The Fate of Cholesterol Exiting Lysosomes*

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Cholesterol released from ingested low density lipoproteins in lysosomes moves both to the plasma membrane and to the endoplasmic reticulum (ER) where it is re-esterified. Whether cholesterol can move directly from lysosomes to ER or first must traverse the plasma membrane has not been established. To examine this question, the endocytic pathway of rat hepatoma cells was loaded at 18 °C with low density lipoproteins (LDL) labeled with [3H]cholesteryl linoleate, and the label then was chased at 37 °C. The hydrolysis of the accumulated ester proceeded linearly for several hours. Almost all of the released [3H]cholesterol moved to the plasma membrane rapidly and without a discernable lag. In contrast, the re-esterification in the ER of the released [3H]cholesterol showed a characteristic lag of 0.5–1 h. These data are inconsistent with direct cholesterol transfer from lysosomes to ER; rather, they suggest movement through the plasma membrane.

Furthermore, we found that progesterone, imipramine and 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) strongly inhibited the re-esterification of lysosomal cholesterol in the ER. However, contrary to previous reports, they did not block transfer of [3H]cholesterol from lysosomes to the cell surface. Therefore, the site of action of these agents was not at the lysosomes. We suggest instead that their known ability to block cholesterol movement from the plasma membrane to the ER accounts for the inhibition of lysosomal cholesterol esterification.

These findings are consistent with the hypothesis that cholesterol released from lysosomes passes through the plasma membrane on its way to the ER rather than proceeding there directly. As a result, ingested cholesterol is subject to the same homeostatic regulation as the bulk of cell cholesterol, which is located in the plasma membrane.

Cell cholesterol levels are tightly regulated by homeostatic mechanisms. For example, when cells ingest cholesterol in the form of low density lipoprotein (LDL),1 sterol biosynthesis is reduced and excess cholesterol is converted to cholesteryl esters for storage (1, 2). While these processes are well understood, the associated pathways of intracellular cholesterol movement are obscure. It appears that cholesterol moves bidirectionally between the plasma membrane and the ER (3).

Furthermore, the cholesterol released from ingested LDL in lysosomes moves rapidly to the plasma membrane (4). Since the cholesterol derived from the degradation of LDL is re-esterified by ACAT (1, 2), lysosomal sterol must also be transported to the ER.

Two recent studies addressed the question of the pathway taken by cholesterol from lysosomes to ER. One suggested direct movement of cholesterol from lysosomes to ER by a mechanism that is inhibited by amphiphiles (5). The other concluded that approximately 70% of lysosomal cholesterol passes through the plasma membrane prior to esterification, the remainder moving directly between the lysosomes and the ER (6).

In the present study, we have analyzed the fate of ingested LDL [3H]cholesterol esters. We found no evidence for a pathway of cholesterol movement from the lysosomes to the ER that does not include the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—[1α-2α(3H)]cholesteryl linolate (48 Ci/mmol), [1,2-3H]cholesterol (40 Ci/mmol), [1-14C]cholesterol (52 mCi/mmol), and [1-14C]oleic acid (58 mCi/mmol) were from Amersham Corp. Cholesterol oxidase (EC 1.1.3.8; Brevibacterium sp.) was from Beckman Clinical Diagnostics (Carlsbad, CA). U18666A was from The Upjohn Co. (Kalamazoo, MI).

Cells—FU5AH rat hepatoma cells and human foreskin fibroblasts derived from primary explants were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Prior to feeding labeled LDL, fibroblasts were incubated for 16–20 h in medium supplemented with 5% lipoprotein-deficient serum to up-regulate LDL receptors (1). Such preincubation did not substantially stimulate LDL uptake in hepatoma cells (data not shown) and was omitted in experiments with these cells. Similar results were obtained with both cell types. However, because they esterify large amounts of cholesterol, hepatoma cells were used in these studies with the exception of the subcellular fractionation of cell homogenates on Percoll gradients where resolution of membranes was much better for fibroblasts.

Preparation of Labeled LDL—Plasma obtained from healthy volunteers was labeled with [3H]cholesteryl linolate as described (7), and the LDL was isolated by ultracentrifugation (8). The product had a specific activity of ~106 dpm/mg protein. Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum as described (9).

Specific Labeling of Cell Surfaces with Sterols—Cell monolayers were rinsed and covered with phosphate-buffered saline plus the radio-labeled cholesterol solubilized in Triton WR1339 (0.025% final). The flasks were incubated for 20 min at 18 °C, the buffer was removed, and the cells were rinsed and layered with medium for further incubation at 37 °C as described in the figure legends.

Pulse-chase Experiments—The medium was removed from replicate flasks of cells, and the cells were rinsed. Medium containing 5% LPDS plus [3H]LDL (50 μg/ml protein) was layered over the cells, and the flasks were incubated for 1.5–2 h at 18 °C (10). The labeled medium was removed, and the cells were rinsed three times with phosphate-buffered saline. Medium containing 10% fetal calf serum or 5% LPDS was then added to the flasks for further incubation at 37 °C.

Subcellular Fractionation—Cell homogenates prepared as described (11) were mixed with 30% Percoll in 5 mM NaPi, pH 7.5, 0.25 mM sucrose, 1 mM EDTA and layered on a 0.2–ml cushion of 56% sucrose in 5 mM

* This work was supported by National Institutes of Health Grant HL 28448. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LDL, low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; ER, endoplasmic reticulum; LPDS, lipoprotein-deficient serum; [3H]LDL, [3H]cholesteryl linolate-labeled LDL; U18666A, 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one; HPLC, high performance liquid chromatography.
been incubated with [3H] LDL and not warmed. The zero time values were measured in cells that had been incubated for 10 min at 37 °C prior to extraction and assay. Cholesterol mass was scored by its appearance in cholesteryl oleate and cholesteryl palmitate. The free [3H]cholesterol was re-esterified and assayed for free [3H]cholesterol and for the incorporation of [3H]cholesterol into [3H]cholesteryl oleate and [3H]cholesteryl palmitate, represented as the sum of unesterified and re-esterified label expressed as a percentage of total label recovered in the flask. Panel C shows the incorporation into esters of [14C]oleic acid (●) given as total dpm/flask.

**FIG. 1.** Time course of metabolism of [3H]LDL by hepatoma cells. Replicate flasks were incubated for 2 h at 18 °C with medium containing 5% lipoprotein-deficient serum and [3H]LDL. The cells were rinsed and fresh medium containing 10% fetal bovine serum was added to the flasks. At the times indicated, the cells were suspended, treated with cholesterol oxidase, and assayed for the radiolabel in esters, cholesteryl esters, and cholesterol as described under "Experimental Procedures." Panel A shows the hydrolysis of [3H]cholesteryl linoleate calculated as the sum of unesterified and re-esterified label expressed as a percentage of the total label recovered in the flask. Panel B shows the sum of [3H]cholesteryl oleate and [3H]cholesteryl palmitate, representing re-esterified label and given as the total dpm in each flask. Panel C shows [3H]cholestenone (●) and free [3H]cholesterol (○) given as the total in each flask. The zero time values were measured in cells that had been incubated with [3H]LDL and not warmed.

**FIG. 2.** Time course of incorporation into cholesteryl esters of [14C]oleic acid and [3H]cholesterol released from LDL in hepatoma cells. Replicate flasks of cells were labeled with [3H]LDL as described in the legend to Fig. 1. The cells were rinsed, and fresh medium containing 10% fetal bovine serum plus 0.07 µCi/ml [14C]oleic acid was added to the flasks. At the times indicated, the cells were dissociated and assayed for [3H]cholesterol and for the incorporation of 3H and 14C into newly synthesized esters as described under "Experimental Procedures." Panel A shows the hydrolysis of LDL [3H]cholesteryl linoleate calculated as described in the legend to Fig. 1A. Panel B shows the incorporation of [3H]cholesterol into [3H]cholesteryl oleate and palmitate expressed as a percentage of total [3H]cholesterol (●), and the incorporation into esters of [14C]oleic acid (○) given as total dpm/flask.

** RESULTS**

**Time Course of Hydrolysis and Re-esterification of LDL Cholesteryl Linoleate—** A pulse of [3H]cholesteryl linoleate-labeled LDL ([3H]LDL) was allowed to accumulate in pre-lysosomal compartments during a preincubation at 18 °C (10). There was little hydrolysis of the [3H]cholesteryl linoleate during the loading period (Figs. 1A and 2A). Shift to 37 °C evoked rapid hydrolysis. The arrival of the released [3H]cholesterol at the ER was scored by its appearance in cholesteryl oleate and cholesteryl palmitate. The free [3H]cholesterol was re-esterified with lagged kinetics (Fig. 2B).

**Movement of Lysoosomal Cholesterol to the Plasma Membrane—** In the experiment shown in Fig. 1, ~87% of the small amount of free [3H]cholesterol released from LDL in the lysosomes was accessible to cholesterol oxidase after the 2-h incubation at 18 °C (Fig. 1C). Approximately the same fraction of total free cholesterol was in the oxidizable pool at each time point during the subsequent 37 °C chase. This finding confirms that lysosomal cholesterol moves rapidly and continuously to the plasma membrane (4). The data also demonstrate that...
plasma membrane [14C]cholesterol had only a small lag, per-
lesterol was again strongly lagged while the esterification of
medium containing 5% lipoprotein-deficient serum plus [3H]LDL. The
Replicate flasks were incubated for 1.5 h at 18 °C with
toma cells.
thereafter, 3H and 14C were determined in free and (re-)ester-
lysosomal pool with ingested LDL [3H]cholesteryl linoleate and
We prelabeled the
than the activity of the enzyme.
These data suggest that the lag in re-esterification of LDL
the plasma membrane pool with [14C]cholesterol. At intervals
cholesteryl esters (13). We compared the kinetics of bulk cho-
lates in the plasma membrane.
almost all of the free cholesterol released from LDL accumu-
lates in the plasma membrane.
The esterification of bulk cell cholesterol in intact cells is
commonly measured by the incorporation of [14C]oleate into
cholesterol esters (13). We compared the kinetics of bulk cho-
esterification with that of the re-esterification of lysos-
omal [3H]cholesterol (Fig. 2). Incorporation of [14C]oleate into
cholesterol esters proceeded linearly while the esterification of the
[3H]cholesterol from lysosomes once again was lagged.
These data suggest that the lag in re-esterification of LDL
cholesterol reflected the kinetics of its delivery to ACAT rather
than the activity of the enzyme.
Esterification of Plasma Membrane Cholesterol Compared
with Cholesterol Released in the Lysosomes—We prelabeled the
lysosomal pool with ingested LDL [3H]cholesteryl linoleate and
the plasma membrane pool with [14C]cholesterol. At intervals
thereafter, [3H] and [14C] were determined in free and (re-ester-
fied) cholesterol. The re-esterification of ingested LDL [3H]
cholesterol was again strongly lagged while the esterification of
plasma membrane [14C] cholesterol had only a small lag, per-
haps reflecting recovery from the 18 °C incubation (Fig. 3A).
With increasing time of chase, the fraction of each label ester-
ified approached the same value (Fig. 3B).
Effect of Amphiphiles on the Movement of Lysosomal Choles-
terol to the Plasma Membrane—A variety of amphiphiles in-
hibit cholesterol esterification, apparently by blocking move-
ment of plasma membrane cholesterol to the ER (14–16),
perhaps through interaction with a site resembling the multi-
drug-resistance P-glycoprotein (16). Although there is evidence
that progesterone inhibits the transport of nascent cholesterol
to caveolae (17), other studies have shown that the amphiphiles
have no effect on sterol movement from the ER to the plasma
membrane (14, 18). We now have tested their effect on transfer
of [3H]cholesterol from lysosomes to the plasma membrane and
ER.
None of the amphiphiles tested had a significant impact on
either the hydrolysis of the labeled LDL esters or on the move-
ment of the liberated [3H]cholesterol to the cholesterol oxidase
sensitive compartment (the plasma membrane) (Fig. 4). In
some experiments (e.g., Fig. 6), and at higher concentrations,
imipramine and U18666A inhibited the hydrolysis of [3H]LDL,
perhaps by reducing lysosomal acidity (19). However, in no case
did the agents affect the rate of appearance of [3H]cholesterol
in the oxidizable pool.
The effect of progesterone on the transfer to the plasma
membrane of [3H]cholesterol released from the lysosomes of
human fibroblasts was analyzed by subcellular fractionation on
Percoll gradients (Fig. 5). The lysosomes (Fig. 5A, marked by
β-galactosidase) were well resolved from plasma membranes
(Fig. 5, B and C, marked by cholesterol mass). Furthermore,
the distribution of these markers was the same for treated
and untreated cells. The re-esterification of the [3H]cholesterol
released from lysosomes was found in association with the plasma
membranes both in control cells (Fig. 5B) and progesterone-treated
cells (Fig. 5C). Similar results also were obtained with imipramine (not shown). These findings are consistent
with the results obtained with cholesterol oxidase (Fig. 4), which suggested that progesterone and imipramine had no
lysosomal cholesterol. Cells were loaded with $[^3H]$LDL for 2 h at 37 °C in the presence of various amphipaths and then chased for a further 4 h at 37 °C prior to assay of $[^3H]$cholesteryl linoleate hydrolysis and re-esterification. We found that 38% of the $[^3H]$cholesteryl linoleate was hydrolyzed in the control cells, and that 7.5% of this became re-esterified (Fig. 6). Progesterone, imipramine, and U18666A all caused a moderate decrease in the amount of labeled $[^3H]$cholesteryl linoleate hydrolyzed, but profoundly inhibited the re-esterification of the released $[^3H]$cholesterol. We have shown by measuring esterification in cell homogenates supplemented with saturating amounts of cholesterol delivered in Triton WR-1339 that 12 μM progesterone inhibits ACAT activity by less than 20% (15). Similar experiments showed that U18666A and imipramine have no effect on ACAT activity at 2.7 and 50 μM, respectively (data not shown); these were the concentrations used in Fig. 6. That U18666A does not inhibit ACAT also was concluded from the observation that the amphiphile had no effect on the synthesis of cholesteryl oleate in cells treated with 25-hydroxycholesterol (5).

**DISCUSSION**

It is well established that progesterone (21) and hydrophobic amines such as imipramine and U18666A (22) cause the dramatic accumulation of cholesterol in the lysosomes of cultured fibroblasts. This finding was explained by the subsequent observation that these compounds appeared to inhibit cholesterol movement from lysosomes to the plasma membrane (21, 23). The latter hypothesis was supported by more recent studies in which cholesterol oxidase was used to distinguish cholesterol in the plasma membrane from that in intracellular pools (5). In

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contrast, the results shown in Figs. 4 and 5 suggest that the amphiphiles have no effect on cholesterol transfer from lysosomes to plasma membrane. The reason for the discrepancy between our results and the earlier ones is not clear. Since it appears that the amphiphiles do not inhibit cholesterol egress from lysosomes (Figs. 4 and 5), its accumulation in the lysosomes of treated cells (21, 22) must have another explanation. In this regard, we have shown recently that treating cells with these amphiphiles induces the accumulation of excess sterol at the cell surface, which then is transferred to lysosomes in a process not normally visible.2 Thus, the large amount of lysosomal cholesterol found in amphiphile-treated cells is not trapped there but appears to be dynamically derived from the plasma membrane.

The second major finding presented here relates to the fate of [3H]cholesterol released from LDL [3H]cholesteryl linolate in lysosomes. The lag in esterification of the sterol suggests that it passes through an intermediate compartment prior to esterification in the ER. This compartment could be intracellular; however, we shall argue below that it is most likely to be the plasma membrane.

Fig. 1C shows that most of the [3H]cholesterol released in lysosomes moves immediately to the plasma membrane (also see Ref. 4). About 85% of cell-free [3H]cholesterol was associated with the plasma membrane at all times during the chase. The fraction of cell cholesterol mass in the oxidizable pool was also 85% (not shown, but see Ref. 20). Of the ~15% of unoxidized cell cholesterol mass, a major fraction has been shown to be endocytic (20). Thus, the data suggest that most, and perhaps all, of the [3H]cholesterol emerging from the lysosomes immediately moved to the cell surface and its derivatives. The question then becomes whether there is evidence for a direct pathway from lysosome to ER, however minor in magnitude. As outlined below, our findings suggest not.

It can be argued from the kinetic data in Figs. 1–3 that the cholesterol emerging from the lysosomes mixed with cholesterol from the plasma membrane prior to re-esterification in the ER. If, upon release from the lysosomes, cholesterol had moved directly to the ER, the time course of its esterification would have been hyperbolic, as was the time course of its arrival at the plasma membrane (Fig. 1C). However, this was not observed (Fig. 1B). We interpret the lag in the re-esterification of the ingested [3H]cholesterol to reflect the profound isotope dilution that this probe experiences upon delivery to the plasma membrane. The accelerating time course would then reflect the progressive rise in the specific labeling of the plasma membrane cholesterol pool. Furthermore, the rate of esterification of lysosomal cholesterol still was increasing sharply after approximately 1 h of chase, even though the rate of hydrolysis of [3H]cholesteryl esters had begun to level off (Fig. 1, A and B). In contrast, the kinetics of movement of [3H]cholesterol to the plasma membrane paralleled the hydrolysis of the [3H]cholesteryl linolate (Fig. 1, A and C), consistent with a direct relationship between the two processes.

If lysosomal cholesterol had moved directly to the ER, its representation in the nascent ester pool would initially have been at least as great as that of plasma membrane cholesterol. That this was not the case is shown in Fig. 3, B. It is also significant that while the esterification of plasma membrane [14C]cholesterol initially was proportionately greater than that of lysosomal [14C]cholesterol, the contributions from the two pools equalized after 2–3 h of chase (Fig. 3, B). This result suggests that both labels had entered the same substrate pool, namely the plasma membrane.

The inhibition of the esterification of lysosomal cholesterol by progesterone, U18666A, and imipramine (Fig. 6) is unlikely to be due to a block in the export of cholesterol from lysosomes since transfer to the plasma membrane is unaffected by these agents (Fig. 4). Nor can it be attributed to inhibition of ACAT activity that is essentially unaffected by the concentrations used in these experiments. Rather, we suggest that the block in esterification is due to the inhibition of movement of plasma cholesterol to the ER by the amphiphiles (14). That is, the most parsimonious hypothesis is that these compounds act at a single site. In this case, there is no need to postulate a direct route of cholesterol movement from lysosomes to ER.

The analysis presented above assumes that if cholesterol movement from lysosomes to ER occurs, it is as rapid as is transfer to the plasma membrane. The amphiphiles could, in principle, inhibit transfer through an intracellular membrane in the pathway from lysosomes to ER. Nonetheless, the simplest hypothesis that explains all our data is that only one major intermediate compartment is involved, the plasma membrane.

While the bulk of cellular free cholesterol resides in the plasma membrane, the proteins that regulate cholesterol homeostasis are in the ER. We have proposed that the abundance of cholesterol in the plasma membrane (and hence in the cell) is communicated to the ER by a stream of cholesterol that moves bi-directionally between these two compartments (14, 24). It makes sense in terms of this model that lysosomal cholesterol does not move directly to the ER but first enters the plasma membrane. In this way, ingested cholesterol is integrated into the bulk pool that determines the extent of cholesterol transfer to the ER for esterification and other regulatory reactions (24).

Acknowledgements—We thank Dr. T. L. Steck for helpful discussions and critical reading of the manuscript, for which we are also grateful to Kristen Page.

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