Regulation of cholesterol biosynthesis and esterification by 25-hydroxycholesterol in a macrophage-like cell line: uncoupling by progesterone

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Abstract The coordinated control of cholesterol biosynthesis and esterification by 25-hydroxycholesterol was studied in the macrophage-like cell line P388D$_1$. Since 25-hydroxycholesterol rapidly stimulated incorporation of [^{3}H]oleate into the cholesteryl ester fraction of these cells, we have tested the possibility that the well-known inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) by 25-hydroxycholesterol might be the indirect consequence of an increased cholesterol esterification rather than a direct effect on HMG-CoA reductase. The experimental results show that progesterone, an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), when added together with 25-hydroxycholesterol, abolished the increased cholesterol esterification without affecting the inhibition of HMG-CoA reductase by 25-hydroxycholesterol. Thus, uncoupling cholesterol esterification had no effect on 25-hydroxycholesterol's ability to inhibit HMG-CoA reductase. Unexpectedly, pretreatment of P388D$_1$ cells with 25-hydroxycholesterol resulted in no elevation of ACAT activity as measured in broken cell preparations. Therefore, the possibility that 25-hydroxycholesterol stimulated cholesteryl ester formation by increasing the amount of cholesterol available for esterification, rather than by acting directly on ACAT activity, was considered. Labeling experiments using [^{14}C]-cholesterol have provided evidence for this assumption.—**Miller, S. C., and G. Melnykovych.** Regulation of cholesterol biosynthesis and esterification by 25-hydroxycholesterol in a macrophage-like cell line: uncoupling by progesterone. *J. Lipid Res.* 1984. **25:** 991–999.

Supplementary key words HMG-CoA reductase • acyl-CoA:cholesterol acyltransferase • cholesteryl ester

Kandutsch, Chen, and Heiniger (1) originally advanced the hypothesis that oxygenated sterols rather than cholesterol regulate cholesterol biosynthesis. Data from a number of laboratories (2–6) have provided evidence, both biochemical and genetic, supporting this hypothesis. Most investigators examining regulation of cholesterol metabolism by oxygenated sterols have used 25-hydroxycholesterol because of its ready availability and high potency. Several studies have shown reciprocal regulation of HMG-CoA reductase and ACAT by 25-hydroxycholesterol (for review see 7), which suggests that there may be some common mediator, perhaps free cholesterol, acting on both enzymes in a reciprocal manner. It is of interest that in studies in microsomes where both HMG-CoA reductase and ACAT activities were measured, an increase in the free cholesterol concentration of microsomes resulted in a decreased HMG-CoA reductase activity and an increase in ACAT activity (8). However, recently published results by van Heusden and Wirtz (9) are in disagreement with this work. In spite of apparently identical experimental conditions, they could not show a decrease in HMG-CoA reductase following cholesterol loading of depleted microsomes. However, such loading was reflected in an increase in ACAT activity.

The data presented in this report show that cholesterol biosynthesis and esterification are acutely regulated by 25-hydroxycholesterol in a macrophage-like cell line P388D$_1$. We have previously used this cell line as a model system to investigate the effect of glucocorticoids (10) and 25-hydroxycholesterol (11) on fluid-phase endocytosis. These studies have demonstrated a rapid inhibitory effect of 25-hydroxycholesterol on endocytosis that could not be prevented by exogenous mevalonate. The present experiments using [^{14}C]acetate and [^{3}H]-oleate have extended this work, confirming the results obtained in hepatocytes by Drevon, Weinstein, and

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl-CoA:cholesterol acyltransferase; MEM, Eagle's minimum essential medium; NCS, neonatal calf serum; BSA, bovine serum albumin.

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Steinberg (12) who showed rapid stimulatory effect of 25-hydroxycholesterol on cholesterol esterification. However, contrary to our expectations, the 25-hydroxycholesterol-mediated increase in cholesterol esterification measured in vivo did not correspond with an increase in the activity of ACAT measured in microsomes. The data suggest that 25-hydroxycholesterol may stimulate the formation of cholesteryl esters in $P388D_1$ macrophage-like cells by increasing the rate of entry of free cholesterol into the ACAT substrate pool.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Acetate (56 mCi/mmol) was obtained from Research Products International Corp., Mount Prospect, IL; [3-^{14}C]HMG-CoA (57.6 mCi/mmol), [5-^{3}H]mevalonate (5 Ci/mmol, DBED salt), [1-^{14}C]oleoyl CoA (59.7 mCi/mmol), [9,10-^{3}H]oleate (8.5 Ci/mmol), [4-^{14}C]cholesterol (59.4 mCi/mmol), and [cholesteryl 1,2,6,7,-^{3}H]cholesteryl oleate (77 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Ion exchange resin (AG 1-X8, formate form, 200 to 400 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA. Routinely used chemicals were from Sigma Chemical Corp., St. Louis, MO.; 25-hydroxycholesterol and 7-ketocholesterol were obtained from Steraloids, Wilton, NH. Purity of sterols was checked by thin-layer chromatography using hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) on silica gel G preadsorbent plates from Analtech Inc., Newark, DE.

Cell culture

The $P388D_1$ mouse macrophage-like cell line was originally isolated by Dawe and Potter (13) from a methylcholanthrene-induced lymphoma (P388) of a DBA/2 mouse. Koren, Handwerger, and Wunderlish (14) have shown the morphological and physical properties of these cells to be similar to, if not characteristic of, normal macrophages. The cells were provided by Dr. T. Suzuki, Department of Microbiology, University of Kansas Medical Center. The $P388D_1$ cell line was routinely carried in monolayer cultures in Eagle's minimum essential medium (MEM) supplemented with 10% neonatal calf serum ($MEM_{10}NCS$). Serum was obtained from Biocell Laboratories, Carson, CA. Subcultivation was by scraping the cells with a rubber policeman from T-75 plastic flasks (Corning).

Incorporation of [^{14}C]acetate and [^{3}H]oleate into free cholesterol and cholesteryl esters

Cells were plated at an initial density of 2×10^6 cells per 60-mm dish in $MEM_{10}NCS$ and were grown for 24 hr. Ethanol (0.1% final concentration) or 25-hydroxycholesterol in ethanol were then added directly to dishes. The[^{14}C]acetate made up in $MEM_{10}NCS$ was added to give 1 μCi/ml, 20 μM final concentration. The [^{3}H]oleic acid and unlabeled oleic acid were combined with an equimolar amount of 1 N KOH and then dissolved in MEM_0 containing 10% fatty acid-free BSA to give 5 mM oleate. Molar ratio of fatty acid to bovine serum albumin was 3.31. Forty μl of this solution was added to 4 ml of medium/60-mm dish giving 50 μM final concentration of oleate and 1 μCi/ml [^{3}H]oleate with a specific activity of 4.4×10^4 dpm/nmol. Incorporation of [^{3}H]oleate into cholesteryl oleate was linear for 6 hr. After labeling for the appropriate length of time, the cell cultures were placed on ice and washed three times with cold 0.85% NaCl. The cells were scraped off with a rubber policeman, triturated in 1 ml of 0.85% NaCl, and a portion was removed for protein determination by the method of Lowry et al. (15). The rest of the cell suspension was transferred to a screw-cap tube, the dish was washed once with another 1 ml of 0.85% NaCl, and the rinse was combined with the suspension. The cells were pelleted at 800 *g* in an International centrifuge (Model PR-6) for 10 min at 4°C. The supernatant was removed by aspiration and 2 ml of chloroform–methanol 2:1 (v/v) was added and the samples were vortexed for 15 sec. After phase separation the chloroform phase was washed twice according to the method of Folch, Lees, and Sloane Stanley (16). Samples were taken to dryness under N_2 and 200 μl of chloroform was added and vortexed and 150 μl was removed. A mixture (20 μl) of cholesterol and cholesteryl oleate (40 μg of each) was added to the 150 μl of the extract and the sample was evaporated to dryness under N_2. The lipids were redissolved in 50 μl of chloroform followed by a 20-μl rinse and applied to silica gel G preadsorbent TLC plates. Plates were developed in hexane–diethyl ether–acetic acid 80:20:1 (v/v/v). In order of increasing R_f values, phospholipids (which remained at the origin), free cholesterol, fatty acids, triglycerides, and cholesteryl esters were visualized with I_2 vapor and identified by reference to appropriate standards. With this solvent system 25-hydroxycholesterol esters are resolved at an R_f greater than free cholesterol (12) and less than fatty acids. The free cholesterol and cholesteryl esters were scraped into counting vials and 5 ml of Econofluor scintillation fluid was added. The samples were counted in a Packard Tri-Carb liquid scintillation counter. Counting efficiency of ^{3}H was 42% and of ^{14}C was 81%. Double label corrections were calculated according to a program in a PDP-11 digital computer (Digital Equipment Co.). Statistical analysis was by Student's *t* test for unpaired samples.

Measurement of HMG-CoA reductase

Cells were grown and processed as described above but cell pellets were frozen until the day of assay. The

cell pellets were resuspended in homogenization buffer (1 mM potassium phosphate, pH 7.3, 0.32 M sucrose, 1 mM $MgCl_2$) and disrupted by sonication. A sample for protein determination was removed and the remainder was kept at 4°C. HMG-CoA reductase activity was determined as previously described (17). Briefly, the assay mixture consisted of a total volume of 0.22 ml containing 20 μmol of potassium phosphate, pH 7.3, 4 μmol of glucose-6-phosphate, 0.5 μmol of NADP, 1.0 unit of glucose-6-phosphate dehydrogenase, 1.8 μmol of dithiothreitol, and 100 μg or 150 μg of sample protein. The reaction mixture was preincubated at 37°C for 10 min and the reaction was started by adding [^{14}C]HMG-CoA. The reaction was routinely run for 60 min and stopped by addition of 25 μl of 6 N HCl. The rest of the procedure was exactly as described previously (17).

Measurement of acyl-CoA:cholesterol acyltransferase activity in microsomes

Cell pellets were suspended in homogenization buffer (1 mM potassium phosphate, pH 7.3, 0.32 M sucrose, 1 mM $MgCl_2$) and disrupted by 25 strokes in a Dounce stainless steel homogenizer. The homogenate was centrifuged at 800 *g* (Sorvall RC2-B) for 10 min and the supernatant was removed and centrifuged at 12,000 *g* (Sorvall RC2-B) for 10 min. The supernatant was again removed and centrifuged at 105,000 *g* (Beckman L5-65) for 60 min at 4°C. The resulting microsomal pellet was resuspended in homogenization buffer to approximately 10 mg/ml protein concentration. Aliquots of 100 μl each were frozen until the time of assay.

The ACAT activity in microsomes was determined as described by Spector et al. (18) in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.3, containing 0.4 mg/ml fatty acid-free BSA. Microsomal protein (50–400 μg) was added in 30 μl of homogenization buffer. The reaction mixture was preincubated at 37°C for 6 min and the reaction was started by adding 20 μl of phosphate buffer containing 5 nmol of oleoyl CoA (1.1×10^5 dpm); sp act 10 μCi/μmol; the final concentration of oleoyl CoA per assay was 16.67 μM. Studies on the effect of oleoyl CoA on enzyme activity showed optimum ACAT activity at this concentration. The reaction was linear for 10 min and was proportional to protein up to 400 μg of microsomal protein. The assay was routinely run at 37°C for 10 min. The reaction was stopped by the addition of 2 ml of chloroform–methanol 2:1 (v/v). The lipids were extracted and washed as described by Folch et al. (16) and the cholesteryl [^{14}C]oleate was isolated by thin-layer chromatography as described above. In some experiments [^{3}H]cholesteryl oleate was added as an internal standard for recovery. Greater than 90% of the added radioactivity was consistently recovered.

RESULTS

Effect of time of incubation with 25-hydroxycholesterol on incorporation of [^{14}C]acetate and [^{3}H]oleate into free cholesterol and cholesteryl esters

The time required for 25-hydroxycholesterol to alter cholesterol metabolism was examined by measuring the incorporation of [^{14}C]acetate and [^{3}H]oleate into free and esterified cholesterol separated by thin-layer chromatography. At 0 hr 25-hydroxycholesterol was added directly to cultures. The time points illustrated in **Fig. 1A, B** represent the time when both labels were added

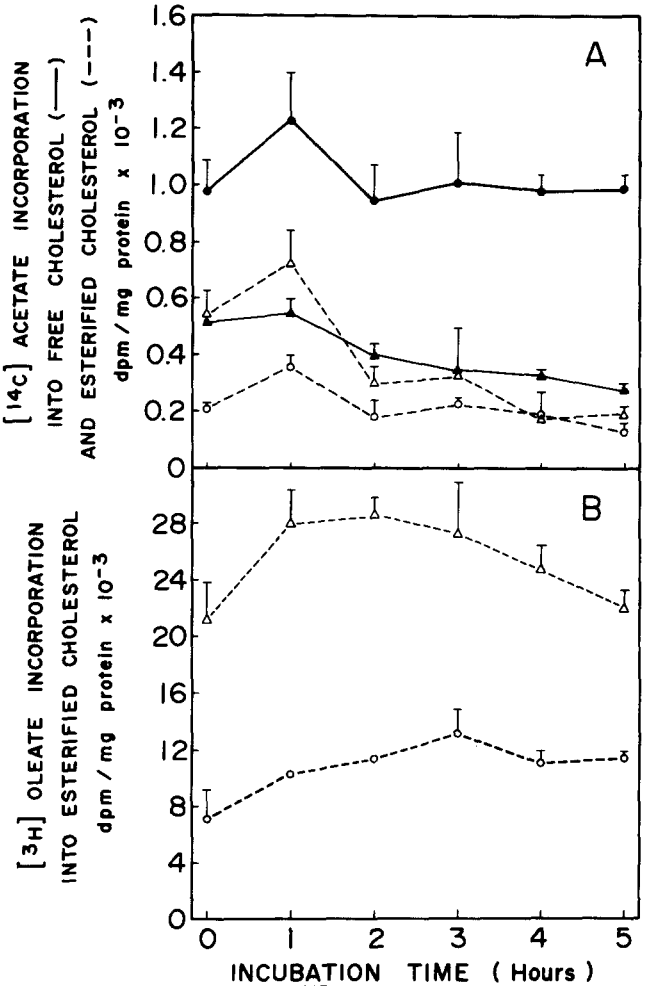

Fig. 1. Effect of 2.5 μM 25-hydroxycholesterol on the rate of (A) [^{14}C]acetate incorporation into free and esterified cholesterol and (B) [^{3}H]oleate incorporation into cholesteryl esters. Cells were plated at 2×10^6 cells per 60-mm dish in $MEM_{10}NCS$ and pregrown for 24 hr before 25-hydroxycholesterol (▲, △) or ethanol (●, ○) were added directly to cultures. At the indicated time points both [^{14}C]acetate and [^{3}H]oleate were added and the amount of radioactivity incorporated during a 1-hr pulse was determined. A, Incorporation of [^{14}C]acetate into free cholesterol (● —— ●, ▲ —— ▲) and cholesteryl esters (○ —— ○, △ —— △). B, Incorporation of [^{3}H]oleate into cholesteryl esters (○ - - - ○, △ - - - △). Data points represent the mean ± SD from triplicate cultures.

to the cultures after 25-hydroxycholesterol addition. The duration of the pulse was 1 hr. As shown in Fig. 1A, the incorporation of [^{14}C]acetate into free cholesterol was significantly reduced ($P < 0.01$) in 25-hydroxycholesterol-treated cultures and continued to decrease throughout the 5-hr incubation. In contrast, the incorporation of [^{14}C]acetate into esterified cholesterol was significantly stimulated at the two earliest time points ($P < 0.01$), but was no longer stimulated ($P < 0.2$) at later time points. As shown in Fig. 1B, the incorporation of [^{3}H]oleate into cholesteryl esters was also stimulated by 25-hydroxycholesterol by the first time point and this stimulation progressively decreased from 3- to 1.9-fold after 5 hr of incubation. We should point out that the double-label experiments were done in the presence of 50 μM unlabeled oleate and that 25-hydroxycholesterol did not alter the incorporation of either [^{14}C]acetate or [^{3}H]oleate into other lipid classes (data not shown).

Concentration effect of 25-hydroxycholesterol on cholesterol synthesis and esterification

Using the double-label approach described above, the incorporation of both [^{14}C]acetate and [^{3}H]oleate into free cholesterol and cholesteryl esters was determined as a function of 25-hydroxycholesterol concentration. After addition of the indicated concentrations of 25-hydroxycholesterol (**Table 1**), the cultures were immediately pulsed with both labels for 1 hr. There was no effect on protein/culture by 25-hydroxycholesterol treatment. In the absence of any 25-hydroxycholesterol, 23% of the total ^{14}C-labeled cholesterol was esterified. Increasing the 25-hydroxycholesterol concentration stimulated [^{14}C]acetate incorporation into esterified cholesterol, such that 75% of the total ^{14}C-labeled cholesterol was esterified. The stimulation of [^{14}C]acetate incorporation into cholesteryl esters reached a maximum at 2.5 μM 25-hydroxycholesterol. However, the incorporation of [^{14}C]acetate into free cholesterol continued to decrease with increasing concentrations of 25-hydroxycholesterol, reaching the lowest value at 12.5 μM 25-hydroxycholesterol. In contrast, the incorporation of [^{3}H]oleate into free and esterified cholesterol *both* reached maximum effects at 12.5 μM 25-hydroxycholesterol. In the absence of 25-hydroxycholesterol 67% of the total ^{3}H-labeled cholesterol was esterified. Increasing the concentration of 25-hydroxycholesterol to 12.5 μM stimulated [^{3}H]oleate incorporation into esterified cholesterol, such that 97% of the ^{3}H was in cholesteryl esters. The results from the double-label approach are complicated by the likely metabolism of [^{3}H]oleate through β-oxidation to cholesterol. The data in Table 1 present a qualitative picture on the ability of 25-hydroxycholesterol to *rapidly* alter the incorporation of [^{14}C]acetate and [^{3}H]oleate into both free and esterified cholesterol. Thus, the double-labeling approach allows one to measure the 25-hydroxycholesterol-mediated regulation of both HMG-CoA reductase and ACAT activities. Overall, the pattern of both precursors appears to be quite similar except for the incorporation of [^{14}C]acetate into esterified cholesterol, which reached a maximum effect at 2.5 μM 25-hydroxycholesterol. The most obvious explanation for these results obtained with [^{14}C]acetate is the suppressing effect of 25-hydroxycholesterol on HMG-CoA reductase and thus on the de novo synthesis of ^{14}C-labeled free cholesterol.

Effect of 25-hydroxycholesterol on HMG-CoA reductase versus ACAT activity

We next examined the time of incubation with 25-hydroxycholesterol required to bring about changes in the activities of both HMG-CoA reductase and ACAT. Although HMG-CoA reductase activity was reduced after 1 hr incubation with 2.5 μM 25-hydroxycholesterol and the activity decreased further with time of incuba-

TABLE 1. Dose-response of 25-hydroxycholesterol on incorporation of both [^{14}C]acetate and [^{3}H]oleate into free cholesterol and cholesteryl ester

	[^{14}C]Acetate Incorporation				[^{3}H]Oleate Incorporation			
25-OH	Free Cholesterol	Cholesteryl Ester	Free Cholesterol plus Cholesteryl Ester	[^{14}C]Cholesteryl Ester as a Percent of Total [^{14}C]Cholesterol	Free Cholesterol	Cholesteryl Ester	Free Cholesterol plus Cholesteryl Ester	[^{3}H]Cholesteryl Ester as a Percent of Total [^{3}H]Cholesterol
μM	*dpm/culture* × 10^{-3}				*dpm/culture* × 10^{-4}			
0	1.73 ± 0.18	0.50 ± 0.02	2.15 ± 0.17	23.3	0.84 ± 0.03	2.34 ± 0.03	3.49 ± 0.35	67.0
0.25	1.47 ± 0.02	0.52 ± 0.12	1.99 ± 0.13	26.1	0.68 ± 0.3	2.83 ± 0.12	3.51 ± 0.14	80.6
2.5	0.30 ± 0.03	0.92 ± 0.08	1.78 ± 0.10	51.7	0.55 ± 0.02	6.14 ± 0.20	6.70 ± 0.18	91.6
12.5	0.30 ± 0.03	0.81 ± 0.18	1.11 ± 0.21	73.0	0.27 ± 0.04	8.97 ± 0.30	9.24 ± 0.34	97.1
25	0.29 ± 0.06	0.91 ± 0.10	1.20 ± 0.18	75.8	0.32 ± 0.09	9.69 ± 0.53	10.01 ± 0.47	96.8

Triplicate cell cultures were incubated for 1 hr in the presence of both [^{14}C]acetate and [^{3}H]oleate. In addition, the cultures contained either 25-hydroxycholesterol in ethanol or an equivalent amount of ethanol (0.1% final concentration) for the same length of time during pulse. Results are expressed as mean ± SD.

tion, the activity of ACAT was not stimulated at any time point examined (data not shown). However, the results in Fig. 1B clearly demonstrated that 2.5 μM 25-hydroxycholesterol rapidly stimulated [^{3}H]oleate incorporation into cholesterol esters ~2- to 3-fold. This disparity prompted us to examine the ability of 25-hydroxycholesterol to stimulate ACAT activity in sonicates, homogenates, and in microsomes. Under no conditions did we find increased ACAT activity after pretreatment with 25-hydroxycholesterol up to 25 μM (data not shown).

Chase of endogenous versus exogenous labeled cholesterol: effect of 25-hydroxycholesterol during chase

Considering that our results with ACAT showed no stimulation by 25-hydroxycholesterol, it was reasonable to speculate that 25-hydroxycholesterol may stimulate esterification of cholesterol in intact cells by increasing the entry of free cholesterol into the ACAT substrate pool. This possibility was examined by incubating cultures with [^{14}C]acetate for 3 hr to form endogenous ^{14}C-labeled cholesterol. The labeling medium was removed and one set of cultures received fresh medium with 0.1% ethanol while another set received fresh medium with 2.5 μM 25-hydroxycholesterol in the same volume of ethanol. Samples were harvested throughout 16 hr of incubation. As shown in **Table 2,** the 0-hr sample represents the end of the labeling period. At 0 hr the ^{14}C-labeled cholesteryl esters represented 22% of the total ^{14}C-labeled cholesterol. One hour into the chase, cultures that received 25-hydroxycholesterol had 51% of the total ^{14}C-labeled cholesterol esterified while only 25% was esterified in control cultures. The 25-hydroxycholesterol stimulation of synthesis of ^{14}C-labeled cholesteryl esters reached a maximum after 4 hr. Through 16 hr of incubation, cultures treated with 25-hydroxycholesterol showed an increase in ^{14}C-labeled cholesteryl esters which was balanced by a decrease in ^{14}C-labeled free cholesterol such that total ^{14}C-labeled cholesterol was unaltered. After 16 hr, protein increased from 0.54 ± 0.01 to 0.77 ± 0.04 mg protein/culture in control versus 0.81 ± 0.04 mg protein/culture in 25-hydroxycholesterol-treated cultures.

The uptake and esterification of exogenous [^{14}]cholesterol of high specific activity (59.4 mCi/mmol) was examined under our standard conditions, i.e., medium supplemented with 10% NCS. As shown in **Fig. 2,** when cultures were incubated with [^{14}C]cholesterol for 20 hr ~16% of the total [^{14}C]cholesterol was esterified. When the labeling medium was removed and replaced with fresh medium, the amount of [^{14}C]cholesteryl esters increased from 28.82 ± 0.42 to 36.88 ± 1.85 pmol/culture and this amount remained constant throughout 8 hr of incubation. Cultures that received fresh medium containing 12.5 μM 25-hydroxycholesterol had more [^{14}C]cholesteryl esters after 1 hr of incubation. The amount of [^{14}C]cholesteryl esters continued to increase in 25-hydroxycholesterol-treated cultures resulting in a 3.2-fold stimulation over control cultures after 8 hr of

TABLE 2. Incorporation of [^{14}C]acetate into free and esterified cholesterol: effect of 2.5 μM 25-hydroxycholesterol during chase

Chase	±25-OH (2.5 μM)	Free Cholesterol	Cholesteryl Ester	Free Cholesterol plus Cholesteryl Ester
hr			*dpm/mg protein* × 10^{-3}	
0	−	12.50 ± 0.73	3.59 ± 0.19	16.09 ± 0.88
1	−	14.24 ± 1.33	4.66 ± 0.72	18.91 ± 2.05
	+	9.31 ± 0.89	9.60 ± 0.99	18.91 ± 1.88
4	−	11.44 ± 0.49	6.23 ± 0.35	17.67 ± 0.83
	+	6.14 ± 0.24	11.46 ± 0.33	17.60 ± 0.46
8	−	11.70 ± 0.18	6.61 ± 0.09	18.30 ± 0.27
	+	5.94 ± 0.68	11.48 ± 0.83	17.43 ± 1.46
12	−	9.98 ± 1.70	5.15 ± 0.61	15.13 ± 2.27
	+	6.10 ± 0.92	9.21 ± 1.73	15.31 ± 2.64
16	−	8.83 ± 0.99	4.52 ± 0.39	13.35 ± 1.38
	+	5.64 ± 0.61	7.26 ± 1.04	12.90 ± 1.65

Cells were plated at 2 × 10^6 cells per 60-mm dish in MEM_{10}NCS and pregrown for 24 hr. Cultures were pulse-labeled for 3 hr with [^{14}C]acetate. At 0 hr the medium was removed and replaced with fresh medium ± 2.5 μM 25-hydroxycholesterol. At indicated times during the chase, cultures were removed and the amount of radioactivity incorporated into free and esterified cholesterol was determined. Results are expressed as mean ± SD from triplicate cultures.

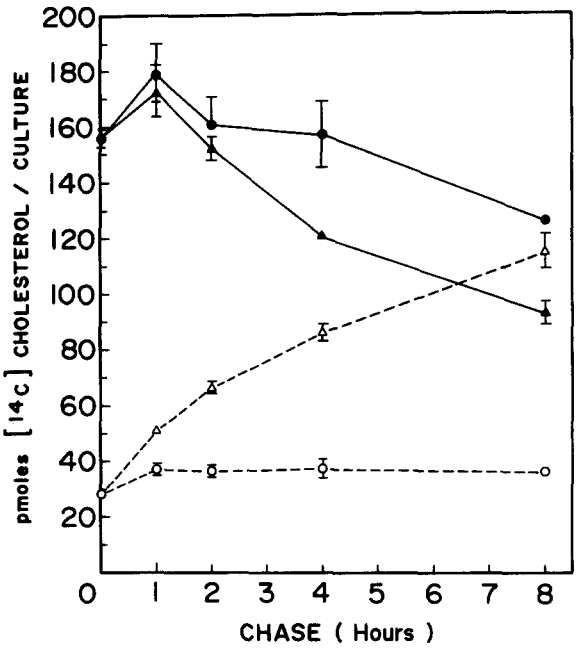

Fig. 2. Incorporation of exogenous [^{14}C]cholesterol into cellular free and esterified cholesterol: effect of 12.5 μM 25-hydroxycholesterol during chase. Cells were plated at 5 × 10^5 cells per 35-mm dish in $MEM_{10}NCS$ and pregrown for 24 hr. Cultures were then labeled with 0.1 μCi/ml [^{14}C]cholesterol (59.4 mCi/mmol) for 20 hr. At 0 hr the medium was removed and replaced with fresh medium containing 25-hydroxycholesterol (▲, △) or an equivalent amount of ethanol (●, ○). At indicated times during the chase cultures were put on ice and the medium was removed with a Pasteur pipette. The medium was extracted by the method of Folch et al. (15) and a portion of the lipid-soluble fraction was counted as a measure of [^{14}C]cholesterol efflux. Cellular free [^{14}C]cholesterol (● —— ●, ▲ —— ▲) and esterified cholesterol (○ - - - ○, △ - - - △) were determined as described in Methods. Data points represent the mean ± SD from duplicate cultures.

incubation. After an initial increase, the amount of free [^{14}C]cholesterol decreased in both control and 25-hydroxycholesterol-treated cultures. After 4 hr of incubation with 25-hydroxycholesterol, free [^{14}C]cholesterol was significantly decreased.

Time-course for effect of 25-hydroxycholesterol on HMG-CoA reductase versus cholesterol esterification

Experiments designed to examine whether the 25-hydroxycholesterol effect on cholesterol esterification preceded the effect on HMG-CoA reductase activity were performed next. The time-course of the 25-hydroxycholesterol effect was examined after short intervals. As shown in **Fig. 3,** addition of 12.5 μM 25-hydroxycholesterol resulted in a rapid stimulation of the incorporation of [^{3}H]oleate into cholesteryl esters with a maximum rate measurable after incubation for 7.5 min. This increased rate of incorporation proceeded linearly for at least 60 min. Incorporation in control cultures increased more slowly. The effect of 25-hydroxycholesterol on HMG-CoA reductase was also very rapid, but there was no significant decrease ($P < 0.05$) until 30 min of incubation with 25-hydroxycholesterol.

Effect of progesterone on regulation of cholesterol metabolism by 25-hydroxycholesterol

Although there was no clear separation in time, the experiment shown in Fig. 3 suggested that 25-hydroxycholesterol stimulated cholesterol esterification before suppressing activity of HMG-CoA reductase. To test the possibility that cholesteryl ester synthesis stimulated by 25-hydroxycholesterol provided a signal to reduce the activity of HMG-CoA reductase, it was first necessary to show that progesterone inhibited the 25-hydroxycholesterol-mediated increase in cholesterol esterification. As previously shown by others (3, 19, 20), the simultaneous addition of increasing amounts of progesterone with 25-hydroxycholesterol resulted in progessively lower amounts of [^{3}H]oleate incorporated into cholesteryl esters (data not shown). Having established that progesterone could attenuate the 25-hydroxycholesterol effect on

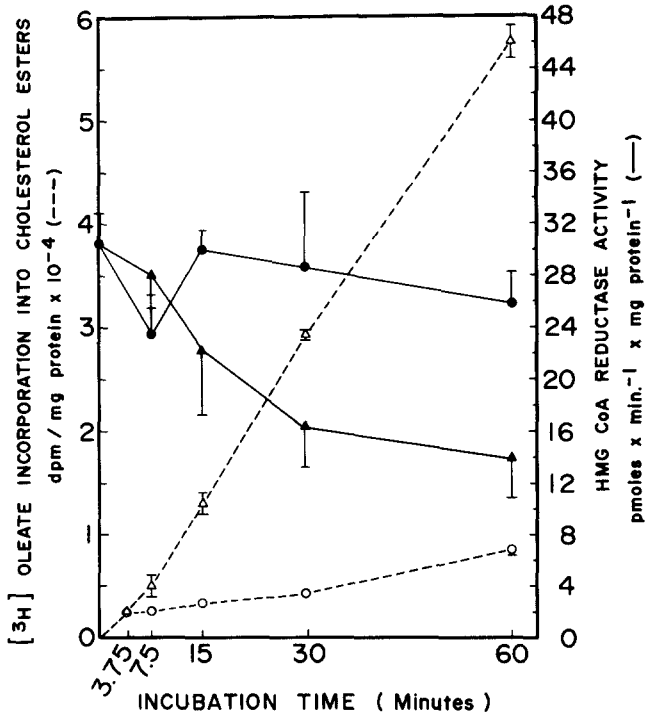

Figure 3. Effect of 12.5 μM 25-hydroxycholesterol on the incorporation of [^{3}H]oleate into cholesteryl esters and HMG-CoA reductase activity versus time of incubation. Conditions are identical to those described in the legend to Fig. 1. The incorporation of [^{3}H]oleate into cholesteryl esters was measured in duplicate cultures and HMG-CoA reductase activity was determined in triplicate from cells pooled from triplicate cultures. One-hundred and fifty μg of protein from cell sonicates was used. Data points represent mean ± SD. Cholesteryl ester synthesis: control (○ - - - ○); and 25-hydroxycholesterol-treated (△ - - - △). HMG-CoA reductase activity: control (● —— ●); and 25-hydroxycholesterol-treated (▲ —— ▲).

cholesterol esterification, we repeated the experiment described in Fig. 3 but included two new variables: effect of *1*) 25 μM progesterone and *2*) 25 μM progesterone in combination with 12.5 μM 25-hydroxycholesterol. The results from this experiment (**Fig. 4**) demonstrate that the increased esterification of cholesterol by 25-hydroxycholesterol was linear for 2 hr. The addition of 25 μM progesterone completely abolished the increased incorporation of [^{3}H]oleate into cholesteryl esters by 12.5 μM 25-hydroxycholesterol. However, the activity of HMG-CoA reductase was significantly decreased by 25-hydroxycholesterol ($P < 0.01$) regardless of progesterone addition. It is noteworthy that glucocorticoids, which in other cells elevate HMG-CoA reductase (12), failed to have any effect on ACAT.

DISCUSSION

The results of these experiments show that in P388D$_1$ cells 25-hydroxycholesterol *rapidly* stimulates esterification of cholesterol. This effect does not appear to involve a direct stimulation of ACAT, as indicated by our inability to demonstrate increased activity of ACAT in sonicates, homogenates, or in microsomal fractions prepared from cells grown in the presence of 25-hydroxycholesterol. Similarly, no stimulation of ACAT activity was seen when 25-hydroxycholesterol was added directly to the reaction mixture (S. C. Miller and G. Melnykovych, unpublished results).

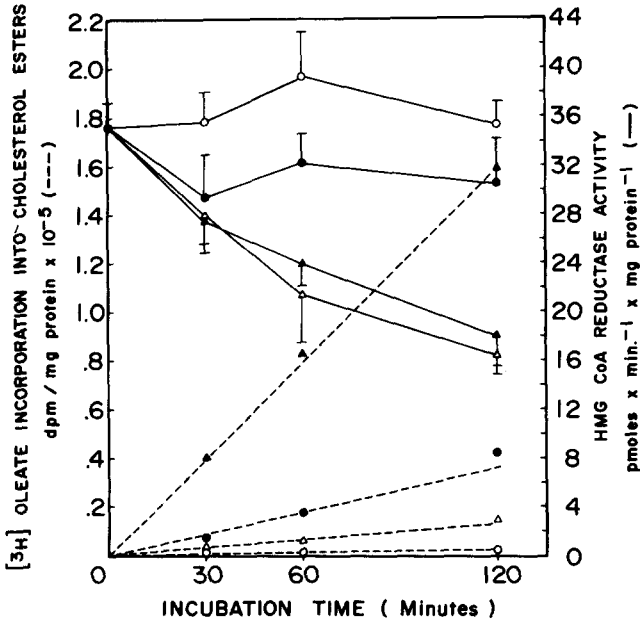

Figure 4. Effect of 25 μM progesterone on the ability of 12.5 μM 25-hydroxycholesterol to stimulate cholesterol esterification and suppress HMG-CoA reductase activity versus time of incubation. Conditions are identical to those described in the legend to Fig. 3. [^{3}H]Oleate incorporation into cholesteryl esters: control (● - - - ●); 25 μM progesterone (○ - - - ○); 12.5 μM 25-hydroxycholesterol (▲ - - - ▲); progesterone plus 25-hydroxycholesterol (△ - - - △). HMG-CoA reductase activity: control (● —— ●); 25 μM progesterone (○ —— ○); 12.5 μM 25-hydroxycholesterol (▲ —— ▲); or 25 μM progesterone plus 12.5 μM 25-hydroxycholesterol (△ —— △). Data points represent mean ± SD.

The pulse chase experiments using [^{14}C]acetate as cholesterol precursor have suggested the possibility that 25-hydroxycholesterol might increase the availability of cellular cholesterol for esterification by ACAT. The cells were labeled for 3 hr and were then chased in the presence or absence of 25-hydroxycholesterol. This caused an immediate elevation of esterification which leveled off at 4 hr and persisted throughout the time of incubation (Table 2). At all time points, the 25-hydroxycholesterol-mediated increase in ^{14}C-labeled cholesterol was balanced by a corresponding decrease in the label in the free cholesterol fraction. The stimulatory effect of 25-hydroxycholesterol on cholesterol esterification was confirmed in another experiment in which exogenous [^{14}C]cholesterol was used (Fig. 2). Again there was an immediate increase in the labeled cholesteryl esters in 25-hydroxycholesterol-treated cultures with a concomitant decrease in free cholesterol label.

The results of Field and Mathur (21) support our interpretation of these results. They observed that the effect of 25-hydroxycholesterol on ACAT was dependent on the availability of cholesterol to the enzyme. Significantly, when ACAT was saturated by its substrate, viz. cholesterol, 25-hydroxycholesterol was without effect. Only at concentrations below ACAT saturation was 25-hydroxycholesterol stimulatory.

Our inability to detect stimulation of ACAT activity after pretreatment of cells with 25-hydroxycholesterol was unexpected. With microsomes prepared from nontreated cells, 25-hydroxycholesterol also failed to stimulate ACAT. These findings contradict other studies, notably that of Drevon et al. (12). The most plausible explanation for our findings in broken cell preparations is that under our conditions ACAT was saturated with respect to cholesterol. When saturated with cholesterol, ACAT activity would not increase further after treatment with 25-hydroxycholesterol. Drevon et al. (12) might have detected the stimulation of ACAT activity by direct addition of 25-hydroxycholesterol because the microsomal pool of cholesterol could have been suboptimal for ACAT activity. It must be restated that it was consideration of the above findings that led us to experiments designed to ask whether 25-hydroxycholesterol could mobilize or increase cholesterol availability to ACAT. Our findings with [^{14}C]cholesterol (Fig. 2) support this idea. The same argument would apply to the alternate mechanism for increased esterification of cholesterol resulting from a stimulation of oleoyl CoA synthesis. However, in the latter case we should have

observed an *early* increase in labeling of triglyceride and phospholipid fractions. This we could not show (unpublished results).

Such results also suggest caution in equating changes in the in vivo incorporation of labeled oleate with changes in microsomal ACAT activity. In spite of numerous reports indicating stimulation of ACAT by free cholesterol (8, 22, 23) and by 25-hydroxycholesterol (24), the molecular mechanism of this effect remains obscure. The manner of presentation of cholesterol to the enzyme as shown by Billheimer et al. (25) might be involved. In their study, when cholesterol was added as a solution in acetone it had no effect on ACAT, but when added in a dispersion in Triton WR-1339 it increased ACAT activity in proportion of concentration.

The direct inhibition of ACAT by progesterone, reported by others (3, 19, 20) and confirmed in our experiments (Fig. 4), has provided us with a means of separating the stimulatory effect of 25-hydroxycholesterol on ACAT from the inhibition of HMG-CoA reductase. These results exclude cholesteryl esters as being involved in the regulation of HMG-CoA reductase since, in such case, progesterone should have diminished the effect of 25-hydroxycholesterol on both ACAT and HMG-CoA reductase. Indeed, earlier information of Bell, Sargeant, and Watson (26) has demonstrated that the increased cholesterol esterification is not a precondition for sterol-mediated suppressive effect on HMG-CoA reductase.

The mechanism whereby 25-hydroxycholesterol inhibits HMG-CoA reductase activity is currently under investigation in several laboratories. Kandutsch and Shown (27) have proposed a model in which the action of 25-hydroxycholesterol is mediated by a putative cytosolic receptor protein, thus conforming to the generally accepted concept of steroid hormone action (28).

It would be of interest to determine whether cell variants resistant to 25-hydroxycholesterol in terms of the effect on HMG-CoA reductase would also show a diminished response toward the stimulatory effect of 25-hydroxycholesterol on cholesterol esterification. To the best of our knowledge there are no thorough studies of this problem. In one investigation Sinensky, Duwe, and Pinkerton (6) have examined cholesterol esterification in a 25-hydroxycholesterol-resistant variant by measuring the microsomal ACAT activity with endogenous cholesterol as the substrate. No difference in ACAT activity between the parent culture and the resistant strain was found. Our results suggest that in vivo measurement of cholesterol esterification by [^{3}H]oleate incorporation would be necessary before one could conclude whether or not cholesterol esterification is affected in 25-hydroxycholesterol-resistant cell variants.

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