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Stimulation of Acyl-CoA: Cholesterol Acyltransferase Activity by Hyperlipemic Serum Lipoproteins (39838)[1]

G. H. ROTHBLAT, M. NAFTULIN, AND L. Y. ARBOGAST

Medical College of Pennsylvania, Department of Physiology and Biochemistry, 3300 Henry Avenue, Philadelphia, Pennsylvania 19129

Our previous studies (1) on the metabolism of cholesteryl ester (EC) in hepatoma cell cultures demonstrated that growth of these cells in the presence of hyperlipemic rabbit serum (HRS) lipoproteins resulted in a marked accumulation of EC. EC accumulation was not observed during culture in equivalent free cholesterol (FC) concentrations of normolipemic sera (NRS) or lipoproteins, indicating a response of the cells to new or modified lipoproteins in HRS. Quantitation of the source of the accumulated EC demonstrated a contribution of both exogenous EC and FC to cellular EC (2). Growth of the hepatoma in HRS resulted in increased cellular incorporation of FC which was closely correlated with an increase in the amount of FC esterified. Previous studies with this hepatoma indicate that at least 40% of the cellular esterified cholesterol that accumulates in these cells can be derived from the esterification of free cholesterol, thus demonstrating the importance of this process in cellular cholesteryl ester accumulation (2).The present study, therefore, was conducted to investigate the influence of HRS lipoproteins on the synthesis of EC by the acyl-CoA: cholesterol acyltransferase (ACAT) (EC. 2.3.1.26) from hepatoma cells. This enzyme has been found in various tissues with marked elevation of activity in atherosclerotic aorta (3-7).

Methods. Cells, growth conditions, and serum lipoproteins. The Fu5 rat hepatoma cell lines were originally derived from the Reuber H35 rat hepatoma. Monolayers of stock cells were grown in Falcon plastic flasks (75 cm^2) containing 15 ml of Eagle's minimal essential medium (MEM) formulated as previously described (1) and supplemented with 10% fetal bovine serum (FBS) (Flow Labs). Cells were exposed to 2.5% HRS or NRS for 24 hr prior to harvest when the monolayers were approaching confluency. Cell yield per flask averaged 6 mg of cell protein. Cells were scraped from the flask, pelleted, and washed by recentrifugation in buffered salt solution. Procedures used for obtaining HRS and NRS lipoproteins from cholesterol-corn oil-fed rabbits have been previously described (1). The density (<1.019 g/ml) used to isolate very low-density lipoprotein (VLDL) was such as to include the "intermediate" or "remnant" particles present in HRS (8, 9).

Preparation of microsomes, preincubation conditions, and enzyme assay. Washed cell pellets were resuspended in 10^{-3} *M* phosphate buffer (pH 7.4) using 2 ml of buffer/flask and were disrupted by sonication with a Branson sonifier (microtip setting No. 1 for 10 sec). Cell homogenates were then diluted with an equal volume of 0.5 *M* sucrose and were centrifuged as 12,000*g* for 15 min. Each flask yielded an average of 4 mg of protein in the 12,000*g* supernate ($S_{12,000}$). The $S_{12,000}$ fraction was preincubated with isolated NRS or HRS lipoprotein for 2 hr at 37° by adding lipoproteins to give 1 mg of lipoprotein FC/4 mg of $S_{12,000}$ protein. A microsomal fraction was then pelleted by centrifugation at 100,000*g* for 45 min and was resuspended by sonication in 0.1 *M* phosphate buffer (pH 7.1). The suspension volume was adjusted to 100 μg of microsomal protein 0.45 ml of buffer, using the method of Lowry *et al.* (10) for protein determination. All procedures were carried out at 4°. In designated experiments (Table 2, Nos. 1 and 2), isolated microsomes (1 mg of protein) were preincubated with VLDL (1 mg of FC), reisolated, and washed by

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centrifugation. In all experiments, untreated $S_{12,000}$ or isolated microsomal controls were preincubated in the absence of added lipoprotein.

The method used for the assay of cellular ACAT activity is a modification of the procedure of Hashimoto and Dayton (5). The incubation mixture for this assay consisted of 0.5 ml of 0.1 *M* phospahte buffer (pH 7.1) containing 100 μg of microsomal protein, 2.5 μM $MgCl_2$, 0.5 μM dithiothreitol, and 0.6 mg of fatty acid-free bovine albumin (Sigma). [1-^{14}C]Palmityl-CoA (New England Nuclear, 57.8 mCi/mmole), adjusted to a specific activity of 1.16 μCi/μmole, was added at concentrations ranging from 0.5 to 30 nmole as indicated in the text. Incubations were at 37° for 30 min unless otherwise stated. Values are presented as the average of at least two replicate assays.

The reaction was stopped by the addition of chloroform–methanol, and the lipids were extracted by the method of Bligh and Dyer (11). Total lipid extracts were spotted on silica gel G thin-layer chromatographic (tlc) plates and were developed with petroleum ether/ethyl ether/acetic acid (85/15/1). The lipid bands on the tlc plate were counted by liquid scintillation techniques. The efficiency of recovery of radiolabeled lipids was >95%. Palmityl-CoA was assayed using the method described by Barnes and Wakil (12). Quantitation of free and esterified cholesterol was achieved by gas–liquid chromatographic techniques as previously described (1, 2).

Results. ACAT activity in Fu5AH. Microsomal ACAT activity reached a broad maximum between pH 7.1 and 8.1, with no activity below pH 6.3. No ACAT activity could be detected at any pH in the 100,000*g* supernatant fraction or in boiled microsomal controls. Maximum levels of [^{14}C]palmitate were incorporated into EC at substrate concentrations between 100 and 200 nmole of palmityl-CoA/1 mg of microsomal protein (incubation time: 10 and 30 min). Some inhibition was observed at 300 nmole/1 mg of protein. Incubation of [1-^{14}C]palmitate with the microsomal preparation resulted in negligible formation of ^{14}C-labeled cholesteryl palmitate. Partial activity was restored upon addition of ATP and CoA as shown by Hashimoto *et al.* (13).

As shown in Table 1, EC synthesis in microsomal fractions from cells grown in HRS was 2.5 times greater than in control material obtained from cells grown in NRS. In addition, the FC concentration of the microsomal fraction isolated from cells grown in HRS was elevated when compared to similar material obtained from cells grown in normolipemic sera. The stimulation of EC synthesis was directly related to the concentration of HRS added to the growth medium, with maximum stimulation obtained at concentrations above 1.0% serum in the growth medium (data not shown).

Effect of preincubation of microsomes with hyperlipemic VLDL (H-VLDL). Because of the known stimulatory effect of H-VLDL on FC esterification in whole cells (1, 2) the time course of the utilization of [^{14}C]palmityl-CoA by untreated control microsomes and microsomes preincubated with H-VLDL was quantitated. Figure 1 shows the utilization of 100 and 200 nmole of [^{14}C]palmityl-CoA/1 mg of microsomal protein. Preincubation with H-VLDL stim-

TABLE 1. UNESTERIFIED CHOLESTEROL CONTENT AND ACAT ACTIVITY OF MICROSOMAL FRACTIONS FROM FU5AH CELLS GROWN IN HYPERLIPEMIC AND NORMAL RABBIT SERA

	Serum[a]	
	NRS	HRS
Cholesteryl palmitate[b] (nmole/mg of protein)	2.00 ± 0.18 (16)[c]	4.50 ± 0.35 (18)
Unesterified cholesterol (μg/mg of protein)	52.1 ± 2.6 (8)	73.5 ± 2.4 (10)

[a] Cells grown in medium supplemented with 2.5% normal rabbit serum (NRS) or hyperlipemic rabbit serum (HRS) for 24 hr prior to assay.

[b] Thirty-minute assay with 100 nmole of [^{14}C]palmityl-CoA/1 mg of microsomal protein.

[c] Values given as nanomoles ± SE. Value within parentheses is number of determinations.

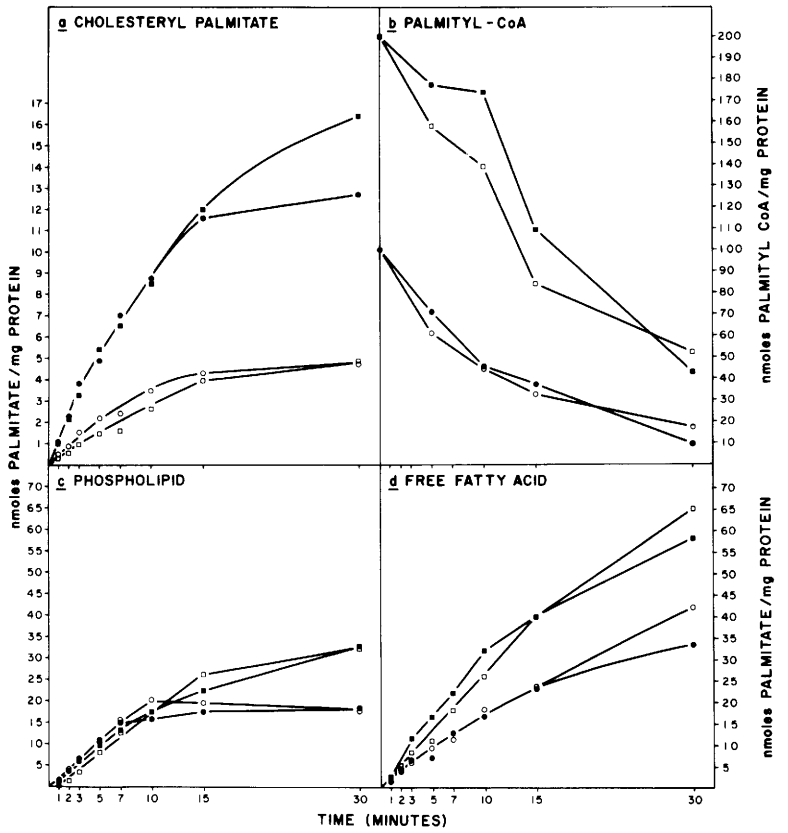

FIG. 1. Utilization of palmityl-CoA by microsomal preparations from Fu5AH cells. $S_{12,000}$ fraction preincubated either with or without H-VLDL prior to isolation of microsomal fraction: (●——●) 100 nmole of palmityl-CoA/1 mg of protein, preincubated with H-VLDL; (■——■) 200 nmole of palmityl-CoA/1 mg of protein, preincubated with H-VLDL; (○——○) 100 nmole of palmityl-CoA/1 mg of protein, no VLDL treatment; (□——□) 200 nmole of palmityl-CoA/1 mg of protein, no VLDL treatment. Panel a: incorporation into cholesteryl palmitate; panel b: utilization of palmityl CoA; panel c: incorporation into phospholipids; panel d: recovery as palmitic acid.

ulated [^{14}C]palmitate incorporation into EC (Fig. 1a) at all time points. EC synthesis in untreated microsomal fractions was similar at both substrate concentrations during the entire 30-min incubation period. Esterification in H-VLDL-treated microsomal fractions was similar at both concentrations of substrate during the first 15 min of incubation. Between 15 and 30 min only a small increase in labeled EC was observed at 100 nmole of palmityl-CoA/1 mg of microsomal protein, in contrast to a continued increase at 200 nmole. Exposure of microsomes to H-VLDL had no significant effect on palmitate incorporation into phospholipid (PL) (Fig. 1c), deacylation of palmityl-CoA to free fatty acids (FFA) (Fig. 1d), or the overall utilization of labeled substrate as measured by the amount of unreacted palmityl-CoA (Fig. 1b). Increased substrate concentration resulted in increased palmitate recovery as PL and FFA at the later time points. The stimulatory effect of H-VLDL was dependent on the amount of H-VLDL added to the $S_{12,000}$ fraction. Maximum stimulation was achieved at H-VLDL concentrations above 0.5 mg of lipoprotein FC/4 mg of $S_{12,000}$ protein (data not shown).

An increase in ACAT activity could be elicited by adding H-VLDL directly to isolated microsomal fractions (Table 2, Nos. 1 and 2), indicating that no factor in the $S_{12,000}$ was essential for ACAT stimulation. Washing the treated microsomal preparations by

recentrifugation after incubation with VLDL had no significant effect on ACAT activity. Table 2 also shows the results from a series of experiments in which normal microsomes from Fu5AH cells (as $S_{12,000}$) were preincubated at 0° (No. 3), 37° (No. 4), and at 37° in the presence of VLDL from either normal (N-VLDL, No. 5) or hyperlipemic (H-VLDL, No. 6) rabbit sera. In the absence of lipoprotein, preincubation at 37° stimulated activity 1.8-fold over that at 0°. The addition of N-VLDL to the preincubation mixture had no significant effect on EC synthesis, whereas H-VLDL, added at similar FC concentrations, elicited a 2.8-fold increase over the control preincubated microsomal fraction. Exposure of microsomal preparations to other lipoproteins isolated from HRS also resulted in increased incorporation of palmitate into EC (Table 3).

ACAT activity in Fu5-5 microsomal fractions. In a previous comparative study it was observed that the Fu5-5 rat hepatoma, a cell line closely related to Fu5AH, exhibited elevated EC content and FC esterification when grown in HRS (2), although the level of each was lower than that seen with Fu5AH. As with Fu5AH, growth of Fu5-5 cells in 2.5% HRS stimulated EC synthesis; 1.85 ± 0.12 of as opposed to 0.96 ± .06 nmole/mg of protein/30 min for NRS. Table 2 (Nos. 4 and 6) demonstrates that increased EC synthesis was obtained with the Fu5-5 microsomes pretreated with H-VLDL, with the amount of cholesteryl palmitate synthesized being less than that seen with equivalent material obtained from Fu5AH cells.

Microsomal cholesterol content. Table 4 shows the FC content of the microsomal fraction obtained from FBS-grown cells after preincubation in the presence of either N-VLDL or H-VLDL. Exposure of the microsomal preparation to normal lipoprotein had no effect on unesterified cholesterol content, whereas incubation in the presence of H-VLDL produced a dose-dependent net increase in microsomal FC content.

Discussion. The accumulation of EC in any cell type might be due to increased esterification of FC, to decreased cellular hydrolysis of incorporated exogenous EC, or to a combination of both processes. Evidence has been obtained demonstrating re-

TABLE 2. MICROSOMAL CHOLESTERYL PALMITATE SYNTHESIS IN Fu5 CELLS[a]

No.	Material	Preincubation conditions	Cholesteryl Palmitate (nmole/mg of microsomal protein)	
			Fu5AH cells	Fu5-5 cells
1	Microsomal fraction	− VLDL	5.31 ± 0.48 (9)	
2	Microsomal fraction	+ H-VLDL	11.76 ± 1.11 (10)	
3	$S_{12,000}$	− VLDL, 0°	2.75 ± 0.14 (5)	
4	$S_{12,000}$	− VLDL	5.01 ± 0.25 (20)	0.96 ± 0.06 (6)
5	$S_{12,000}$	+ N-VLDL	5.83 ± 0.40 (7)	
6	$S_{12,000}$	+ H-VLDL	11.36 ± 0.72 (20)	2.12 ± 0.19 (6)

[a] N- and H-VLDL added at 1 mg of lipoprotein FC/4 mg of $S_{12,000}$ protein, followed by isolation of the microsomal fraction (Nos. 3–6); or 1 mg of lipoprotein FC/1 mg of microsomal fraction, followed by reisolation of microsomes (Nos. 1 and 2). Preincubations were at 37° (No. 3: 0°) for 2 hr. Thirty-minute assay with 100 nmole of [^{14}C]palmityl-CoA/1 mg of microsomal protein. Values given as nanomoles ± SE, with number of determinations indicated in parentheses.

TABLE 3. EFFECT OF HYPERLIPEMIC RABBIT LIPOPROTEINS ON HEPATOMA ACAT ACTIVITY[a]

Lipoprotein	No lipoprotein (control)	VLDL	LDL[b]	HDL[c]
Cholesterol palmitate (nmole/mg of microsomal protein/30 min)	5.13	11.92	9.08	7.81
Stimulation (treated/control)		2.3	1.8	1.5

[a] Individual lipoproteins preincubated 2 hr with $S_{12,000}$ from Fu5AH hepatoma cells prior to isolation of microsomal fraction. All lipoproteins added at 1 mg of lipoprotein FC/4 mg of $S_{12,000}$ protein. Substrate concentration = 100 nmole of [^{14}C]palmityl-CoA/1 mg of microsomal protein.

[b] LDL, Low-density lipoproteins.

[c] HDL, High-density lipoproteins.

TABLE 4. UNESTERIFIED CHOLESTEROL CONTENT OF MICROSOMAL FRACTIONS FROM Fu5AH

Microsomal fraction (preincubation)[a]	Lipoprotein concentration[b]	Microsomal FC content (μg/mg of microsomal protein)[c]
Control	0	42.2
H-VLDL	0.2	55.6
	0.5	58.6
	0.8	63.0
	1.0	75.9
N-VLDL	0.2	41.3
	0.8	41.6

[a] Lipoproteins preincubated for 2 hr with $S_{12,000}$ prior to isolation of microsomal fraction.

[b] Milligrams of lipoprotein FC per 4 mg of $S_{12,000}$ proteins.

[c] Average of at least two determinations.

duced hydrolysis of EC in lipid-laden lysosomes from rabbit aortic cells (14) and in fibroblasts derived from patients with cholesteryl ester storage disease (15), suggesting that EC accumulation, at least in part, is due to a decrease in cellular hydrolysis. Other studies have demonstrated an increased EC content in atherosclerotic aorta directly correlated with increased ACAT activity (3–7). Our previous studies have shown that Fu5AH and Fu5-5 hepatoma cells accumulate EC when exposed to HRS. These accumulated esters are stored in anisotropic lipid inclusions similar to those isolated from atherosclerotic human aorta (16). Quantitative determinations of the source of these accumulated esters in Fu5AH cells indicate that at least 40% of the cellular EC is derived from the esterification of FC, and that esterification of FC is markedly increased when these cells are exposed to HRS (2).

The data from the present experiments indicate that both the microsomal fractions isolated from Fu5AH rat hepatoma cells grown on HRS and the microsomal fractions preincubated *in vitro* with hyperlipemic lipoproteins synthesize more cholesteryl palmitate than do untreated controls. The results obtained with isolated microsomal preparations closely parallel the increased incorporation and esterification of serum lipoprotein FC shown by cells cultured in HRS (2). The higher ACAT activity of treated and untreated microsomes from Fu5AH cells, as compared to that from the closely related Fu5-5 hepatoma cells, was also of the same order of magnitude as previously observed with whole cells (2).

Although an increase in cholesterol esterification has been demonstrated in atherosclerotic aortas (4–7, 17) and in cultures of fibroblasts exposed to LDL and oxygenated sterols (18, 19), the mechanism responsible for increased EC synthesis has not been elucidated. A number of mechanisms could be proposed to explain the apparent increase in ACAT activity in hepatoma cells grown in HRS. The ability of HRS to stimulate EC accumulation and FC esterification in cells in which protein synthesis has been blocked by treatment with cycloheximide (1, 2), together with the stimulation of EC synthesis in isolated microsomal fractions incubated with H-VLDL, indicates that increased enzyme activity is not due to increased enzyme synthesis. The data suggest that increased EC synthesis in both whole cells and isolated microsomes is linked to greater substrate availablility. The present experiments use endogenous FC as the acyl acceptor, and Tables 1 and 4 show that growth of cells in HRS or preincubation of a microsomal fraction with H-VLDL, but not N-VLDL, can increase the microsomal FC content. The reduced synthesis of EC in untreated microsomes, where the acyl donor is not limiting, could be due to the reduced availability of FC. This interpretation is consistent with the recent observation of Nilsson (20) that stimulated synthesis of cholesterol in hepatocytes was correlated with increased esterification.

Nervi *et al.* (21) has recently reported that, in rats, net cholesterol uptake from circulating intestinal lipoproteins occurs only in the liver. It was suggested that the specificity of uptake by the liver, as opposed to peripheral tissues, could reside either in the presence of specific lipoprotein recognition sites on hepatocyte cell membranes or in the greater ability of the hepatocyte to esterify cholesterol. Data obtained from our studies on hepatoma cells indicate that cholesterol accumulation in the hepatoma is a function both of the ability of a serum lipoprotein to deliver cholesterol to cells and to the level of cellular ACAT activity. The enhanced uptake of cholesterol from lipo-

proteins derived from hyperlipemic as opposed to normal sera (1, 2) could occur in cells by either endocytosis of the whole lipoprotein or by transfer of cholesterol at the cell surface. The greater availability of FC in cells exposed to HRS or, as in our recent studies FC/lecithin dispersions with molar ratios greater than one (22), could result in greater substrate availability and enhanced esterification. The present observations that the FC content of isolated microsomal fractions is increased by incubation with H-VLDL, but not N-VLDL, demonstrates that H-VLDL can more readily transfer FC to these membranes and suggests that a similar phenomenon could occur when cellular plasma membranes are exposed to hyperlipemic lipoproteins. The differences in ACAT activity in similar microsomal preparations from Fu5-5 and Fu5AH cells correlate well with differences in esterification of FC and accumulation of EC observed in whole cells. Although exposure to HRS results in a stimulation of FC esterification in both cell types, the enhanced ability of Fu5AH to accumulate EC may be a reflection of the greater ACAT activity of the Fu5AH cells.

Summary. Cholesteryl ester synthesis by the microsomal fraction from rat hepatoma cells was stimulated by preincubation of the microsomal fraction with lipoproteins obtained from hyperlipemic rabbit sera. Lipoproteins from normolipemic sera had no stimulatory effect. Growth of cells in hyperlipemic rabbit sera also increased microsomal cholesteryl ester synthesis. Increased cholesteryl ester synthesis is accompanied by an increased microsomal-free cholesterol content.

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