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Esterification of Low Density Lipoprotein Cholesterol in Human Fibroblasts and Its Absence in Homozygous Familial Hypercholesterolemia

(cholesterol synthesis/atherosclerosis/hyperlipidemia/low density lipoprotein receptors/ hydroxymethylglutaryl-coenzyme A reductase)

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ABSTRACT **A new mechanism is described for the cellular esterification of cholesterol derived from extracellular lipoproteins. Incubation of monolayers of cultured fibroblasts from normal human subjects with low density lipoproteins led to a 30- to 40-fold increase in the rate of incorporation of either [^{14}C]acetate or [^{14}C]oleate into the fatty acid fraction of cholesteryl [^{14}C]esters. This stimulation of cholesteryl ester formation by low density lipoproteins occurred despite the fact that endogenous synthesis of free cholesterol was completely suppressed by the lipoprotein. Thus, exogenous cholesterol contained in low density lipoproteins, rather than endogenously synthesized sterol, appeared to provide the cholesterol substrate for this cellular esterification process. High density lipoproteins and the lipoprotein-deficient fraction of serum neither stimulated cholesteryl ester formation nor inhibited cholesterol synthesis. Both the low density lipoprotein-dependent increase in cholesterol esterification and decrease in free cholesterol synthesis required the interaction of the lipoprotein with its recently described cell surface receptor. Cells from homozygotes with familial hypercholesterolemia, which lack specific low density lipoprotein receptors, showed neither lipoprotein-dependent cholesterol esterification nor suppression of cholesterol synthesis. The reciprocal changes in free cholesterol synthesis and cholesteryl ester formation produced by low density lipoprotein-receptor interactions may play an important role in the regulation of the cholesterol content of mammalian cells.**

Normal human fibroblasts possess, on their cell surface, a receptor that binds low density lipoproteins (LDL) with high affinity and specificity (1, 2). Binding of LDL to this receptor regulates cholesterol metabolism in two ways: (*i*) it suppresses cholesterol synthesis by reducing the rate of synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), the rate-controlling enzyme in cholesterogenesis (1), and (*ii*) it increases the rate at which fibroblasts degrade the protein component of LDL (2). Cells from homozygotes with the autosomal dominant disorder, familial hypercholesterolemia (FH), show a severe deficiency in the number of functional LDL receptor molecules (1–3). As a result of this primary genetic defect, these mutant cells manifest two secondary abnormalities: overproduction of cholesterol (4–6) and deficient catabolism of LDL protein (1–3).

We now report a third function of the LDL receptor. Binding of LDL to this receptor appears to initiate a cellular process for the esterification of cholesterol derived from extracellular LDL. In cells from homozygotes with FH, the deficiency in LDL receptors leads to an inability to esterify LDL-cholesterol.

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; FH, familial hypercholesterolemia; LPDS, lipoprotein-deficient serum.

METHODS

Cells. Skin biopsies were obtained with informed consent, and fibroblast cultures were established in our laboratory as described (7, 8). In all experiments except those in Table 3, the normal cells were derived from the foreskin of a healthy newborn, and the mutant cell line was obtained from J.P., a 12-yr-old female subject with the homozygous form of FH (4). All cells were grown in monolayer, maintained in Eagle's minimum essential medium supplemented as described (1), and used between the 5th and 25th passage. For all experiments, cells from stock flasks were dissociated with trypsin–EDTA solution (1) and were seeded (day 0) at a concentration of 1×10^5 cells per dish into 60×15-mm dishes (Falcon) containing 3 ml of growth medium with 10% fetal calf serum. On day 3, the medium was replaced with fresh medium containing 10% fetal calf serum. On day 6, when the cells were in late logarithmic growth, the monolayer was washed with 2 ml of Dulbecco's phosphate-buffered saline, after which 2 ml of fresh medium containing 5% (v/v) lipoprotein-deficient human serum (LPDS) was added (final protein concentration, 2.5 mg/ml). All experiments were initiated on day 7 after the cells had been grown for 24 hr in the presence of LPDS.

Lipoproteins. Human LDL, high density lipoprotein (HDL), and LPDS were obtained from healthy subjects and prepared by differential ultracentrifugation as described (5). The protein concentration in the LDL preparations averaged 60% of the cholesterol concentration.

Preparation of [^{14}C]Acetate and [^{14}C]Oleate. Sodium [*1*-^{14}C]-acetate in ethanol (New England Nuclear Corp., 53 mCi/mmol) was evaporated to dryness and resuspended in a solution of nonradioactive sodium acetate to the indicated specific activity. [*1*-^{14}C]oleic acid in hexane (New England Nuclear Corp., 51.8 mCi/mmol) was evaporated to dryness and resuspended in a solution containing 12.7 mM nonradioactive sodium oleate (Applied Science) in complex with 12% bovine albumin (Fraction V, Armour) in 0.9% sodium chloride prepared as described by Van Harken *et al.* (9). In calculating specific activity, no correction was made for the small amount of endogenous fatty acid bound to the albumin.

Measurement of Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Oleate into Lipids. On day 7, the medium containing 5%

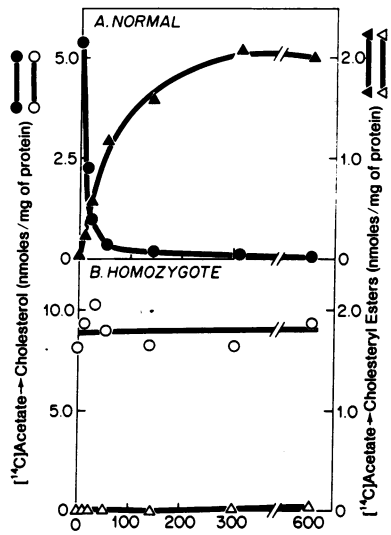

FIG. 1. Effect of LDL on incorporation of [^{14}C]acetate into free cholesterol (circles) and cholesteryl esters (triangles) in normal (*A*) and mutant (*B*) cells. Cell monolayers were preincubated for 6 hr in LPDS-medium containing the indicated concentration of LDL-cholesterol, after which [^{14}C]acetate (0.97 mM, 2800 cpm/nmole) was added. After a further 20-hr incubation, the cells from two dishes were pooled in 0.2 ml of phosphate-buffered saline and the content of free [^{14}C]cholesterol and cholesteryl [^{14}C]esters was determined as described in *Materials and Methods*.

LPDS was replaced with 2 ml of medium containing 5% LPDS and the indicated concentration of either LDL or nonlipoprotein cholesterol dissolved in ethanol. After the indicated time of preincubation, either the [*1*-^{14}C]acetate or the [*1*-^{14}C]oleate-albumin solution was added in volumes up to 40 μl. Cells were then incubated at 37° in a humidified CO_2 incubator for the indicated time, after which each monolayer was washed twice with 2 ml of cold phosphate-buffered saline and the cells were harvested by scraping. After centrifugation (900 × *g*, 3 min, 24°), the cell pellet was suspended in 0.1 ml of 0.9% NaCl, and a 10-μl aliquot was removed for protein determination (10). To the remaining suspension was added 4 ml of chloroform–methanol (2:1) containing 3.8 × 10^4 cpm of [*1,2*-^{3}H]cholesterol (New England Nuclear Corp., 58 Ci/mmol), 40 μg of cholesterol, and 40 μg of cholesteryl oleate. The mixture was then agitated for 30 sec and allowed to stand at 24° for at least 30 min. To separate the phases, 0.9 ml of H_2O was added (11), and each tube was centrifuged (900 × *g*, 10 min, 24°). The bottom phase was evaporated to dryness under air, and the lipids were resuspended in 100 μl of hexane and spotted on plastic-backed thin-layer plates coated with silica gel G without gypsum (Brinkman). The plates were developed in heptane–ethyl ether–acetic acid (85:15:2), and the lipid spots were identified with iodine vapor (cholesterol, R_F 0.2; cholesteryl esters, R_F 0.9) and counted in 10 ml of 0.5% 2,5-diphenyloxazole and 10% methanol in toluene. For each dish a correction was made for the recovery of the internal [^{3}H]cholesterol standard, which averaged 75%. In experiments in which radioactivity in the medium was measured, the volume of chloroform–methanol was appropriately adjusted (11). In all experiments, each value represents the mean of duplicate incubations using either one or two dishes for each assay. All results are expressed as the pmoles or nmoles of [^{14}C]acetate or [^{14}C]oleate incorporated into the indicated [^{14}C]lipid.

TABLE 1. *Effect of lipoproteins on [^{14}C]acetate incorporation into lipids in normal fibroblasts*

Addition to incubation medium	[^{14}C]Acetate incorporation into lipids (pmoles/dish)				
	[^{14}C]-Fatty acids	[^{14}C]Triglycerides	[^{14}C]-Phospholipids	Free [^{14}C]cholesterol	Cholesteryl [^{14}C]-esters
None	165	32	414	700	18
LDL	177	34	461	32	198
HDL	125	30	409	635	13

Cell monolayers were preincubated in LPDS-medium containing no lipoproteins, LDL (50 μg of protein per ml), or HDL (50 μg of protein per ml). After 6 hr, [^{14}C]acetate (0.5 mM, 14,100 cpm/nmole) was added. After a further 20-hr incubation, the medium and cells from two dishes were pooled together in a final volume of 4 ml and the content of [^{14}C]lipids was determined.

RESULTS

When normal fibroblasts were incubated with increasing amounts of LDL, the incorporation of [^{14}C]acetate into free cholesterol decreased and became nearly undetectable at LDL-cholesterol levels above 100 μg/ml (Fig. 1*A*). We have previously shown that this reduction in cholesterol synthesis is due to a specific suppression of HMG CoA reductase activity (4, 5, 7). In contrast, the incorporation of [^{14}C]acetate into cholesteryl esters, which was nearly undetectable in the absence of LDL, was stimulated more than 40-fold in its presence. When the ^{14}C-labeled cholesteryl esters formed in the presence of LDL were subjected to alkaline hydrolysis, more than 95% of the radioactivity was found in the fatty acid fraction. In cells from a homozygote with FH, the addition of LDL neither suppressed the synthesis of free cholesterol nor did it induce the formation of cholesteryl esters (Fig. 1*B*).

LDL had no effect on the incorporation of [^{14}C]acetate into fatty acids, triglycerides, or phospholipids in normal cells (Table 1). Unlike LDL, HDL did not suppress [^{14}C]acetate incorporation into cholesterol, nor did it stimulate incorporation into cholesteryl esters (Table 1).

The LDL effect on cholesterol and cholesteryl ester formation in normal and mutant cells was not affected by the omission of serum from the incubation medium (Table 2). Lipoprotein-deficient serum did promote the excretion from the cells of an appreciable fraction of the newly synthesized free cholesterol, but not the cholesteryl esters (Table 2).

To assay the LDL-dependent esterification more directly, cells were first preincubated with LDL and then incubated with [^{14}C]oleate bound to albumin. In normal cells, the rate of [^{14}C]oleate incorporation into cholesteryl esters was maximal at an oleate concentration of 0.1 mM (Fig. 2*A*). In the presence of LDL the increasing incorporation of radioactivity with increasing amounts of [^{14}C]oleate was apparently due to a progressive increase in the specific radioactivity of the intracellular oleate pool rather than an increase in the absolute amount of cholesteryl esters formed. Using gas liquid chromatography, we have shown that in normal cells incubated in LPDS the addition of LDL for 24 hr caused a 10-fold increase

TABLE 2. *Distribution of [^{14}C]cholesterol and cholesteryl [^{14}C]esters formed after incubation of normal and homozygous familial hypercholesterolemic fibroblasts with [^{14}C]acetate*

Additions to incubation medium	[^{14}C]Acetate incorporation (pmoles/dish)			
	Free [^{14}C]cholesterol		Cholesteryl [^{14}C]esters	
	Cells	Medium	Cells	Medium
Exp. A.: Normal cells				
No serum	1869	19	19	1.7
+ LDL	106	21	389	2.2
LPDS	1613	227	16	5.6
+ LDL	107	102	342	3.4
Exp. B.: Homozygous FH cells				
No serum	643	7.1	4.1	0.0
+ LDL	1219	78	6.0	0.6
LPDS	1453	135	5.2	1.8
+ LDL	1473	330	6.0	1.6

Cell monolayers were preincubated in medium containing either no serum or 5% LPDS in the absence or presence of LDL (50 μg of cholesterol per ml). After 6 hr, [^{14}C]acetate (0.5 mM, 11,100 cpm/nmole) was added. After a further 20-hr incubation, the medium and cells were separated, duplicate samples of each were pooled, and the content of free [^{14}C]cholesterol and cholesteryl [^{14}C]esters was determined.

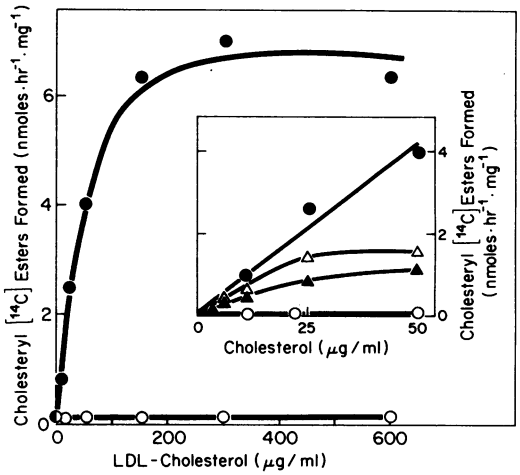

FIG. 3. Rate of incorporation of [^{14}C]oleate into cholesteryl esters as a function of the LDL concentration in normal (closed symbols) and mutant (open symbols) cells. Cell monolayers were preincubated in LPDS-medium containing the indicated concentration of LDL-cholesterol. After 17 hr, [^{14}C]oleate (0.1 mM, 25,000 cpm/nmole) was added and the cells were harvested 1 hr later. The cellular content of cholesteryl [^{14}C]esters was determined. The inset compares these results (●,○) with those obtained after the addition of nonlipoprotein cholesterol added in 20 μl of ethanol (▲,△).

in cholesteryl ester content (from 1.4 to 14 μg of sterol per mg of cell protein), whereas no significant change was seen in the mutant cells (from 0.67 to 0.46 μg of sterol per mg of cell protein). The addition of 0.1 mM oleate did not further increase the content of cholesteryl esters in either cell line (manuscript in preparation). At 0.1 mM oleate, ^{14}C-labeled cholesteryl ester formation was linear with time for at least 4 hr (Fig. 2*B*). In the mutant cells, cholesteryl ester formation was virtually undetectable at all levels of oleate despite the presence of a large amount of LDL in the medium (Fig. 2*A*).

The material isolated as ^{14}C-labeled cholesteryl esters in the standard thin-layer system migrated identically with authentic cholesteryl oleate in two additional thin-layer silica gel systems: 100% benzene (R_F 0.7) and tetralin–hexane (1:1, R_F 0.55). After alkaline hydrolysis, more than 95% of the radioactivity migrated as fatty acids in silica gel in heptane–ethyl ether–acetic acid (85:15:2).

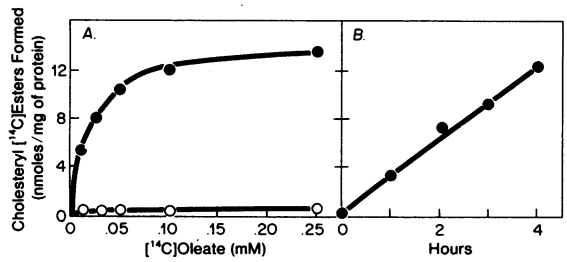

FIG. 2. [^{14}C]Oleate incorporation into cholesteryl esters after incubation with LDL as a function of oleate concentration (*A*) and time (*B*) in normal (●) and mutant (○) cells. Cell monolayers were preincubated 17 hr in LPDS-medium containing LDL (Exp. *A*, 60 μg of cholesterol per ml; Exp. *B*, 150 μg of cholesterol per ml). In Exp. *A*, the indicated concentration of [^{14}C]oleate (2300 cpm/nmole) was then added and the cells were incubated for a further 3 hr before harvest. In Exp. *B*, [^{14}C]-oleate (0.1 mM, 24,000 cpm/nmole) was added and the cells were harvested at the indicated time. The cellular content of cholesteryl [^{14}C]esters was determined as described in *Materials and Methods*.

In normal cells the rate of cholesterol esterification with [^{14}C]oleate rose as the concentration of LDL in the medium was increased up to a saturating level of about 150 μg/ml of LDL-cholesterol (Fig. 3). The half-maximal rate was obtained at 40 μg/ml of LDL-cholesterol (equivalent to 24 μg/ml of LDL-protein). This value is similar to the half-maximal concentration for LDL binding (10 μg/ml of LDL protein) (1, 2). In the absence of LDL, the rate of esterification in the mutant cells was low and equal to that in the normal. However, the mutant cells showed no stimulation by LDL at any level. In other experiments, HDL-cholesterol in concentrations up to 200 μg/ml (1000 μg/ml of HDL-protein) did not stimulate [^{14}C]oleate incorporation into cholesteryl [^{14}C]-esters in normal cells.

The addition of cholesterol to the medium in a nonlipoprotein form increased the rate of esterification in both the normal and mutant cells (Fig. 3, inset). In the normal cells, at equal cholesterol concentrations the rate of esterification was higher with LDL than with nonlipoprotein cholesterol, whereas the converse was true in the mutant cells, indicating that the latter do not lack the ability to esterify cholesterol provided the LDL receptor can be bypassed.

When LDL was added at a nonsaturating level, the rate of esterification remained low for 3 hr, increased sharply between 3 and 8 hr, and remained constant over the next 24 hr (Fig. 4). At a saturating level of LDL, the 3-hr lag was not observed. When the normal cells were incubated with nonlipoprotein cholesterol, the rate of esterification increased gradually over a 24-hr period but never reached the level seen with LDL (Fig. 4, inset).

Similar rates of formation of cholesteryl [^{14}C]esters were observed in the cell lines from four normal subjects of different

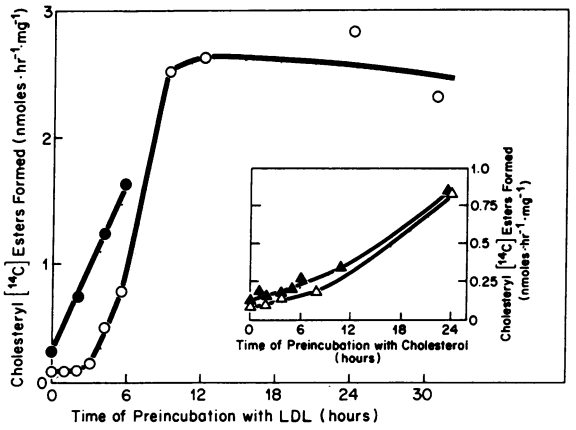

FIG. 4. Rate of [^{14}C]oleate incorporation into cholesteryl esters in normal cells as a function of duration of preincubation with LDL (circles) and nonlipoprotein cholesterol (triangles, inset). Cell monolayers were preincubated for the indicated time in LPDS-medium containing LDL (○, 60 μg of cholesterol/ml; ●, 150 μg of cholesterol/ml) or cholesterol added in 20 μl of ethanol (△, 30 μg/ml; ▲, 60 μg/ml). At the indicated time, [^{14}C]-oleate (0.1 mM, 24,000 cpm/nmole) was added and the cells were harvested 1 hr later. The cellular content of cholesteryl [^{14}C]-esters was determined as described in *Materials and Methods*.

ages and sexes (Table 3). In contrast, in the fibroblasts from four homozygotes with FH the rate of esterification was 35-fold lower than in the normal subjects and was not significantly different from values in normal and mutant cells in the absence of LDL.

To study the turnover of cholesteryl [^{14}C]esters, normal cells were preincubated with [^{14}C]oleate and LDL for 24 hr, after which the radioactive oleate was replaced with unlabeled oleate (Fig. 5*A*). In the absence of lipoproteins, the radioactivity in cholesteryl esters decreased by about 50% between 6 and 24 hr. The addition of HDL had no effect on

TABLE 3. *Rate of incorporation of [^{14}C]oleate into cholesteryl [^{14}C]esters by cells from normal subjects and homozygotes with familial hypercholesterolemia*

Subjects	Sex	Age (years)	Site of skin biopsy	Cholesteryl [^{14}C]-esters formed, nmoles·hr^{-1}·mg of protein^{-1}
Normal				
1	M	Newborn	Foreskin	3.7
2	F	20	Deltoid	1.5
3	M	25	Deltoid	3.9
4	F	40	Deltoid	2.6
			Mean	2.9
Homozygote				
5	F	12	Abdominal	0.06
6	M	10	Deltoid	0.09
7	F	23	Deltoid	0.10
8	F	6	Deltoid	0.07
			Mean	0.08

Cell monolayers were preincubated in LPDS-medium containing LDL (150 μg of cholesterol per ml). After 17 hr, [^{14}C]oleate (0.1 mM, 24,120 cpm/nmole) was added and the cells were harvested 2 hr later. The cellular content of cholesteryl [^{14}C]esters was determined as described in *Materials and Methods*.

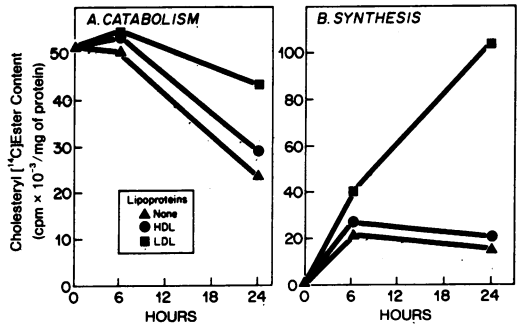

FIG. 5. Effect of lipoproteins on the synthesis and catabolism of cholesteryl [^{14}C]esters in normal cells. Cell monolayers were preincubated in LPDS-medium containing LDL (30 μg of protein per ml) and either [^{14}C]oleate (0.1 mM, 2190 cpm/nmole) (Exp. *A*) or 0.1 mM nonradioactive oleate (Exp. *B*). After 17.5 hr (zero time), the cell monolayers in Exp. *A* were washed with phosphate-buffered saline and switched to medium containing 0.1 mM nonradioactive oleate and no lipoproteins (▲), 60 μg of protein per ml of HDL (●), or 30 μg of protein per ml of LDL (■). At the same time, the cell monolayers in Exp. *B* were washed with phosphate-buffered saline and switched to medium containing [^{14}C]oleate (0.1 mM, 2190 cpm/nmole) and the lipoproteins corresponding to those in Exp. *A*. After the indicated time, the cells were harvested for determination of their content of cholesteryl [^{14}C]esters.

this catabolic process (Fig. 5*A*). When the initial incubation was performed in the presence of unlabeled oleate and the cells were then switched to medium containing [^{14}C]oleate, the synthesis of cholesteryl esters ceased after 6 hr both in the absence of lipoproteins and in the presence of HDL (Fig. 5*B*). On the other hand, when LDL was present in the medium, [^{14}C]oleate continued to be incorporated into cholesteryl esters (Fig. 5*B*). This continued synthesis precluded an accurate assessment of the effect of LDL on catabolism since the unlabeled material being formed in Exp. 5*A* caused an unknown dilution of the specific radioactivity of the cholesteryl ester pool. To obviate this problem, cells were incubated with LDL and [^{14}C]oleate continuously for 96 hr. Under these conditions the content of cholesteryl [^{14}C]oleate was maximal at 24 hr and gradually declined over the next 72 hr (Fig. 6). This decline occurred despite a continued synthesis of cholesteryl esters as indicated by the 24-hr pulse label experiments conducted at 48 and 72 hr (Fig. 6). These data suggest that when normal cells are exposed to LDL, a continual synthesis and hydrolysis of cholesteryl esters occurs so that the cellular cholesteryl ester content is determined by a balance between the rates of these two processes.

DISCUSSION

Cholesterol is believed to be esterified in mammalian tissues by two major mechanisms (12, 13). One of these processes occurs in plasma and involves the transfer of fatty acids from lecithin to cholesterol through the action of the plasma enzyme lecithin:cholesterol acyltransferase, which acts specifically on HDL-cholesterol (12). In solid tissues, esterification of cholesterol appears to involve microsomal enzyme systems that catalyze the transfer of fatty acids from their acyl coenzyme A thiol esters to cholesterol (13). In contrast to the requirement for specific lipoproteins in the plasma esterification system, a role for lipoproteins in the cellular process has not hitherto been clearly defined (13).

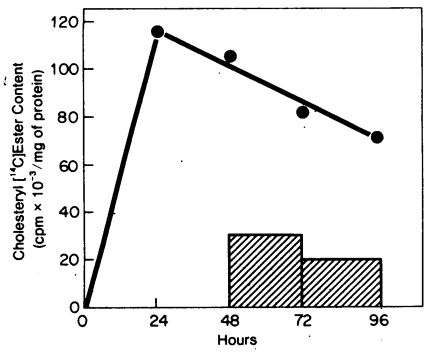

FIG. 6. Turnover of cholesteryl [^{14}C]esters in normal cells in the presence of LDL. Cell monolayers were incubated in LPDS-medium containing LDL (150 μg of cholesterol per ml) and [^{14}C]-oleate (0.1 mM, 2190 cpm/nmole). Every 24 hr the medium was replaced with fresh medium containing LDL and [^{14}C]oleate at the original concentrations. At the indicated time (●), the cells were harvested for determination of their content of cholesteryl [^{14}C]esters. Another group of cells was treated identically except that the medium contained nonradioactive 0.1 mM oleate in place of the [^{14}C]oleate. At 48 or 72 hr, these cells were switched to medium containing 0.1 mM [^{14}C]oleate and were harvested after 24 hr. The bars indicate the amount of [^{14}C]oleate incorporated during each of the 24-hr labeling periods.

In the present studies we have described a mechanism for cholesterol esterification in human fibroblasts that appears to be specific for cholesterol derived from LDL. The unique feature of this reaction is that it requires the presence of a cell surface receptor that recognizes the protein component of LDL. Evidence for the role of the LDL receptor in this esterification mechanism stems from two observations: (*i*) the LDL saturation curve for esterification is similar to that previously demonstrated for LDL-receptor binding (1, 2), and (*ii*) fibroblasts from subjects with homozygous FH, which lack the LDL receptor (1–3), show no increase in cholesterol esterification in the presence of LDL.

The reciprocal decrease in free cholesterol synthesis and increase in cholesteryl ester formation induced by LDL in normal cells raise the possibility that the increase in cholesteryl esters is an intermediate event necessary for suppression of hydroxymethylglutaryl CoA reductase activity. Consistent with this hypothesis is the observation that nonlipoprotein cholesterol, which acts in the absence of the LDL receptor (5, 14), induces cholesterol esterification (Fig. 3) and suppresses hydroxymethylglutaryl CoA reductase activity equally well in both normal and homozygous FH cells (5, 14).

Since about 70% of the cholesterol in LDL normally exists in an esterified form (13), the incorporation of [^{14}C]oleate most likely occurs after an initial cleavage of the LDL-cholesteryl esters. Such hydrolysis and reesterification may be required for the uptake of cholesteryl esters by the cell and might proceed by the following sequence of events: LDL binds to its receptor on the cell surface → cholesteryl esters in LDL are then hydrolyzed → free cholesterol crosses the cell membrane → and the sterol is finally reesterified with fatty acids. An alternative hypothesis that must be considered is that the cholesteryl esters in LDL enter the cell intact through the action of the LDL receptor and that the incorporation of [^{14}C]oleate represents an enzymatic exchange of the esterified fatty acid with fatty acids present in the cell. No matter which of these mechanisms is operative, when normal cells are exposed to LDL the net result is an increase in the cellular content of cholesteryl esters (manuscript in preparation).

If the findings in cultured fibroblasts bear relation to the mechanism of cellular cholesteryl ester formation *in vivo*, then this LDL-dependent reaction may be important in the cholesteryl ester deposition characteristic of the usual form of human atherosclerosis that occurs when plasma LDL levels are normal (15). On the other hand, because of their defect in esterification, the cholesteryl esters that accumulate in the arterial tissues of homozygotes with FH are likely derived not from cellular synthesis but from entrapment of plasma LDL when it rises to enormous levels.

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