Effects of Cholestyramine on Low Density Lipoprotein Binding Sites on Liver Membranes from Rabbits with Endogenous Hypercholesterolemia Induced by a Wheat Starch-Casein Diet*

Yu-sheng Chao, Ting-Ting Yamin, and Alfred W. Alberts
From the Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065

Rabbits fed a wheat starch-casein diet develop a marked hypercholesterolemia with a lipoprotein distribution similar to that of humans. Approximately 76% of the total cholesterol is carried in the low density lipoprotein (LDL) fraction (1.006 < d < 1.063 g/ml). Inclusion of 1% cholestyramine in the diet prevents the increase in plasma cholesterol. The cholestryramine effect is mediated through an increased fractional catabolic rate of LDL. In order to determine the potential role of hepatic LDL receptors in the removal of LDL from the plasma, binding of 125I-LDL and 125I-β-VLDL (β-migrating very low density lipoproteins) to hepatic membranes prepared from livers of rabbits fed the wheat starch-casein diet with or without cholestyramine supplementation was investigated. Membranes from livers of the cholestyramine-supplemented animals exhibit high levels of specific EDTA-sensitive binding of either of the 125I-labeled lipoproteins. Very little EDTA-sensitive binding occurs on liver membranes from wheat starch-casein-fed rabbits that have not been treated with cholestyramine. These results indicate that the hypercholesterolemia in rabbits associated with the wheat starch-casein diet is wholly or partially the result of a decreased number of specific hepatic LDL receptors and thus a decreased catabolism of plasma cholesterol. The response of the liver to the inclusion in the diet of the bile acid sequestrant, cholestyramine, is to maintain or increase the number of specific LDL binding sites, thus promoting catabolism of plasma cholesterol.

There is a specific high affinity receptor for low density lipoproteins on the cell surface of a variety of cultured cells (1) which recognizes apoprotein B and apoprotein E (2) and is regulated by the concentration of LDL in the culture medium (1). One of the mechanisms that causes elevated LDL levels in familial hypercholesterolemia is believed to be the defect in the gene for the LDL receptor (1). Cholesterol, the major component of LDL, is degraded mainly by the liver. Recently, attention has been focused on the regulation of hepatic LDL receptor. Several lines of evidence show that LDL receptors exist on rat hepatic plasma membranes. Administration of pharmacological doses of 17α-ethinyl estradiol increases the number of high affinity binding sites for rat LDL, rat hepatic very low density lipoproteins, and chylomicron remnants in vitro (3). The increased number of binding sites is also accompanied by increased hepatic catabolism of 125I-labeled LDL and apo-E-containing HDL in rats and in isolated perfused rat livers (4). Autoradiographic studies show that, in the rat, hepatic uptake of LDL follows a pathway that closely resembles that of LDL receptor in cultured cells (5). Slater et al. (6) have attributed increased catabolism of LDL and lowered levels of plasma cholesterol in rabbits fed cholestyramine to an increase in the receptor-mediated hepatic uptake of LDL in rabbits. A high affinity LDL binding site that is also present in cultured pig (7) and rabbit hepatocytes (8) and in liver membranes prepared from dogs (9).

Recently, Kita et al. (10) demonstrated the presence of specific binding sites for LDL in rabbit liver membranes. Unlike the binding site described in other cells, this binding site consists of two components, one EDTA-sensitive and one EDTA-resistant. They further demonstrated that in homozygous Watanabe-heritable hyperlipidemic rabbits, the hepatic EDTA-sensitive binding activity is either lost or greatly reduced, whereas the EDTA-resistant binding activity is not affected (10). Feeding rabbits with cholesterol results in hypercholesterolemia and decreased binding of 125I-β-VLDL to hepatic membranes (11).

Rabbits can also become hypercholesterolemic when they are fed cholesterol-free, semisynthetic diets which contain casein (12-14). Carroll and associates (12, 13) have shown that such diets result in reduction in bile acid synthesis and fecal steroid excretion. In the current studies, we investigate the hepatic LDL receptor activity in rabbits fed a cholesterol-free, semisynthetic diet and the regulation of the receptor activity by cholestyramine treatment.

**EXPERIMENTAL PROCEDURES**

**Rabbits and Diets**—Male New Zealand White rabbits weighing 1.5-2.0 kg were used in all experiments. The diet consisted of wheat starch (33.3%), Cellulofour (30%), casein (27%), minerals (4%), molasses (2%), water (2%), corn oil (1%), and vitamin (0.7%) (24). This diet is referred to as wheat starch-casein diet in this manuscript. Cholesterol-fed rabbits were fed milled rabbit chow supplemented with 0.5% cholesterol and 10% corn oil for 2 months. All rabbits were housed individually and were fed a chow diet for 1 week before being changed to the experimental diets.

**Preparation of Lipoproteins**—β-VLDL (d < 1.006 g/ml) from cholesterol-fed rabbits and LDL (1.02 < d < 1.063 g/ml) from cholesterol-fed rabbits were isolated by sequential ultracentrifugation (15) at 7°C in a Ti-60 or a 40.3 rotor in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc.). All lipoproteins were recentrifuged once under the same conditions and dialyzed against 0.15 M NaCl and 0.015 EDTA, pH 7.4. β-VLDL is greatly enriched in cholesteryl esters.
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while containing only 5% of triglycerides and migrates as a single \( \beta \) band on agarose electrophoresis. The major apolipoproteins of \( \beta \)-VLDL are apo-B and apo-E. There are only trace amounts of apo-C present in this fraction as judged by electrophoresis in 10% polyacrylamide gel in the presence of 0.2% sodium dodecyl sulfate. More than 98% of the apolipoprotein in the LDL preparations is apo-B as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lipoprotein preparations were labeled with \(^{125}\text{I}\) by the iodine monochloride method as previously described (16). Free iodide was removed by dialysis against 0.15 M NaCl, 0.01% EDTA, pH 7.4. For LDL and \( \beta \)-VLDL, averages of 3 and 10% of the radioactivity, respectively, were extractable into chloroform/methanol. All lipoproteins (labeled and unlabeled) that were used in the binding assays were dialyzed against 100 volumes of buffer A (50 mM Tris, 25 mM NaCl, 0.5 mM CaCl\(_2\), pH 8.0) for 24 h at 4°C. \(^{125}\text{I}\)-labeled LDL could be stored at 4°C for up to 1 week without loss of binding activity. The binding activity of \(^{125}\text{I}\)-labeled LDL decreased with time of storage and, therefore, was used immediately after preparation. LDL and \(^{125}\text{I}\)-LDL were passed through a Millex-HA 0.45-\( \mu \)m filter (Millipore) before use.

Preparation of Liver Membranes—Rabbit liver membranes were prepared essentially as described by Kovanen et al. (17). Rabbits were killed by injection of 3 ml of pentobarbital (Nembatal, Abbott Laboratories) via an ear vein. The liver was removed, placed in ice-cold 0.15 M NaCl solution, and homogenized in buffer A with a Polytron (Brinkmann Instruments). The 8,000-100,000 × g pellet was prepared as described (17). The pellet was washed once with buffer A and centrifuged at 100,000 × g. The pellets of the membrane preparations were stored at -70°C. On the day of an experiment, the pellet was suspended in Buffer A by flushing five times through a 22-gauge needle. The suspension was sonicated for 45 s at 4°C and diluted with buffer A to a protein concentration of 10 mg/ml.

Binding of \(^{125}\text{I}\)-Lipoproteins to Liver Membranes—The binding of \(^{125}\text{I}\)-lipoproteins to liver membranes was measured as described (17). The assay was conducted in 80 pl of buffer B (50 mM Tris, 25 mM NaCl, 0.5 mM CaCl\(_2\), 20 mg/ml of bovine serum albumin, pH 8.0) containing 100 \( \mu \)g of membrane protein in the absence or presence of excess unlabeled lipoproteins and in the absence or presence of 30 mM EDTA. After incubation at 0°C in an ice water bath for 60 min, membrane-bound \(^{125}\text{I}\)-lipoproteins were separated from free \(^{125}\text{I}\)-lipoprotein by layering 50 pl of the incubation mixture on 150 pl of fetal bovine serum in a 250-\( \mu \)l Beckman ultracentrifuge tube and centrifuged at 8,000-100,000 × g for 100 min at 4°C. The supernatant was removed by aspiration. The pellet was washed once with 200 pl of buffer B and centrifuged once at 100,000 × g for 30 min. After removing the supernatant, the area containing the pellet was sliced off with a razor blade and counted in a Packard \( \gamma \) counter.

The difference between the amount of lipoprotein bound in the absence and presence of EDTA was defined as EDTA-sensitive binding. The difference between the amount of lipoprotein bound in the presence of EDTA and in the presence of unlabeled lipoprotein was defined as EDTA-resistant binding.

Analyses—Protein was determined by a modified procedure of Lowry with bovine serum albumin as a standard (18). Serum cholesterol concentration was determined by the cholesterol oxidase method as previously described (16). Free iodide was removed by dialysis against 0.15 M NaCl, 0.01% EDTA, pH 7.4. For LDL and \( \beta \)-VLDL, averages of 3 and 10% of the radioactivity, respectively, were extractable into chloroform/methanol. All lipoproteins (labeled and unlabeled) that were used in the binding assays were dialyzed against 100 volumes of buffer A (50 mM Tris, 25 mM NaCl, 0.5 mM CaCl\(_2\), pH 8.0) for 24 h at 4°C. \(^{125}\text{I}\)-labeled LDL could be stored at 4°C for up to 1 week without loss of binding activity. The binding activity of \(^{125}\text{I}\)-labeled LDL decreased with time of storage and, therefore, was used immediately after preparation. LDL and \(^{125}\text{I}\)-LDL were passed through a Millex-HA 0.45-\( \mu \)m filter (Millipore) before use.

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Analyses—Protein was determined by a modified procedure of Lowry with bovine serum albumin as a standard (18). Serum cholesterol concentration was determined by the cholesterol oxidase method using Agent assay reagent from Abbott Laboratories. The equilibrium-dissociation constant (KJ) and maximal bound lipoprotein (Bmax) were calculated from the slope and intercept of the Scatchard plots (19).

Removal of \(^{125}\text{I}\)-LDL in Intact Rabbits—\(^{125}\text{I}\)-LDL, containing 200 \( \mu \)g of apo-LDL, was injected into fed rabbits between 10 and 11 a.m. through an ear vein. Blood samples were obtained from an ear artery at intervals during the next 3 days. \(^{125}\text{I}\) was determined in 100-\( \mu \)l samples of serum and the results were expressed as percentage of \(^{125}\text{I}\) in the sample taken 10 min after the injection.

RESULTS

Rabbits fed a diet consisting of wheat starch as the sole carbohydrate source and casein as the sole protein source and without any exogenous cholesterol developed hypercholesterolemia (Fig. 1). Inclusion of a bile acid sequestrant, cholestyramine, at a level of 1% in the wheat starch-casein diet prevents the increase in serum cholesterol levels. The distribution of cholesterol among serum lipoproteins was determined in three animals from each of the two groups (Table I). In the cholestyramine-treated group, approximately two-thirds of the cholesterol is found in the HDL fraction with only 5% in the LDL fraction. This is similar to that found in rabbits fed a normal rabbit chow diet (data not shown). VLDL, LDL, and HDL cholesterol levels are increased in the rabbits fed the wheat starch-casein diet in the absence of cholestyramine. However, the bulk of the increase is associated with the LDL fraction which in this group accounts for approximately 76% of the plasma cholesterol level.

The effect of cholestyramine on clearance of LDL in rabbits fed the wheat starch-casein diet is illustrated in Fig. 2. The inclusion of cholestyramine in this diet results in a more rapid removal of injected \(^{125}\text{I}\)-labeled rabbit LDL from plasma.

To investigate the role of the liver in the removal of \(^{125}\text{I}\)-LDL in rabbits, we studied the binding of \(^{125}\text{I}\)-LDL to liver membranes prepared from rabbits fed the wheat starch-casein diet alone or this diet supplemented with cholestyramine. It has been shown that in normal rabbit liver membranes there are two binding sites for \(^{125}\text{I}\)-LDL, an EDTA-sensitive and an EDTA-resistant site (10). The experiments in Fig. 3 show binding of \(^{125}\text{I}\)-LDL to liver membranes derived from a representative animal from each group. Similar results were obtained with the other animals of the groups. In liver membranes from the wheat starch-casein-fed rabbits, total binding can be inhibited by excess amounts of unlabeled LDL, but can only be slightly inhibited by EDTA (Fig. 3A). The results
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Fig. 3. Binding of $^{125}$I-LDL to liver membranes from wheat starch-casein-fed (A) and cholestyramine-treated rabbits (B). Each assay tube contained 100 μg of membrane protein and the indicated concentration of $^{125}$I-LDL (514 cpm/mg of protein) in the absence (○) or presence of either 30 mM EDTA (▲) or unlabeled rabbit LDL at 1 mg/ml (●).

Fig. 4. Binding of $^{125}$I-LDL to the EDTA-sensitive (A) and to the EDTA-resistant (B) binding sites of liver membranes from either wheat starch-casein-fed (○) or cholestyramine-treated (●) rabbits.

Fig. 5. Binding of $^{125}$I-β-VLDL to liver membranes from wheat starch-casein-fed (A) and cholestyramine-treated (B) rabbits. Each assay tube contained 100 μg of membrane protein and the indicated concentration of $^{125}$I-β-VLDL (161 ng/mg of protein) in the absence (○) or presence of either 30 mM EDTA (▲) or unlabeled β-VLDL at 1 mg/ml (●).

Fig. 6. Binding of $^{125}$I-β-VLDL to the EDTA-sensitive (A) and to the EDTA-resistant (B) binding sites of liver membranes from either wheat starch-casein-fed (○) or cholestyramine-treated (●) rabbits.

The above data show that treatment with cholestyramine can prevent the loss of hepatic EDTA-sensitive binding site for $^{125}$I-LDL. In order to investigate whether cholestyramine treatment can actually induce this binding site, three rabbits were made hypercholesterolemic by feeding them the wheat starch-casein diet for 60 days. The average serum cholesterol level in these animals was 188 mg/dl. Subsequent inclusion of cholestyramine in the diet for an additional 14 days resulted in a marked decrease in serum cholesterol levels to an average of 41 mg/dl. Binding studies with hepatic membranes from these rabbits clearly demonstrated the presence of the EDTA-sensitive binding site for $^{125}$I-LDL with similar $K_d$ and maximal binding values as those seen in liver membranes prepared from rabbits fed cholestyramine from the start of wheat starch-casein feeding. These results demonstrate that cholestyramine not only prevents repression of hepatic $^{125}$I-LDL binding sites but can also induce these binding sites when they are repressed.

It has been shown that the LDL binding site of rabbit liver membranes also recognizes β-VLDL, a cholesterol-rich lipoprotein that accumulates in plasma of cholesterol-fed rabbits.
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We studied the binding of $^{125}$I-β-VLDL to liver membranes from the two groups of rabbits. Fig. 5 shows that the amount of $^{125}$I-β-VLDL bound to EDTA-sensitive binding sites is considerably greater in rabbits fed the wheat starch-casein diet supplemented with cholestyramine than in the animals fed the wheat starch-casein diet alone. The EDTA-sensitive binding of $^{125}$I-β-VLDL to liver membranes from both groups of rabbits is shown in Fig. 6A. Scatchard analysis of the binding to membranes from cholestyramine-treated rabbits gave a single component with a calculated apparent $K_d$ of 0.3 μg/ml and maximal binding of 128 ng/mg of protein. Significant EDTA-sensitive binding of $^{125}$I-β-VLDL to liver membranes from wheat starch-casein-fed rabbits also occurs but to a much lesser extent than in the cholestyramine-treated rabbits. As seen with binding of $^{125}$I-IDL-L, $^{125}$I-β-VLDL binds to the EDTA-resistant site and this binding is not affected by the dietary treatment (Fig. 6B).

DISCUSSION

It has been shown that hypercholesterolemia can be induced in rabbits by feeding either a cholesterol diet or a cholesterol-free, semisynthetic diet, and that both diets are equally atherogenic in rabbits (20). In contrast to the cholest-sterol diet, hypercholesterolemia induced by a cholesterol-free, semisynthetic diet is primarily due to endogenous synthesis of cholesterol. In the current study, we demonstrated that while the level of plasma cholesterol raised, the hepatic EDTA-sensitive binding activity for LDL and β-VLDL was either lost or greatly reduced in wheat starch-casein-fed rabbits. Since cholesterol is degraded mainly in the liver, a reduction of a binding site for cholesterol-carrying lipoprotein may also cause the accumulation of cholesterol in plasma.

Cholesterol is catabolized in the liver by conversion to bile acids. It has been shown that cholestyramine, a bile acid sequestrant, lowers plasma cholesterol by increasing the fractional catabolic rate of LDL in rabbits (6), dogs (9), and in humans (21). The current studies show that when cholestyramine is added to the wheat starch-casein diet, the level of plasma cholesterol is markedly lowered and the removal rate of LDL from rabbit plasma is increased. The data presented also show that the hepatic EDTA-sensitive binding site for LDL and β-VLDL is either lost or greatly reduced in rabbits fed a wheat starch-casein diet. This binding site is present in rabbits fed cholestyramine. Scatchard analysis of this binding data gives a $K_d$ of 0.8 μg/ml and a maximal binding of 42 ng/mg for LDL and a $K_d$ of 0.3 μg/ml and a maximal binding of 128 ng/mg for β-VLDL. They are similar to the binding site described in chow-fed rabbits; the $K_d$ and maximal binding are 1.0 μg/ml and 35 ng/mg, respectively, for LDL, and 0.5 μg/ml and 150 ng/mg, respectively, for β-VLDL (10). These results suggest that the EDTA-sensitive binding site observed in cholestyramine-fed rabbits is the same binding site observed in chow-fed rabbits. Hui et al. (22) have demonstrated that in young dogs there are hepatic apo-B and apo-B-apo-E receptors, whereas in adult dogs, only the apo-E receptor exists. However, the hepatic apo-B-apo-E receptor in adult dogs can be induced by feeding the dogs cholestyramine (22). Since apo-E also binds to the apo-B binding site, it is not known from this study whether cholestyramine affects the apo-B-apo-E receptor or the combination of apo-B-apo-E and apo-B-apo-E receptors in rabbits.

The mechanism by which cholestyramine induces hepatic LDL binding sites in these rabbits is not known. Since the wheat starch-casein diet is known to decrease the fecal excretion of bile acids in rabbits (12, 13), the addition of a bile acid sequestrant to the diet may promote the excretion of bile acids, thereby increasing the demand for cholesterol for bile acid synthesis in the liver. This demand can be supplied either by an increased level of de novo synthesis or from plasma LDL. The increase in hepatic binding sites for $^{125}$I-β-LDL coupled with marked lowering of serum cholesterol levels by cholestyramine treatment suggest that the increase in LDL binding sites plays a major role in supplying the need for cholesterol in the liver for bile acid synthesis. Experiments are in progress to study the effects of cholestyramine on the de novo synthesis of bile acids and cholesterol in this model.

The membranes used in this research were prepared from whole livers which consist of different cell types including hepatocytes, endothelial cells, and Kupffer cells. The membranes are relatively crude preparations that include internal as well as plasma membranes. Since liver biosynthesizes the apoproteins, the observed EDTA-sensitive binding activity in cholestyramine-treated rabbits may also reflect systems involved in biosynthesis and secretion of lipoproteins in addition to binding to plasma membranes.

Our results show that the hepatic EDTA-sensitive binding site for LDL in rabbits is regulated by the dietary manipulation and drug treatment, and that an increase in the number of hepatic LDL receptors may lower serum cholesterol. The observation that mevinolin, a hydroxymethylglutaryl-CoA reductase inhibitor (23), also reduces serum cholesterol levels in rabbits fed the wheat starch-casein diet (24) suggests that rabbits with diet-induced endogenous hypercholesterolemia provide an attractive model for the study of lipoprotein metabolism.

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