# Unexpected Inhibition of Cholesterol 7α-Hydroxylase by Cholesterol in New Zealand White and Watanabe Heritable Hyperlipidemic Rabbits

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## Abstract

**We investigated the effect of cholesterol feeding on plasma cholesterol concentrations, hepatic activities and mRNA levels of HMG-CoA reductase and cholesterol 7α-hydroxylase and hepatic LDL receptor function and mRNA levels in 23 New Zealand White (NZW) and 17 Watanabe heritable hyperlipidemic (WHHL) rabbits. Plasma cholesterol concentrations were 9.9 times greater in WHHL than NZW rabbits and rose significantly in both groups when cholesterol was fed. Baseline liver cholesterol levels were 50% higher but rose only 26% in WHHL as compared with 3.6-fold increase with the cholesterol diet in NZW rabbits. In both rabbit groups, hepatic total HMG-CoA reductase activity was similar and declined > 60% without changing enzyme mRNA levels after cholesterol was fed. In NZW rabbits, cholesterol feeding inhibited LDL receptor function but not mRNA levels. As expected, receptor-mediated LDL binding was reduced in WHHL rabbits. Hepatic cholesterol 7α-hydroxylase activity and mRNA levels were 2.8 and 10.4 times greater in NZW than WHHL rabbits. Unexpectedly, cholesterol 7α-hydroxylase activity was reduced 53% and mRNA levels were reduced 79% in NZW rabbits with 2% cholesterol feeding. These results demonstrate that WHHL as compared with NZW rabbits have markedly elevated plasma and higher liver cholesterol concentrations, less hepatic LDL receptor function, and very low hepatic cholesterol 7α-hydroxylase activity and mRNA levels. Feeding cholesterol to NZW rabbits increased plasma and hepatic concentrations greatly, inhibited LDL receptor-mediated binding, and unexpectedly suppressed cholesterol 7α-hydroxylase activity and mRNA to minimum levels similar to WHHL rabbits. Dietary cholesterol accumulates in the plasma of NZW rabbits, and WHHL rabbits are hypercholesterolemic because reduced LDL receptor function is combined with decreased catabolism of cholesterol to bile acids. (*J. Clin. Invest.* 1995. 95:1497–1504.) Key words: HMG CoA reductase • LDL receptor • hypercholesterolemia • bile acids • biosynthesis**

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## Introduction

It is well known that rabbits and rats respond differently to dietary cholesterol: plasma cholesterol levels increase markedly when cholesterol is fed to rabbits while plasma cholesterol concentrations hardly change when rats consume large amounts of cholesterol in the diet (1). This difference may be related to the effect of cholesterol on the function of hepatic LDL receptors and bile acid synthesis in the two animal species. In cholesterol-fed rabbits (2) but not rats (3, 4), hepatic LDL receptors are downregulated so that the liver in cholesterol-fed rabbits clears less LDL from the plasma. In rats, cholesterol feeding increased bile acid synthesis by upregulating cholesterol 7α-hydroxylase activity and mRNA levels (4–7), whereas the effect of cholesterol feeding on bile acid synthesis in rabbits has not yet been entirely defined.

The Watanabe heritable hyperlipidemic (WHHL)[1] rabbit model was first described by Watanabe, a veterinarian at Kobe University in Japan in 1975 (8). It is a strain of rabbit with consistently high plasma cholesterol levels associated with elevated plasma concentrations of β-very low density lipoprotein (β-VLDL) attributed to the deficiency of LDL receptors that is inherited as a dominant trait (9). The abnormal LDL receptor gene from WHHL rabbits has been transferred to "D × L," English Half-lop (EHL-WW) rabbits who then manifest marked hypercholesterolemia in homozygotes (10).

At least four mechanisms are responsible for cholesterol homeostasis and affect plasma cholesterol concentrations: (*a*) synthesis from acetate as regulated by the formation of mevalonic acid from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) that is catalyzed by the rate-limiting enzyme HMG-CoA reductase (11); (*b*) expression of LDL receptors especially in the liver where more than half of the receptors are located (12); (*c*) dietary intake of cholesterol (13); and (*d*) the transformation of cholesterol to bile acids, the major catabolic pathway for cholesterol that is regulated by the formation of 7α-hydroxycholesterol and is catalyzed by the hepatic microsomal enzyme, cholesterol 7α-hydroxylase (14).

The aim of this paper is to evaluate the role of bile acid synthesis in cholesterol homeostasis in normocholesterolemic New Zealand White (NZW) and hypercholesterolemic WHHL rabbits. The effect of feeding cholesterol and its C-24 ethyl analogue, sitosterol, which blocks the intestinal absorption of cholesterol, was compared by measuring plasma and liver cho-

1. *Abbreviations used in this paper:* EHL-WW, English Half-lop; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NZW, New Zealand White; WHHL, Watanabe heritable hyperlipidemic.

lesterol concentrations, function and mRNA levels of hepatic LDL receptors, and the activities and mRNA levels of HMG-CoA reductase and cholesterol 7$\alpha$-hydroxylase in both rabbit groups. The results confirm that hypercholesterolemia was associated with diminished LDL receptor function in WHHL and cholesterol-fed NZW rabbits. However, the importance of diminished bile acid synthesis in increasing plasma cholesterol concentrations was emphasized by the inhibition of the rate-controlling enzyme, cholesterol 7$\alpha$-hydroxylase, in the cholesterol-treated NZW rabbits and the hypercholesterolemic WHHL rabbits.

## Methods

### *Materials*

Cholesterol (cholest-5-en-3$\beta$-ol), 5$\alpha$-cholestane, cholestanol (5$\alpha$-cholestan-3$\beta$-ol), and sitosterol (24B-ethylcholest-5-en-3$\beta$-ol) were obtained from Sigma Chemical Co. (St. Louis, MO) and used as standards for the measurements of sterols by capillary gas-liquid chromatography. Normal rabbit chow (Purina Mills Inc., St Louis, MO) contained < 0.001% wt/wt cholesterol as determined by capillary gas-liquid chromatography. Rabbit chow containing 0.2% and 2% cholesterol and 0.2% sitosterol (the sitosterol for preparing the feeding chow was a gift of Eli Lilly Co., Indianapolis, IN) was added to the normal chow and was also prepared by Purina Mills Inc. The experimental chow diets all contained 0.57% (wt/wt) saturated fatty acids, 0.66% (wt/wt) monounsaturated fatty acids, and 0.97% polyunsaturated fatty acids.

### *Experimental plan*

The experiments were carried out in 23 NZW rabbits (Hazleton Labs, Denver, PA) weighing 5.5–6.8 lb and 17 WHHL rabbits (EHL-WW rabbits, weighing 5.5–6.5 lb) which were bred in The Lipid Lab, Rogosin Institute, Cornell University Medical School, New York (15). The ages of these rabbits were all between 6.5 and 7.5 mo. The control rabbits (eight NZW and six WHHL rabbits) were fed normal rabbit chow. The 0.2% cholesterol chow diet (~300 mg/d) was fed to four NZW and eight WHHL rabbits; the 2% cholesterol chow diet (~3 g/d) was fed to five NZW rabbits, and 0.2% sitosterol chow (~300 mg/d) was fed to six NZW and three WHHL rabbits. 2% cholesterol fed to WHHL rabbits caused severe toxicity and data were not used. The rabbits were fed the experimental diets for 10 d. Blood samples (2–3 ml) were taken with the anticoagulant ethylenediaminetetraacetate (EDTA) immediately before and at the completion of the feeding period from each rabbit for determination of plasma cholesterol concentrations and liver function tests (plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin levels). Animals were killed under anesthesia (ketamine 20–30 mg/kg body wt combined with xylazine 2–3 mg/kg body wt intramuscularly), liver was removed, and a portion was immediately frozen for measurements of the microsomal activities and mRNA levels of HMG-CoA reductase and cholesterol 7$\alpha$-hydroxylase and membrane LDL receptor function and mRNA levels. The animal protocol was approved by the Subcommittee on Animal Studies at the VA Medical Center (East Orange, NJ) and the Institutional Animal Care and Use Committee at UMD-New Jersey Medical School (Newark, NJ).

### *Chemical analysis*

*Assays for sterols.* Neutral sterols were extracted with hexane from 1 ml plasma or 1 g (wet) pieces of liver after saponification in 1 N ethanolic NaOH. Trimethylsilyl ether derivatives were prepared and quantitated by capillary gas-liquid chromatography (model 5890; Hewlett-Packard, Palo Alto, CA) equipped with a 25-m fused silica CP-Sil 5-CB capillary column as described previously (16, 17). The retention times of the sterol trimethylsily either derivatives relative to the internal standard, 5$\alpha$-cholestane (retention time 14.62 min) were: cholesterol, 1.40; cholestanol, 1.42, and sitosterol, 1.81. For determination of free/ester cholesterol in the liver, 1 g (wet) pieces of liver were extracted into chloroform/methanol (2:1). Free and ester cholesterol were then separated by TLC method as described previously (18).

*Assays for LDL receptor function.* LDL ($1.019 < d < 1.063$) was isolated from blood of untreated NZW rabbits by ultracentrifugation (19) and labeled with $^{125}$I (New England Nuclear, Boston, MA) by the iodine monohydrochloride method (20). Receptor-mediated LDL binding to rabbit liver membranes was assayed by methods described previously by Nguyen et al. (21) and Kovanen et al. (22), except that cold phosphate-buffered saline containing 2% bovine serum albumin was used, instead of fetal bovine serum, to wash the membranes after incubation with the labeled ligand. Receptor-mediated LDL binding to the liver membranes was determined as the difference between total binding of [$^{125}$I]LDL (assayed in the absence of unlabeled LDL) and nonspecific binding (assayed in the presence of 40-fold excess unlabeled LDL).

*Assays for hepatic microsomal total HMG-CoA reductase and cholesterol 7$\alpha$-hydroxylase activities.* Hepatic microsomes were prepared by differential ultracentrifugation (23), and the protein determined according to Lowry et al. (24). The assay for HMG-CoA reductase activity was based on the methods by George et al. (25) and Nguyen et al. (21). Briefly, 50–200 $\mu$g of microsomal protein was preincubated at 37°C for 5 min in a final volume of 150 $\mu$l buffer (50 mM $K_2HPO_4$, 30 mM EDTA, 10 mM dithiotreitol, 70 mM KCl, pH 7.4) containing an NADPH generating system (34 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.3 U glucose-6-phosphate dehydrogenase) and [$^3$H]-mevalonolactone (40,000 dpm) as internal recovery standard. The reaction was started with the addition of 30 nmol [3-$^{14}$C]HMG-CoA (Amersham Corp., Arlington Heights, IL; sp act, 30 dpm/pmol) and stopped after 15 min at 37°C with the addition of 20 $\mu$l 6 N HCl. Zero-time controls were run with each experiment. After lactonization at 37°C for 30 min, the products were separated by thin-layer chromatography, and mevalonolactone quantitated by liquid scintillation counting (21).

Cholesterol 7$\alpha$-hydroxylase activity was measured in hepatic microsomes after removal of endogenous lipid by acetone and reconstituting the microsomal protein with cholesterol and optimum amounts of cofactors by the isotope incorporation method of Shefer et al. (23).

*Northern analysis.* Total RNA from samples of frozen liver was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (26). Portions of frozen liver, 0.2 g, were homogenized in 2 ml of room temperature TRI reagent (Molecular Research Center, Cincinnati, OH) using a Polytron tissue disrupter at full speed for 5–10 s. After 5 min, 0.4 ml of chloroform was added, and the sample was mixed vigorously and centrifuged at 12,000 *g* for 15 min at 4°C. The aqueous phase was removed and 1 ml of isopropanol was added. The samples were mixed and allowed to stand at room temperature for 5–10 min and then centrifuged at 12,000 *g* for 10 min at 4°C. The pelleted RNA was then washed in 75% ethanol. The total RNA pellet was dissolved in 100 $\mu$l of diethylpyrocarbonate-treated water. Poly ($A^+$) RNA was isolated by oligo (dT) cellulose chromatography (27). The relative levels of HMG-CoA reductase, cholesterol 7$\alpha$-hydroxylase, and LDL receptor mRNAs were quantitated by Northern blot analysis as previously described (28) except that a Red Roller hybridization oven from Hoefer Scientific Instruments (San Francisco, CA) was used. $\beta$-Actin served as the internal standard. Several exposures of each blot were made to ensure that measurements were made within the linear response of the Ultrascan laser densitometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The $\beta$-actin signal was always stronger than that for HMG-CoA reductase, cholesterol 7$\alpha$-hydroxylase, and LDL receptor. The cDNAs for hamster HMG-CoA reductase, pRED 227, and human $\beta$-actin, HHCl 89, were purchased from American Type Culture Collection (Rockville, MD). The cDNA for rat liver cholesterol 7$\alpha$-hydroxylase, 7$\alpha$6, was a gift from Dr. J. Y. L. Chiang (Northeastern Ohio University, Rootstown, OH). The cDNA for the rat hepatic LDL receptor, LDLR-1, was a generous gift from Dr. Richard Tanaka (Bristol Myers Squibb, Princeton, NJ).

*Table I. The Effect of Cholesterol Feeding on Plasma and Liver Cholesterol Levels in NZW amd WHHL Rabbits*

| | Plasma | | Liver | |
|---|---|---|---|---|
| | *mg/dl* | | *mg/g (% ester)* | |
| | NZW | WHHL | NZW | WHHL |
| Controls | 33±5 ($n$ = 8) | 326±83 ($n$ = 6) | 2.6±0.5 (7.1) ($n$ = 5) | 3.9±0.5 (7.1) ($n$ = 5) |
| 0.2% Cholesterol* | 68±28 ($n$ = 4) | 742 ± 100[‡] ($n$ = 8) | 9.3±0.7[§] (57.1) ($n$ = 4) | 4.9±1.0 (29.4) ($n$ = 5) |
| 2% Cholesterol* | 871±259[‖] ($n$ = 5) | N/A[¶] | 25.5±2.7[§] (68.3) ($n$ = 5) | N/A |
| 0.2% Sitosterol* | 19±2[‖] ($n$ = 6) | 447±8 ($n$ = 3) | 2.3±0.5 (6.2) ($n$ = 5) | 3.4±0.5 (6.8) ($n$ = 3) |

Data presented as mean±SD. * Rabbits were fed 0.2 or 2% cholesterol or 0.2% sitosterol, respectively, for 10 d. [§] $P < 0.001$ compared with the control value for NZW rabbits. [‡] $P < 0.001$ compared with the control value for WHHL rabbits. [¶] Not available. [‖] $P < 0.01$ compared with the control value for NZW rabbits.

### *Statistical study*

Baseline data were compared statistically by the unpaired Student's $t$ test. Where control values were used several times, the Bonferroni correction was used. The BMDP Statistical Software (BMDP Statistical Software, Inc., Los Angeles, CA) was used for statistical evaluations.

## Results

Plasma and liver cholesterol levels are listed for NZW and WHHL rabbits in Table I. Plasma concentrations of cholesterol were 9.9 times greater ($P < 0.0001$) in WHHL than NZW rabbits fed the control diet that was virtually devoid of cholesterol. When the chow containing 0.2% cholesterol (300 mg/d) was fed for 10 d, plasma concentrations increased about twofold in both rabbit groups, and rose 26 times in NZW rabbits who were fed 2% cholesterol (3,000 mg/d) in the diet for 10 d. Of interest, plasma cholesterol levels were similar in NZW rabbits (871 mg/dl) fed 2% cholesterol and WHHL rabbits (742 mg/dl) that received 0.2% cholesterol in the diet.

When 0.2% sitosterol was fed for 10 d, plasma cholesterol concentrations declined 42% ($P < 0.01$) in NZW rabbits but did not decrease in the hypercholesterolemic WHHL rabbits compared with their respective control groups. It is noteworthy that despite the large intake, 300 mg/d, the plasma sitosterol concentration remained low, i.e., < 1 mg/dl in the NZW rabbits (data not shown).

Liver cholesterol concentrations in NZW and WHHL rabbits are presented as milligrams per gram of liver. Hepatic cholesterol concentrations were 50% higher ($P < 0.005$) in the WHHL as compared with NZW rabbits fed the same almost cholesterol-free control diet. When 0.2 and 2% cholesterol were fed, liver cholesterol rose 3.6- and 9.8-fold ($P < 0.001$), respectively, in NZW rabbits but only increased 26% ($P$ = NS) in WHHL rabbits with 0.2% cholesterol in the diet for 10 d. The proportion of hepatic esterified cholesterol increased with hepatic cholesterol in both groups. Sitosterol feeding did not change hepatic cholesterol levels significantly in the NZW and WHHL rabbits.

Measurements of hepatic microsomal total HMG-CoA reductase and cholesterol 7$\alpha$-hydroxylase activities are reported in Table II. In both NZW and WHHL rabbits fed the cholesterol-deficient control diet, HMG-CoA reductase activities were similar although plasma and liver cholesterol concentrations were greater in WHHL rabbits. Feeding cholesterol, as expected, inhibited HMG-CoA reductase activities in both rabbit groups. Total enzyme activity was reduced 18% ($P$ = NS) with 0.2% cholesterol but decreased 68% ($P < 0.001$) with 2% cholesterol feeding in NZW and 64% ($P < 0.05$) with 0.2% cholesterol in WHHL rabbits. In contrast, dietary sitosterol (0.2%), which acts in the intestine to block cholesterol absorption, increased hepatic HMG-CoA reductase activity 2.5 times ($P < 0.05$) in the NZW rabbits but produced only a small rise in enzyme activity in the WHHL rabbits.

Hepatic microsomal cholesterol 7$\alpha$-hydroxylase activity was almost threefold ($P < 0.0001$) higher in the NZW than WHHL rabbits at baseline when fed the cholesterol-deficient control diet. Unexpectedly, both 0.2 and 2% cholesterol in the diet progressively reduced cholesterol 7$\alpha$-hydroxylase activity ($P < 0.01$) 41 and 53%, respectively, in the NZW rabbits but did not change the already low activity in the WHHL rabbits. When sitosterol was fed, cholesterol 7$\alpha$-hydroxylase activity

*Table II. Effect of Cholesterol Feeding on Hepatic Total HMG-CoA Reductase and Cholesterol 7α-Hydroxylase Activity in Rabbits*

| | HMG-CoA reductase | | Cholesterol 7α-hydroxylase | |
|---|---|---|---|---|
| | *pm/mg per min** | | *pm/mg per min** | |
| | NZW | WHHL | NZW | WHHL |
| Controls | 28±3 ($n$ = 6) | 36±17 ($n$ = 5) | 34±8 ($n$ = 8) | 12±2 ($n$ = 6) |
| 0.2% Cholesterol[‡] | 23±7 ($n$ = 4) | 13± 7[§] ($n$ = 8) | 20±4[‖] ($n$ = 4) | 13±3 ($n$ = 8) |
| 2% Cholesterol[‡] | 9±4[¶] ($n$ = 5) | N/A** | 16±6[‖] ($n$ = 5) | NA |
| 0.2% Sitosterol[‡] | 70±25[§] ($n$ = 3) | 48±7 ($n$ = 3) | 28±4 ($n$ = 3) | 15±1 ($n$ = 3) |

* Mean ±SD. [‡] Rabbits were fed 0.2 or 2% cholesterol or 0.2% sitosterol, respectively, for 10 d. [¶] $P < 0.001$ compared with the control value for NZW rabbits. [§] $P < 0.05$ compared with the relevant control values. [‖] $P < 0.01$ compared with the control value for NZW rabbits. ** Not available.

*Table III. Effect of Cholesterol Feeding on Hepatic HMG-CoA Reductase and Cholesterol 7α-Hydroxylase mRNA Levels in Rabbits*

| | HMG-CoA reductase mRNA* | | Cholesterol 7α-hydroxylase mRNA* | |
|---|---|---|---|---|
| | NZW | WHHL | NZW | WHHL |
| Controls | 0.55±0.37 ($n$ = 4) | 0.48±0.06 ($n$ = 3) | 29.1±15.6 ($n$ = 3) | 2.8±0.4 ($n$ = 3) |
| 0.2% Cholesterol‡ | | 0.48±0.19 ($n$ = 5) | | 7.2±2.2 ($n$ = 3) |
| 2% Cholesterol‡ | 0.48±0.04 ($n$ = 5) | | 6.2±3.7§ ($n$ = 4) | |

* Relative densitometric units (mean±SD) corrected by recovery of the internal standard, β-actin. ‡ Rabbits were fed 0.2 or 2% cholesterol, respectively, for 10 d. § $P < 0.05$ compared with relevant control values.

was not inhibited and remained at baseline levels in NZW and WHHL rabbits.

Hepatic HMG-CoA reductase mRNA levels as measured by Northern blot analysis and densitometeric scanning were similar in both NZW and WHHL rabbits when fed the control cholesterol-deficient diet (Table III). Moreover, enzyme mRNA levels did not decrease in both rabbit groups when cholesterol was added to the diet despite the drop in HMG-CoA reductase activity (Table II) and comparable elevated plasma cholesterol concentrations (Table I).

Hepatic cholesterol 7α-hydroxylase mRNA levels were 10 times greater and corresponded to higher enzyme activity in NZW than WHHL rabbits fed the control cholesterol-deficient diet ($P < 0.05$). Unexpectedly, 2% cholesterol feeding inhibited cholesterol 7α-hydroxylase mRNA levels 79% ($P < 0.05$) in NZW, whereas in WHHL rabbits the initially low mRNA levels rose slightly with 0.2% cholesterol feeding. Fig. 1 shows a representative Northern blot illustrating the inhibitory effect of cholesterol feeding on cholesterol 7α-hydroxylase mRNA levels in NZW rabbits.

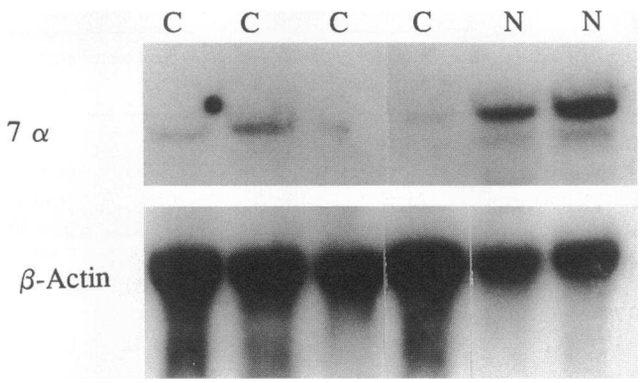


*Figure 1.* Northern blot of cholesterol 7α-hydroxylase mRNA (7α) in NZW rabbits fed the control diet ($N$) and the diet with 2% cholesterol ($C$). On the control diet, a major transcript at 4.0 kb is noted that virtually disappears in the cholesterol-fed rabbits. β-Actin is used as internal standard.

*Table IV. Effect of Cholesterol Feeding on Hepatic LDL Receptor-mediated Binding and mRNA Levels in Rabbits*

| | Hepatic receptor-mediated LDL binding | | Hepatic LDL receptor mRNA* | |
|---|---|---|---|---|
| | *ng/mg protein* | | | |
| | NZW | WHHL | NZW | WHHL |
| Control | 175±29 ($n$ = 7) | 112±25 ($n$ = 6) | 0.61±0.02 ($n$ = 3) | 0.45±0.06 ($n$ = 3) |
| 0.2% Cholesterol‡ | 115±32 ($n$ = 4) | 108±32 ($n$ = 6) | | 0.55±0.09 ($n$ = 5) |
| 2% Cholesterol‡ | 89±36§ ($n$ = 5) | | 0.55±0.18 ($n$ = 5) | |
| 0.2% Sitosterol‡ | 228±40 ($n$ = 3) | 110±58 ($n$ = 3) | | |

Data presented as mean±SD. * Releative densitometric units corrected by recovery of the internal standard, β-actin. ‡ Rabbits were fed 0.2 or 2% cholesterol or 0.2% sitosterol, respectively, for 10 d. § $P < 0.01$ compared with the control values for NZW rabbits.

Receptor-mediated hepatic LDL binding and mRNA levels are reported in Table IV. As expected, WHHL rabbits fed the control diet showed 36% less ($P < 0.005$) receptor-mediated LDL binding than NZW rabbits. When NZW rabbits were fed 0.2 and 2% cholesterol diets, receptor-mediated LDL binding declined 34 and 49% ($P < 0.01$), respectively. In contrast, 0.2% cholesterol feeding to WHHL rabbits did not further decrease receptor-mediated LDL binding. Sitosterol feeding, in distinction, stimulated LDL receptor binding in NZW rabbits consistent with the decrease in plasma cholesterol concentrations (Table I). In comparison, WHHL rabbits failed to express additional LDL receptors when fed sitosterol.

In both rabbit groups fed the control cholesterol-deficient diet, similar amounts of LDL receptor mRNA were present and did not decrease with dietary cholesterol feeding (Table IV). Thus, WHHL and cholesterol-fed NZW rabbits show decreased hepatic LDL receptor binding despite unchanged mRNA levels.

Plasma liver function tests and body weights are listed in Table V. Normal values for plasma alkaline phosphatase, glutamic-pyruvic acid transaminase, and bilirubin were present in the NZW and WHHL rabbits on the control cholesterol-deficient diet and remained normal with cholesterol feeding. The weights of the animals were similar before and after treatment in all groups. Percent weight gain was 14% in controls, 17% in WHHL fed 0.2% cholesterol, and 20% in NZW rabbits fed 2% cholesterol. Liver histology showed only increased deposition of fat without hepatocyte destruction, inflammation, or disruption of the architecture (Fig. 2) when cholesterol was fed for 10 d to both rabbit groups.

## Discussion

The results of this investigation demonstrate major differences in cholesterol metabolism that involve plasma and liver cholesterol concentrations, expression of hepatic LDL receptors, baseline hepatic cholesterol 7α-hydroxylase activities and mRNA levels, and response to cholesterol and sitosterol feeding be-

*Table V. Serum Alkaline Phosphatase and Glutamic-Pyruvic Transaminase Activities before and after Cholesterol Feeding*

| | Alkaline phosphatase | | Glutamic-pyruvic transaminase | | Bilirubin | | Weight | |
|---|---|---|---|---|---|---|---|---|
| | *U* | | *IU* | | *mg/dl* | | *lb* | |
| | Before | After | Before | After | Before | After | Before | After |
| Normal | 20–140 | | 20–80 | | 0.1–0.5 | | 6.3±0.3 | 7.2±0.8 |
| 0.2% Cholesterol* | 101±17 | 105±10 | 55±18 | 34±5 | 0.1±0.1 | 0.2±0.1 | 6.3±0.5 | 7.4±0.4 |
| (WHHL) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) |
| 2% Cholesterol‡ | 118±5 | 113±2 | 54±13 | 56±13 | 0.1±0.1 | 0.2±0.1 | 6.1±0.2 | 7.3±0.6 |
| (NZW) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) | ($n$ = 5) |

* 0.2% cholesterol in the diet was fed to WHHL rabbits for 10 d. ‡ 2% cholesterol in the diet was fed to NZW rabbits for 10 d.

tween WHHL and NZW rabbits. Marked plasma hypercholesterolemia is the hallmark feature of WHHL rabbits and has been attributed to the inherited deficiency of LDL receptors. The abnormal LDL receptor gene has been transferred to EHL-WW rabbits that manifest hypercholesterolemia (15) and were used in our experiments. On the cholesterol-deficient control diet, plasma and liver cholesterol concentrations were 9.9 times and 50% greater in the WHHL rabbits than NZW rabbits (Table I) and correspond to the reduction in receptor-mediated LDL binding (Table IV). As expected, cholesterol feeding not only increased hepatic total cholesterol but raised the proportion of esterified liver cholesterol. The interesting point is that the cholesterol-fed NZW rabbits reproduce most of the abnormalities in cholesterol metabolism and LDL receptor function found in WHHL rabbits: markedly elevated plasma cholesterol levels in combination with reduced receptor-mediated LDL binding.

The rabbit chow used in these studies contained the same small amounts of saturated (0.57%), monounsaturated (0.66%), and polyunsaturated (0.97%) fatty acids. No additional oil was added to solubilize the cholesterol and sitosterol

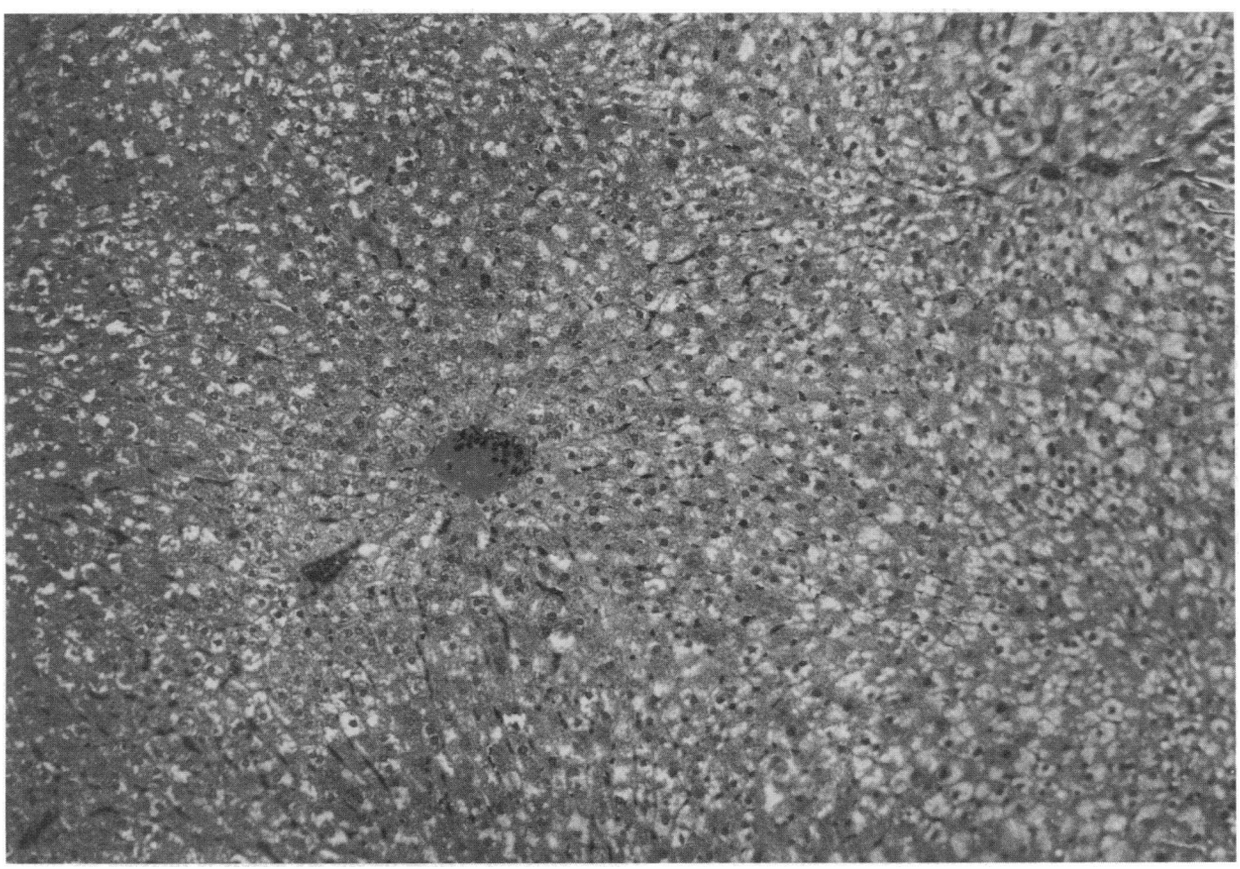

*Figure 2.* Photomicrograph of hepatic histology (× 100, hematoxylin and eosin stain) in NZW rabbits fed 2% cholesterol in the diet. The pericentral hepatocytes show vacuolar change consistent with deposition of fat. Hepatic lobular architecture was preserved. Similar picture is seen in WHHL rabbits.

incorporated into the diets. All rabbit groups consumed similar amounts of food, and thus fatty acid intake and caloric consumption were equal throughout the experimental periods. As a result, both the NZW and WHHL rabbits gained similar weight during the 10 d of feeding studies. In other reports (29), oils containing saturated fats plus cholesterol when added to the diet caused much higher plasma cholesterol levels and greater downregulation of LDL receptor function than with unsaturated fats. This indicated a separate role for triglycerides independent of cholesterol in modulating LDL receptor function (29) and was not a confounding factor in our studies. In addition, since all liver function tests and hepatic histology from the rabbits fed the cholesterol-containing diets were normal, our results were not caused by toxicity due to cholesterol feeding. Dietschy et al. (30) have studied cholesterol synthesis in the WHHL rabbits and found that plasma cholesterol concentrations and cholesterol synthesis rates were affected by the animals' age. In our experiments, WHHL and NZW rabbits were of similar age ($\sim$ 7 mo old) and size ($\sim$ 6 lb) so that the effects of age and size could be equalized.

A major new discovery in this study was the demonstration that hepatic cholesterol 7$\alpha$-hydroxylase activity and mRNA levels were substantially higher in NZW than WHHL rabbits. Since bile acid synthesis represents the major catabolic path for the elimination of cholesterol, reduced cholesterol 7$\alpha$-hydroxylase activity and mRNA levels in the WHHL rabbits signify diminished bile acid synthesis and provide an additional mechanism for the development of hypercholesterolemia in these animals. In a recent study by Krause et al. (31), endogenous hypercholesterolemia was produced in NZW rabbits by feeding low-fat, cholesterol-free diets that contained casein as the sole protein source. In these hypercholesterolemic rabbits, mRNA levels of cholesterol 7$\alpha$-hydroxylase were also reduced. However, it is not clear whether the low levels of cholesterol 7$\alpha$-hydroxylase in the baseline chow-fed WHHL rabbits represent a primary defect that is coupled to the inherited deficiency of LDL receptor function or is secondary to feedback suppression by the enlarged plasma and hepatic cholesterol pools. In this regard, it is noteworthy that hepatic HMG-CoA reductase was equally active in the NZW and WHHL control rabbits. Thus, the larger hepatic cholesterol pool did not inhibit hepatic cholesterol biosynthesis in the WHHL rabbits although cholesterol 7$\alpha$-hydroxylase was suppressed. This suggests that newly synthesized cholesterol, in this case, was not involved in the inhibition of cholesterol 7$\alpha$-hydroxylase activity. However, feeding 0.2% cholesterol significantly inhibited HMG-CoA reductase activity in both NZW and WHHL rabbits. This suggests that HMG-CoA reductase activity is regulated by a hepatic cholesterol pool that is sensitive to dietary cholesterol.

Feeding 2% dietary cholesterol to NZW rabbits unexpectedly inhibited cholesterol 7$\alpha$-hydroxylase activity and reduced mRNA levels substantially as well as downregulated LDL receptor function and was associated with elevated plasma cholesterol concentrations that were as high as seen in WHHL rabbits. Liver cholesterol was also markedly enhanced. Thus, the absorbed dietary cholesterol accumulated in the liver and suppressed LDL receptor expression and also probably inhibited cholesterol 7$\alpha$-hydroxylase activity and reduced mRNA levels in the NZW rabbits. As a result, marked plasma hypercholesterolemia developed. Some 30 yr earlier, Hellström (32) reported that cholesterol feeding to adult male albino rabbits resulted in a reduction in turnover and fecal elimination of deoxycholic acid as measured by isotope dilution techniques and is consistent with our idea that cholesterol inhibits cholesterol 7$\alpha$-hydroxylase (Table II) and bile acid synthesis.

It seems likely that the larger hepatic cholesterol pool plays a role in the inhibition of cholesterol 7$\alpha$-hydroxylase since liver cholesterol concentrations were elevated at baseline in WHHL and cholesterol-fed NZW rabbits. That feeding 2% cholesterol to NZW rabbits suppressed cholesterol 7$\alpha$-hydroxylase activity and mRNA levels to the baseline levels found in WHHL rabbits, but that 0.2% cholesterol feeding did not further decrease the enzyme activity and mRNA levels in WHHL rabbits suggests that the baseline enzyme levels in WHHL rabbits might be at the minimum for cholesterol 7$\alpha$-hydroxylase. However, it is important to note that in NZW rabbits 0.2% cholesterol inhibited cholesterol 7$\alpha$-hydroxylase activity 41%, which was still significantly higher than minimum levels in the WHHL rabbits although hepatic cholesterol levels in these cholesterol-fed NZW rabbits were nearly two times larger than in WHHL rabbits. Therefore, a metabolically active pool of cholesterol that regulates cholesterol 7$\alpha$-hydroxylase probably exists in the liver but cannot be defined by measuring total hepatic cholesterol concentrations.

The inhibitory effect of dietary cholesterol on cholesterol 7$\alpha$-hydroxylase in NZW rabbits is unexpected and opposite to the effect of cholesterol feeding in Spraque-Dawley rats (4–7). Both cholesterol 7$\alpha$-hydroxylase activity and mRNA levels were stimulated severalfold and bile acid outputs were increased in the cholesterol-fed rats. Plasma cholesterol levels did not rise in rats, although cholesterol accumulated mostly as esters in the liver. Apparently, cholesterol-fed rats respond differently and are able to convert excess dietary cholesterol to bile acids without raising plasma concentrations. Similar results to cholesterol-fed rats have been reported by Poorman et al. (33) to occur in specially bred hypercholesterolemia-resistant rabbits. In these animals, baseline activity and mRNA levels of cholesterol 7$\alpha$-hydroxylase were much higher than in normal NZW rabbits and were not inhibited by cholesterol feeding. One point is clear: that, in rabbits, dietary cholesterol does not upregulate bile acid synthesis by causing intestinal bile acid malabsorption as has been suggested to occur in the cholesterol-fed rat by Björkhem et al. (34). However, it is important to emphasize that $\sim$ 90% of plasma cholesterol is transported by HDL in the rat as compared with only 50% in the rabbit (35). Thus, the return of cholesterol to the liver in different lipoprotein fractions may ultimately determine its utilization as substrate for bile acid synthesis.

When 0.2% sitosterol was added to the diet, plasma cholesterol levels declined 42% ($P < 0.01$) in the NZW but not in the WHHL rabbits. Apparently, sitosterol, which is poorly absorbed, competitively blocks intestinal cholesterol absorption and decreases the return to the liver. Consequently, hepatic LDL receptor expression is upregulated and HMG-CoA reductase activity is stimulated (Table II), but the increase of LDL receptors may be greater than the input of newly synthesized cholesterol by the liver in the NZW rabbits fed sitosterol. As a result, plasma cholesterol levels decline significantly. In contrast, receptor-deficient WHHL rabbits are unable to increase the number of LDL receptors when fed sitosterol and, consequently, plasma cholesterol levels did not decrease.

With respect to the expression of LDL receptors, WHHL

rabbits show low receptor-mediated binding but normal amounts of mRNA consistent with an inherited translational abnormality of receptor protein. Not only is binding deficient, but the internalization of the receptor-bound cholesterol may also be impaired to account for the high level of plasma cholesterol. However, the higher than expected baseline receptor-mediated binding in our WHHL rabbits probably relates to nonspecific binding to liver membranes and may be in part due to the high background levels for these measurements. The higher cholesterol concentrations circulating in the plasma and increased hepatic cholesterol concentrations in the WHHL rabbits do not suppress hepatic HMG-CoA reductase activity or mRNA levels. However, feeding cholesterol to both rabbit groups strongly downregulated HMG-CoA reductase activity without decreasing mRNA levels. Thus, dietary cholesterol probably inhibits HMG-CoA reductase posttranscriptionally in rabbits as has been reported to occur in rats fed cholesterol (4, 5, 28). In NZW rabbits, dietary cholesterol also inhibits hepatic LDL receptor function without changing LDL receptor mRNA levels. Both HMG-CoA reductase and LDL receptors in NZW rabbits appear to be coordinately regulated and respond similarly to dietary cholesterol at a posttranscriptional level. This point is supported by the sitosterol feeding experiment where decreasing the return of intestinal cholesterol to the liver stimulated hepatic HMG-CoA reductase activity and tended to increase the expression of LDL receptors in the NZW rabbits.

Recently, Rudel et al. (36) reported that dietary cholesterol inhibited cholesterol 7$\alpha$-hydroxylase activity and mRNA levels in African green monkeys. The authors related the downregulation of cholesterol 7$\alpha$-hydroxylase to the accumulation of dietary cholesterol in the liver and suggested that reduced bile acid synthesis might decrease cholesterol absorption to limit the further uptake of dietary cholesterol. The effect of dietary cholesterol on cholesterol 7$\alpha$-hydroxylase in the African green monkeys is similar to the results of this study in NZW rabbits fed cholesterol. Both of these experiments emphasize the role of increased hepatic cholesterol concentrations to diminish cholesterol 7$\alpha$-hydroxylase that contributes to hypercholesterolemia in these two species.

The inhibitory effect of dietary cholesterol on cholesterol 7$\alpha$-hydroxylase (bile acid synthesis) in rabbits might help us to explain why some humans are so sensitive to dietary cholesterol and increased plasma concentrations. These subjects might respond to dietary cholesterol like NZW rabbits with diminished bile acid synthesis contributing to elevated plasma cholesterol levels and a reduction in LDL receptors. Alternatively, other people respond to dietary cholesterol like rats where bile acid synthesis is stimulated and plasma cholesterol levels do not rise. Identifying these individuals should help clarify their atherosclerosis risk and may make more sensible recommendations for dietary treatment. Nevertheless, these studies suggest that diminished cholesterol 7$\alpha$-hydroxylase and bile acid synthesis are related to elevated hepatic cholesterol concentrations and together with reduced LDL receptors increase plasma cholesterol levels when cholesterol is fed to NZW rabbits or where LDL receptors are abnormal in WHHL rabbits.

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