FH was reduced from

988 mg/dl to 184 mg/dl by the transplantation of normal liver which possessed normal LDL receptor activity, suggesting that LDL receptor activity in the liver primarily determines the plasma LDL cholesterol levels (24). In addition, Shiomi et al. (4) reported that hepatic LDL receptor activity was increased 12-fold in homozygous WHHL rabbits during pregnancy, resulting in 78% decrease in LDL cholesterol and that the cause of LDL receptor induction might be due to the action of estrogens. Estrogens are reported to induce the LDL receptor and lower LDL cholesterol in animal species (25–27). These findings support the current data; the mutant LDL receptors in homozygous WHHL rabbits have the ability to reduce plasma LDL cholesterol, when induced by any treatment.

The LDL receptor mutation in WHHL rabbits is classified as Class 2 in human FH (28), in which the precursor protein is synthesized normally, but is not susceptible to processing, failing to be transferred to the cell surface (22). However, in homozygous WHHL rabbits, only a small portion of the LDL receptor precursor protein is processed to the mature form (22). Yamamoto et al. estimated (21) that the mutant protein could function. Drug treatments such as those in this experiment might stimulate the precursor processing to the mature form, since the increases in LDL binding activity were much more than those in mRNA for the LDL receptor (Figs. 1 and 2). Another explanation for the phenomenon might be possible: the half life ($t_{1/2}$) of LDL receptor mRNA is considered to be several hours (29), whereas that of LDL receptor protein is more than 24 hours (30). Since the homozygous WHHL rabbits used in this experiment were sacrificed at 24 hours after the final administration of pravastatin, the induction ratio of hepatic LDL receptor mRNA could be lower than that of hepatic LDL receptor activity at that point.

As reported previously (31, 32), the induction of hepatic HMG-CoA reductase activity by inhibition of the enzyme or bile acid sequestrant is considered to be a compensatory phenomenon caused by depletion of cholesterol. In the present study, hepatic HMG-CoA reductase activity was induced by 6.6- and 27.1-fold in groups 2 and 3 (Fig. 1), respectively, suggesting that the hepatic cholesterol was depleted by the inhibitor of HMG-CoA reductase and the bile acid sequestrant resin. On the other hand, the induction ratio of hepatic LDL receptor activity in group 3 was not much higher than that in group 2 (13.9 vs. 11.2, Fig. 1). This might be explained by a compensatory mechanism: the hepatic cholesterol deficiency was compensated by the highly induced HMG-CoA reductase activity in group 3. Another explanation may be possible that these induction ratios show the maximum ability for homozygous WHHL rabbits to induce its hepatic LDL receptor, because Shiomi et al. (4) also reported the same order of LDL receptor induction (12-fold) in homozygous WHHL rabbits during pregnancy.

Synthesis of the mRNA for LDL receptor was not induced in the adrenal gland, whereas it was induced in the intestine as well as the liver (Fig. 2). Kume et al. also reported (6) that mRNA for LDL receptor in adrenal gland was not affected by the treatment of pravastatin in heterozygous WHHL rabbits. Pravastatin selectively inhibited the sterol synthesis in the liver and intestine, but only weakly inhibited it in other organs including the adrenal gland (9). The present results seem to be consistent with the finding mentioned above: the induction of the synthesis of mRNA for the LDL receptor occurred only in tissues where cholesterol synthesis was inhibited. We can not, however, rule out the following possibilities: (i) mRNA for the LDL receptor in the adrenal gland might be affected to a lesser extent than those in the liver and intestine by the treatment of HMG-CoA reductase inhibitors, (ii) any compensatory mechanisms would not be observed in the adrenal gland after the treatment of cholesterol lowering drugs, because of sufficiently high levels of mRNA for the LDL receptor in this organ. The clarification of these possibilities are now under investigation.

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