Impaired receptor-mediated catabolism of low density lipoproteins in fasted rabbits

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Abstract Plasma cholesterol levels of New Zealand white rabbits, fasted for 9 days, increased 4-fold owing to elevated levels of low density lipoproteins and intermediate density lipoproteins. Estimates of the turnover of radioiodinated low density lipoproteins and methyl-low density lipoproteins using the Matthews model showed that clearance of low density lipoprotein by receptor-dependent pathways was reduced by 80%. Receptor-independent removal of low density lipoprotein was unchanged. The absolute catabolic rate of low density lipoprotein was not affected by fasting. EDTA-sensitive binding of ^{125}I-labeled low density lipoproteins was selectively lost from liver membranes isolated from fasted rabbits. These results are consistent with the hypothesis that the hypercholesterolemia of fasted rabbits is the result of down-regulation of the hepatic low density lipoprotein receptor.—**Stoudemire, J. B., G. Renaud, D. M. Shames, and R. J. Havel.** Impaired receptor-mediated catabolism of low density lipoproteins in fasted rabbits. *J. Lipid Res.* 1984. **25:** 33–39.

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Lipoprotein concentrations in blood plasma of the New Zealand white (NZW) rabbit can be manipulated by a variety of means. Addition of cholesterol to the diet results in a large increase in plasma cholesterol levels owing to elevated levels of very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) (1, 2). Hypercholesterolemia associated mainly with increased low density lipoproteins (LDL) has been described in rabbits fed a cholesterol-free, semi-synthetic diet containing casein or certain other proteins (3–5). When rabbits are fasted for 3 or more days (the time required for the stomach to empty), plasma cholesterol levels increase progressively, leveling off after 7–9 days (6–8). This increase is associated with an elevation of lipoproteins with densities between 1.006 and 1.063 g/ml (8). The hypercholesterolemia of cholesterol or casein-feeding is accompanied by delayed clearance of LDL from the plasma, owing to down-regulation of the high affinity LDL receptor (9–11). Deficiency of the LDL receptor also underlies the genetically determined hyperlipidemia of the Watanabe heritable hyperlipidemic (WHHL) rabbit (12–15). The current study was undertaken to evaluate activity of the LDL receptor in fasted rabbits. The activity has been examined in vivo and in liver cell membranes.

METHODS AND MATERIALS

Rabbits

Male New Zealand white (NZW) rabbits weighing 2–2.5 kg were used in all experiments. Control animals were fed Purina laboratory rabbit chow. Food was withdrawn from fasted animals for 9 days. Weight loss during the fast was 538 ± 124 g (mean ± SD). All animals were given water ad libitum.

Lipoproteins

Blood was taken from the femoral artery of fed or fasted rabbits anesthetized with xylazine (Cutter) at 7 mg/kg and Ketamine (Bristol) at 80 mg/kg and mixed with Na_2Edetate (EDTA), 1 mg/ml. Lipoproteins were prepared from fresh plasma by sequential ultracentrifugation (16) at the following background densities: very low density lipoproteins (VLDL), 1.006 g/ml; intermediate density lipoproteins (IDL), 1.006–1.019 g/ml; low density lipoproteins (LDL), 1.019–1.063 g/ml, and high density lipoproteins (HDL), 1.063–1.21 g/ml. LDL used for iodination were recentrifuged once at d 1.063 g/ml and dialyzed overnight at 4°C against 0.15 M NaCl, 0.01% EDTA, and 5 μg/ml gentamycin, pH 7.4. The LDL was then iodinated with either ^{125}I or ^{131}I by a modification (17) of the method of McFarlane (18). Unreacted iodine

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; NZW, New Zealand white; WHHL, Watanabe heritable hyperlipidemic; EDTA, sodium salt ethylenediamine tetraacetic acid; apo, apolipoprotein; FCR, fractional catabolic rate; ACR, absolute catabolic rate.

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was removed by chromatography on Sephadex G-50 followed by dialysis overnight at 4°C against 0.15 M NaCl. Lipid-labeling was uniformly less than 8%, and more than 98% of the label was precipitated by 10% TCA. Less than 2% of the total ^{125}I or ^{131}I was soluble in 1,1,3,3-tetramethylurea. Specific activity was 360–540 cpm/ng protein.

Reductive methylation

The radiolabeled LDL was chemically modified by reductive methylation as described by Weisgraber, Innerarity, and Mahley (19), with a 60-min reaction sequence. The reaction mixture was chromatographed on Sephadex G-50 and the modified LDL (methyl-LDL) was dialyzed overnight at 4°C against 0.15 M NaCl. More than 85% of lysyl residues was routinely methylated as estimated with a colorimetric assay based on the reaction of 2,4,6-trinitrobenzene-1-sulfonic acid with primary amines (20). The distribution of ^{125}I or ^{131}I among protein and lipids was unchanged by methylation.

LDL turnover

In some experiments, native LDL from fed rabbits and native LDL from fasted rabbits, one labeled with ^{125}I and the other with ^{131}I, were injected simultaneously into a marginal ear vein. In other experiments, alternately labeled native LDL and methyl-LDL were similarly injected simultaneously. Twenty-two to 85 μCi of each isotope was injected into each animal. Blood samples (2 ml) were taken at intervals from the opposite ear vein into 2 mg EDTA. Plasma was separated and a portion was assayed for ^{125}I and ^{131}I in a Searle gamma counter (Model 1185) with automatic ratio correction for double-label counting. To determine TCA-soluble radioactivity, 0.5 ml of 20% TCA was added to 0.5 ml of plasma, the mixture was filtered through glass wool, and a portion of the filtrate was counted.

Kinetic analysis

The plasma disappearance curves of TCA-insoluble radioiodine were analyzed by the method of Matthews (21), which assumes a simple two-compartment model where plasma apoLDL equilibrates with an extravascular compartment and where all irreversible loss of apoLDL occurs from the plasma compartment. All curve-fitting was performed with the SAAM-27 computer program (22). Statistical uncertainties were estimated from the covariance matrix in the neighborhood of the least squares fit. The fractional catabolic rate (FCR), pools per day, was derived from the best fit of the compartmental model to the tracer data. The plasma volume of each rabbit was estimated from the dilution of the injected ^{125}I in a blood sample taken 3 min after injection. The concentration of protein in LDL was estimated by multiplying the measured value for LDL-cholesterol at the end of the experiment by the average cholesterol:protein ratio found in LDL of fed or fasted rabbits. The plasma pool of apoLDL was calculated as the product of apoLDL and plasma volume. The absolute catabolic rate (ACR) was calculated by multiplying the FCR (pools/day) by the apoLDL pool size.

Binding of ^{125}I-LDL to liver cell membranes

Cell membranes were prepared from livers of fed and fasted rabbits exactly as described by Kovanen, Brown, and Goldstein (23). The final pellets were stored at −70°C. For binding assays, the membranes were thawed and resuspended in 50 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCL, pH 8.0, by flushing three times through a 25-gauge needle, followed by sonication at 0°C for 45 sec with a Bronson cell disruptor, power setting 6. The suspension was then assayed for protein and diluted to a final concentration of 10 mg protein/ml in 20 mM NaCl, 0.63 mM $CaCl_2$, 50 mM Tris-HCL, 20 mg/ml bovine serum albumin, pH 8.0. The binding assay was conducted at 0°C as described by Kovanen et al. (23) except that the incubation mixture was centrifuged in a Beckman Ti 42.2 rotor (11). EDTA-sensitive binding was defined as the difference between the amount of ^{125}I-LDL bound in the absence and presence of EDTA. EDTA-resistant binding was defined as the difference in the amount of ^{125}I-LDL bound in the presence of EDTA and excess unlabeled LDL.

Other analyses

Total cholesterol and triglycerides were estimated in plasma and lipoprotein fractions by an automated method (24). Protein (25), phospholipids (26), and free and esterified cholesterol (27) were measured in lipoprotein samples that had been purified by recentrifugation at the appropriate density. Apolipoprotein B (apoB) was estimated by the difference between the total protein of the LDL fraction and the protein soluble in 1,1,3,3-tetramethylurea (28). ApoE was measured in plasma by specific radioimmunoassay (29). Lipids in extracts (30) of livers perfused with 200 ml of ice-cold 0.15 M NaCl were estimated by the methods used for lipoproteins.

Statistical analysis

Residual variances obtained from the least squares fit of the Matthews' model for FCR (21) to averaged data (native and methyl-LDL in fed and fasted rabbits) were tested statistically with the Fisher F test. For comparison of other unpaired data, use was made of Student's t test. The significance of a difference in the values obtained with fasted rabbits relative to the fed rabbits was tested at the $P < 0.05$ level and, if significant, is indicated in the tables.

TABLE 1. Concentration of total cholesterol and triglycerides in plasma and lipoprotein fractions of fed and fasted NZW rabbits

	Plasma	VLDL	IDL	LDL	HDL
			mg/dl		
Total cholesterol					
Fed[a]	58.5 ± 1.9[b]	17.0 ± 7.2	7.8 ± 3.5	11.5 ± 3.3	22.3 ± 4.4
Fasted	209.0 ± 57.9[c]	15.9 ± 8.0	29.1 ± 7.2[c]	110.3 ± 8.5[c]	11.7 ± 2.0
Triglycerides					
Fed[a]	213.3 ± 27.4	128.5 ± 37.0	14.0 ± 5.8	15.5 ± 3.9	24.8 ± 4.9
Fasted	76.0 ± 23.9[c]	30.9 ± 12.6[c]	10.0 ± 3.0	29.5 ± 11.1	7.4 ± 1.9[c]

[a] Taken from Havel et al. (29).
[b] Values are mean ± SD (n = 4).
[c] Significantly different from fed ($P < 0.05$; Students' t test; unpaired).

RESULTS

Plasma and lipoprotein cholesterol and triglyceride levels were measured in a group of rabbits fasted for 9 days (**Table 1**). As compared with rabbits fed Purina laboratory rabbit chow ad libitum, plasma cholesterol levels of fasted rabbits were increased 4-fold owing to elevated levels of LDL and IDL. The VLDL cholesterol levels were unchanged and those of HDL were reduced. Levels of triglycerides in plasma, VLDL, and HDL were reduced in fasted rabbits, whereas those of IDL and LDL were unchanged.

As compared with fed rabbits, VLDL, IDL, and LDL were enriched in cholesteryl esters at the expense of triglycerides (**Table 2**). The content of protein, phospholipids, and cholesterol in all the lipoprotein fractions was similar in the two groups except cholesterol of LDL, which was increased in fasted rabbits. The calculated diameter of the VLDL of fed rabbits was approximately 30 Å greater than that of the VLDL of fasted rabbits, whereas the diameters of IDL and LDL were unchanged (31). Concentration of apolipoprotein E in plasma was also increased in the fasted as compared to fed rabbits (4.1 ± 1.0 and 1.7 ± 0.2 mg/dl, respectively; n = 3). ApoB constituted a larger fraction of the total protein of VLDL in fasted than in fed rabbits. More than 90% of the total protein in LDL of both groups was apoB.

The total cholesterol concentration in the liver of fasted rabbits was increased owing to an increase in esterified cholesterol (**Table 3**). Triglycerides were also increased, whereas concentrations of phospholipid and free cholesterol were unchanged.

For all turnover data, fits of the compartmental model to the data sets were good with very small statistical uncertainties. All estimates of FCR had fractional standard deviations of 0.05 or less. Terminal slopes were always well determined with 36 hr of tracer data.

In order to evaluate the role of the LDL receptor in fasting hypercholesterolemia, we derived the turnover of radiolabeled native LDL and methyl-LDL simultaneously in three fed and three fasted rabbits. Results are shown in **Fig. 1** and **Table 4** (Exp. 1). The mean FCR for native LDL, which reflects clearance by receptor-mediated and receptor-independent processes, was 65% lower in fasted rabbits than in the fed rabbits. The receptor-independent removal of LDL, estimated by the clearance of methyl-LDL, did not differ significantly in the two groups. Receptor-dependent removal of LDL, estimated by the difference between the FCR for native LDL and methyl-LDL, was decreased by 80% in the fasted as compared

TABLE 2. Composition of plasma lipoproteins in fed and fasted NZW rabbits (% mass)

Component	VLDL		IDL		LDL		HDL	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
	%		%		%		%	
Cholesteryl esters	8.1 ± 1.7[a]	18.2 ± 6.8[b]	18.3 ± 4.9	37.1 ± 5.5[b]	25.1 ± 8.8	42.2 ± 2.4[b]	19.4	21.4 ± 0.7
Triglycerides	60.4 ± 2.9	47.0 ± 6.1[b]	30.1 ± 3.4	14.0 ± 5.1[b]	22.3 ± 7.2	5.4 ± 3.7[b]	7.5	4.4 ± 0.8
Cholesterol	3.8 ± 0.5	4.4 ± 1.2	5.0 ± 0.4	7.8 ± 1.0	5.7 ± 1.1	9.3 ± 1.0[b]	2.7	4.4 ± 1.1
Phospholipids	15.3 ± 2.3	14.3 ± 0.8	19.7 ± 7.3	16.2 ± 2.8	20.7 ± 2.0	20.5 ± 1.9	22.6	22.2 ± 0.6
Protein	12.8 ± 1.0	16.3 ± 4.6	26.7 ± 4.3	25.4 ± 6.7	26.2 ± 1.0	21.0 ± 1.0[b]	47.5	47.2 ± 2.2
Apoprotein B (% of total protein)	40.9 ± 6.1	55.4 ± 7.2[b]	72.7 ± 3.2	80.6 ± 3.6	91.3 ± 1.1	90.2 ± 1.0		

[a] Values are mean ± SD (n = 4) except for HDL of fed rabbits (n = 2).
[b] Significantly different from fed ($P < 0.05$; Students' t-test, unpaired).

TABLE 3. Concentration of hepatic lipids in fed and fasted NZW rabbits

	Phospholipid	Cholesterol	Cholesteryl Esters	Triglycerides
		mg/g liver		
Fed	30 ± 6.7	2.6 ± 0.63	1.1 ± 1.1	26.0 ± 13
Fasted	33 ± 3.0	2.7 ± 0.30	4.8 ± 4.2[a]	89.0 ± 63[a]

[a] Significantly different ($P < 0.05$; Students' t-test; unpaired).

to fed rabbits. Receptor-dependent removal accounted for 62% of total removal in fed and 32% of total removal in fasted rabbits. In one fasted rabbit no receptor-dependent removal could be detected. The FCR's of LDL isolated from fed and fasted rabbits were virtually identical when they were injected simultaneously into fed and fasted recipient rabbits (Table 4, Exp. 2). The mean pool size of apoLDL in blood plasma increased 2-fold in fasted rabbits. The mean ACR was almost identical in the two groups.

In order to determine the relationship between LDL metabolism in vivo and the activity of the hepatic LDL-receptor, we measured the binding of ^{125}I-LDL to liver cell membranes prepared from fed and fasted rabbits. Total binding of ^{125}I-LDL to liver membranes from fed rabbits was inhibited by EDTA and by an excess amount of unlabeled LDL (**Fig. 2A**). Total binding of ^{125}I-LDL to liver membranes of fasted rabbits was inhibited by excess unlabeled LDL, but only slightly by EDTA (Fig. 2B). In agreement with the findings of others (10, 14), liver membranes from fed rabbits displayed two classes of specific LDL binding sites. A portion of the specific binding was displaced by excess unlabeled LDL, and was also prevented by the addition of EDTA (Fig. 2C). This EDTA-sensitive binding is thought to reflect the activity of the classical LDL receptor (32). EDTA-sensitive binding at 10 mg of LDL protein per ml with membranes from fed rabbits was 16.7 ± 10.5 ng LDL protein/mg membrane protein (mean ± SD, n = 3), in the range reported by others (11, 14). EDTA-sensitive binding was virtually undetectable in the membranes from the fasted rabbits (0.8 ± 3.6 ng/mg membrane protein at 10 mg LDL protein per ml, n = 3). An additional component of specific LDL binding was sensitive to excess unlabeled LDL but not to EDTA. This EDTA-resistant binding was not saturable within the range of ^{125}I-LDL concentrations used in the assay (Fig. 2D). EDTA-resistant binding was similar in liver membranes from fed and fasted rabbits.

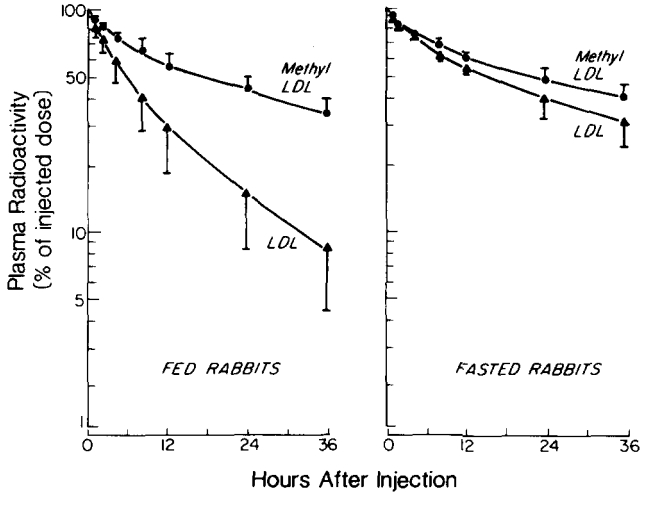

Fig. 1. Disappearance of total plasma TCA-soluble radioactivity after the intravenous injection of radiolabeled native and methyl-LDL in fed rabbits (left panel) and fasted rabbits (right panel). Values for native LDL are means and 1 SD for six fed and six fasted rabbits; those for methyl-LDL are for three fed and three fasted rabbits.

DISCUSSION

In the current study we found, in confirmation of the results of others (6–8), that plasma cholesterol concentration increases 4-fold when NZW rabbits are fasted for 9 days. This increase was primarily the result of a 10-fold increase in LDL cholesterol, but IDL cholesterol levels also increased 4-fold. Klauda and Zilversmit (8) observed greatly reduced fecal excretion of bile acids and endogenous sterols in fasted rabbits and proposed that the increased plasma cholesterol concentration was caused by reduced clearance of beta lipoproteins from the blood. Our studies demonstrate that LDL is removed from the plasma of fasted rabbits more slowly than in fed rabbits. LDL receptor activity has been found in liver membranes isolated from several mammalian species (14, 23, 33, 34). Several lines of evidence indicate that the activity of the hepatic LDL receptor influences the level of plasma LDL. Treatment of rats with 17α-ethinyl estradiol stimulates hepatic LDL receptor activity and hepatic LDL catabolism concurrently (23, 34). The increase in hepatic LDL receptors produced by cholestyramine treatment is also coupled to reduction of plasma LDL levels in rabbits (11) and dogs (33).

Based on the original observations of Mahley and associates (35), we have used the difference between the clearance of native LDL and methyl-LDL to estimate receptor-dependent catabolism of LDL in fed and fasted rabbits. In confirmation of the results of Bilheimer, Watanabe, and Kita (36), we found that approximately 62% of the clearance of LDL in normal rabbits can be attributed to the LDL receptor. Our results in fasted rabbits indicate that LDL clearance is reduced as a result of a decrease in receptor-mediated clearance of LDL. Although the LDL receptor is down-regulated in fasted

TABLE 4. Kinetic data for apoLDL and apo-methyl-LDL turnover in fed and fasted rabbits

	Rabbit	Weight	Plasma Volume	ApoLDL Pool	FCR, pools/day			ACR of ApoLDL		
					Total	Receptor-Independent	Receptor-Dependent	Total	Receptor-Independent	Receptor-Dependent
		kg	*ml*	*mg/kg*					*mg kg^{-1} day^{-1}*	
Fed										
Exp. 1	A	2.38	100	8.7	2.93	0.79	2.14	25.6	6.9	18.7
	B	2.43	109	8.9	1.70	0.70	1.00	15.1	6.2	8.9
	C	2.29	85	8.3	1.46	0.65	0.81	12.1	5.4	6.7
Exp. 2	D	2.57	112	7.7	1.80 (1.76)[a]			13.9		
	E	2.40	98	9.3	2.16 (2.14)			20.4		
	F	2.30	94	9.2	3.29 (3.30)			30.3		
Mean ± SD		2.40 ± 0.10	99.7 ± 9.9	8.7 ± 0.60	2.24 ± 0.73	0.71 ± 0.07[b]	1.32± 0.72	19.6 ± 7.2	6.2 ± 0.8	11.4 ± 6.4
Fasted										
Exp. 1	A	1.64	74	12.7	0.82	0.41	0.41	31.4	15.7	15.7
	B	1.52	58	14.2	0.53	0.53	0.00	18.3	18.3	0.0
	C	1.57	58	12.1	1.13	0.62	0.51	22.8	12.5	10.3
Exp. 2	D	2.09	80	31.9	0.60 (0.59)			19.1		
	E	2.14	80	22.8	0.70 (0.71)			15.9		
	F	1.94	67	19.2	0.91 (0.91)			17.4		
Mean ± SD		1.82 ± 0.27[c]	69.5 ± 10.1[c]	18.8 ± 7.6[c]	0.78 ± 0.22[c]	0.52 ± 0.11	0.31 ± 0.27[c]	20.8 ± 5.7	15.5 ± 2.9[c]	8.7 ± 8.0

[a] Values in parentheses refer to native LDL from fasted rabbits labeled with ^{131}I; all other values are for ^{125}I-labeled LDL from fed rabbits.
[b] Significantly different from total FCR at $P < 0.05$.
[c] Significantly different from fed at $P < 0.05$.

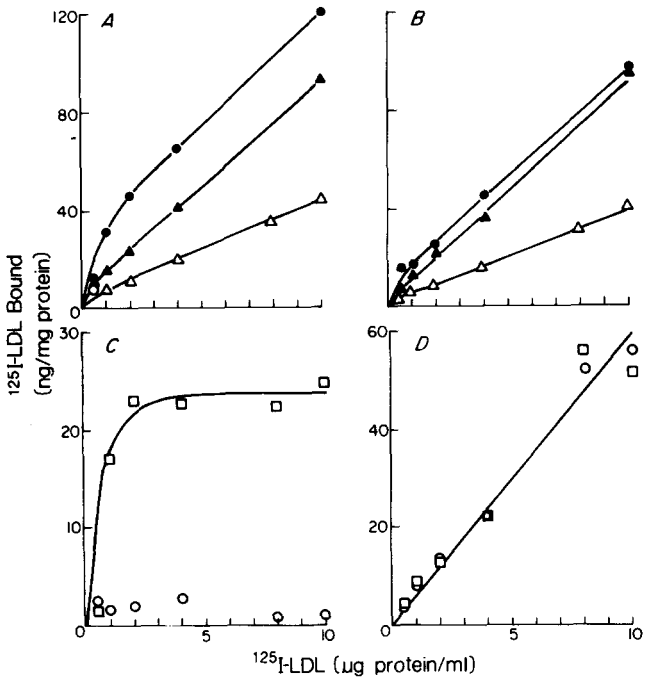

Fig. 2. Binding of ^{125}I-LDL to liver membranes from fed (A) and fasted (B) rabbits. Each assay tube contained 100 μg of membrane protein and the indicated concentration of ^{125}I-LDL (385 cpm/ng protein) in the absence (●) or presence of either 3 mM EDTA (▲) or unlabeled rabbit LDL at 1 mg/ml (△). Calculated EDTA-sensitive (C) and EDTA-resistant (D) binding of LDL to membranes from fed (□) and fasted (○) rabbits. Values represent the mean of two assays on a single set of membrane preparations.

rabbits, we found appreciable removal of LDL by the receptor-dependent pathway in two of these animals studied. The residual receptor-dependent removal of LDL could reflect the activity of extrahepatic LDL receptors. Alternatively, some active receptors may remain in the liver, which cannot be detected in our binding assay (small amounts of EDTA-sensitive binding may be difficult to detect in the presence of large components of EDTA-resistant and nonspecific binding).

The pool size of apoLDL was greater in the fasted rabbits than the fed, but the FCR of methyl-LDL was not altered. This observation suggests that receptor-independent uptake of LDL was not saturated at the levels of LDL that occur during fasting. Receptor-independent uptake of LDL does not appear to be saturated even at the levels of LDL that occur in the WHHL rabbit (36).

Unlike patients with homozygous familial hypercholesterolemia (37) and WHHL rabbits (36), in whom the ACR of apoLDL is increased 2 to 5-fold, the rate of production and removal of apoLDL was not altered in our fasting rabbits. One factor that may account for this difference is a reduced rate of secretion of hepatogenous VLDL in fasting rabbits.[2]

[2] Hornick, C. A., and R. J. Havel. Unpublished observations.

The characteristics of binding of ^{125}I-LDL to liver membranes isolated from fasted rabbits have provided additional evidence that the reduced FCR is the result of down-regulation of the LDL hepatic receptor. The liver membranes isolated from fed rabbits had two specific LDL binding sites. One was resistant to addition of EDTA and the other, analogous to the classical LDL receptor (32), was EDTA-sensitive. EDTA-sensitive binding to liver membranes from fasted rabbits was virtually undetectable. As observed by others (7, 8), we found that cholesterol accumulates (as esters) in the liver of fasted rabbits. Such accumulation, owing to reduced conversion of cholesterol to bile acids, could lead to down-regulation of the hepatic LDL receptor (38).

VLDL, IDL, and LDL were all enriched in cholesteryl esters in fasting rabbits. This could be the result of a decreased FCR for these lipoproteins. An increased residence time in the plasma would allow for increased transfer of cholesteryl esters synthesized by lecithin:cholesterol acyltransferase to these lipoproteins, as suggested for WHHL rabbits (29). Recent studies by Kita et al. in the WHHL rabbit (39) and studies in our laboratory (unpublished) in fasted rabbits indicate that the FCR of VLDL and IDL is reduced as well as that of LDL. These observations suggest that VLDL remnants are also taken up in the liver via the LDL receptor. The fasted rabbit may provide a useful model to study the effects of down-regulation of the hepatic LDL receptor. The fasted rabbit may have some advantages over the WHHL rabbit in that LDL production is not increased and there is no associated hypertriglyceridemia. Rabbits in which LDL receptors are down-regulated or genetically absent should provide useful models in which to evaluate the role of the hepatic LDL receptor in the conversion of VLDL to LDL.

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