Role of Lysosomal Acid Lipase in the Metabolism of Plasma Low Density Lipoprotein

OBSERVATIONS IN CULTURED FIBROBLASTS FROM A PATIENT WITH CHOLESTERYL ESTER STORAGE DISEASE*

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The hydrolysis of cholesteryl esters contained in plasma low density lipoprotein was reduced in cultured fibroblasts derived from a patient with cholesteryl ester storage disease, an inborn error of metabolism in which lysosomal acid lipase activity is deficient. While these mutant cells showed a normal ability to bind low density lipoprotein at its high affinity cell surface receptor site, to take up the bound lipoprotein through endocytosis, and to hydrolyze the protein component of the lipoprotein in lysosomes, their defective lysosomal hydrolysis of the cholesteryl ester component of the lipoprotein led to the accumulation within the cell of unhydrolyzed cholesteryl esters, the fatty acid distribution of which resembled that of plasma lipoprotein. When the cholesteryl ester storage disease cells were incubated with low density lipoprotein, the reduced rate of liberation of free cholesterol by these mutant cells was associated with a delay in the occurrence of two lipoprotein-mediated regulatory events, suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, and activation of endogenous cholesteryl ester formation. In contrast to their defective hydrolysis of exogenously derived lipoprotein-bound cholesteryl esters, the cholesteryl ester storage disease cells showed a normal rate of hydrolysis of cholesteryl esters that had been synthesized within the cell. These data lend support to the concept that in cultured human fibroblasts cholesteryl esters entering the cell bound to low density lipoprotein are hydrolyzed within the lysosome and that one of the functions of this intracellular organelle is to supply the cell with free cholesterol.

Cultured human fibroblasts possess a specific mechanism for the net uptake of cholesterol derived from plasma low density lipoprotein (LDL),† the major cholesterol-carrying protein in human blood (1-6). Previous data suggest that this mechanism involves the following sequences of events: (a) LDL binds to a specific receptor on the cell surface (1, 2); (b) the surface-bound LDL becomes incorporated into endocytotic vesicles; (c) the internal endocytotic vesicles containing bound LDL fuse with lysosomes (4, 5); (d) the cholesteryl ester and protein components of LDL are hydrolyzed by lysosomal enzymes to products, including free cholesterol and amino acids (2, 4, 5); and (e) the liberated free cholesterol is transferred from lysosomes to cellular membranes (6). The resultant accumulation of cholesterol within the cell regulates two events in cellular cholesterol metabolism: (a) cholesteryl ester formation is stimulated through an activation of a membrane-bound fatty acyl-CoA:cholesterol acyltransferase (7, 8) and (b) cholesterol synthesis is reduced through a suppression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (9). Formulation of this model of LDL metabolism in human fibroblasts has been facilitated by a comparison of the behavior of normal fibroblasts with that of mutant cells from subjects with the homozygous form of familial hypercholesterolemia, which lack the cell surface LDL receptor and thus fail to manifest all of the metabolic consequences of high affinity LDL binding (1-9).

A critical aspect of the above model involves the role of the lysosome in hydrolyzing the cholesteryl ester and protein components of LDL so as to make free cholesterol available to the cell. This requirement for lysosomes has been inferred from studies in which lysosomal hydrolytic activity has been blocked in intact cells through the use of inhibitors such as...
chloroquine (4, 5). Monolayers of fibroblasts treated with chloroquine are unable to hydrolyze the LDL taken up from the medium and as a consequence their cellular cholesteryl ester formation is not activated and HMG-CoA reductase activity is not suppressed (4).

To investigate further the role of the lysosome in cellular LDL metabolism, the present studies were conducted utilizing mutant fibroblasts that are deficient in lysosomal acid lipase (11). Monolayers of cells treated with chloroquine (4, 5) has confirmed that: (a) lysosomal acid lipase plays a major role in hydrolyzing cholesteryl esters that enter the cell bound to LDL (12), and (b) the reciprocal regulation of cellular cholesteryl ester formation and cholesterol synthesis initiated by LDL binding to its receptor is dependent upon the subsequent liberation of free cholesterol from LDL by the action of this lysosomal enzyme.

**EXPERIMENTAL PROCEDURE**

**Materials**

- 3-Hydroxy-3-methyl-[3-14C]butyrate (11.1 mCi/mmol), [1-14C]oleic acid (51.8 mCi/mmol), [1,2-14C]cholesterol (52 Ci/mmol), and [4-14C]cholesterol (50 mCi/mmol) were purchased from New England Nuclear Corp. 
- Sodium (carrier-free in 0.05 M NaOH) was obtained from Schwarz/Mann. 
- Cholesterol, 25-hydroxycholesterol, cholesteryl esters, methyl esters of fatty acids, 14% boron trifluoride-methanol, and gas-liquid chromatographic columns were obtained from Applied Science and Supelco. 
- Chloroquine diphosphate was purchased from Sigma Chemical Co. 
- Linoleoyl chloride was purchased from Nu Chek Prep., Inc. 
- Dimethylsulfoxide was obtained from Mallinckrodt.

**Cells**

The normal human fibroblast strain used in this study was the same strain (D. S.) that has been used in our previous studies (10–12). The regulation of lipoprotein and cholesterol metabolism in these cells is typical of that in 20 other normal fibroblast strains that have been studied in our laboratory and that were derived from skin biopsies of healthy adults and children. The fibroblasts from the subject with cholesteryl ester storage disease were derived from a 12-year-old girl (J. R.); these cells have been previously shown to be severely deficient in lysosomal acid lipase activity (12). All cells were grown in monolayer and were used between the 8th and 20th passage. Cell lines were maintained in a humidified incubator (5% CO₂) at 37°C in 75 cm² stock flasks containing Eagle's minimum essential medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 20 mM Tris-chloride, pH 7.4, 24 mM NaHCO₃, 1% (v/v) nonessential amino acids, and 10% (v/v) fetal calf serum. Unless otherwise stated, all experiments were carried out using a standard format: confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin/0.02% EDTA solution and were seeded on Day 0 at a concentration of 1 x 10⁶ cells/dish into Petri dishes (60 x 15 mm) containing 3 ml of growth medium with 10% fetal calf serum. On Day 6, when the cells were in late logarithmic growth, each monolayer was washed with 2 ml of Dulbecco’s phosphate-buffered saline, after which 2 ml of fresh medium containing 5% (v/v) human lipoprotein-deficient serum (LPDS) were added (final protein concentration, 2.5 mg/ml). All experiments were initiated on Day 7 after the cells had been incubated for 24 hours in the presence of human LPDS. All incubations were carried out at 37°C.

**Lipoproteins**

- Human LDL (density 1.019 to 1.063 g/ml) and human LPDS (density >1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (9). Lipoprotein-deficient total serum was prepared as previously described (10). The concentration of LDL is expressed in terms of its protein content. The mass ratio of total cholesterol to protein content in LDL was 1.61:1, and 70% of the total cholesterol was in an esterified form. 
- [1,2-3H]Cholesterol from [1,2-3H]cholesteryl linoleate-LDL (5) was isolated and quantified as in the monolayer assay (9).

**Preparation of [1,2-3H]Cholesteryl Linoleate-LDL**

[1,2-3H]Cholesterol was reacted with linoleoyl chloride in dry pyridine, the mixture extracted as described by Goodman (15), and the resultant [1,2-3H]cholesteryl linoleate was purified by thin layer chromatography (5). The [1,2-3H]cholesteryl linoleate bound to LDL ([1,2-3H]CL-LDL) was prepared by incubation of LDL with [1,2-3H]cholesteryl linoleate in the presence of 10% dimethylsulfoxide as previously described (5). The specific activity of multiple batches of [1,2-3H]CL-LDL ranged between 90,000 and 1,600,000 cpm/nmol of total cholesteryl in LDL. Direct measurements indicated that 1 ng of LDL protein was associated with approximately 0.56 pmol of total cholesteryl (5). For experiments, [1,2-3H]CL-LDL was diluted with native LDL to give the final specific activity indicated in the legends. When [1,2-3H]CL-LDL was prepared by this method, all of its radioactivity was precipitated by an antibody to LDL (5). Moreover, when this material was incubated with normal cells, its uptake and hydrolysis paralleled that of the protein component of LDL labeled with ¹¹C (Ref. 5 and Fig. 3). In addition, fibroblasts from patients with homozygous familial hypercholesterolemia, which have a reduced ability to bind LDL, showed a reduced ability to liberate free [1,2-3H]cholesterol from [1,2-3H]CL-LDL (5). While these preparations of [1,2-3H]CL-LDL have provided a useful marker for studying the cellular fate of LDL-bound cholesteryl esters, it has not yet been established whether the [1,2-3H]cholesteryl linoleate is actually incorporated into the apolar core of the LDL or whether it exists bound to the surface of the lipoprotein.

**Preparation of Cell-free Fibroblast Extracts**

The cells from 30 dishes (60 x 15 mm) were scraped with a rubber policeman into 30 ml of chilled buffer containing 50 mM Tris-chloride, pH 7.4, and 0.15 M NaCl. After centrifugation (900 x g, 5 min, 4°C), the pooled pellet was resuspended in 5 ml of the same buffer, washed once more in the same manner, and the final pellet suspended in 1.5 ml of water. The extract was sonicated using a Branson Sonitron with a microprobe (4, 5) and then centrifuged (2500 x g, 20 min, 4°C); the resulting supernatant was used for enzyme assays.

**Hydrolysis of [1,2-3H]Cholesteryl Linoleate-LDL by Cell-free Extracts**

Each assay contained the following in a final volume of 50 µl: 0.1 mM sodium acetate (pH 3 to 6.5) or 0.1 mM Tris-Cl (pH 7 to 9) at the indicated pH; 0.5 mg/ml of bovine serum albumin; 5 mM 2-mercaptoethanol; 1 mM EDTA; the indicated concentration of [1,2-3H]CL-LDL, and 5 to 125 µg of extract protein. At 37°C for the indicated time, each reaction was terminated with 1 ml of chloroform/methanol (2/1) containing 30 µg of ¹¹C cholesterol (500 cpm). The free [1,2-3H]cholesterol was isolated and quantified as in the monolayer assay described below. Hydrolytic activity is expressed as the nanomoles of [1,2-3H]cholesterol formed from [1,2-3H]CL-LDL per milligram of extract protein, this calculation being based on the measured specific radioactivity of the [1,2-3H]cholesteryl linoleate in each LDL preparation (5). A blank value, which was determined by parallel incubation containing boiled extracts, was subtracted from each value. In the normal cell extract, the blank value was less than 2% of the measured value (5) and in the mutant cell extract the blank value was no more than 50% of the measured value.

**Proteolytic Degradation of [¹¹C]LDL by Cell-free Extracts**

Each assay contained the following in a final volume of 100 µl: 0.1 mM sodium acetate, pH 4.0; 5 mM dithiothreitol; 1 mM EDTA; the indicated concentration of [¹¹C]LDL; and 60 to 75 µg of extract protein. After incubation at 37°C for the indicated time, each reaction was terminated with 2.4 ml of 15% trichloroacetic acid, after which 0.1 ml of solution containing 10 mg of bovine serum albumin was added. The precipitate was removed by centrifugation, the supernatant solution was extracted with chloroform in the presence of hydrogen peroxide to
remove free iodine, and an aliquot of the aqueous phase was counted to determine the amount of \(^{125}\text{I}\)-labeled acid-soluble material formed (2, 4). Previous studies have shown that the bulk of this acid-soluble material is \(^{125}\text{I}\)-iodotyrosine (2, 4). Proteolytic activity is expressed as the micrograms of \(^{125}\text{I}\)-LDL degraded to acid-soluble material per milligram of extract protein. A blank value (\(<0.01\%\) of total added radioactivity) was determined by parallel incubations of \(^{125}\text{I}\)-LDL in the absence of extract.

Uptake and Hydrolysis of \(^{3}\text{H}\)-Cholesteryl Linoleate-LDL by Intact Fibroblast Monolayers

On Day 7 of cell growth after 24 hour incubation in medium containing human LPDS, monolayers were incubated with \[^{3}\text{H}]
cholesterol linoleate-LDL in growth medium containing 5% human LPDS as previously described (5). Where indicated, 0.02 or 0.1 mg chloroauric acid was added to 10 or 20 \(\mu\)l of a 10 \(\mu\)m stock solution made up in Eagle's minimum essential medium adjusted to pH 7. After incubation at 37\(^\circ\)C for the indicated time, each monolayer was washed twice at 4\(^\circ\)C with 3 ml of solution containing 0.15 M NaCl and 50 mM Tricloro-acide, pH 7.4, and 2 mg/ml of bovine serum albumin, followed by a third wash with 3 ml of solution containing 0.15 M NaCl and 50 mM Tricloro-acide, pH 7.4. The cells were then scraped from the dish and extracted with chloroform/methanol, an internal standard containing \[^{3}\text{H}]
cholesterol (50 \(\mu\)g, 5.1 cpmp), and unlabeled cholesteryl linolate (50 \(\mu\)g was added), and the free and esterified \[^{3}\text{H}]
cholesterol were separated by thin layer chromatography on silicic gel sheets sequentially developed with benzene/ethyl acetate (2/1), followed by petroleum ether/benzene (2/1) as previously described (5). Hydrolytic activity is expressed as picomoles or nanomoles of \[^{3}\text{H}]
cholesterol formed from \[^{3}\text{H}]
cholesterol-LDL per millgram of total cell protein. Uptake is expressed as the picomoles or nanomoles of \[^{3}\text{H}]
cholesteryl linolate contained within the cell per milligram of total cell protein. The calculations for both free and esterified \[^{3}\text{H}]
cholesterol were corrected for the recovery of \[^{3}\text{H}]
cholesterol from each dish, which averaged 85%. This method was validated by preliminary experiments showing that: (a) no detectable free \[^{3}\text{H}]
cholesterol was released from the cells into the medium during the first 8 hours of incubation and thereafter less than 5% of the total \[^{3}\text{H}]
cholesterol that was formed appeared in the medium, and (b) the radioactivity in the cholesteryl ester spot on the chromotogram represented almost entirely unhydrolyzed \[^{3}\text{H}]
cholesteryl linolate (5).

Binding and Degradation of \(^{125}\text{I}\)-LDL by Intact Fibroblast Monolayers

On Day 7 of cell growth, monolayers were incubated with \(^{125}\text{I}\)-LDL in growth medium supplemented with 5% human LPDS as previously described (1,2). After the indicated interval, the monolayers were washed with trichloroacetic acid, treated with chlorofom/methanol, and hydrogen peroxide to remove free iodine, and an aliquot of the aqueous phase was counted to determine the amount of \(^{125}\text{I}\)-labeled acid-soluble material formed by the cells and released to the medium (2). Previous studies have demonstrated that the bulk of this acid-soluble material is \(^{125}\text{I}\)-iodotyrosine (2, 4). Degradation activity represents the cell-dependent rate of proteolysis and is expressed as the micrograms of \(^{125}\text{I}\)-LDL degraded to acid-soluble material per mg of total cell protein. A blank value due to the presence of small amounts of acid-soluble material (\(<0.01\%\) of total added radioactivity) was determined in the absence of extract. The preparation was determined at the appropriate LDL concentration for each time interval by incubations of \(^{125}\text{I}\)-LDL in the absence of cells (2). To determine \(^{125}\text{I}\)-LDL binding, the cell monolayers were washed six times and dissolved in 0.1 N NaOH as previously described (1, 2). An aliquot of the dissolved cell solution was counted to determine the cellular content of \(^{125}\text{I}\)-LDL and another aliquot was used to measure cell protein content. Binding activity is expressed as the micrograms of \(^{125}\text{I}\) radioactivity contained within the cell per milligram of total cell protein.

Incorporation of \(^{125}\text{I}\)-Oleate into Cholesteryl Esters by Intact Fibroblast Monolayers

On Day 7 of cell growth, monolayers were incubated with \(^{125}\text{I}\)-oleate bound to albumin in growth medium supplemented with 5% human LPDS (7). After the indicated interval, the cells were washed and harvested, extracted with chloroform/methanol, and the cholesteryl \(^{125}\text{I}\)-oleate isolated by thin layer chromatography as previously described (7). Esterification activity is expressed as picomoles of cholesteryl \[^{125}\text{I}\]oleate formed per mg of total cell protein.

Assay of HMG-CoA Reductase Activity

The rate of conversion of \[^{14}\text{C}]
HMG-CoA to \[^{14}\text{C}]
mevalonate was measured in extracts of detergent-solubilized cells as originally described (9) except that after lactonization the mixture was taken to dryness and the mevalonolactone was extracted once with 0.1 ml of chloroform/methanol (2/1) and spotted directly on the thin layer sheets (16). Enzyme activity is expressed as the picomoles of \[^{14}\text{C}]
mevalonate formed per min per mg of soluble protein.

Measurement of Steroids and Fatty Acids

The content of free and esterified cholesterol in fibroblasts and in LDL was determined by a previously described method in which the steroids were extracted from either washed cell pellets or isolated LDL with chloroform/methanol. The free and esterified cholesterol fractions were separated on silicic acid/Cellite columns, and the cholesterol content in each fraction was measured by gas-liquid chromatography (6). Correction for procedural losses (which averaged 25%) was made by utilizing \[^{3}\text{H}]
cholesterol, cholesteryl \[^{14}\text{C}]
oleate, and stigmastanol as internal standards (6).

The relative composition of the fatty acyl component of the cholesteryl esters and triglycerides in fibroblasts and LDL was determined by two methods.

Method I—A chloroform/methanol extract was subjected to thin layer chromatography on silica gel sheets impregnated with silver nitrate. The silver nitrate impregnation was accomplished by dipping precasted plastic sheets of gel without pyrogen (Brinkmann) into a solution containing 5% (w/v) AgNO₃ in methanol/water (2/1). The dipped sheets were air-dried and then heated at 80\(^\circ\)C for 30 min. When the activated sheets were developed in benzene, this chromotography resolved cholesteryl esters according to the degree of unsaturation of the fatty acid component. Typical Rₚ values were as follows: free cholesterol, 0.07; diunsaturated cholesteryl esters, 0.51; monounsaturated cholesteryl esters, 0.50; and saturated cholesteryl esters, 0.65. Each isolated fraction was eluted from the silver nitrate/silica gel by successive 2-ml washings with chloroform/methanol (2/1), hot benzene/ethyl ether (1/1), and benzene. Following alkaline hydrolysis, the cholesteryl content of each fraction was determined by gas-liquid chromatography (6). The presence of residual AgNO₃ did not affect the chromatographic process. Tracer amounts of \[^{3}\text{H}]
cholesteryl linolate and cholesteryl \[^{14}\text{C}]
oleate were used to correct for procedural losses, which averaged 38%.

Method II—A chloroform/methanol extract was subjected to thin layer chromatography on silicic gel using benzene/ethyl acetate (2/1). This resolved a fraction of triglycerides and mixed cholesteryl esters according to the degree of unsaturation of the fatty acid component. Typical Rₚ values of 0.45 and 0.71, respectively. The lipids were eluted from the silica gel as described in Method I and subjected to transesterification with boron trifluoride/methanol to yield fatty acid methyl esters (17), which were then analyzed by gas-liquid chromatography using a Hewlett-Packard model 3575 research chromatograph with a flame ionization detector and Hewlett-Packard model 3370 B integrator. The methyl esters were methylated on 250 C 100/120 mesh Gas-chrom Q. The rate of the nitrogen carrier gas was 18 ml/min. Average elution times were: methyl palmitate, 299 s; methyl palmitoleate, 368 s; methyl stearate, 502 s; methyl oleate, 605 s; and methyl linolate, 790 s. The sum of the integrated areas of all of the methyl ester peaks was obtained, and the area of each individual component was expressed as a percentage of the total.

Other—Unless otherwise stated, each value in all of the experiments represents the mean of duplicate assays or incubations. The protein concentrations of extracts and whole cells were determined by a modification of the method of Lowry et al. (18), using bovine serum albumin as a standard.

RESULTS

Cell-free extracts of fibroblasts from a patient with cholesteryl ester storage disease were severely deficient in their ability to hydrolyze \[^{3}\text{H}]
cholesteryl linolate bound to LDL (Fig. 1). At pH 4, the degree of reduction in hydrolytic activity toward this lipoprotein-bound substrate was similar to the 95% reduction in hydrolytic activity previously observed in this cell strain utilizing detergent-solubilized cholesteryl \[^{3}\text{H}]
oleate or sym-
While this agent had no effect on the initial rate of lysosomal degradative processes that has previously been shown to block the hydrolysis of both the protein and lytic degradation of '*'I-LDL in both cell strains (Fig. 30).

The metabolic results of the LDL metabolism in fibroblasts were incubated with LDL labeled either in its protein moiety with '*'I or in its cholesteryl ester portion with '*' showing the metabolism of the LDL-bound cholesterol as a function of the concentration of LDL. Experiment A, varying amounts of [H]CL-LDL (52,262 cpm/nmol of cholesteryl linoleate) were incubated for 1 hour at 370 with the indicated extract (normal, 57 pg of protein; mutant, 75 pg of protein) in a final volume of 50 µl at pH 4.3 under standard assay conditions. The amount of free [H]cholesterol formed was determined as described under "Experimental Procedure." The conversion of '*'I-LDL to '*'I-degraded '*'I-LDL was similar in the normal and mutant cells (Fig. 4A). In both cell strains, the cellular content of '*'I-LDL reached a plateau by 2 hours and this level remained constant throughout the subsequent 8 hours (Fig. 4A). As previously reported (2, 4), the steady state content of '*'I-LDL reflects a zero level of hydrolytic activity in these intact mutant cells with '*'I-LDL over the subsequent 8-hour interval (Fig. 4B).

Whereas in the normal cells the metabolism of the cholesteryl ester component of LDL paralleled the metabolism of the protein component, in the mutant cells the metabolism of the cholesteryl esters was selectively reduced (Fig. 4E to 4H). In normal cells, the content of '*'I-cholesterol linoleate reached a steady state after 2 hours, reflecting equal rates of uptake and hydrolysis (Fig. 4E). As it did with the protein component, chloroquine inhibited the hydrolysis of the '*'I-cholesterol linoleate component of LDL (Fig. 4H) and thus caused a progressive increase in its cellular content (Fig. 4F). In the mutant cells incubated without chloroquine, hydrolysis of [*H]CL-LDL showed an initial lag phase (Fig. 4G) during which the cellular content of unhydrolyzed [*H]-cholesterol linoleate continued to accumulate (Fig. 4E). When the cellular content of [*H]-cholesterol linoleate had reached a level 2- to 3 fold higher than in normal cells, free [*H]-cholesterol began to be formed but at a rate that was only about one-third of that of the normal cells (Fig. 4G). As in the normal cells, the hydrolysis of [*H]-cholesterol linoleate in the mutant cells was inhibited by chloroquine (Fig. 4H), suggesting that the low level of hydrolytic activity in these intact mutant cells was due to the residual acid lipase activity demonstrable in vitro (Figs. 1 and 2). Overall, the metabolism of the LDL bound cholesterol linoleate in the untreated mutant cells bore a partial resemblance to that observed in the normal cells treated with chloroquine. However, unlike the situation in chloroquine-treated normal cells in which acid lipase activity was completely inhibited, the residual acid lipase activity in untreated mutant cells was sufficient to allow an appreciable rate of...
cholesteryl ester hydrolysis once the level of substrate (i.e., the \([^{3}H]\)cholesteryl linoleate) had accumulated to abnormally high levels (Fig. 3E).

The degree of reduction in cholesteryl ester hydrolytic activity in the mutant cells was sufficiently pronounced to permit an analysis of the relationship between hydrolysis of the cholesteryl esters in LDL and the subsequent LDL-mediated regulatory events in cellular cholesterol metabolism. Fig. 4 shows the relationship between uptake and hydrolysis of \([^{3}H]\)CL-LDL and the subsequent stimulation of endogenous cholesteryl ester formation. In the normal cells, the hydrolysis of \([^{3}H]\)CL-LDL (Fig. 4B) was followed by an enhancement of cholesterol ester formation as monitored by the incorporation of \([^{14}C]\)oleate into cholesteryl esters (Fig. 4C). The mutant cells lagged behind the normal cells in the formation of free \([^{14}C]\)cholesterol (Fig. 4B) and this lag in hydrolysis was mirrored by a similar delay in the formation of endogenous cholesteryl esters from \([^{14}C]\)oleate (Fig. 4C). \(^3\)

When intact cells were incubated for 4 hours in the presence of increasing concentrations of \([^{3}H]\)CL-LDL and cholesteryl ester hydrolysis was measured, the shape of the LDL concentration curve in the normal and mutant cells was similar (Fig. 5). In both cell strains, the half-maximal value for hydrolysis

\(^{3}\)In the course of these studies, we have observed in the same cell strain a day-to-day variability in the absolute amounts of cellular uptake and hydrolysis of \([^{3}H]\)CL-LDL. Despite this day-to-day variability in absolute rates of uptake and hydrolysis, the same relative differences between the normal and mutant cells have been noted in each of 15 experiments in which the two cell strains have been compared on the same day under similar growth conditions. The major source of this variability relates to the length of time in which the cells are incubated with human LPDS prior to the experiment, cf. Fig. 3 (cells in LPDS for 48 hours) and Fig. 4 (cells in LPDS for 24 hours). Recent studies indicate that the number of LDL receptors per cell is a regulated function. The number of receptors progressively increases for 48 hours following the removal of lipoproteins from the medium and the rate of this increase is inversely proportional to the content of LDL in the fetal calf serum in which the cells have been grown (M. S. Brown and J. L. Goldstein (1975) Cell, in press).
was achieved at approximately 10 μg/ml, a value similar to that previously reported for half-maximal binding of LDL to its receptor (1, 2). Thus, the saturating concentration for hydrolysis of $[^{3}H]CL-LDL$ was much lower in intact cells than in cell-free extracts (Fig. 2), suggesting that in the intact cell LDL binding to its receptor was rate-limiting for cholesteryl ester hydrolysis. At the 4-hour time point studied and at saturating LDL levels, a 7-fold difference in hydrolysis of $[^{3}H]CL-LDL$ was noted between the normal and mutant cells. At a similar early time point, the maximal rate of endogenous cholesteryl ester formation in the mutant cells was also reduced approximately 7-fold at saturating LDL levels (Fig. 6), again suggesting that the rate of endogenous cholesteryl ester formation was dictated by the rate of hydrolysis of exogenous cholesteryl esters contained in LDL.

The LDL-mediated suppression of HMG-CoA reductase activity could also be correlated with the hydrolysis of the cholesteryl esters in LDL (Fig. 7). Thus, at 6 hours, a time when hydrolysis of $[^{3}H]CL-LDL$ in the mutant cells was markedly reduced (Fig. 4B), there was relatively little suppression of HMG-CoA reductase activity in these mutant cells (Fig. 7A). However, at 24 hours after considerable free $[^{3}H]$cholesterol had been formed from LDL (Fig. 4B), HMG-CoA reductase activity in the mutant cells was almost completely suppressed (Fig. 7B). In contrast to their retarded response to LDL, the mutant cells promptly suppressed HMG-CoA reductase activity and developed enhanced cholesteryl esterification capacity in a manner identical with normal cells when treated with 25-hydroxycholesterol (data not shown).

We have previously reported that incubation of normal fibroblasts with LDL resulted in a marked increase in the cellular content of cholesteryl esters (6). The current data suggest that this net accumulation is due to a hydrolysis of the incoming LDL-bound cholesteryl esters by a lysosomal acid lipase, followed by a re-esterification of the liberated free cholesterol within the cell. To further assess the role of the lysosomal acid lipase in this process, the relative fatty acid composition of the cholesteryl esters that accumulated in fibroblasts incubated with LDL was compared with that of LDL itself. Table I shows that the predominant fatty acid in the cholesteryl esters of plasma lipoproteins is well known and has been attributed to the formation of these esters by the plasma enzyme, lecithin:cholesterol acyltransferase, which preferentially transfers polyunsaturated fatty acids from position 2 of lecithin to cholesterol (22). In contrast to its

![Fig. 5](image1.png)

**Fig. 5.** Effect of increasing LDL concentrations on the hydrolysis of $[^{3}H]$cholesterol linolate bound to LDL, by normal (●) and mutant (▲) fibroblast monolayers. Cells were grown under standard conditions, and after 24 hours in human LPDS (Day 7) each monolayer received 2 ml of growth medium containing 5% human LPDS and the indicated concentration of $[^{3}H]CL-LDL$ (176,000 cpm/mmol of cholesteryl linolate), and either no chloroquine or 100 μM chloroquine. After incubation at 37°C for 4 hours, the cellular content of free $[^{3}H]$cholesterol was determined as described under “Experimental Procedure.” Each plotted value represents the percent difference between the amount of $[^{3}H]$cholesterol formed in the absence and presence of chloroquine (5).

![Fig. 6](image2.png)

**Fig. 6 (left).** Effect of increasing LDL concentrations on the rate of cholesteryl ester formation in normal (●) and mutant (▲) fibroblast monolayers. Cells were grown under standard conditions, and after 24 hours in human LPDS (Day 7), each monolayer received 2 ml of growth medium containing 5% human LPDS and the indicated concentration of LDL. After incubation at 37°C for 5 hours, each monolayer was pulse-labeled for 2 hours with 0.1 μCi $[^{3}H]$oleate-albumin (18,431 cpm/mmol), after which the cells were harvested for determination of the cellular content of cholesteryl $[^{3}H]$esters as described under “Experimental Procedure.”

![Fig. 7](image3.png)

**Fig. 7 (center).** Effect of increasing LDL concentrations on suppression of HMG-CoA reductase activity in normal (●) and mutant (▲) fibroblasts. Cells were grown under standard conditions, and after 24 hours in human LPDS (Day 7), each monolayer received 2 ml of growth medium containing 5% human LPDS and the indicated concentration of LDL. After incubation at 37°C for either 6 hours (A) or 24 hours (B), cells were harvested for measurement of HMG-CoA reductase activity as described under “Experimental Procedure.” The data are expressed as a percentage of HMG-CoA reductase activity in control cells that received no LDL. These control values (pmol·min⁻¹ mg of protein⁻¹) were: normal (●), 283 at 6 hours and 246 at 24 hours; mutant (▲), 149 at 6 hours and 231 at 24 hours.

**Fig. 8 (right).** Effect of increasing concentrations of LDL on the content of free (A) and esterified (B) cholesterol in normal (●) and mutant (▲) fibroblasts. Cells were seeded (Day 0) at a concentration of 3 x 10⁵ cells/100-mm Petri dish in 7 ml of growth medium containing 10% fetal calf serum. On Day 3, the medium was replaced with 7 ml of fresh growth medium containing 10% fetal calf serum. On Day 6 each cell monolayer was washed with 7 ml of phosphate-buffered saline after which were added 7 ml of growth medium containing 5% human LPDS. After 24 hours in LPDS (Day 7), the medium was replaced with 5 ml of fresh growth medium containing 5% human LPDS and the indicated concentration of LDL. After incubation at 37°C for 30 hours, cell monolayers were washed, harvested, pooled, and the cellular content of cholesterol and cholesteryl esters was determined as described under “Experimental Procedure.” Each data point represents the mean obtained from three dishes.
abundance in LDL, cholesteryl linoleate comprised only 12% of the cholesteryl esters that accumulated in normal cells after incubation with LDL. The distribution of the fatty acids in cellular cholesteryl esters resembled the distribution in cellular triglycerides, suggesting that both classes of esters were synthesized within the cell and that their fatty acids had originated from a common precursor pool. These data lend further support to the concept that when LDL is taken up by cells its cholesteryl esters are hydrolyzed and the liberated free cholesterol is re-esterified within the cell.

If, as suggested by the previous data, this hydrolysis occurs in lysosomes, then the inhibition of lysosomal hydrolysis with chloroquine should result in the cellular accumulation of cholesteryl esters, the fatty acid composition of which resembles that of the incoming plasma LDL rather than that of endogenous synthesis. As shown in Table II, chloroquine had no effect on the total amount of cholesteryl esters that accumulated when cells were incubated with LDL. However, this agent prevented the shift in the relative distribution of mono-unsaturated and di-unsaturated fatty acids contained in cholesteryl esters so that the resultant cellular pattern resembled that of plasma LDL.

Since the cells from the patient with cholesteryl ester storage disease bore a partial resemblance to chloroquine-treated normal cells in their metabolism of [3H]CL-LDL (Fig. 3), it was of interest to determine whether the fatty acid composition of their cellular cholesteryl esters, like those of the chloroquine-treated cells, resembled those of plasma LDL. Fig. 8 shows that when mutant and normal cells were incubated for 30 hours in LPDS with varying concentrations of plasma LDL, the content of tree and esterified cholesterol rose in both cell strains. However, as shown by the data in Table III, after incubation with LDL the ratio of mono-unsaturated to di-unsaturated fatty acids in cholesteryl esters in the mutant cells resembled more closely that of plasma LDL than did the cellular esters of the normal cells. On the other hand, when cells were incubated in the absence of all exogenous cholesteryl esters and cellular cholesteryl ester formation was stimulated by the presence of 25-hydroxycholesterol (8), the endogenously synthesized cholesteryl esters in both the normal and mutant cells had a high ratio of mono-unsaturated to di-unsaturated fatty acids, a finding that further indicates that the mutant cells have no primary defect in their ability to synthesize cholesteryl esters.

Two types of studies were conducted to determine whether, in addition to hydrolyzing exogenously derived cholesteryl esters, the lysosomal acid lipase also participated in the hydrolysis of endogenously synthesized cholesteryl esters. In the first set of experiments, normal and mutant cells were incubated with LDL in the presence of [14C]oleate so as to label the fatty acid moiety of endogenously synthesized cholesteryl esters (Fig. 9A). The cells were then switched to medium that contained no LDL and were further incubated either in the presence or absence of chloroquine. As expected from the data in Fig. 6, the content of cholesteryl [14C]oleate at the beginning of the second incubation (zero time in Fig. 9A) was approximately 6-fold higher in the normal cells than in the mutant cells. However, after the removal of LDL the relative rate of hydrolysis was similar in both cell strains, one-half of the cholesteryl [14C]oleate disappearing in 12 hours in the normal cells and 8 hours in the mutant cells. In contrast to its potent inhibition of the hydrolysis of exogenous cholesteryl esters (Fig. 3), chloroquine had no inhibitory effect on the hydrolysis of endogenously synthesized cholesteryl [14C]oleate in either the normal or in the mutant cells (Fig. 9A).

To further study the hydrolysis of endogenously synthesized cholesteryl esters, a second set of experiments was performed after formation of endogenous cholesteryl [14C]oleate was stimulated by 25-hydroxycholesterol (8) (Fig. 9B). Under these conditions, the cellular content of cholesteryl [14C]oleate was similar in both the normal and mutant cells, and again the cholesteryl esters were hydrolyzed at the same rate in both cell strains. Considered together, the experiments in Fig. 9A and B suggest that the lysosomal acid lipase, although essential for the hydrolysis of incoming cholesteryl esters contained in LDL,

### Table I

Relative fatty acid composition of cholesteryl esters and triglycerides contained in plasma LDL and in normal fibroblasts incubated with LDL

| Material analyzed | Distribution of fatty acids |  |  |  |  |
|------------------|-----------------------------|---|---|---|---|
|                  | %                          | <16:0| 16:0| 16:1| 18:0| 18:1| 18:2 |
| Cholesteryl esters |                             |    |    |    |    |    |    |
| Plasma LDL       | 9                          | 14 | 6  | 4  | 19 | 48  |    |
| Fibroblasts      | 16                         | 16 | 12 | 9  | 35 | 12  |    |
| Triglycerides    |                             | 14 | 27 | 12 | 10 | 36  | <1  |

|       |  |  |  |
|-------|---|---|---|
|       | % |  |  |
|       |   |  |  |

* Number of carbon atoms/number of double bonds.

### Table II

Effect of chloroquine on fatty acid composition of cholesteryl esters in normal fibroblasts

| Condition of incubation | Cholesteryl ester content | Distribution of fatty acids | Ratio of (b/c) | µg sterol/mg protein |
|-------------------------|---------------------------|-----------------------------|----------------|---------------------|
|                         |                           | Saturated (%) | Mono-unsaturated (%) | Di-unsaturated (%) |
| + LDL                   | 34.1                      | 30 | 58 | 12 | 4.8 |
| + choloroquine          | 38.1                      | 13 | 39 | 48 | 0.8 |
| Plasma LDL used in these incubations | 14 | 28 | 58 | 0.5 |
Cells were seeded (Day 0) at a concentration of $2 \times 10^8$ cells/100 mm Petri dish in 7 ml of growth medium containing 10% fetal calf serum. On Day 3, each cell monolayer was washed with 4 ml of phosphate-buffered saline, after which were added 5 ml of fresh growth medium containing 10% human LPDS, 10 μg/ml of ethanol, and either 50 μg/ml of LDL or 0.5 μg/ml of 25-hydroxycholesterol plus 20 μg/ml of cholesterol added in ethanol. After incubation for 48 hours (Day 5), the cell monolayers from seven dishes were washed, harvested, and pooled. The cellular content of cholesteryl esters and the distribution of fatty acids esterified to cholesterol were determined as described under “Experimental Procedure” by Method 1.

| Genotype | Condition of incubation | Cholesteryl ester content | Distribution of fatty acids | Ratio (b/c) |
|----------|-------------------------|---------------------------|----------------------------|-------------|
|          | LDL                     | 25-Hydroxycholesterol     | Saturated (a) | Mono-unsaturated (b) | Di-unsaturated (c) |       |
| Normal   | +                       | -                         | 17.0 | 19          | 55          | 26          | 2.1  |
| Normal   | -                       | +                         | 24.9 | 30          | 40          | 16          | 2.0  |
| Mutant   | +                       | -                         | 39.9 | 25          | 37          | 38          | 1.0  |
| Mutant   | -                       | +                         | 34.9 | 33          | 53          | 14          | 3.8  |

Plasma LDL used in these incubations

14 28 58 0.5

**DISCUSSION**

The experiment described in this paper support the hypothesis that in human fibroblasts the lysosome is the cellular organelle that hydrolyzes the cholesteryl esters of exogenous LDL and thus provides the cell with free cholesterol, one of its most important constituents. Mutant fibroblasts derived from a patient with the autosomal recessive disorder cholesteryl ester storage disease, which have been reported previously to be severely deficient in lysosomal acid lipase activity (10–12), were shown in the current studies to manifest a reduced ability to hydrolyze the cholesteryl esters contained in LDL. The metabolic defect in these mutant cells was characterized in several different ways. First, using [14C]CL-LDL as a substrate, it was demonstrated that hydrolysis of the [14C]cholesteryl linoleate by intact monolayers was slower in the mutant cells than in normal cells and, moreover, that this hydrolysis was not maximal in the mutant cells until they had accumulated a 3-fold larger intracellular level of the radioactive substrate as compared with normal cells. Second, when incubated with native LDL the mutant cells in the steady state contained a 3-fold larger amount of unhydrolyzed cholesteryl linoleate than did normal cells (cf. the di-unsaturated fatty acyl cholesteryl ester content data in Table III). Third, when LDL was added to cells previously incubated in the absence of lipoproteins, the metabolic consequences that depend upon the liberation of free cholesterol from LDL (i.e., stimulation of endogenous cholesteryl ester formation and suppression of HMG-CoA reductase activity) were delayed in the mutant cells to an extent that was proportional to their slower rate of cholesteryl ester hydrolysis.

An interesting finding emerged when the relative rates of hydrolysis of [14C]CL-LDL were compared in normal and mutant cells using cell free extracts incubated at acid pH and intact cell monolayers. As previously reported (12), the acid lipase deficiency in the extracts of mutant cells was not complete. However, while the cholesteryl esterase activity of the mutant cell extracts was 2.1 and 4.7 μg of sterol/mg of cell protein, respectively. Circles refer to normal cells incubated either in the presence (O) or absence (●) of chloroquine. Triangles refer to mutant cells incubated either in the presence (△) or absence (●) of chloroquine.
mutant extracts was less than one-twentieth that of the normal extracts, the intact mutant cells showed rates of cholesteryl ester hydrolysis that were nearly one-third that of the normal cells. Similar disparities between enzyme activities measured in vitro and in intact cells have been noted in other human genetic disorders involving incomplete enzyme deficiencies (23). In the case of the mutant cells, part of this disparity might be explained by the 3-fold higher substrate concentration (i.e. [3H]cholesterol linoleate) that was present within the intact cells during the time of maximal hydrolysis. Whatever the mechanism for the observed hydrolysis of [3H]CL-LDL in the intact mutant cells, this residual enzyme activity, like the normal, is believed to have occurred within lysosomes since it could be abolished by treatment of the cells with chloroquine.

While the lysosomal acid lipase appears to be required for the hydrolysis of exogenous cholesteryl esters bound to LDL, the data suggest that endogenously formed cholesteryl esters are hydrolyzed by a different mechanism. This conclusion is based on the observations that: (a) the rate of hydrolysis of endogenously synthesized cholesteryl [14C]oleate was the same in the normal and mutant cells and (b) that this hydrolysis was not inhibited by chloroquine. Although the experiments in Fig. 1 using an exogenous substrate (i.e. [3H]CL-LDL) did not demonstrate any hydrolysis at neutral pH, it is possible that such enzyme activity may be demonstrable in vitro by the use of a more physiologic, endogenously synthesized cholesteryl ester substrate.

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