Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during simulation of intracellular cholesterol esterification

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Abstract Very low density lipoproteins, newly secreted by cultured rat hepatocytes into a serum-free medium, contain some cholesteryl esters although the percentage of total cholesterol in ester form is less than that in plasma very low density lipoproteins. When acyl CoA:cholesterol acyltransferase activity in hepatocytes was stimulated by the addition of 25-hydroxycholesterol (10 μg/ml) or mevalonolactone (1 mM), the absolute amount of esterified cholesterol secreted in very low density lipoproteins increased significantly, but the amount of free cholesterol decreased or showed no change. Thus the percentage of very low density lipoprotein cholesterol in ester form increased, in some experiments to as much as 50% of the total. These results provide additional evidence that hepatic acyl CoA:cholesterol acyltransferase plays a role in the generation of some of the cholesteryl ester in newly-secreted lipoproteins. They further suggest that changes in the activity of the enzyme can potentially regulate the fraction of cholesterol secreted in esterified form.—**Drevon, C. A., S. C. Engelhorn, and D. Steinberg.** Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during stimulation of intracellular cholesterol esterification. *J. Lipid Res.* 1980. **21:** 1065–1071.

Supplementary key words acylCoA:cholesterol acyltransferase · lecithin:cholesterol acyltransferase · 25-hydroxycholesterol · mevalonolactone

Much of the cholesteryl ester moiety in plasma lipoprotein fractions is probably formed within the plasma through the action of lecithin:cholesterol acyltransferase (LCAT) (1). In patients with an inherited LCAT deficiency, only 5–17% of total plasma cholesterol is present as cholesteryl ester (1) compared to about 75% in normal subjects. Almost all the cholesteryl ester in the plasma of these patients can be recovered in the very low density lipoprotein (VLDL) fraction (2). Since there is no measurable LCAT activity in the plasma of these patients, there must be alternative sources of this plasma VLDL cholesteryl ester, including secretion by liver and/or intestine of VLDL (or VLDL precursors) already containing cholesteryl esters. It has been previously demonstrated that VLDL isolated from the Golgi apparatus in rat liver contains some cholesteryl ester (3), and that nascent lipoproteins secreted by perfused rat liver (4–7) and cultured rat hepatocytes (8) also contain a significant amount of cholesteryl ester. When guinea pigs (9–11) or rats (12) are given dietary supplements of cholesterol, there is an increase in acylCoA:cholesterol acyltransferase (ACAT) activity in microsomes of intestinal mucosal cells and an increase in the amount of cholesteryl ester in the triglyceride-rich lipoproteins of intestinal lymph. Since LCAT activity in intestinal lymph is very low (10, 13), these data suggest that intracellular ACAT may play a role in the production of cholesteryl esters for incorporation into intestinal VLDL prior to its secretion. ACAT activity in cultured rat hepatocytes can be increased by incubation with 25-hydroxycholesterol or with high concentrations of mevalonolactone (14). We present data in this report showing that the rate of secretion of newly-synthesized ester cholesterol into VLDL is increased when ACAT activity is increased in cultured hepatocytes and that the net cholesteryl ester content of nascent VLDL is also increased.

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; ACAT, acylCoA:cholesterol acyltransferase; LCAT, lecithin:cholesterol acyltransferase; FCS, fetal calf serum; DME, Dulbecco's modified Eagle's medium.

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MATERIALS AND METHODS

Chemicals

DL-[2-^{14}C]Mevalonolactone (sp act 27.3 mCi/mmol) was obtained from New England Nuclear Corp., Boston, MA and [2-^{3}H]glycerol (sp act 5 Ci/mmol) from Amersham, Arlington Heights, IL. Contur plastic petri dishes, 60 or 100 mm diameter, were purchased from Lux Inc., Newberg Park, CA. Routinely used chemicals and collagenase (type 1) were obtained from Sigma Chemical Co., St. Louis, MO. β-Sitosterol and 25-hydroxycholesterol were from Steraloids, Wilton, NH and their purity was checked by thin-layer chromatography and gas–liquid chromatography. β-Sitosterol and 25-hydroxycholesterol were found to be ≧99 and 98% pure, respectively. Fetal calf serum (FCS, Irvine Scientific Sales Co., Fountain Valley, CA) was heat-inactivated at 56°C for 30 min before use. Penicillin G, streptomycin sulfate, and Dulbecco's phosphate-buffered saline (15) were obtained from Grand Island Biological Co., Berkeley, CA.

Perfusion and incubation media

Rat livers were perfused with a modified calcium-free Hanks solution (16) as described earlier (8). An arginine-free Dulbecco's modified Eagle's medium (DME) (8), supplemented with glucose (40 mM), ornithine (0.4 mM), and 10 mM HEPES buffer from Grand Island Biological Co., Santa Clara, CA was used as the standard incubation medium.

Isolation and cell culture of hepatocytes

The isolation of rat heptocytes was based upon the method described by Berry and Friend (17), except that sterile conditions were maintained throughout. The detailed procedures for cell culture and for isolation of medium VLDL have been described previously (8). The cells were plated in 60- or 100-mm Contur dishes at 3×10^6 and 9×10^6 cells per dish, respectively. The amount of medium (arginine-free DME medium containing FCS (20%), insulin (10 μg/ml), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml)) was 3 ml for 60-mm and 9 ml for 100-mm dishes, and the cells were maintained at 39°C in a 95% air–5% CO_2 atmosphere. The cells were incubated for 5 to 24 hr under these conditions before changing to the standard medium without FCS for measurement of lipoprotein secretion over the following 5 to 20 hr.

Measurement of intracellular cholesterol esterification and VLDL secretion

Cholesterol esterification in intact hepatocytes was measured by adding [2-^{14}C]mevalonolactone dissolved in ethanol to the culture medium; the final ethanol concentration both in control and experimental dishes was 0.5%. The medium and the cells were harvested at the indicated time points. The cells were washed with PBS, centrifuged at 1,000 *g* for 3 min and the buffer was decanted. The cells were resuspended in 500 μl of distilled water and vigorously agitated on a vortex mixer to break the cells and form a homogeneous suspension. A sample for protein determination was taken and the remainder was extracted with chloroform–methanol 2:1 (v/v), as described by Folch, Lees, and Sloane Stanley (18). Cholesteryl esters, triglycerides, free cholesterol, and phospholipids were separated using silica gel thin-layer chromatography plates, developed in hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) and scraped into counting vials containing a toluene-based liquid scintillation fluid as previously described (8).

The medium was centrifuged at d 1.006 g/ml to isolate VLDL secreted by the liver cells (8, 19). The top fraction was then extracted with chloroform–methanol as described above, and the major lipid classes were quantified as described below. The recovery of previously prepared rat plasma VLDL by centrifugation was about 90% and the recovery of labeled cholesterol and triglycerides through the TLC system was also 90%.

Chemical analysis

Protein was measured according to Lowry et al. (20). Free cholesterol and ester cholesterol were determined by gas–liquid chromatography using β-sitosterol as an internal standard. A Varian Aerograph, series 2100, equipped with a 6 ft, 2 mm I.D. column, packed with 3% SP 2250 on 80/100 mesh Supelcoport (Supelco) was used at 295°C with argon (30 ml/min) as carrier gas. Sterols, triglycerides, and phospholipids were also measured using a charring method as described by Marsh and Weinstein (21).

RESULTS

It has been shown previously that when rat hepatocytes are incubated with [^{3}H]glycerol, more than 95% of the labeled triglycerides secreted are recovered in the d < 1.006 g/ml fraction and that ^{3}H-labeled triglyceride secretion is linear as a function of time for 3–5 hr (8). Thus the rate of VLDL secretion can be closely approximated from the total ^{3}H-labeled triglyceride appearing in the medium over 5 hr. As shown in **Table 1**, addition of 25-hydroxycholesterol to the medium had little or no effect on

TABLE 1. Effects of 25-hydroxycholesterol on incorporation of [^{3}H]glycerol and of [^{14}C]mevalonolactone into medium and cell lipids during a 5-hr incubation with cultured rat hepatocytes

	Total Radioactivity Incorporated (dpm/mg cell protein)			
	Cell Lipids		Medium Lipids	
Additions to Basic Medium	Triglycerides [^{3}H] × 10^{-3}	Cholesteryl Esters [^{14}C]	Triglycerides [^{3}H] × 10^{-3}	Cholesteryl Esters [^{14}C]
None	281 ± 19	983 ± 136	25.8 ± 0.9	62 ± 1
25-Hydroxy-cholesterol	353 ± 13	1600 ± 110	30.6 ± 3.6	157 ± 16

Rat hepatocytes were cultured for 24 hr in 3 ml of DME containing heat-inactivated FCS (20%), insulin (10 μg/ml), streptomycin sulfate (100 μg/ml), and penicillin G (100 units/ml) in 60-mm dishes. Then fresh serum-free medium was added containing, in addition, 25 μCi [2-^{3}H]glycerol per dish (2.1×10^4 dpm/pmol) and 0.2 μCi [2-^{14}C]mevalonolactone per dish (60 dpm/pmol) dissolved in ethanol (0.5% final concentration) with or without 25-hydroxycholesterol (10 μg/ml). Cells were harvested after 5 hr incubation at 39°C. Medium and all lipids were extracted and fractionated as described under Methods. Data represent the mean ± SD for three dishes.

the amount of [^{3}H]glycerol appearing in medium triglycerides, implying that there was little or no increase in overall VLDL secretion rate. The incorporation of [^{14}C]mevalonolactone into cholesteryl esters in the medium, however, was markedly stimulated by 25-hydroxycholesterol (+150%). Incorporation into cellular cholesteryl esters was also increased but to a lesser degree (+60%), suggesting that net cholesteryl ester secretion into the medium might be increased by 25-hydroxycholesterol. Incorporation of [^{3}H]glycerol into cell triglycerides was slightly increased in this experiment but in a larger series of similar studies 25-hydroxycholesterol did not significantly affect triglyceride synthesis (14).

In the experiment summarized in **Table 2**, newly-secreted VLDL (d < 1.006 g/ml) was separated from denser lipoproteins at the end of the incubation with [^{14}C]mevalonolactone. Again the addition of 25-hydroxycholesterol increased incorporation into hepatocyte cholesteryl esters (+165%) but the effect on incorporation into VLDL cholesteryl esters was much greater (+680%). Incorporation into cholesteryl esters in the d > 1.006 g/ml fraction was increased to an intermediate degree. In all fractions the recovery of radioactivity in free cholesterol was decreased, compatible with increased cellular ACAT activity. In three experiments under the conditions described in Table 2, net VLDL triglyceride secretion measured in the absence and in the presence of 25-hydroxycholesterol (10 μg/ml) was unchanged (11.4 versus 10.7, 7.3 versus 7.4, and 8.0 versus 10.0 μg/mg cell protein).

While these isotopic studies suggested *both* an increase in cholesteryl ester synthesis in the cells *and* an increase in the amount of cholesteryl ester secreted in VLDL, the latter conclusion required additional supporting evidence. The pools of cholesteryl ester in the hepatocyte may be heterogeneous with respect to specific radioactivity and the specific radioactivity of the precursor pool delivering cholesteryl ester to nascent VLDL is not known. Therefore net measurements of VLDL free cholesterol and ester cholesterol were made after incubation in the absence or in the presence of 25-hydroxycholesterol. As shown in **Table 3**, incubation with 25-hydroxycholesterol caused a highly significant increase in the total cholesterol content of nascent VLDL and all of the increase was accounted for by the increase in the ester cholesterol fraction, which more than doubled. The

TABLE 2. Effects of 25-hydroxycholesterol on incorporation of [^{14}C]mevalonolactone into free and esterified cholesterol in newly-secreted VLDL (d < 1.006 g/ml), in the fraction of d > 1.006 g/ml, and in intracellular lipids

	Cell Lipids		Medium Lipids in d < 1.006 Fraction		Medium Lipids in d > 1.006 Fraction	
Additions to basic medium	Free Cholesterol	Cholesteryl Esters	Free Cholesterol	Cholesteryl Esters	Free Cholesterol	Cholesteryl Esters
	(dpm/mg protein/hr)					
None	7,740	1,380	39.6	24.0	44.4	4.2
25-Hydroxy-cholesterol	3,660	3,660	29.4	187.2	16.2	18.6

After preincubation as described in the footnote to Table 1, [2-^{14}C]mevalonolactone (0.1 μCi/ml; sp act, 60 dpm/pmol) was added and cells were incubated for 3 hr in the presence or absence of 25-hydroxycholesterol, 10 μg/ml. Fresh medium of the same composition was added and incubation was continued for an additional 8 hr. The medium was harvested and centrifuged at 3,000 *g* to remove cell debris. The supernatant fraction was centrifuged at d 1.006 g/ml for 18 hr at 4°C (100,000 *g*). The lipids were extracted from the top (d < 1.006 g/ml) and bottom (d > 1.006 g/ml) and from the harvested cells as described under Methods. Free cholesterol and cholesteryl esters were isolated by thin-layer chromatography for radioassay. Medium from six plates was pooled and divided into duplicate samples for fractionation and analysis; cell data represent mean values for three individual dishes.

TABLE 3. Effect of 25-hydroxycholesterol on cholesterol content of nascent VLDL secreted by cultured rat hepatocytes

Additions	Total Cholesterol	Free Cholesterol	Ester Cholesterol	Percentage of Cholesterol in Ester Form
		(μg cholesterol/mg cell protein)		
None	0.86 ± 0.04	0.63 ± 0.05	0.23 ± 0.07	26.7%
25-Hydroxy-cholesterol	1.17 ± 0.05	0.60 ± 0.03	0.57 ± 0.03	48.7%

The hepatocytes were plated in DME medium containing 20% FCS. After 5 hr incubation the medium was changed to serum-free DME medium with or without 25-hydroxycholesterol (10 μg/ml) and incubation was continued for 1 hr. The VLDL secreted during this first hour would probably show less than the maximal effect of the 25-hydroxycholesterol since a significant amount of preformed VLDL is secreted during the initial hour. Therefore, the medium harvested at 1 hr was discarded. After another 5 hr incubation at 39°C, the medium was harvested and centrifuged at d 1.006 g/ml at 4°C for 18 hr to isolate VLDL. The mass of free and total cholesterol was determined by gas–liquid chromatography using β-sitosterol as an internal standard. The data represent means ± S.D. of 12 determinations.

percentage of total cholesterol present in ester form increased from 26.7% to 48.7%.

High concentrations of mevalonolactone in the medium also increase hepatocyte ACAT activity (14). Measurements of net free and ester cholesterol secretion in VLDL were made in the presence and in the absence of 1 mM mevalonolactone (**Table 4**). Incubation was extended to 20 hr to increase the yield of VLDL for mass measurements. In each of three experiments, incubation with mevalonolactone increased the net amount of VLDL ester cholesterol secreted. There was a concomitant decrease in free cholesterol content, resulting in a marked increase in the percentage of total cholesterol in ester form. However, there was no consistent change in total VLDL cholesterol.

It was necessary to consider the possibility that some of the cholesteryl ester in newly-secreted VLDL might be attributable to the action of lecithin: cholesterol acyltransferase (LCAT). Hepatocytes were incubated for 20 hr in serum-free DME medium and the medium from 32 plates was combined. This was divided into two equal aliquots. From one aliquot, the VLDL fraction was isolated and analyzed for cholesteryl ester content. The second aliquot of harvested medium was distributed into 16 empty culture dishes and incubated for an additional 20 hr in the absence of cells and then VLDL was isolated for

TABLE 4. Effects of mevalonolactone on cholesterol content of nascent VLDL secreted by cultured rat hepatocytes

	Total Cholesterol	Free Cholesterol	Ester Cholesterol	Percentage of Cholesterol in Ester Form
		(μg cholesterol/mg cell protein)		
Experiment 1				
Control	3.05 ± 0.08	2.49 ± 0.12	0.55 ± 0.11	18%
Mevalonolactone	2.53 ± 0.12	1.81 ± 0.07	0.72 ± 0.08	28.5%
Experiment 2				
Control	4.28 ± 0.39	2.92 ± 0.11	1.36 ± 0.19	31.8%
Mevalonolactone	4.59 ± 0.30	2.40 ± 0.07	2.19 ± 0.28	47.7%
Experiment 3				
Control	5.27 ± 0.15	3.00 ± 0.04	2.23 ± 0.19	42.3%
Mevalonolactone	5.30 ± 0.25	2.51 ± 0.06	2.95 ± 0.15	55.7%

Hepatocytes were plated in DME containing 20% FCS as described in the Method section. After 5 hr incubation the medium was changed to serum-free DME with or without mevalonolactone (1 mM) and incubated for 20 hr at 39°C. The medium was harvested and centrifuged d 1.006 g/ml at 4°C for 18 hr to isolate VLDL. The mass of free and total cholesterol was determined by gas-liquid chromatography using β-sitosterol as an internal standard. The data represent means ± S.D. of 3–6 determinations.

analysis. The amount of VLDL ester cholesterol secreted during the first 20-hr incubation was 1.80 ± 0.12 μg/mg of cell protein. The ester cholesterol in the VLDL isolated from the medium incubated an additional 20 hr without cells corresponded to 2.04 ± 0.44 μg/mg cell protein. Thus, the increment in VLDL ester cholesterol over the second 20-hr interval was 0.24 μg/mg cell protein, an increase of only 13%. Thus, it appears that the rate of ester cholesterol formation by action of LCAT on secreted lipoproteins can account for only a small fraction at most of the total ester cholesterol isolated.

As a further test of the possible significance of secreted LCAT, hepatocytes were incubated for 24 hr in serum-free DME medium alone or in medium containing 25-hydroxycholesterol (10 μg/ml) or DL-mevalonolactone (1 mM). At the end of the incubation, the activity of LCAT in the medium was assayed by the method of Glomset and Wright (22). The LCAT activity recovered in the medium was less in the presence of 25-hydroxycholesterol or mevalonolactone than in the control dishes, showing that the increase in VLDL ester cholesterol induced by these substances cannot be attributed to a greater rate of secretion of LCAT in their presence.

DISCUSSION

The data presented in this report demonstrate that incubation of rat hepatocytes in the presence of 25-hydroxycholesterol or mevalonolactone increases the fraction of newly-secreted VLDL cholesterol present in ester form. Both 25-hydroxycholesterol and mevalonolactone have previously been shown to increase ACAT activity by rat hepatocytes (14, 23). Thus, the simplest interpretation of the results is that changes in ACAT activity can potentially regulate the cholesteryl ester content of newly-secreted VLDL. It has been shown previously that cholesterol feeding increases ACAT activity in the liver (24, 25) and that the cholesteryl ester content of VLDL secreted by perfused livers from cholesterol-fed rats can be markedly increased (7). It has been proposed on the basis of kinetic evidence that esterification of cholesterol within the liver may be responsible for most of the cholesteryl esters found in the plasma of cholesterol-fed rabbits (26). In livers of cholesterol-fed animals, the lipid content, and particularly the cholesteryl ester content, is enormously increased and these stores of preformed esters might be responsible for the higher ester cholesterol content of secreted lipoprotein. The results of the present studies, particularly those experiments lasting only 5 hr, suggest that an increase in the activity of ACAT, without much change in net liver cholesteryl ester content, is sufficient to influence the cholesteryl ester content of nascent VLDL.

In the present studies hepatocytes were incubated in the absence of any added serum or serum lipoproteins. This does not in itself necessarily rule out the possibility that some of the ester cholesterol found in VLDL might have arisen from the action of lecithin:cholesterol acyltransferase (LCAT) activity secreted from the hepatocytes into the medium and acting on lipoproteins also secreted by the hepatocytes into the medium. Several considerations made it a priori unlikely that this could account for much of the VLDL cholesteryl ester. Marsh (5) used single-pass perfusion through the rat liver and chilled the perfusate immediately to prevent LCAT activity. The ester cholesterol in the nascent VLDL accounted for 23% of the total. In view of the range of values reported, the percentage may have been as high as 44% in some perfusions. Hamilton et al. (6) analyzed VLDL secreted by rat livers perfused with a serum-free medium and found that the fraction of ester cholesterol in VLDL was not altered by the addition of an inhibitor of LCAT. They found that approximately 30% of the cholesterol was present as ester with or without the addition of 5,5′-dithionitrobenzoic acid. In the present studies, the percentage of cholesterol present in ester form was 26.7 in 5-hr incubations and ranged from 18–42 in 20-hr incubations. Davis et al. (8) determined the ester cholesterol content of VLDL accumulating during the first 24 hr of hepatocyte culture and found the value to be 13%; when the VLDL was allowed to accumulate over 3 days of incubation, which should have allowed maximal opportunity for LCAT activity to convert free cholesterol to ester, only 23% of the cholesterol was present in ester form. The possible contribution of secreted LCAT was tested directly and, as reported above, it was shown that cholesterol esterifying activity in the medium would account for less than 15% of the total ester cholesterol recovered and that neither 25-hydroxycholesterol nor mevalonolactone increased the amount of LCAT in the medium. Thus the stimulation of ester cholesterol secretion in VLDL observed cannot be attributed to esterification of free cholesterol after secretion into the medium.

The percentage of total cholesterol present in ester form in nascent VLDL was not as high as that found in rat plasma VLDL. Presumably a significant fraction of the cholesteryl ester present at steady state in vivo derives from activity of LCAT in the plasma compartment (1). In man it is generally accepted that most of the plasma cholesteryl esters are formed by

the action of LCAT. Indeed, it has been reported that human liver contains no measurable ACAT activity (27). However, it has been recently demonstrated by Balasubramaniam et al. (28), and by studies in this laboratory,[2] that there is significant ACAT activity in human liver. Moreover, the presence of a significant amount of cholesteryl ester in the plasma VLDL of patients with total LCAT deficiency implies that either hepatic or intestinal ACAT must be involved in formation of at least some of the cholesteryl esters in plasma.

The sequence of events by which free cholesterol and ester cholesterol become incorporated into nascent VLDL is not known. In the present studies it appeared that stimulation of ACAT increased the percentage of VLDL cholesterol in ester form but did not change the total cholesterol content significantly (Table 4). In other words, it appeared that a certain amount of cholesterol was targeted for incorporation into VLDL and that stimulation of ACAT simply resulted in conversion of some of that cholesterol from the free to the esterified form. The VLDL secreted by livers of cholesterol-fed rats shows a marked increase in ester cholesterol content and a concomitant decrease in VLDL triglyceride content (7), implying a replacement of triglycerides by cholesteryl esters in the apolar lipid core of the lipoproteins. However, in the present studies, 25-hydroxycholesterol increased the ester cholesterol in VLDL without significantly affecting VLDL triglyceride content. It will be of interest to study the size and structure of these ester cholesterol-rich VLDL.

The investigation was supported by National Institutes of Health Research Grant HL 14197, awarded by the National Heart, Lung, and Blood Institute, PHS/DHHS.

Manuscript received 13 February 1980, in revised form 12 May 1980, and in re-revised form 7 July 1980.

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