Cholesterol metabolism in fibroblasts from rabbits resistant to diet-induced hypercholesterolemia

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Abstract We have previously described a colony of New Zealand White rabbits that are resistant to hypercholesterolemia when fed a cholesterol-enriched diet. The present studies used skin fibroblasts obtained from normal and hypercholesterolemia-resistant rabbits to investigate cholesterol metabolism and lipid composition in vitro. The lipid compositions of the two cell lines after incubation in either fetal calf serum or lipoprotein-deficient serum were similar. The conversion of radiolabeled acetate into sterol and phospholipids was higher in resistant fibroblasts than in normal fibroblasts. In contrast, incorporation of radiolabeled oleic acid into cholesteryl ester was significantly lower in resistant fibroblasts than in normal cells. In parallel experiments, the 3-hydroxy-3-methylglutaryl coenzyme A reductase activity was higher and acyl-coenzyme A:cholesterol acyltransferase activity was lower in resistant cells compared to normal cells. Furthermore, binding, uptake, and degradation of normal rabbit ^{125}I-labeled LDL (low density lipoproteins) were 30% higher in resistant than in normal fibroblasts. These observations are consistent with results from previous studies of cholesterol metabolism in the liver membranes of these rabbits. The results indicate that extrahepatic cells (such as fibroblasts) from the resistant rabbit exhibit the same altered cholesterol metabolism as that found in the hepatic tissues of these rabbits. These studies suggest that the resistant rabbit may provide an in vivo and in vitro system for studying the mechanisms by which some individuals of a species can minimize the effect of dietary cholesterol on the development of hypercholesterolemia and atherosclerosis. —**Soma, M. R., J. D. Morrisett, A. M. Gotto, Jr., D. S. Loose-Mitchell, J. A. Poorman, S. A. Smith, and M. L. Overturf.** Cholesterol metabolism in fibroblasts from rabbits resistant to diet-induced hypercholesterolemia. *J. Lipid Res.* 1990. **31:** 985–994.

Supplementary key words fibroblasts • LDL receptor • HMG-CoA reductase • ACAT

The rabbit has several features that make it an attractive model for studying human atherosclerosis. When fed a cholesterol-supplemented diet over several weeks, the normal New Zealand White rabbit develops a dramatically elevated plasma concentration of cholesterol, most of which is transported in the form of cholesteryl ester-rich very low density lipoproteins (CER-VLDL) (1). The elevated plasma cholesterol concentration leads to extensive deposition of cholesterol in the liver, eyes, intercostal spaces, heart, and blood vessels. If the cholesterol-enriched diet is continued, this hypercholesterolemia leads to severe atherosclerosis. There are also strains of rabbits that carry genetic traits leading to spontaneous atherosclerosis (2–5). For example, the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, which lacks a functional apolipoprotein B/E receptor, exhibits traits similar to those of the human type IIa hypercholesterolemic subject (2).

We have developed a colony of rabbits that are resistant to diet-induced hypercholesterolemia and atherosclerosis. When fed a 0.1% cholesterol diet for up to 8 weeks, the resistant animals exhibit plasma cholesterol levels of <70 mg/dl, whereas normal animals develop levels of >700 mg/dl. No difference in cholesterol absorption between the two rabbit strains was detectable; however, an increased bile acid secretion was observed in the resistant animals (M. L. Overturf; unpublished data). The lipoprotein profiles of resistant and normal rabbits fed a cholesterol-supplemented diet reflect the differences in plasma cholesterol. CER-VLDL is the major lipoprotein in cholesterol-fed normal animals, whereas HDL is the

Abbreviations: CER-VLDL, cholesteryl ester-rich very low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl-coenzyme A:cholesterol acyltransferase; LDL, low density lipoprotein; HDL, high density lipoprotein; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; NADP, β-nicotinamide adenine dinucleotide phosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; BSA, bovine serum albumin; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; Tris, Tris[hydroxymethyl]aminomethane; WHHL rabbit, Watanabe Heritable Hyperlipidemic rabbit.

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most abundant lipoprotein in the resistant rabbit (6). In addition, larger amounts of rabbit ^{125}I-labeled LDL are internalized and degraded by peripheral mononuclear cells isolated from resistant rabbits compared to cells from normal animals (6). Differences in cholesterol metabolism have also been demonstrated in studies of liver membranes from these animals (7).

To determine whether the resistance to hypercholesterolemia is mediated by quantitative and/or qualitative differences in the metabolic parameters governing cellular cholesterol homeostasis in extrahepatic tissue, we have studied cholesterol metabolism and LDL receptor function in cultured skin fibroblasts from resistant and normal rabbits. The studies indicate that when incubated in lipoprotein-deficient serum (LPDS), cells from the resistant rabbit exhibit a significantly higher rate of LDL catabolism. Moreover, the resistant fibroblasts show higher HMG-CoA reductase activity, higher levels of sterol synthesis, lower ACAT activity, and lower levels of cholesteryl ester formation than normal cells do. These results suggest that intracellular cholesterol homeostasis in resistant cells differs from that in normal cells. These differences in extrahepatic cholesterol metabolism are consistent with differences observed in studies with rabbit liver membranes. Thus, the phenotype of the resistant rabbit is expressed by multiple cell types.

MATERIALS AND METHODS

Materials

Na[^{125}I] (17 Ci/mg) and [5-^{3}H]mevalonolactone (21 Ci/mmol) were obtained from New England Nuclear (DuPont Co., Boston, MA). [4,5-^{3}H]Cholesteryl oleate, Na[1-^{14}C]acetate (55 mCi/mmol), Na[1-^{14}C]oleate (58 mCi/mmol), L-[4,5-^{3}H(N)]leucine (5 Ci/mmol), 3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (52 mCi/mmol), [7-^{3}H]cholesterol (5 Ci/mmol), and [1-^{14}C]oleoyl coenzyme A (52 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle's medium, fetal calf serum, trypsin-EDTA solution, and gentamicin sulfate were obtained from Gibco (Grand Island, NY). Drugs and hormones were purchased from Sigma Co. (St. Louis, MO). Silica gel G thin-layer chromatography plates were Whatman brand, purchased from Fisher Scientific (Houston, TX).

Cells

Fibroblast strains were established from explants of rabbit skin biopsies (8, 9). Stock cells were grown in monolayer in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 mg/ml), and supplemented with gentamicin (50 μg/ml), glutamine (300 μg/ml), and 10% FCS (fetal calf serum). Cells were used between the 8th and 20th generations, and any comparative study between the two cell strains was performed simultaneously at the same population doubling. There was no significant difference in the amount of cellular protein and number of cells per culture dish for all experiments comparing the behavior of the two cell lines: 1.932 ± 0.337 mg cell protein/60-mm dish for normal fibroblasts versus 2.134 ± 0.328 mg cell protein/dish for resistant cells and $1.1 \times 10^6 \pm 4 \times 10^5$ cells/plate for normal fibroblasts versus $1.2 \times 10^6 \pm 3 \times 10^5$ cells/plate for resistant fibroblasts. On day 0, a total of 5×10^4 fibroblasts were seeded into 60-mm Petri dishes containing 3 ml of growth medium including 10% FCS. When the cells were in the late logarithmic growth phase (day 3 or 4), each monolayer was washed with 3 ml of PBS. Media (2 ml) containing 10% LPDS or FCS was added and the cells were incubated for 24 h or 48 h depending on the experiment performed.

Lipoprotein isolation

LDL (d 1.019–1.063 g/ml) from the plasma of normal rabbits fed normal diet was isolated by sequential preparative ultracentrifugation (10). LDL was iodinated with ^{125}I by the monochloride procedure of McFarlane as modified for lipoproteins (sp act 200–400 cpm/ng LDL protein) and sterilized by filtration (11). Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of fetal calf serum (12).

Determination of binding, uptake, and degradation of normal rabbit ^{125}I-labeled LDL by normal and resistant fibroblasts

The amount of surface-bound ^{125}I-labeled LDL, intracellular ^{125}I-labeled LDL, and degraded ^{125}I-labeled LDL was measured in intact fibroblast monolayers essentially as described by Goldstein, Basu, and Brown (12). After incubating the cells from 5 h in the presence of various amounts of rabbit ^{125}I-labeled LDL for the specific binding, and ^{125}I-labeled LDL and a 25-fold excess of unlabeled rabbit LDL for the nonspecific binding, cell monolayers were washed 5 times with 50 mM Tris–150 mM NaCl buffer, pH 7.4, containing 2 mg/ml BSA, and twice with buffer without BSA. The cells were then incubated for 1 h with 2 ml of 50 mM NaCl, 1 mM HEPES, pH 7.4, and dextran sulfate (4 mg/ml) at 4°C with shaking. The amount of LDL bound was determined from the dextran sulfate-releasable radioactivity in the medium. The cells were then washed, and dissolved in 100 mM NaOH for determination of LDL internalization and protein content. Degradation of ^{125}I-labeled LDL was determined on aliquots of medium after TCA precipitation and chloroform extraction (12).

Incorporation of [^{14}C]acetate into lipids

Since the lipid pool sizes in the two different cell lines were virtually the same (see Table 2), we utilized [^{14}C] acetate for lipid synthesis analysis.

Sterol, fatty acid, and phospholipid synthesis were each measured by the incorporation of [^{14}C]acetate (2 μCi/dish) into cellular sterols, triglycerides, and phospholipids, respectively, during a 2-h incubation at 37°C. After total lipids were extracted, various lipid classes were analyzed by TLC (13, 14) or HPLC (15) for incorporation of radioactivity.

For each experimental condition, the study was conducted in triplicate. After washing (5 times with 150 mM NaCl, pH 7.4), the cells were harvested by scraping and pelleted by centrifugation at 800 *g*. Pellets were resuspended in 150 mM NaCl. In the samples for the TLC analysis, 10,000 dpm of [^{3}H]cholesterol was added as an internal standard, and the recovery of [^{3}H]cholesterol was always greater than 90% and virtually identical for normal and resistant cell extracts. Both lines of cells were then extracted with acetone–diethylacetone 1:2 (v/v) as described (15). The lower phase was removed and evaporated to dryness under a stream of N_2. The residues were then resuspended in petroleum ether and spotted on silica gel G TLC plates. After chromatography (isooctane–diethyl ether–acetic acid 75:25:2 v/v/v), the lipids were visualized by exposure to iodine vapors, identified by reference to standards, scraped from the plates, and placed in scintillation vials. Scintillation fluid (10 ml) was then added and vials were counted in a scintillation spectrometer for ^{3}H and ^{14}C content until a 2-sigma significance was achieved.

Incorporation of [^{14}C]oleate into cholesteryl ester

Incorporation of [^{14}C]oleate was assessed by incubating confluent fibroblasts for 2 h at 37°C with 2 μCi of a solution containing [^{14}C]oleate–albumin complex (5% albumin/0.9% NaCl/20 mM sodium oleate, 200 μCi/ml). The cellular content of [^{14}C]oleate incorporated into lipids was analyzed by TLC analysis after lipid extraction as described above.

Cellular localization of [^{3}H]cholesterol in normal and resistant fibroblasts

Normal rabbit LDL (protein:cholesterol = 0.87) were labeled with [^{3}H]cholesteryl oleate using a modified technique previously described (16). A stable dispersion of cholesteryl oleate was obtained by injecting 100 μCi of [^{3}H]cholesteryl oleate into 300 μl of 50 mM Tris, 150 mM NaCl (pH 7.4) buffer maintained at 60°C. This dispersion was then cooled to 37°C and incubated with 500 μl of normal rabbit plasma d 1.21 g/ml infranatant for 10 min after which 500 μl of normal rabbit LDL (5 mg of protein; cholesterol:protein = 1.13) was added and the incubation was continued for an additional 24 h. The cholesteryl ester-labeled LDL were then re-isolated by centrifugation; more than 90% of the radioactivity was incorporated in the 1.063 g/ml density supernatant, and the specific activity of the LDL preparation was 52 dpm cholesteryl ester/ng LDL cholesterol.

Normal and resistant fibroblasts were grown for 4 days in medium containing 10% FCS. Cell monolayers were then washed with sterile PBS and incubated for 24 h either in 10% FCS or 10% LPDS medium, after which 2 μg protein/ml of [^{3}H]cholesteryl oleate-labeled LDL was added and incubations were continued for an additional 24 h. Cells were then rapidly washed 5 times with cold PBS and collected with a rubber policeman in 3 ml of cold PBS. Cell membranes and cytosol were separated by centrifugation at 100,000 *g* for 60 min at 4°C. The pellet fractions were resuspended in a volume of PBS equal to the volume of the supernatant. The lipids in the pellet and supernatant fractions were then extracted and separated by TLC as described above.

Lipid analysis by high pressure liquid chromatography

HPLC was performed as described by Hoffman and Pownall (15) with a Spectra Physics 8000B liquid chromatograph (Santa Clara, CA). A radial compression module equipped with a Radial Pak cartridge (5 mm i.d.) containing 10 μm silica (Millipore-Waters Division, Amherst, MA) was used. Chromatography was performed using a gradient of solvents as described (15).

Lipid and protein quantitation

Cellular phospholipid concentration was determined as described by Bartlett (17) using a multiplication factor of 25. Triglycerides, total cholesterol, and cholesteryl esters of cells were measured using enzymatic assay kits from Boehringer-Mannheim Diagnostic. Protein concentration was determined by the method of Lowry et al. (18).

Incorporation of [^{3}H]leucine into proteins

Incorporation of [^{3}H]leucine into proteins was performed by standard techniques. After the incubation of cells for 2 h in the presence of [^{3}H]leucine (2 μCi/ml), they were rapidly washed with cold PBS and collected by centrifugation. The cell pellets obtained after 800 *g* centrifugation were resuspended in cold PBS and the proteins were precipitated by a 10% TCA solution on Whatman 3 MM filter paper circles. The filters were washed with absolute ethanol followed by water, cut into small pieces, and counted.

Acyl-coenzyme A:cholesterol acyltransferase assay

Microsome preparation. Microsomes were prepared from confluent cells that had been maintained in media supplemented with 10% fetal calf serum, or 10% lipoprotein-deficient serum, for 2 days. Cells were washed and harvested by scraping into PBS and pelleted by centrifugation. The pellet was resuspended in 2 volumes of 50 mM K_2HPO_4 (pH 7.4), 5 mM DTT, 1 mM EDTA and incubated on ice for 5 min. The suspension was then homo-

genized with a Dounce A homogenizer and the extracts were stored at −80°C.

Enzyme assay. Samples of fibroblast microsomes (25–200 μg) were assayed (19) in 225 μl of 0.2 M K_2HPO_4 buffer containing 5 mg/ml BSA and 67.68 μM oleoyl CoA (7.68 μM [^{14}C]oleoyl CoA, plus 60 μM unlabeled oleoyl CoA). All experiments used a minimum of two microsomal protein concentrations for activity analysis. Microsomes were preincubated at 37°C in the phosphate-BSA buffer for 5 min before addition of the substrate. Substrate was then added and the reaction mixture was incubated at 37°C for 5 min and the reaction was terminated by addition of 5 ml chloroform–methanol 2:1. Approximately 10,000 dpm of [^{3}H]cholesteryl oleate was added to each sample in order to estimate recovery. After the incubation period, the reaction mixture was acidified by addition of 1.05 ml (pH 3.0) water, mixed thoroughly, and left at 4°C overnight. The mixture was then spun in a Damon HN-S II centrifuge at 1200 *g* for 10 min and the upper phase was removed and discarded. The lower phase was washed twice with 1.2 ml fresh upper phase (chloroform–methanol–water 3:48:47). The addition of less than 1 ml methanol resulted in solubilization of the remaining interface before the organic phase was dried in air. The residue was resuspended in hexane, spotted on a TLC plate, and chromatographed a distance of 10 cm in hexane–ethyl acetate 9:1. After autoradiography, the band corresponding to cholesteryl oleate was isolated and counted in a scintillation counter. Recovery of cholesteryl oleate was greater than 40%.

3-Hydroxy-3-methylglutaryl coenzyme A reductase assay

Cell-free extracts. Cells were initially plated in 60-mm Petri dishes at 2 × 10^4 cells/ml in media supplemented with 10% FCS. Four days later the media were removed and the cells were incubated in fresh media supplemented with 10% FCS or 10% LPDS. After 24 h the cells were harvested. Cells were washed twice with 3 ml of 50 mM Tris-HCl, 150 mM NaCl (pH 7.4), and then scraped into 1 ml of the same buffer. The suspension was centrifuged at low speed, the supernatant was discarded, and the cell pellet was frozen in liquid nitrogen and stored until the time of the assay. Cell pellets were extracted with 100 μl of 50 mM potassium phospate, 5 mM dithiothreitol, 5 mM disodium EDTA, 200 mM KCl, and 0.25% Brij96, as described (12).

Enzyme assay. Aliquots of the cell-free extracts (60–100 μg) were assayed (9) in a buffer containing 0.2 M K_2HPO_4 (pH 7.4), 100 mM glucose-6-phosphate, 15 mM NADP, 37.5/ml units glucose-6-phosphate dehydrogenase, 25 mM DTT, and 20 μM HMG-CoA (90,000 dpm/sample [^{14}C]HMG-CoA) in a total volume of 200 μl. Microsomes were preincubated in the reaction buffer at 37°C for 10 min before the addition of the HMG-CoA and then incubated for 120 min at 37°C with moderate shaking. The reaction was stopped by the addition of 20 μl of 5 N HCl, and 90,000 dpm of [^{3}H]mevalonolactone standard was added to measure recovery. The reaction solution was then incubated at 37°C for 30 min to allow lactonization of the mevalonate. The mixture was extracted twice with 10 ml (20 ml total) of diethyl ether. The upper phase was transferred to a 50-ml conical tube, and the combined upper phases were dried and the residue was resuspended in acetone and spotted on a TLC plate and chromatographed in acetone–benzene 1:1. Recovery of labeled mevalonolactone was more than 40%.

RESULTS

Experiments were carried out on skin fibroblasts obtained from normal and hypercholesterolemia-resistant rabbits. The plasma cholesterol concentrations of the rabbits from which the cells were obtained are shown in **Table 1**.

We first analyzed the lipid composition of normal and resistant rabbit fibroblasts incubated in 10% FCS or LPDS. These results are summarized in **Table 2**. Total lipid content was similar for both normal and resistant fibroblasts when incubated in FCS (normal 254, resistant 262 μg/mg cell protein) or LPDS (normal 252, resistant 259 μg/mg cell protein). Both normal and resistant fibroblasts, incubated in LPDS compared to fibroblasts maintained in FCS, showed a slight decrease in triglycerides and free cholesterol, a more pronounced decrease in cholesteryl ester, and an increase in phospholipid content.

We then compared the capacity of fibroblasts from resistant (R) and normal (N) rabbits to synthesize sterols, fatty acids, and phospholipids from [^{14}C]acetate. Both normal and resistant rabbit skin fibroblasts were grown for 3 days in medium containing 10% FCS. Cells were

TABLE 1. Plasma cholesterol of rabbits from which skin fibroblasts were obtained

Rabbit	Sex	Plasma Cholesterol	
		Regular Diet	Cholesterol Diet
		mg/dl	
Normal			
N-1	M	26	1335
N-2	M	33	768
Resistant			
R-1	M	28	68
R-2	M	14	33

Plasma lipids of rabbits from which skin fibroblasts were obtained. N and R refer to normal and resistant rabbits, respectively. The plasma cholesterol was determined when rabbits where fed a normal chow diet and then a 0.25% cholesterol-enriched diet for 8 weeks.

TABLE 2. Lipid content of normal and resistant fibroblasts

Medium	Lipid Content	
	Normal	Resistant
	μg/mg cell protein	
Fetal calf serum		
Triacylglycerols	74.8 ± 13.5 (29.4%)	74.1 ± 12.1 (28.3%)
Phospholipids	146.2 ± 11.6 (57.5%)	155.0 ± 12.7 (59.1%)
Free cholesterol	29.4 ± 4.8 (11.6%)	29.8 ± 2.3 (11.4%)
Esterified cholesterol	4.0 ± 1.4 (1.5%)	3.2 ± 0.8 (1.2%)
Total cholesterol	33.4 ± 3.5	33.0 ± 2.3
Total lipid	254.4 ± 22.8 (100.0%)	262.2 ± 23.0 (100.0%)
Lipoprotein-deficient serum		
Triacylglycerols	67.2 ± 2.8 (26.6%)	67.4 ± 2.2 (25.9%)
Phospholipids	159.6 ± 5.1 (63.3%)	167.0 ± 8.0 (64.3%)
Free cholesterol	24.4 ± 2.1 (9.7%)	24.7 ± 2.3 (9.5%)
Esterified cholesterol	1.1 ± 0.5 (0.4%)	0.7 ± 0.2 (0.3%)
Total cholesterol	25.5 ± 2.1	25.4 ± 2.3
Total lipid	252.3 ± 7.8 (100.0%)	259.8 ± 9.4 (100.0%)

Confluent fibroblast monolayers from normal and resistant rabbits were incubated in FCS or LPDS medium for 24 h. Cells were harvested, and the lipids were extracted and analyzed as described in the Materials and Methods. Values are means ± SD (standard deviation) from five Petri dishes. The values in parentheses represent percentage composition.

then placed in medium containing either 10% FCS or 10% LPDS and maintained for 48 h before the incorporation experiments were begun. The incorporation of acetate into total sterols (cholesterol and cholesteryl esters), is illustrated in **Fig. 1A**. Incorporation of acetate into sterols was significantly greater in resistant than in normal rabbit fibroblasts, when the cells were preincubated in either FCS or LPDS. Sterol synthesis in the resistant cells was 4.5 and 2.5 times greater than in normal cells cultured in either FCS or LPDS, respectively. This enhanced incorporation was consistently observed in the resistant rabbit cell lines. Fatty acid synthesis, measured as incorporation of acetate into triglyceride, was nearly equal in both of the cell lines (Fig. 1B). Interestingly, incorporation of [^{14}C]acetate into phospholipids was consistently 30% higher in resistant fibroblasts compared to normal cells, when cells were incubated in either FCS or LPDS (Fig. 1C)

Incorporation of [^{14}C]acetate into cell lipids was also assayed by HPLC. **Fig. 2** shows two spectra obtained from normal (Fig. 2A) and resistant (Fig. 2B) cells, preincubated in LPDS and then incubated for 2 h in the presence of [^{14}C]acetate. The HPLC analysis confirmed the results from TLC. Incorporation of labeled acetate into triglycerides was nearly equal in both of the cell lines. The acetate incorporation into both sterols and phospholipids, however, was significantly higher in the resistant cells than in normal fibroblasts. The HPLC analysis revealed that, of phospholipids labeled by acetate, incorporation occurred mostly into phosphatidylcholine and phosphatidylethanolamine, and was higher in the resistant compared to normal fibroblasts.

To determine whether the different rates of sterol synthesis by normal and resistant cells were dependent on the stage of cell growth, we determined the rate of protein synthesis by measuring the incorporation of [^{3}H]leucine and the rate of sterol synthesis by incorporation of [^{14}C]acetate. **Fig. 3B** shows that the incorporation of radiolabeled L-leucine was similar in the two different cell lines, with maximum incorporation occurring during the

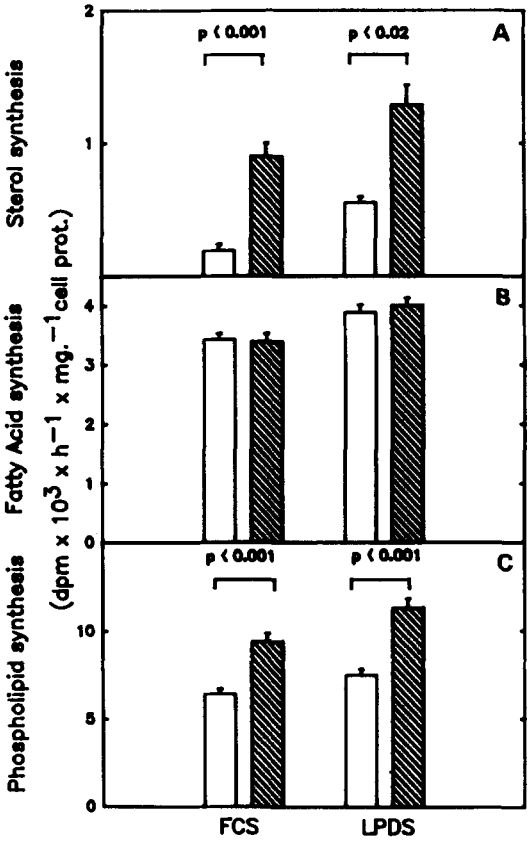

Fig. 1. Sterol, fatty acid, and phospholipid synthesis in normal and resistant rabbit skin fibroblasts. Normal (open bars) and resistant (filled bars) rabbit fibroblasts were grown with medium containing 10% FCS for 3 days. Cells were then incubated for 48 h either in 10% FCS or LPDS media. Incorporation of [^{14}C]acetate into sterols (cholesterol (95%) + cholesteryl ester (<5%)) (A), fatty acids (B), and phospholipids (C) were determined as described in Materials and Methods section. Values are means ± SD of five different determinations, and have been normalized to mg of cell protein as indicated.

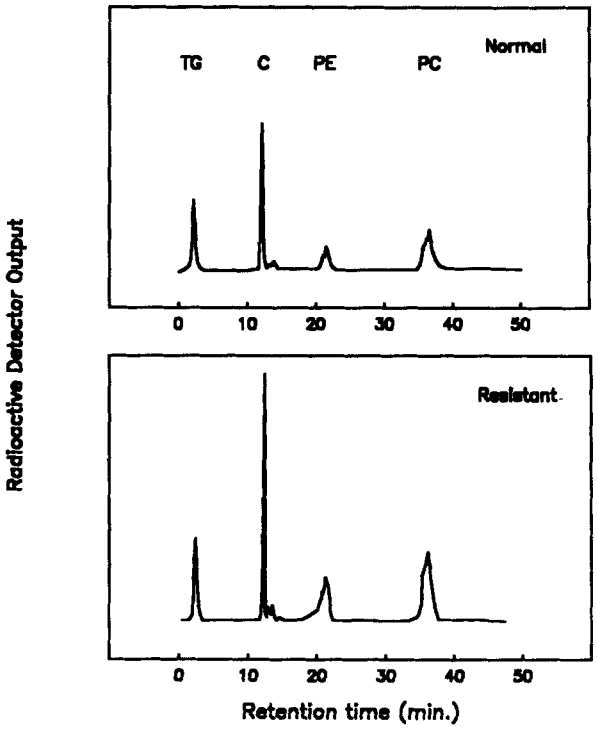

Fig. 2. HPLC separation of radiolabeled lipids. Fibroblasts, at day 3 after seeding were incubated for 48 h in 10% LPDS and then [^{14}C]acetate was incorporated into lipids during a 2-h incubation as described in Materials and Methods. Lipid extracts from equal amounts of cell protein from the two cell lines were analyzed by injecting 150 μl of each; TG, triglycerides; C, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

log phase of growth (day 3 after seeding). The incorporation of acetate into sterol was higher in resistant cells incubated with LPDS than in normal cells under the same conditions (Fig. 3A). These data suggest that the higher rate of sterol synthesis in the resistant cells compared to normal cells was not the result of a difference in cell growth.

HMG-CoA reductase activity, as a specific index of cholesterol synthesis capacity, was measured in normal and resistant cells preincubated in either FCS or LPDS (**Fig. 4**). The activity of HMG-CoA reductase was increased in both normal and resistant cells preincubated in LPDS media for 24 h; however, the level of enzyme activity in both FCS and LPDS was approximately threefold higher for resistant cells compared to normal fibroblasts.

The rate of cholesteryl ester formation in resistant and normal cell lines was also investigated. For these studies, cells were grown to confluence (day 5); then the medium in both normal and resistant cells was replaced with fresh medium containing either 10% FCS or 10% LPDS. [^{14}C]Oleate incorporation into cellular cholesteryl ester over 2 h was then measured. In normal rabbit cells, depletion of cholesterol concentration in the medium (i.e., media supplemented with LPDS) caused a sharp reduction (40%) in cholesteryl ester formation (**Fig. 5A**). The formation of cholesteryl ester by resistant cells was significantly lower than in normal cells, especially when the cells were grown in LPDS. Cholesteryl ester formation was also determined by measuring the acyl CoA:cholesterol acyltransferase activity in cell-free extracts. ACAT activity in normal cells was significantly higher than in resistant fibroblasts when cells were incubated either in FCS or LPDS (Fig. 5B). ACAT activity in resistant cells was virtually unaffected by incubation with either FCS or LPDS.

The capacity of normal and resistant fibroblasts to metabolize lipoproteins via the apoB/E receptor was investigated using rabbit LDL. Binding, uptake, and degradation of ^{125}I-labeled LDL by normal and resistant fibroblasts are shown in **Fig. 6** (A–C). Incubation of cells with 0.25–10 μg/ml radiolabeled ^{125}I-labeled LDL was carried out after preincubation for 24 h with either FCS or LPDS media. Saturable binding was observed in both cell lines; nonspecific binding was assessed in parallel incubations by addition of a 25-fold excess of unlabeled

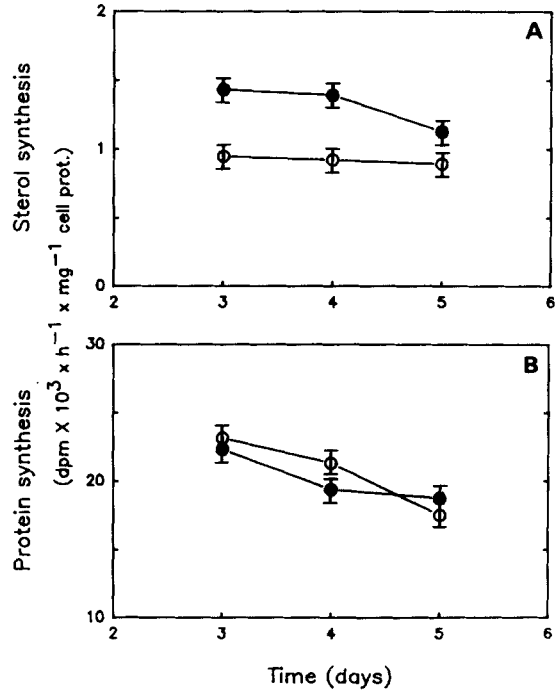

Fig. 3. Incorporation of [^{14}C]acetate into sterols and [^{3}H]leucine into protein by normal (○)and resistant (●) cells. Normal and resistant fibroblasts were grown in medium containing 10% FCS. On days 3, 4, and 5 after seeding, incorporation of [^{14}C]acetate (panel A) and [^{3}H]leucine (panel B) was determined after preincubating the cells with 10% LPDS for 24 h. Incorporation of [^{14}C]acetate and [^{3}H]leucine was determined as described in the Materials and Methods section. Values are means of three different determinations.

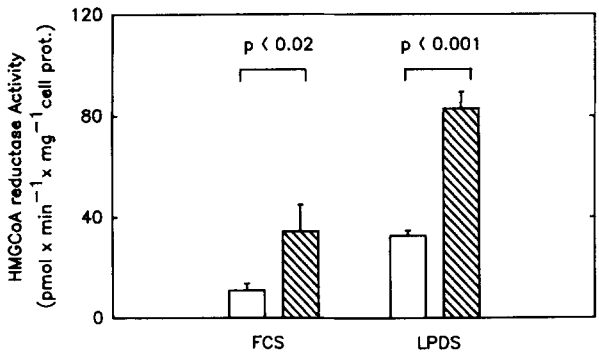

Fig. 4. HMG-CoA reductase activity in normal and resistant fibroblasts. Normal (open bars) and resistant (filled bars) rabbit fibroblasts were grown in medium containing 10% FCS for 3 days. Cells were then incubated for 48 h either in 10% FCS or LPDS media. HMG-CoA reductase activity was then assayed on cell extracts as described in Materials and Methods. Values are means ± SD of four different determinations.

LDL. Binding, uptake, and degradation of rabbit LDL was increased 3- to 4-fold by incubation of normal and resistant cells in medium containing LPDS. These results demonstrated that rabbit LDL interacts with specific cell surface sites in both cell lines. High affinity binding, uptake, and degradation of LDL in resistant fibroblasts preincubated in LPDS was about 1.5-fold higher than in normal cells. The receptor activity was always higher in resistant cells than in normal fibroblasts.

Distribution of [^{3}H]cholesterol between the cell membranes and the cytosol was assessed in cells preincubated with [^{3}H]cholesteryl oleate-labeled LDL in FCS- or LPDS-containing medium (**Table 3**). In resistant fibroblasts grown continuously in 10% FCS, 91.1% and 4.3% of the total [^{3}H]cholesterol absorbed was found in the free cholesterol of the pellet (membranes) and the supernatant (cytosol) fractions, respectively; the percentages of [^{3}H]cholesteryl ester were 3.4% for the pellet and 1.2% for the supernatant. In normal cells, the radioactivity values associated with free cholesterol and cholesteryl ester in the pellet were 84.4% and 5.7%, while in the supernatant the values were 7.3% and 2.6%, respectively. When cells were preincubated for 24 h in medium containing LPDS, there was a 4- to 5-fold increase in the uptake of labeled LDL, but the overall distribution of the [^{3}H]cholesterol in the two different cell lines was similar to the distribution observed in the same cells incubated in FCS. When compared with normal cells, resistant fibroblasts grown in either FCS or LPDS contained a lower level of cholesteryl ester in both the cell pellet and the supernatant fractions. However, the free [^{3}H]cholesterol was 7–8% higher in the pellet fraction, and ~3% lower in the cytosolic fraction compared to normal cells.

DISCUSSION

The results from these studies indicate that fibroblasts isolated from hypercholesterolemia-resistant animals, compared to those from normal animals, exhibit altered intracellular cholesterol metabolism and homeostasis. This view is supported by the following observations. *1*) Cholesterol synthesis assessed by [^{14}C]acetate incorporation was higher in resistant fibroblasts than in normal cells incubated either in FCS or LPDS. This difference was evident at 2 h, and persisted over a 48-h period (data not shown). *2*) Cholesterol esterification determined either by the incorporation of [^{14}C]oleate into intracellular cholesteryl ester or by the measurement of ACAT activity was lower in the resistant cells than in the normal cells incubated in either FCS or LPDS. *3*) Binding, inter-

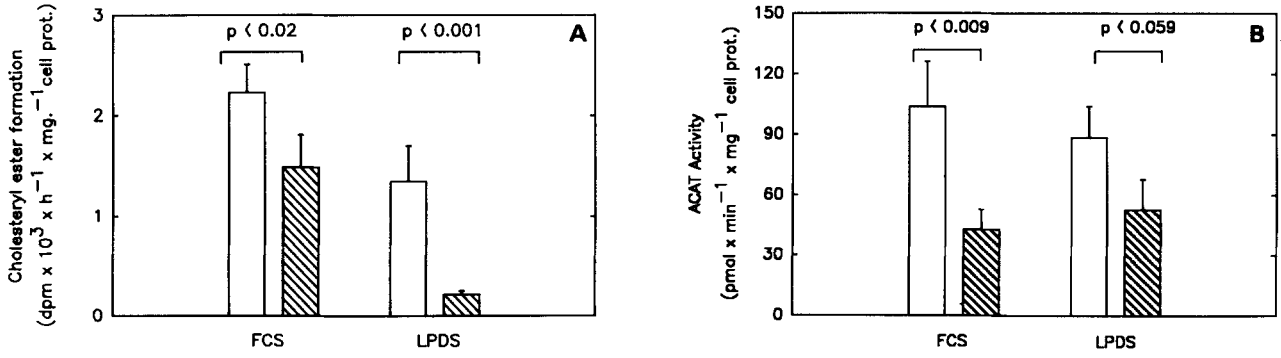

Fig. 5. A: Incorporation of [^{14}C]oleate into cholesteryl ester in normal and resistant rabbit fibroblasts. Rabbit skin fibroblasts were grown in medium containing 10% FCS for 4 days. Cells were then incubated for 24 h either in 10% FCS or LPDS media. Formation of cholesteryl esters was determined by the incorporation of [^{14}C]oleate by normal (open bars) or resistant (filled bars) fibroblasts as described in Materials and Methods. Values are means ± SD of five determinations. B: ACAT activity in normal and resistant fibroblasts. Normal (open bars) and resistant (filled bars) rabbit fibroblasts were grown to confluence with medium containing 10% FCS. Cells were then incubated for 24 h either in 10% FCS or LPDS media. ACAT activity was measured on cell microsomes as described in Materials and Methods. Values are means ± SD of six different determinations.

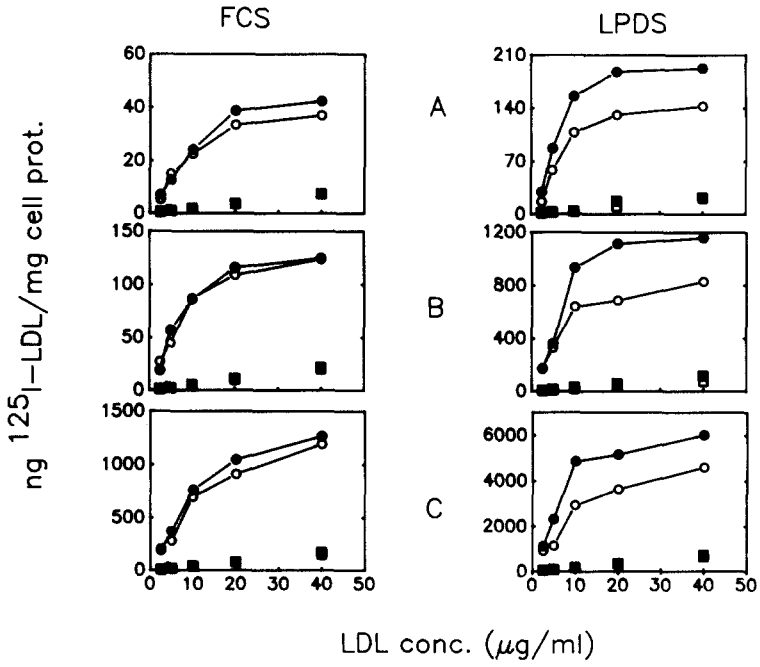

Fig. 6. Saturation kinetics of binding (A), uptake (B) and degradation (C) of rabbit ^{125}I-labeled LDL in normal (○, □) and resistant (●, ■) rabbit fibroblasts. Four days after seeding, fibroblasts were preincubation for 24 h in 10% FCS or LPDS media. Cells were then incubated for 5 h at 37°C with the indicated concentration of ^{125}I-labeled LDL in the presence (□, ■) or absence (○, ●) of a 25-fold excess of cold rabbit LDL.

TABLE 3. Distribution of [^{3}H]cholesterol between cell membranes and cytosolic fraction of normal and resistant fibroblasts

	Normal		Resistant	
Medium	FC	CE	FC	CE
	dpm		*dpm*	
Fetal calf serum				
Cell pellet	58152 (± 3125) 84.4%	3927 (± 327) 5.7%	66863 (± 2143) 91.1%	2498 (± 225) 3.40%
Supernatant	5030 (± 724) 7.3%	1791 (± 177) 2.6%	3159 (± 238) 4.3%	882 (± 54) 1.2%
Lipoprotein-deficient serum				
Cell pellet	235575 (± 5423) 85.3%	12980 (± 1228) 4.73%	394780 (± 3147) 93.7%	5056 (± 846) 1.2%
Supernatant	21541 (± 1740) 7.8%	6076 (± 186) 2.2%	19381 (± 326) 4.6%	2107 (± 158) 0.5%

Cells were grown as described in Materials and Methods. At day 4, cells were incubated for 24 h with either FCS or LPDS medium, after which 2 μg/ml of [^{3}H]cholesteryl oleate-labeled rabbit LDL (123 dpm/ng LDL protein) was added. After 24 h incubation, cells were collected and the cell pellet and supernatant fractions were isolated as described in Materials and Methods. Distribution of radioactivity between cholesterol and cholesteryl ester in the two cellular fractions was determined by TLC as described in Materials and Methods; n = 5. Values are dpm [^{3}H]cholesterol/mg cell protein; FC, free cholesterol; CE, cholesteryl ester.

nalization, and degradation of rabbit LDL by cells incubated in LPDS were significantly higher in resistant cells than in normal cells. The contrasting behavior of the two cell lines was not the result of either a difference in cell growth or of altered cellular lipid composition, as evidenced by the observation that the [^{3}H]leucine incorporation into protein was virtually the same in both cell lines (Fig. 3B), and the lipid composition of the two different lines was very similar (Table 2).

The differing capacities of normal and resistant cells, incubated in either FCS- or LPDS-containing media, to regulate lipid synthesis could lead to differing lipid compositions, differing cholesterol/phospholipid ratios, resulting in differing membrane fluidities and metabolic behavior (20). However, our data did not show any significant differences in cellular lipid composition that would support this hypothesis (Table 2 and Fig. 2).

In spite of the high rate of cholesterol synthesis in resistant cells, cholesterol esterification was lower than that seen in normal cells (7). In FCS the incorporation of [^{14}C]oleate into cholesteryl ester by resistant fibroblasts was 75% of the level seen in normal cells; resistant cells incubated in LPDS had only 20% of the incorporation seen in normal cells in the same media. ACAT activity in resistant fibroblasts was essentially unchanged when the cells were incubated in LPDS. In both LPDS and FCS the resistant cells had 40% of the level of ACAT activity detected in normal cells.

A comparison of the metabolism of rabbit LDL by normal and resistant fibroblasts demonstrated that when cells were incubated in LPDS, a larger amount of ^{125}I-labeled LDL was metabolized by the resistant fibroblasts. The LDL metabolism by resistant cells was mediated by the high affinity receptor, since the nonspecific binding, uptake, and degradation rates of ^{125}I-labeled LDL were nearly equal for the normal and resistant cell lines. Scatchard analysis of the binding data indicated that this higher LDL catabolic rate was due to an increased number of cell surface receptors, and not to a different receptor affinity for LDL (the K_d for the LDL receptor in normal and resistant cells was 5×10^{-10} M and 9×10^{-10} M, respectively).

When cells were incubated in cholesterol-depleted (LPDS) medium, LDL receptor expression and HMG-CoA reductase activity were higher and ACAT activity was lower in resistant than in normal cells, respectively. Interestingly, this behavior of resistant rabbit fibroblasts is similar to that of fibroblasts from subjects with familial hypercholesterolemia (21). Exposure of the resistant cells to media containing cholesterol (FCS medium) causes some down-regulation of cellular cholesterol synthesis (Fig. 4) and LDL receptor synthesis (Fig. 6), and stimulation of cellular cholesterol esterification (Fig. 5A), but not to the levels seen in the normal fibroblasts. The observed differences between normal and resistant cells suggest that, in the resistant cell, cholesterol might be stored in a compartment that does not directly participate in the normal regulation of its own metabolism. This hypothesis is supported by the data obtained from the ^{3}H distribution of the cellular [^{3}H]cholesterol between the pellet and the cytosolic fractions. Lower concentrations of [^{3}H]cholesterol were found in the cytosol of resistant cells than in normal fibroblasts, and a 7% higher concentration of [^{3}H]cholesterol was found in the particulate fraction of the resistant cells compared to normal fibroblasts (Table 3). Another possibility is that cholesterol efflux from the resistant cell is accelerated. This hypothesis is supported by our previous observation that plasma HDL levels remain high and virtually unchanged in resistant animals fed a high-cholesterol diet (6).

The anomalous cholesterol metabolism observed in this hypocholesterolemic rabbit colony is probably due to genetic differences that are manifest in both extrahepatic and hepatic tissues. It is not yet clear how the altered cholesterol metabolism seen in fibroblasts and in hepatic tissue of the resistant rabbit contributes to its resistance to hypercholesterolemia. Nevertheless, this animal model does appear to have excellent potential for revealing new metabolic mutations that afford protection against atherosclerosis.

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