Cholesterol movement between the plasma membrane and the cholesteryl ester droplets of cultured Leydig tumour cells

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The present studies characterize the turnover of plasma membrane cholesterol in MA-10 Leydig tumour cells. Plasma membrane cholesterol of MA-10 cells was slowly internalized and converted into cholesteryl ester. Low-density lipoprotein (LDL) stimulated, in a dose- and time-dependent fashion, plasma membrane cholesterol conversion into intracellular esters. Stimulation of membrane internalization was not simply the consequence of accelerated uptake of membrane with LDL, since binding and internalization of epidermal growth factor and transferrin had no effect on turnover of plasma membrane cholesterol. The protein of LDL is unimportant as well, since delipidated LDL had no effect on membrane turnover. The action of LDL on cholesterol turnover was explained entirely by its contribution to cholesteryl ester stores. The degree of plasma membrane cholesterol internalization and esterification was directly proportional to the size of cellular ester stores.

INTRODUCTION

MA-10 Leydig tumour cells utilize stores of free cholesterol and cholesteryl esters as substrates for steroid hormone synthesis [1–5]. These stores are the most important source of substrate for maintaining short-term (0–4 h) steroidogenesis [1] and can be correlated with the output of progesterone by these cells [2]. The free cholesterol used for steroidogenesis comes predominantly from the plasma membrane [5,6]. Stimulation of MA-10 cells causes plasma membrane cholesterol depletion by two mechanisms [5]. Cholesterol that would normally be transported to the plasma membrane is directed into the steroidogenic pathway, and plasma membrane cholesterol is internalized directly.

Recently it was shown that plasma membrane cholesterol can be induced to internalize by other mechanisms [7,8]. LDL or 25-hydroxycholesterol causes internalization and esterification of cholesterol oxidase-susceptible cell-surface cholesterol in J774 macrophages [7]. Treating fibroblasts with sphingomylinase causes internalization and subsequent esterification of plasma membrane cholesterol [8].

Tabas and co-workers [7] pointed out that a pathway linking acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) with the plasma membrane cholesterol pool may reduce foam cell formation and act as a defence against atherosclerosis. The work of Slotte & Bierman [8] suggests that such a pathway is probably not unique to macrophages but may be generalized. Since we are interested in cholesterol traffic within cells, we wished to determine whether such a pathway exists in MA-10 cells and to clarify the regulation of the pathway. The present studies were directed towards understanding the pathway connecting the plasma membrane and the intracellular cholesteryl ester droplets.

MATERIALS AND METHODS

Materials

[1,2,6,7-³H]Cholesterol (90 Ci/mmol), [26-¹⁴C]cholesterol (54 Ci/mmol), [26-¹⁴C]cholesteryl oleate (54 Ci/mmol) and [1,2,6,7-³H]cholesteryl linoleate were from DuPont/New England Nuclear, and ⁵⁹FeCl (15.9 Ci/g of Fe) was purchased

from Amersham. Human transferrin was from Calbiochem Corp. Eastman Kodak silica thin layer plates were obtained from Fisher Scientific Co. Cholesterol oxidase was purchased from Beckman Instruments. Cholest-4-en-3-one (cholestenone) was from Steraloids Inc. Mouse epidermal growth factor (mEGF) was generously supplied by Dr. Stanley Cohen (Vanderbilt University).

Cell culture

Cell culture conditions for MA-10 cells have been described previously [9,10]. Cells were subcultured on day 0. At 24 h before the experiment the medium was changed to lipoprotein-deficient medium. All experiments were initiated on day 3 by aspirating the medium and washing the dishes with 2 × 2 ml of warm assay medium [Waymouth MB 752/1 with 20 mM-Hepes, NaHCO₃ (1.2 g/l) and BSA (1 mg/ml), pH 7.4]. Culture conditions for human foreskin fibroblasts were identical. All experiments were performed in assay medium.

Preparation of lipoproteins

Human low-density lipoprotein (LDL) was prepared as described [1]. Reconstructed LDL was made using the method of Krieger *et al.* [11] with the modifications described previously [5].

Measurement of cell membrane internalization

Assay medium (2 ml) containing 1 μCi of [³H]cholesterol (90 Ci/mmol; added in ethanol solution) was added to each of 60 × 15 mm dish of cells. After 2 h at 37 °C the dishes were washed with 2 × 2 ml of assay medium. Each dish then received 4 ml of assay medium containing the indicated additions and was returned to the 37 °C incubator until the cells were harvested. Dibutyl-cyclic AMP was added as a 40 × concentrated stock dissolved in a pH 7.4 solution of 10 mM-sodium-phosphate-buffered saline containing 1 mg of BSA/ml. LDL was added after dialysis against 100 vol. of 0.1 M-NaCl to remove EDTA. For harvesting, all dishes were washed with 2 × 2 ml of Hanks balanced salt solution, scraped into tubes and centrifuged at 800 g. Pellets were extracted in 4 ml of chloroform/methanol

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; LDL, low-density lipoprotein; (m)EGF, (mouse) epidermal growth factor.

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(2:1, v/v) containing [^{14}C]cholesterol, [^{14}C]cholesteryl esters, and in some cases [^{14}C]cholestenone as internal recovery standards.

Oxidation procedure

Cellular cholesterol was subdivided into cholesterol oxidase-sensitive and -resistant components using our modification [5] of the method of Lange & Matthies [12].

Lipid methods

Cell extracts were dried and the lipids were separated by t.l.c. as described [5]. In most experiments the area of the t.l.c. plate corresponding to the reference standard was cut out and placed in vials with scintillation cocktail. All data were corrected for procedural losses using internal recovery standards. In some experiments cholesterol and cholesteryl esters were eluted from the t.l.c. plate and the mass was determined. Cholesteryl esters were hydrolysed by KOH in ethanol (3.3% by mass). Mass was determined by gas chromatography [5].

Preparation of the ^{59}Fe -transferrin

To remove the iron, 2 mg of transferrin was dialysed for 3×12 h; first against 0.01 M-EDTA/acetate buffer, pH 7.4, then against 0.1 M- NaClO_4 /0.02 M- NaHCO_3 , and finally against distilled water. The sample was lyophilized and labelled by the method of DiPaola *et al.* [13] using a ^{59}Fe -nitilotriacetate complex.

Determination of ^{59}Fe -transferrin binding

The amount of bound and/or internalized transferrin was measured using cells incubated in assay medium with ^{59}Fe -transferrin. Surface binding was determined at 4 °C. Non-specific binding was determined in the presence of a 200-fold excess of unlabelled transferrin. To terminate incubations, cells were washed with 3×2 ml of ice-cold Hanks salt solution, followed by a single wash with 0.9% NaCl. The cells were then dissolved in 1 ml of 0.5 M-NaOH solution and transferred to plastic tubes. Radioactivity was determined with a Tracer γ -radiation counter.

Other methods

Protein was determined by the method of Bradford [14]. Cholesterol was determined by the method of Rudel & Morris [15] or by gas chromatography.

RESULTS

Effect of dibutyl-cyclic AMP, LDL, or both treatments on the internalization and esterification of membrane-bound [^3H]cholesterol

MA-10 cells treated with dibutyl-cyclic AMP utilize membrane cholesterol as substrate for steroid hormone synthesis [5]. In these experiments [^3H]cholesterol was employed as a marker for the plasma membrane. This label is 84–87% susceptible to cholesterol oxidase [5]. This estimate of the proportion of label on the cell surface is probably too low. Cell fractionation experiments indicated that the large mitochondrial cholesterol pool was not labelled with [^3H]cholesterol and that the label was recovered in cell fractions in proportion to 5'-nucleotidase activity [6]. These latter results indicate that the label and enzyme are in the same plasma membrane location. Fig. 1(a) shows that even unstimulated cells internalized a small but significant portion (about 20%) of membrane [^3H]cholesterol and converted it to cholesteryl ester. Stimulation by dibutyl-cyclic AMP caused a time-dependent loss of both surface [^3H]cholesterol and intracellular [^3H]cholesteryl esters (Fig. 1b). By 6 h only 40% of the initial radioactivity could be detected on the cell surface. Thus dibutyl-cyclic AMP increased the internalization of

Fig. 1. Effects of LDL and dibutyl-cyclic AMP on plasma membrane internalization and esterification

MA-10 cells were grown and the cell surface was labelled with $1 \mu\text{Ci}$ of [^3H]cholesterol as described in the Materials and methods section. After surface labelling, dishes were washed twice with assay medium and further incubated with assay medium alone (a), with 1 mM-dibutyl-cyclic AMP (b), with $50 \mu\text{g}$ of LDL cholesterol/ml (c), or with 1 mM-dibutyl-cyclic AMP plus $50 \mu\text{g}$ of LDL cholesterol/ml (d). Radioactivity in free cholesterol (closed symbols) and cholesteryl ester (open symbols) were determined at 2, 4 and 6 h. The 0 h point was harvested immediately after labelling. The data are expressed in radioactivity per dish as averages $\pm$ range of two independent experiments (triplicate measurements in each experiment).

cholesterol by about 3-fold. Treatment of cells with LDL caused a similar (54%) decrease of surface label (Fig. 1c). In this case, however, the radioactivity that disappeared from the surface could be completely recovered as intracellular cholesteryl esters. When LDL and dibutyl-cyclic AMP were administered simultaneously (Fig. 1d), no greater depletion of surface label was induced than with either treatment alone. Radioactivity disappeared from the cholesteryl esters in a manner similar to that in cells treated with dibutyl-cyclic AMP alone. The internalization process was qualitatively unchanged when the cells were grown in lipoprotein-containing medium (results not shown). The absolute amount of bound label was significantly lower. During these studies we also noted that the particular batch of horse serum used in the growth medium affected somewhat the basal internalization rate.

To investigate further the effects of LDL and dibutyl-cyclic AMP, cells were treated with various concentrations of LDL, either with or without dibutyl-cyclic AMP. LDL caused dose-dependent disappearance of cell surface [^3H]cholesterol (Fig. 2a). At the saturation concentration ($50 \mu\text{g}$ of LDL cholesterol/ml) about 40% of the cholesterol from the cell surface was internalized and converted into cholesteryl ester. The addition of dibutyl-cyclic AMP only slightly increased the disappearance of [^3H]cholesterol surface label, but abolished [^3H]cholesterol incorporation into cholesteryl esters (Fig. 2b).

To investigate the possibility that the stimulation of cholesterol internalization by LDL occurred as a consequence of augmented

Fig. 2. Plasma membrane cholesterol internalization in response to increasing concentrations of LDL with or without dibutyl-cyclic AMP

MA-10 cells were grown and the cell surface was labelled as described in the Materials and methods section. Incubations were for 6 h with the indicated amount of LDL cholesterol alone (a) or with 1 mM-dibutyl-cyclic AMP (b). The radioactivity in free cholesterol (closed symbols) and cholesterol ester (open symbols) was determined. The data are expressed, in radioactivity per dish, as averages $\pm$ range of two independent experiments (triplicate measurements in each experiment).

receptor and membrane internalization associated with receptor-mediated endocytosis [16], we examined the effects of EGF and transferrin, ligands known to be internalized by the same process.

Demonstration of specific ^{59}Fe -transferrin binding in MA-10 Leydig cells

We first showed that MA-10 cells specifically bound and internalized transferrin (Fig. 3). Internalization of ^{59}Fe -transferrin was linear for at least 4 h (Fig. 3a). To demonstrate specificity of binding, cells were incubated at 4 °C for 4 h with ^{59}Fe -transferrin tracer and various concentrations of transferrin. The displacement curve (Fig. 3b) showed that 6.62×10^{-6} M-transferrin (28-fold excess) displaced all specific ^{59}Fe -transferrin binding. Analysis of the curve showed one component binding with an IC_{50} (concn. causing 50% inhibition) of 1.79×10^{-7} M. Specific saturable cell surface binding was measured at 4 °C (Fig. 3c) to block internalization. Non-specific binding represented about one-third of total binding.

Analysis of the saturation binding data revealed that the cells bound ^{59}Fe -transferrin with a high affinity receptor (K_d 4.4×10^{-7} M, Hill coefficient 1), with a capacity of 250 000 binding sites/cell. Cell-associated radioactivity was not saturable at 37 °C (Fig. 3d). Comparison of the cell-associated radioactivity at 37 °C and 4 °C (Figs. 3c and 3d) indicated that the cells incubated for 30 min concentrate more than 20 times as much ^{59}Fe as can be bound to the cell surface.

Delipidated LDL is bound and internalized into the cell

The experiments shown in Table 1 indicated that both reconstructed and delipidated LDL were internalized by the cell. After correcting for different specific radioactivities, the internalization of delipidated LDL was no different than that of reconstructed LDL. In both cases, about 6% of the LDL protein was internalized in 6 h. The tracer quantity of cholesteryl ester in the delipidated preparation was also distributed in the cell in the same proportions as the mass quantities of ester contained in the reconstructed preparation.

EGF, transferrin and delipidated LDL do not affect plasma membrane cholesterol turnover

The data in Table 2 show that none of EGF, transferrin or delipidated LDL had any effect on the internalization of plasma

Fig. 3. Characterization of ^{59}Fe -transferrin binding to MA-10 Leydig tumour cells

All binding studies were performed with cells that had been washed twice in assay medium and then incubated in assay medium containing ^{59}Fe -transferrin. Incubation was terminated by placing the dishes on ice, washing three times with 0.9% NaCl, then dissolving the cells in 0.5 M-NaOH. Radioactivity was quantified by counting the radioactivity in the NaOH digest with a γ -radiation counter. Cells were incubated at 37 °C with $0.24 \mu\text{M}$ - ^{59}Fe -transferrin (a). Specific uptake is shown. This was calculated by subtracting the radioactivity associated with the cells incubated with 200-fold excess of unlabelled transferrin from that associated with cells incubated with the tracer alone. Cells were incubated for 4 h at 37 °C with $0.24 \mu\text{M}$ - ^{59}Fe -transferrin plus the indicated amounts of transferrin (b). Cells were also incubated at 4 °C (c) and 37 °C (d) with the indicated concentrations of labelled transferrin for 0.5 h. Specific uptake is shown. All experiments show the averages $\pm$ range of two experiments (two measurements per experiment).

Table 1. MA-10 cells bind and internalize delipidated and reconstructed LDL equally

Dishes were washed twice with assay medium and the incubated for 6 h with delipidated LDL (dLDL) or reconstructed LDL (rLDL). The LDL was delipidated and either reconstructed using mass quantities of cholesteryl linoleate or labelled using tracer quantities of cholesteryl linoleate. The cholesterol/protein ratio of rLDL averaged $0.8 \mu\text{g}$ of cholesterol/ μg of protein, while the cholesterol content of dLDL was undetectable. The specific radioactivities of dLDL were 25 125, 38 860 and 13 360 r.p.m./ μg of protein, and for rLDL these values were 15 648, 9912 and 11 442 c.p.m./ μg . The radioactivity in free cholesterol and cholesterol ester was determined as described in the Materials and methods section. The data are expressed as averages $\pm$ s.d. of three independent experiments, (triplicate measurements in each).

	Total LDL protein uptake (μg /dish)	^3H Cholesterol radioactivity (%)	
		Free cholesterol	Cholesteryl ester
dLDL	21.8 ± 3.2	52	48
rLDL	23.2 ± 6.0	45	55

membrane cholesterol. Reconstructed LDL that contained quantities of cholesterol linoleate similar to those in native LDL caused cholesterol to shift from the plasma membrane to the ester droplets.

Since the internalization and esterification of [3 H]cholesterol was not affected by receptor-mediated endocytosis or by the protein portion of LDL, we investigated the role of the cholesterol carried by LDL.

Table 2. Plasma membrane cholesterol turnover is not stimulated by activation of receptor-mediated endocytosis

The cell surface was labelled with [3 H]cholesterol for 2 h at 37 °C. After washing twice with assay medium, the cells were further incubated for 6 h with mEGF, transferrin, delipidated LDL (dLDL) or reconstructed LDL (rLDL). Radioactivity in free cholesterol and cholesteryl ester was measured. The data are expressed as c.p.m. of incorporated radioactivity (mean $\pm$ s.d.). The numbers in parentheses are percentages of total cholesterol radioactivity. Each group is the average of two independent experiments (triplicate measurements in each).

Treatment	Cholesterol radioactivity (c.p.m.)	
	Free cholesterol	Cholesteryl ester
Control	154 240 $\pm$ 11 105 (84.5)	28 374 $\pm$ 2224 (15.5)
mEGF (40 μ g/ml)	150 388 $\pm$ 17 712 (84.0)	28 537 $\pm$ 5934 (16.0)
Transferrin (200 μ g/ml)	139 960 $\pm$ 15 209 (85.2)	24 248 $\pm$ 2211 (14.8)
dLDL (50 μ g/ml)	138 137 $\pm$ 11 001 (83.4)	27 432 $\pm$ 1823 (16.6)
rLDL (50 μ g/ml)	87 248 $\pm$ 7223 (55.5)	69 922 $\pm$ 6843 (44.5)

Fig. 4. Effect of cellular cholesteryl ester content on plasma membrane cholesterol internalization and esterification

Cells were incubated overnight with the indicated amounts of LDL cholesterol. On the day of the experiment the cells were washed with lipoprotein-deficient medium and then incubated in the same medium for 2 h. The labelling of the cell surface was done as described in the Materials and methods section. The mass of cellular cholesterol and cholesterol ester (a, c) and internalized [3 H]cholesterol and [3 H]cholesteryl ester radioactivity (b, d) were determined. Changes in free cholesterol (●) and cholesteryl esters (■) are shown. Each group is the average of three independent experiments (triplicate measurements per experiment).

Increased cellular cholesteryl ester content increases [3 H]cholesterol internalization and esterification

Overnight incubation of the cells with increasing concentrations of LDL caused the cellular cholesteryl ester content to increase from 3.5 μ g of cholesteryl ester/plate to 12.8 μ g of cholesteryl ester/plate (Fig. 4c). No significant change in cellular free cholesterol content was observed (Fig. 4a). The ratio of cholesteryl ester to cholesterol increased from 0.26 to 0.75. Internalization and esterification of plasma membrane cholesterol label (Figs. 4b and 4d) increased in proportion to the increase in ester mass. Cells with the highest concentration of cholesteryl esters internalized and esterified 2.2-fold more cholesterol than cells that were not preloaded (zero LDL). These measurements were made after the cells were washed free of LDL and after internalized LDL was metabolized [5] so that it was possible to measure the effect of cholesteryl esters rather than LDL internalization.

Table 3. Plasma membrane internalization and esterification are stimulated by LDL in fibroblast cells

Cells were incubated overnight with (preloaded) or without 50 μ g of LDL cholesterol/ml. For experiments, the cells were washed with medium containing lipoprotein-deficient serum and then incubated in this medium for 2 h. At the end of the incubation the cell surface was labelled with 1 μ Ci of [3 H]cholesterol at 37 °C for 2 h. Dishes were then washed twice with assay medium and incubated for 6 h either alone (control, preloaded) or in the presence of 100 μ g of LDL cholesterol/ml (LDL). The data are expressed as means $\pm$ range for two similar independent experiments (triplicate measurements in each). Values in parentheses are percentages of total cholesterol radioactivity.

	Cholesterol radioactivity (c.p.m.)	
	Free cholesterol	Cholesteryl ester
Control	78 127 $\pm$ 4758	253 $\pm$ 29 (0.3)
LDL	78 192 $\pm$ 17 755	2802 $\pm$ 252 (3.5)
Preloaded	63 031 $\pm$ 8172	4263 $\pm$ 673 (6.3)

Fig. 5. Relationship between plasma membrane cholesterol internalization and esterification and cholesteryl ester mass

MA-10 cells (a) or fibroblasts (b) were grown and the cell surface was labelled as described in the Materials and methods section. After surface labelling, cells were washed twice with assay medium and placed in assay medium for 6 h at 37 °C. Then cells were harvested and the mass and radioactivity present in cholesteryl esters were quantified. The data for MA-10 cells are averages $\pm$ s.d. of three experiments, and the data for fibroblasts are the averages $\pm$ range of two determinations.

[³H]Cholesterol internalization and esterification in human fibroblasts

Table 3 shows that we also could detect internalization of [³H]cholesterol in human fibroblasts. Either acute treatment with LDL or cholesteryl ester loading increased the rate of internalization and esterification. In contrast with the data with MA-10 cells, stimulation was greater in preloaded cells than in cells acutely incubated with LDL. This was probably explained by the cholesteryl ester levels in the cells. Cholesteryl esters made up 5%, 15% and 30% of total cholesterol mass respectively of control, LDL-treated and preloaded cells.

Plasma membrane cholesterol internalization and esterification increase proportionally with cellular cholesterol ester stores

Fig. 5 shows the relationship between plasma membrane cholesterol internalization and esterification and the cellular mass of cholesteryl ester. The data in this Figure are from the experiments of Fig. 4 and Table 3, and thus are from both MA-10 cells and fibroblasts. In the MA-10 cells these data gave a straight line with a regression coefficient of 0.993 ($P < 0.001$). Projecting the regression line back to 'zero cholesteryl ester' indicated that a significant basal, cholesteryl-ester-independent, rate existed. The fibroblast data also indicated a linear and proportionate increase in plasma membrane internalization and esterification as a function of cholesteryl ester mass ($r = 0.971$,

$P < 0.05$). The basal rate in the fibroblasts was, however, much lower than that measured in MA-10 cells.

The close correlation between plasma membrane cholesterol internalization and esterification and cholesteryl ester stores could have been a result of the increased stores and greater cholesteryl-ester-derived cholesterol cycling through the plasma membrane, or a consequence of activation of ACAT by the increased stores of cholesteryl esters. Generally, ACAT activity cannot be readily dissociated from the cellular mass of cholesteryl esters [4], so that these two possibilities cannot be differentiated. The experiments of Fig. 6 allow such a differentiation to be made. These experiments indicated that 25-hydroxycholesterol stimulated ACAT activity in a dose-dependent fashion but did not augment plasma membrane cholesterol internalization and esterification. Thus stimulation of ACAT activity *per se* did not cause the plasma membrane cholesterol to internalize.

DISCUSSION

Initially we hypothesized that (1) constitutive membrane cycling caused the basal rate of membrane cholesterol turnover, and (2) the accelerated rate occurred because of greater membrane internalization during receptor-mediated endocytosis. This hypothesis was based on observations showing that plasma membrane proteins rapidly cycle between the cell surface and the cell interior [17] and observations showing that LDL increases the internalization rate of the LDL receptor [16]. Our results completely disproved the second portion of this hypothesis. We examined the effects of EGF and transferrin and found that they did not stimulate this pathway. The processing of mEGF by cells has been well characterized, is known to require mediation of coated pits [18], and has been studied in MA-10 cells [19,20]. The cellular processing of mEGF differs from that of LDL in that, although both receptors are contained in the same coated pits, the mEGF receptor does not recycle. Transferrin was studied because the transferrin receptor is also internalized into the coated vesicles [21] and because this receptor recycles. We characterized the interaction of transferrin with MA-10 cells and found that the MA-10 cells have saturable transferrin receptors of the type found in reticulocytes [22]. The transferrin receptor number and the molar internalization rate for transferrin was about three times greater than for LDL. Neither mEGF nor transferrin, however, stimulated the basal rate of membrane cholesterol turnover, indicating that membrane internalization (or at least receptor internalization) did not induce this stimulation.

Removing core cholesteryl ester from LDL did not prevent uptake of the lipoprotein, but abolished stimulation of plasma membrane cholesterol turnover. These data suggested that the protein portion of LDL was not involved in stimulation, and directed attention toward LDL cholesteryl ester. Internalized LDL is degraded in the lysosome, releasing free cholesterol that can be incorporated into membranes, synthesized into steroid hormones or esterified [5]. Quiescent MA-10 cells can be made to accumulate significant stores of cholesteryl esters after prolonged incubation with LDL ([2,4]; Fig. 4). Processing of internalized LDL is rapid in the MA-10 cells, so that 4 h after removing LDL, the LDL-derived cholesterol has all been transported to its ultimate destination [5]. Using cells preloaded with LDL and subsequently allowed to process the internalized material in lipoprotein-free medium, cells were produced with various levels of cholesteryl esters. Such ester-loaded cells internalized and esterified plasma membrane cholesterol as well as cells acutely incubated with LDL; moreover, the stimulation was directly proportional to the cellular content of cholesteryl ester. Thus cholesteryl ester content seems to drive stimulated membrane

Fig. 6. Effect of 25-hydroxycholesterol on ACAT activity and plasma membrane internalization and esterification

For measurement of plasma membrane internalization (●), cells were grown and the cell surface was labelled with 1 μ Ci of [³H]-cholesterol as described in the Materials and methods section. After surface labelling, the dishes were washed twice with assay medium and placed in assay medium containing the indicated concentrations of 25-hydroxycholesterol. After 6 h at 37 °C the cells were harvested and the radioactivity present in cholesterol and cholesteryl esters was determined. For measurement of ACAT activity (△) cells were washed and placed in assay medium containing the indicated concentrations of 25-hydroxycholesterol. After 5 h at 37 °C, 200 nmol of [³H]oleate was added and the incubation was continued for a further 1 h at 37 °C. At this time cells were harvested and the radioactivity present in cholesteryl esters was quantified. The radioactivity incorporated into cells incubated with 25-hydroxycholesterol represented 2.7 nmol/dish. All data are the means $\pm$ S.D. of six determinations from two independent experiments.

turnover. This observation may not explain the basal rate. The basal rate of internalization seems to be out of proportion to the low levels of cholesteryl ester found in MA-10 cells.

Earlier studies probably identified the same pathway studied here. Cholesterol-labelled vesicles are taken up slowly by smooth muscle cells [23] or fibroblast cells [24], and some of the label gets incorporated into the cellular cholesteryl esters. The rate of incorporation is very slow and is undoubtedly influenced by the interaction of the vesicles with the cells. It seems likely, however, that these investigators detected basal turnover, as we define it here. We did not previously detect a basal internalization rate [5]. In our previous studies we did not, however, quantify radioactivity accumulating in the cholesteryl ester fraction. More recently it has been shown that LDL stimulates plasma membrane cholesterol internalization and esterification in J774 macrophages [7]. These studies also did not detect a basal internalization rate in these largely ester-deficient cells.

There is a difference between our data and previous data that must be mentioned. The turnover of plasma membrane cholesterol in J774 macrophage cells was stimulated by 25-hydroxy-cholesterol [7]. Neither this oxysteroid nor 7-oxo-cholesterol-stimulated turnover in MA-10 cells. It is not obvious why this difference occurred, although our data would tend to refute the earlier contentions that stimulation of ACAT drives this pathway [7]. Since these oxysteroids regulate cholesterol-regulated processes in MA-10 cells [3] and have no effect on membrane turnover, it would imply that activation of ACAT is not important, and again suggests that cholesteryl esters *per se* may be important. Our data are consistent with this view.

Our results with MA-10 cells and with fibroblasts suggested that this pathway is not unique to macrophages, but may be found in many cells. This pathway may exist to remove excess cholesterol from cells. Thus ester droplet cholesterol would be made available to extracellular cholesterol acceptors arriving at the surface of the cell. Such a function would be totally consistent with our observations that the pathway is largely regulated by cholesteryl ester levels. Recently we performed experiments indicating that cholesteryl ester cholesterol passes through the plasma membrane before being converted into progesterone in cyclic AMP-stimulated MA-10 cells [25]. These experiments have relevance to the present experiments for two reasons. First, they clearly show that ester cholesterol is inserted into the plasma membrane. Secondly, they indicate that this cholesterol must pass through the plasma membrane before subsequent utilization by the cell. Thus it would appear that the steroid-synthesizing cell has simply adapted a general cellular mechanism for ridding the cell of cholesterol to its unique need for a rapidly available substrate source.

Cellular ester levels are not the only factor controlling plasma membrane cholesterol turnover. Dibutyl-AMP-stimulated MA-10 cells rapidly internalize plasma membrane cholesterol and then utilize the cholesterol for steroid hormone

synthesis [5]. This occurs at the same time as cellular ester levels are decreasing or have been depleted, so the process is clearly not cholesteryl ester driven. Depleting fibroblast plasma membranes of sphingomyelin results in membrane cholesterol becoming internalized and esterified [8]. LDL also may work by more than one mechanism. Ester-stimulated plasma membrane turnover is generally less than LDL-stimulated turnover (compare Fig. 1c with Figs. 4b and 4d). It seems possible that LDL cholesterol passing directly to the plasma membrane augments the ester-stimulated rate. The LDL may also influence some other membrane control mechanism not specifically tested for in these experiments.

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