Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits

(3-hydroxy-3-methylglutaryl CoA reductase/S1-nuclease assay/regulation of cell surface receptors/Watanabe-heritable hyperlipidemic rabbits)

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ABSTRACT Through the use of a quantitative solution hybridization assay with ^{32}P-labeled cDNA probes, we found that mevinolin, an inhibitor of cholesterol synthesis, elevates the level of mRNA for the low density lipoprotein receptor in livers of hamsters and rabbits. In hamsters the maximal effect (3-fold increase) occurred at 0.1% mevinolin in the diet for 10 days. The same dose produced a maximal induction (10-fold) of mRNA levels for 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme of cholesterol synthesis, and a maximal decrease (80%) in plasma cholesterol. The drug lowered the level of all cholesterol-carrying lipoproteins in plasma. In normal rabbits, mevinolin produced a 90% reduction in plasma low density lipoprotein–cholesterol levels, which was associated with a 2.5-fold increase in low density lipoprotein receptor mRNA levels. A similar induction of receptor mRNA occurred in livers of Watanabe-heritable hyperlipidemic rabbits, although the plasma cholesterol was not reduced to normal, presumably because the receptors produced by the mutant mRNA function poorly. These data are consistent with the hypothesis that mevinolin and other inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase lower plasma cholesterol levels in part by stimulating production of mRNA for the low density lipoprotein receptor in liver.

Inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase; EC 1.1.1.88), the rate-limiting enzyme of cholesterol synthesis, reduce the level of lipoprotein-bound cholesterol in blood of humans and animals (1, 2). In dogs these drugs lower the concentration of all cholesterol-carrying lipoproteins in plasma (3). In humans these drugs selectively reduce low density lipoprotein (LDL) (4–6). One mechanism for the cholesterol-lowering effect involves an increase in the activity of hepatic LDL receptors, which remove LDL from plasma through receptor-mediated endocytosis (3, 5, 7). Administration of mevinolin, a HMG CoA reductase inhibitor, to young dogs produced a 2-fold increase in hepatic LDL receptor activity as measured by direct assay of ^{125}I-labeled LDL binding to liver membranes (3). This was associated with a corresponding increase in the rate of removal of intravenously administered ^{125}I-labeled LDL from plasma and a decrease in the calculated rate of production of LDL (3). In humans with heterozygous familial hypercholesterolemia who have a 50% reduction in LDL receptors, mevinolin increased the rate of removal of ^{125}I-labeled LDL from plasma (5). In some hypercholesterolemic subjects mevinolin also decreased the production of LDL (8). Increasing the hepatic LDL receptors decreases LDL production by enhancing the clearance from plasma of intermediate density lipoprotein (IDL), the precursor of LDL (9).

How does an inhibitor of cholesterol synthesis lead to an increase in LDL receptor activity in the liver? One mechanism has been suggested through studies of cultured fibroblasts in which the number of LDL receptors is subject to negative regulation by cholesterol (9). When intracellular cholesterol levels fall, fibroblasts mount a dual response: they produce increased amounts of mRNA for HMG CoA reductase so as to synthesize additional cholesterol, and they produce increased amounts of mRNA for the LDL receptor so as to accelerate uptake of exogenous sterol (9).

We have suggested (3, 9) that mevinolin may trigger an increase in LDL receptor activity in liver by inhibiting cholesterol synthesis, thereby tending to deplete the liver of cholesterol and eliciting an increase in the mRNA for LDL receptors as well as a compensatory increase in the mRNA for HMG CoA reductase. It has now become possible to test this hypothesis through the use of cDNA probes for the LDL receptor. We have shown (10) that these probes can detect the increase in hepatic LDL receptor mRNA levels that follows treatment of rabbits with 17α-ethinyl estradiol. These measurements were made with a sensitive and quantitative solution hybridization/S1-nuclease digestion assay (10, 11).

In the current studies we have employed this assay to measure the levels of LDL receptor mRNA in livers of hamsters and rabbits fed mevinolin. The results show that high doses of mevinolin can lower plasma cholesterol in these two species and that this decline is accompanied by an increase in mRNA for LDL receptors in liver.

METHODS

Materials. ^{32}P-labeled nucleotides and S1 nuclease were purchased from Dupont–NEN Products and Miles Laboratories, respectively. Mevinolin in the lactone form was kindly provided by Alfred Alberts of Merck Sharp & Dohme. Other materials were obtained from reported sources (10, 12).

Animals. Male Golden Syrian hamsters (100–120 g) were obtained from Sasco (Omaha, NE). Female New Zealand White rabbits (1.3–1.5 kg) were purchased from Hickory Hill Rabbitry (Flint, TX). Female homozygous Watanabe-heritable hyperlipidemic (WHHL) rabbits (2.3–2.7 kg) were raised in Dallas, TX (13). All animals were exposed to 12-hr light/12-hr dark cycle for at least seven days prior to use.

Mevinolin Administration. Hamsters were fed a chow diet (Wayne Research Animal Diets) supplemented with 0.01–0.20% mevinolin for various times. Diets were prepared as described (3). Control hamsters were fed the same chow diet without mevinolin. Hamsters were sacrificed at the end of the

Abbreviations: HDL, high density lipoprotein; HMG CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; WHHL rabbits, Watanabe-heritable hyperlipidemic rabbits.

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dark cycle without fasting. Rabbits were fed Purina Rabbit Laboratory Chow supplemented with 0.01–0.10% mevinolin for various times. Control rabbits were fed the same chow without mevinolin. Rabbits were sacrificed at the end of the dark cycle after 12 hr of fasting.

Measurement of mRNA by S1-Nuclease Protection. Total RNA was isolated from liver by homogenization in guanidinium thiocyanate (15) followed by centrifugation through a cesium chloride cushion (16). A single-stranded ^{32}P-labeled cDNA probe ($\approx 7 \times 10^4$ cpm/fmol) was used to measure hamster LDL receptor mRNA. This probe, which was isolated from a hamster genomic bacteriophage library by colony hybridization with a human LDL receptor cDNA (ref. 17; T.C.S., M.S.B., J.L.G., and D. W. Russell, unpublished data), was prepared as follows: A 1.1-kilobase *Eco*RI fragment from the hamster genomic clone was subcloned into the *Eco*RI site of the M13mp19 vector (18). The 3′ end of the M13 clone (anti-sense strand) contains 115 nucleotides of hamster sequence that corresponds to exon 5 of the human LDL receptor gene (19). A single-stranded ^{32}P-labeled probe containing only the exonic portion of the M13 insert was synthesized with an oligonucleotide primer (20 nucleotides in length, AGGGTGGGAGCAAAGCTTAC) in the presence of 44 μM [α-^{32}P]CTP (10^6 cpm/pmol); 0.25 mM each dTTP, dATP, and dGTP; and the Klenow fragment of *Escherichia coli* DNA polymerase (20). The extended product was digested with *Eco*RI, and the resulting ^{32}P-labeled probe was purified by 7 M urea/5% polyacrylamide gel electrophoresis and hydroxylapatite chromatography (11).

A single-stranded rabbit ^{32}P-labeled cDNA probe ($\approx 3 \times 10^4$ cpm/fmol) was used to measure rabbit LDL receptor mRNA as described (10). A single-stranded hamster ^{32}P-labeled cDNA probe ($\approx 3.5 \times 10^4$ cpm/fmol) was used to measure hamster HMG CoA reductase (12). To estimate β-actin mRNA concentrations in hamster and rabbit liver, a single-stranded DNA template containing a portion of a human β-actin cDNA (21) was prepared by subcloning a 381-base-pair *Sma* I–*Msp* I fragment (nucleotides 124–505) into the *Sma* I–*Acc* I site of the M13mp19 vector. A single-stranded ^{32}P-labeled probe complementary to nucleotides 124–320 was synthesized with an oligonucleotide primer (20 nucleotides in length, sequence complementary to nucleotides 301–320) followed by digestion with *Hin*dIII. The specific activity was $\approx 8.5 \times 10^4$ cpm/fmol.

After hybridization of each probe with various amounts of mRNA and digestion with S1 nuclease, the trichloroacetic acid-precipitable radioactivity was measured (10, 11). Lines were fitted to the mRNA curve either visually or by the method of least squares. The slope of the line was taken to reflect the cpm of protected probe per μg of total liver RNA. For rabbits, values were converted to mRNA copies per liver cell as described (10). The hamster data were calculated similarly using a RNA/DNA (wt/wt) ratio of 4.5 (measured) and an estimated value of 6.9 pg of DNA per cell (22).

Other Assays. Rabbit hepatic LDL receptors were quantified by NaDodSO$_4$/polyacrylamide gel electrophoresis and by ligand blotting with ^{125}I-labeled β-migrating very low density lipoprotein (VLDL) as described (10). Hamster liver HMG CoA reductase activity was assayed in microsomes (23). Cholesterol (10) and protein (24) were measured by the indicated methods.

RESULTS

Fig. 1 illustrates the assay used to measure the amount of LDL receptor mRNA. Increasing amounts of total RNA from the liver of a control and a mevinolin-treated hamster were incubated with a single-stranded uniformly ^{32}P-labeled cDNA probe. Excess probe was destroyed by S1 nuclease, and the hybridized (protected) probe was precipitated with trichloro-
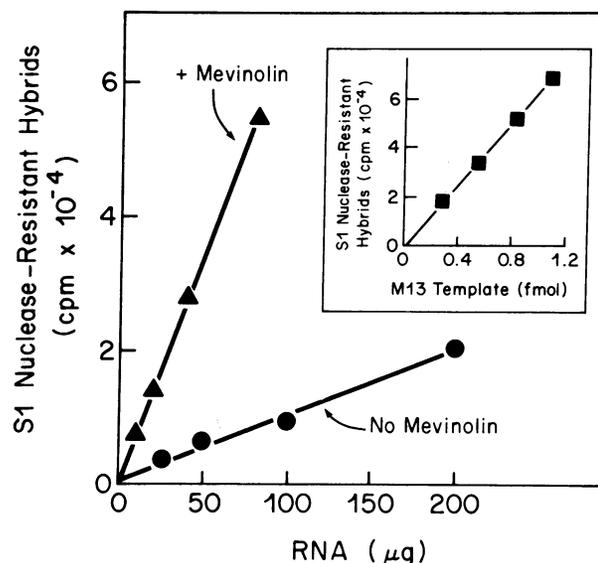

FIG. 1. Hepatic LDL receptor mRNA in untreated (●) and mevinolin-treated (▲) hamsters was measured by the S1-nuclease technique. Mevinolin (0.1%) was fed to one hamster for 17 days (▲). A second animal received the same diet without mevinolin for the same time (●). Various amounts of total liver RNA were hybridized with a single-stranded ^{32}P-labeled hamster LDL receptor cDNA probe. The hybrids were subjected to S1 digestion, and the trichloroacetic acid-precipitable radioactivity was measured. (*Inset*) S1 nuclease-resistant hybrids formed between the ^{32}P-labeled probe and various amounts of the template DNA from which it was synthesized. A blank value of 0.7×10^4 cpm (representing the amount of S1-resistant radioactivity precipitated in the absence of mRNA or template) was subtracted from each value.

acetic acid and subjected to scintillation counting. The slope of each line is proportional to the amount of LDL receptor mRNA in the tissue sample. The specific activity of the cDNA probe was calculated by hybridization with known amounts of the recombinant M13 bacteriophage that was used to generate the probe (Fig. 1, *Inset*). Using an estimate of the amount of total RNA per liver cell, we can express the data as mRNA copies per cell. These units provide a convenient reference point for comparison of levels of different mRNAs. However, the significance of the absolute numbers must be held in question since the receptor mRNA is undoubtedly distributed heterogeneously among liver cells and since we cannot be certain that all ^{32}P-labeled cDNA/mRNA hybrids are totally protected from S1-nuclease digestion.

Fig. 2 shows the results of a 10-day treatment of hamsters with various amounts of mevinolin in the diet. Livers from four hamsters were pooled for each experimental point. A detectable increase in LDL receptor mRNA occurred at a dose of 0.025% mevinolin, which is approximately 16 mg/kg of body weight per day. Maximal levels of receptor mRNA occurred at 0.1% mevinolin in the diet (Fig. 2A). The plasma cholesterol level began to fall at 0.05% mevinolin and reached a nadir at 0.1% mevinolin (Fig. 2A). For comparative purposes, we also measured the enzymatic activity of HMG CoA reductase and the amount of mRNA for HMG CoA reductase in the same livers (Fig. 2B). The relative increase in reductase activity and mRNA levels was much greater than the increase in LDL receptor mRNA. However, the maximal amount occurred at the same dosage level (0.1% mevinolin). Fig. 3A shows that the maximal amount of receptor mRNA occurred 10 days after the onset of treatment with 0.1% mevinolin. The activity of HMG CoA reductase and the amount of reductase mRNA also reached a peak at 10 days (Fig. 3B).
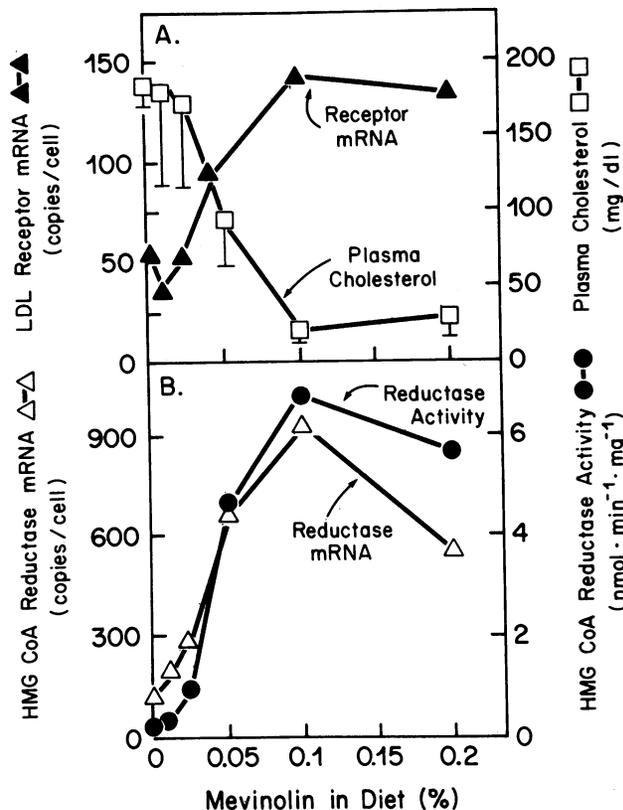
FIG. 2. Hepatic levels of LDL receptor mRNA (▲), HMG CoA reductase mRNA (△), and HMG CoA reductase activity (●) and total plasma cholesterol (□) in hamsters fed with the indicated amount of mevinolin for 10 days. mRNA concentrations were measured on pooled aliquots from four animals. Plasma cholesterol was determined on each of the four animals, and the mean ± SEM is shown.

FIG. 3. Changes in hepatic levels of LDL receptor mRNA (▲), HMG CoA reductase mRNA (△), HMG CoA reductase activity (●) and total plasma cholesterol (□) in hamsters fed 0.1% mevinolin for the indicated time. Measurements were made on four animals as described in Fig. 2.

Fig. 4 shows the correlation between the total plasma cholesterol level and the level of LDL receptor mRNA in livers of mevinolin-treated hamsters, using the data from the dose–response and time course experiments. The control animals in the dose–response experiment had higher plasma cholesterol levels than those in the time course experiment. Nevertheless, in both experiments there was an inverse relation between LDL receptor mRNA levels and plasma cholesterol levels. The correlation coefficient for the pooled data was 0.82. As a control, we also measured the amount of β-actin mRNA in the same livers and found that mevinolin had no consistent effect (Fig. 5). Mevinolin at the highest doses had a tendency to decrease the body weight of the animals (Fig. 5).

To explore the effect of mevinolin on specific lipoproteins, we fed 15 hamsters a diet containing 0.1% mevinolin for 17 days (Table 1). On the last day the animals were fasted and given mevinolin by subcutaneous injection. Plasma samples from groups of two or three of these animals were pooled for lipoprotein fractionation. Hepatic RNA from each group was also pooled and used for measurements of specific mRNA levels. The control group comprised 12 hamsters fed the same diet without mevinolin and used on the same day as the treated animals. Under these conditions mevinolin caused a 4.4-fold increase in LDL receptor mRNA levels (Table 1). The total plasma cholesterol level fell by 80%. Major declines were observed in the levels of cholesterol in VLDL, LDL, and high density lipoprotein (HDL). We also performed NaDodSO₄/polyacrylamide gel electrophoresis on the isolated lipoprotein fractions from these hamsters and visualized the apolipoproteins by staining with Coomassie blue. Mevinolin caused a nearly complete disappearance of apo-
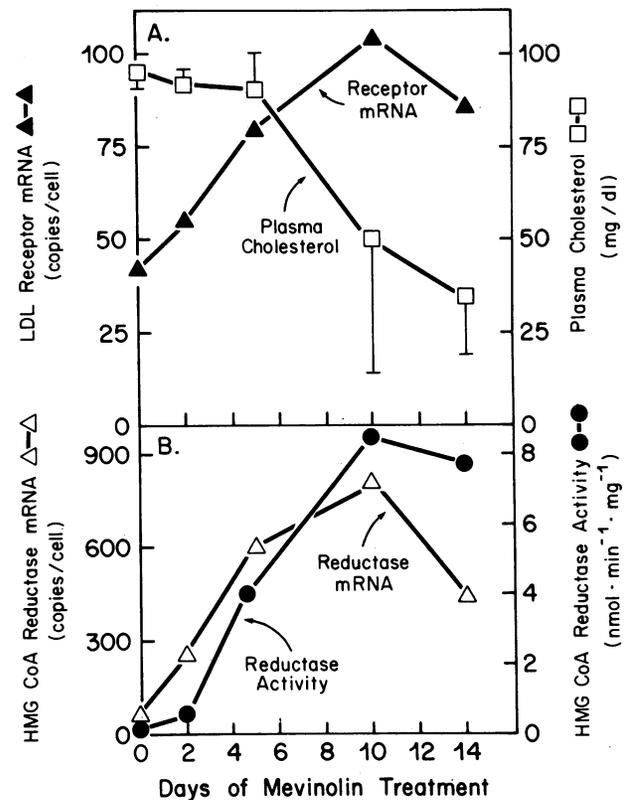
lipoprotein B-100 and apolipoprotein A-I from all fractions (data not shown). The amount of plasma obtained was not sufficient to quantify the levels of the other apolipoproteins.

We next studied the effect of mevinolin on LDL receptors in the rabbit. Using an identical solution hybridization assay but employing a rabbit cDNA probe, we found that mevinolin
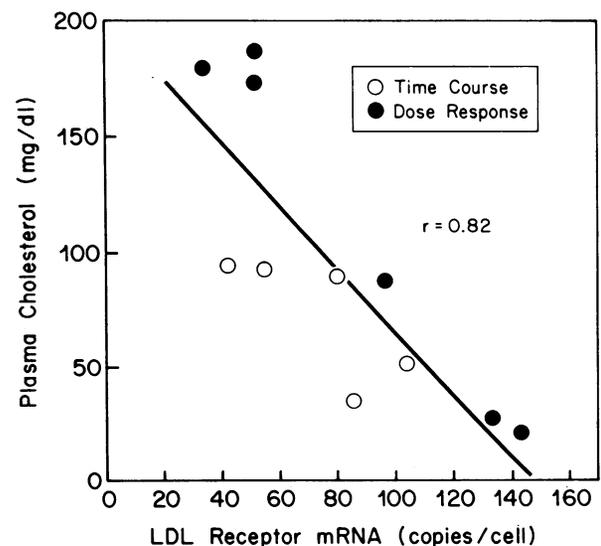

FIG. 4. Correlation between the concentrations of total plasma cholesterol and hepatic LDL receptor mRNA in mevinolin-treated hamsters. These values were derived from the time course curve (○) in Fig. 3 and the dose–response curve (●) in Fig. 2.

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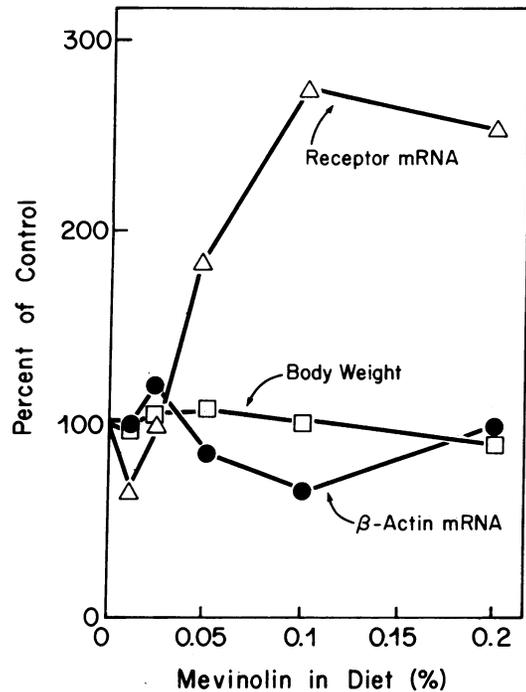
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FIG. 5.   Changes in body weight (□) and hepatic levels of LDL receptor mRNA (△) and of β-actin mRNA (●) after treatment with various amounts of mevinolin in the diet for 10 days. These data are derived from the same animals shown in Fig. 2. The measured amount of β-actin mRNA in livers of control animals was 490 copies per cell (100% value).

FIG. 6.   Changes in hepatic levels of LDL receptor mRNA (▲) and of LDL receptor protein (●) and plasma LDL-cholesterol (□) in normal rabbits treated with the indicated dose of mevinolin in the diet for 8 days. To measure LDL receptor protein levels, detergent-solubilized extracts of liver membranes were subjected to electrophoresis on NaDodSO$_4$/polyacrylamide gels under nonreducing conditions and transferred to nitrocellulose paper for ligand blotting with ^{125}I-labeled β-migrating VLDL. Densitometric scans of the autoradiograms were obtained, and the peak area was determined. Each value is the mean of data from three rabbits. The SEM values for plasma cholesterol are shown.

increased LDL receptor mRNA levels in normal rabbits, with a peak effect between 0.02 and 0.1% in the diet (Fig. 6). There was a corresponding increase in the amount of LDL receptor protein as visualized by NaDodSO$_4$/polyacrylamide gel electrophoresis/nitrocellulose blotting of liver extracts, followed by incubation of the nitrocellulose with ^{125}I-labeled β-migrating VLDL and densitometric scanning of the autoradiogram (Fig. 6). Mevinolin produced a 90% fall in the level of plasma LDL-cholesterol (Fig. 6). The total cholesterol level fell from 65 to 23 mg/dl (data not shown).

Table 2 shows that mevinolin produced a 3-fold increase in the amount of LDL receptor mRNA, but not β-actin mRNA, in livers of WHHL rabbits. These rabbits are homozygous for a mutant LDL receptor gene that has an in-frame deletion of 12 nucleotides, resulting in a poorly functional LDL receptor that reaches the cell surface in reduced amounts (25). As a result, WHHL rabbits have cholesterol levels that are up to 10-fold higher than normal. Although plasma LDL-cholesterol levels fell by 43% in the mevinolin-treated animals, the level after treatment was 304 mg/dl, which is still markedly elevated. That mevinolin can increase LDL receptor mRNA levels in WHHL rabbits even when the plasma
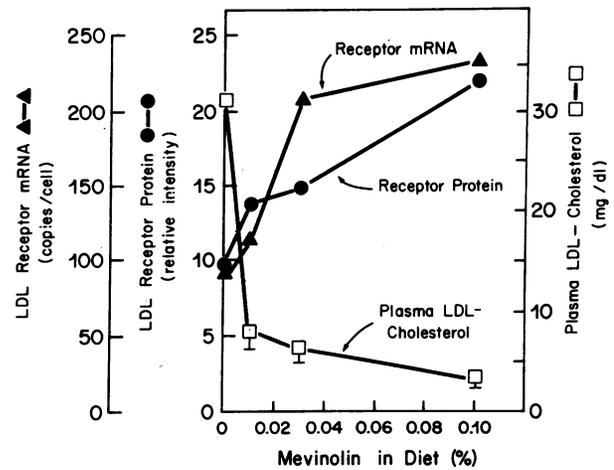
LDL-cholesterol level remains elevated suggests that mevinolin acts directly on the control mechanism governing the mRNA level, and not indirectly through lowering plasma LDL-cholesterol.

DISCUSSION

The current data show that mevinolin, an inhibitor of HMG CoA reductase, can increase the level of mRNA for the LDL receptor in livers of hamsters and rabbits. The dose of mevinolin that was required to lower the cholesterol and increase the receptor mRNA level is much higher than the dose that lowers cholesterol in humans (2, 4–6). The reason why animals such as hamsters and rabbits are relatively resistant to the effects of mevinolin is unknown.

From the data now available, it is impossible to determine how much of the cholesterol-lowering effect is attributable to the elevation in LDL receptors and how much may be due to other effects on cholesterol metabolism. In addition to increasing the rate of catabolism of LDL, which is directly attributable to increased LDL receptors, mevinolin also inhibits the production of LDL (3, 8). This inhibition may also be mediated by the increase in LDL receptors, which enhance the clearance from plasma of IDL, the precursor of LDL (9). However, a direct inhibition of LDL secretion has not been ruled out.

Table 1.   Increased mRNA for LDL receptor in livers of hamsters treated with mevinolin

Animals	No.	Body weight, % of pretreatment	mRNA for LDL receptor, copies per cell	Plasma cholesterol level, mg/dl			
				Total	VLDL	LDL	HDL
Control	12	107 ± 4	34 ± 6.8	132 ± 8	19 ± 1	54 ± 7	56 ± 7
Mevinolin-treated	15	86 ± 1	151 ± 19	26 ± 3	2 ± 0.5	12 ± 2	10 ± 2

Hamsters were treated with or without 0.1% mevinolin in the diet for 17 days. The animals were fasted 18 hr before sacrifice after which two subcutaneous injections of 25 mg of mevinolin per kg of body weight (14) were given at the 18th and 6th hr before sacrifice. Control hamsters received subcutaneous injections of solution without mevinolin. After treatment, the animals were killed, blood was obtained for plasma cholesterol, and hepatic RNA was prepared. Concentrations of LDL receptor mRNA were measured by the standard S1-nuclease assay. Each value is the mean ± SEM of data from the indicated number of animals.

Table 2. Effect of mevinolin on LDL receptor mRNA in liver and on plasma cholesterol levels in WHHL rabbits

WHHL rabbits	Body weight, % of pretreatment	mRNA, copies per cell		Pretreatment plasma cholesterol, mg/dl				Posttreatment plasma cholesterol, mg/dl			
		LDL receptor	β-actin	Total	VLDL	LDL	HDL	Total	VLDL	LDL	HDL
Control	109 ± 2	51 ± 6	431 ± 8	871 ± 103	284 ± 104	556 ± 58	13 ± 0	709 ± 33	211 ± 62	465 ± 57	11 ± 1
Mevinolin	122 ± 5	164 ± 4	324 ± 66	743 ± 101	187 ± 45	536 ± 50	9 ± 1	426 ± 37	85 ± 10	304 ± 32	8 ± 1

WHHL rabbits were treated with or without 0.03% mevinolin in the diet for 10 days. One day prior to treatment, animals were fasted for 12 hr after which blood was obtained from the ear arteries for measurement of pretreatment plasma cholesterol. After treatment with mevinolin or the control diet, the animals were killed after fasting for 12 hr, blood was obtained by cardiac puncture for measurement of posttreatment cholesterol, and hepatic mRNA was prepared. Concentrations of LDL receptor and β-actin mRNA were measured by the standard S1-nuclease assay. Each value is the mean ± SEM of data from three animals.

In hamsters and rabbits mevinolin produced a decrease in all classes of cholesterol-carrying lipoproteins, including HDL, whereas in humans mevinolin produces a selective decrease in LDL and perhaps IDL levels, without lowering HDL (4–6). This difference may be due to the relative abundance of apolipoprotein E in the HDL fractions from the lower animal species as compared with humans. Such apolipoprotein E-containing HDL may bind to the LDL receptor, thus allowing rapid catabolism when LDL receptors are elevated (9). In addition, an increase in LDL receptors might accelerate the clearance of partially catabolized VLDL from plasma, thus leading to a reduced generation of HDL, some of whose lipids are derived from VLDL (26). 17α-Ethinyl estradiol, which increases hepatic LDL receptor mRNA and protein, reduces HDL as well as LDL levels in animals (10). Thus, the observed fall in HDL levels after mevinolin treatment does not exclude an increase in hepatic LDL receptors as the primary mechanism.

It seems likely that the increased mRNA for LDL receptors in liver results from the mevinolin-mediated inhibition of cholesterol synthesis. This inhibition leads to a compensatory increase in the amount of mRNA for HMG CoA reductase, presumably by depleting cells of a sterol that acts as a feedback suppressor of transcription of the HMG CoA reductase gene (27). The same sterol may act as a repressor of the LDL receptor gene, and thus the same mechanism that compensates for the inhibitory effect of mevinolin on cholesterol synthesis may also lead to an increased production of LDL receptors. In the steady state, this compensatory mechanism may restore cholesterol synthesis to a near-normal rate, but only so long as the transcription rate for HMG CoA reductase and the LDL receptor remain elevated. The increased receptors, in turn, may be responsible for the reduced production and enhanced catabolism of LDL.

In hamsters treated with various doses of mevinolin for various times, there was a clear inverse relation between levels of hepatic LDL receptor mRNA and plasma cholesterol (Fig. 4). However, among untreated hamsters there was a poor correlation between levels of plasma total or LDL-cholesterol and hepatic LDL receptor mRNA. Clearly, many variables in addition to the LDL receptor mRNA determine the plasma cholesterol in individual animals. When production of LDL receptors is stimulated through drug therapy, the receptor mRNA level appears to become the dominant factor that is associated with a fall in plasma cholesterol. Conversely, when receptor levels are suppressed by feeding diets rich in saturated fat or cholesterol, the receptors again may become the dominant factor in regulating the plasma cholesterol (28).

It is difficult to extend these findings directly to humans in whom direct measurements of hepatic mRNA levels and LDL receptor binding activity are not possible. Nevertheless, the observed increase in plasma clearance of LDL (5) and the decrease in production of LDL (8) are both consistent

with an increase in hepatic LDL receptor levels in humans as well as animals treated with cholesterol-lowering doses of mevinolin.

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