Evidence for a Cholesterol Transport Pathway from Lysosomes to Endoplasmic Reticulum That Is Independent of the Plasma Membrane*

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We have studied the movement of low density lipoprotein (LDL)-derived cholesterol in cultured Chinese hamster ovary cells. Our hypothesis is that when LDL cholesterol is effluxed from lysosomes, the bulk of LDL cholesterol is mobilized to the plasma membrane, while another pathway delivers LDL cholesterol from lysosomes to acyl-CoA/cholesterol acyltransferase (ACAT) in the endoplasmic reticulum. Three lines of evidence support this model. First, LDL cholesterol transport to ACAT can be blocked without inhibiting the movement of cholesterol from lysosomes to plasma membrane or from plasma membrane to endoplasmic reticulum. Second, LDL cholesterol transport to ACAT is normal in a Chinese hamster ovary mutant with defective plasma membrane-to-ACAT movement. Third, LDL cholesterol is not diluted by the plasma membrane cholesterol pool before reaching ACAT. Our evidence supports a vesicular model of cholesterol transport from lysosomes to the endoplasmic reticulum that is independent of the plasma membrane.

All cells possess specific cell surface receptors capable of binding and internalizing low density lipoproteins (LDLs)3 (1). Upon endocytosis, LDL is transported to lysosomes, where the protein is degraded and the cholesterol ester core is hydrolyzed. Free cholesterol leaves the lysosomes and is transported to the plasma membrane, where most cellular cholesterol resides (2, 3). Cholesterol also moves into the cell interior, where it can be converted to cholesterol ester by the endoplasmic reticulum

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The abbreviations used are: LDL, low density lipoprotein; ER, endoplasmic reticulum; ACAT, acyl-CoA/cholesterol acyltransferase; CHO, Chinese hamster ovary; U18666A, 3-β-(diethylamino)-ethoxy-androst-5-en-17-one; FITC, fluorescein isothiocyanate; 25-HC, 25-hydroxycholesterol; BSA, bovine serum albumin; CL, cholesterol linoleate; HBSS, Hank’s balanced salt solution; IC50, 50% inhibitory concentration; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; CD, 2-hydroxypropyl-β-cyclodextrin; NCS, newborn calf serum; LPDS, lipoprotein-deficient serum; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-di-phenyl tetrazolium bromide.

** Experimental Procedures

Materials

- [1,2-14C]Oleic acid (50 mCi/mmol), [1,2,6,7-2H]cholesterol (46.5 Ci/mmol), [1,2,6,7-2H]cholesterol linoleate (86 Ci/mmol), and cholesterol [1-14C]oleate

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Cultured Cells, Preparation of LDL, Lipoprotein-deficient Serum, Media, and Buffers

LDL was prepared by ultracentrifugation (10). LDL labeled with [3H]cholesterol linoleate (CL) ([3H]CL-LDL) was prepared with an average specific activity of 17,000 cpm/nmol of total cholesteryl linoleate (11). Lipoprotein-deficient serum was prepared as described, omitting the thrombin incubation (10). The following media were prepared: H-5% NCS (Ham's F-12 medium containing 5% (v/v) newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES, pH 7.1); H-5% LPDS and H-1% LPDS (H-5% NCS in which 5% (v/v) newborn calf serum was replaced with 5% (v/v) lipoprotein-deficient calf serum, respectively); H-5% LPDS/mev (H-5% LPDS containing 20 μM mevinolin and 0.5 mM mevalonate); and H-BSA (Ham's F-12 medium containing 0.2% BSA). The corresponding dialysis modified Eagle's medium-based media were prepared using fetal calf serum.

All the buffers were prepared: TBS (50 mM Tris-Cl and 155 mM NaCl, pH 7.4) and PBS (1.5 mM KH₂PO₄, 8 mM NaH₂PO₄, 2.7 mM KCl, and 137 mM NaCl, pH 7.3).

All cells were grown in a monolayer in a humidified incubator (5% CO₂) at 37 °C. CHO-K1 cells and CHO mutants 2-2, 4-4, and 3-6 were seeded into 12-well plates (15,000 cells/well) in H-5% NCS. On day 2, cells were washed with HBSS and fed H-5% LPDS. On day 4, cells were incubated as described in the Fig. 8 legend. After 8 h, cells were washed with TBS. Lipids were extracted with hexane/isopropyl alcohol (3:2), and a chromatography using heptane/diethyl ether/glacial acetic acid (90:30:1) was isolated from cells incubated with [3H]CL-LDL and 25-HC. 25-HC amine inhibition. This could be due to inhibition of an LDL-specific component.

Application of LDL [3H]cholesterol and [14C]oleate into Cholesterol Esters

On day 0, CHO cells were seeded into six-well plates (25,000 cells/well) in H-5% NCS. On day 1, cells were washed in Hanks' balanced salt solution (HBSS) and refed H-5% LPDS. On day 3, cells were refed H-5% LPDS. Experiments were conducted on day 3 or 4 as described in the figure legends. Cells were then washed with TBS, and lipids were extracted with hexane/isopropyl alcohol (3:2). A chromatography standard was added (50 μg of cholesterol, 20 μg of cholesteryl linoleate, 20 μg of cholesteryl oleate, and 0.001 μCi of [14C]cholesteryl oleate) ([3H]cholesterol, [3H]cholesteryl linoleate, and [3H]cholesteryl oleate were quantified as described (12). Radioactivity was measured with liquid scintillation counting using ReadySafe. Lipid extraction, monolayer incubated with 0.1 M NaOH, and aliquots were taken for protein determination (13). The 50% inhibitory concentrations (IC₅₀) were calculated by regression analysis.

Cholesterol Oxidase Treatment

Comparison of CHO and Hepatoma Cells—On day 0, CHO cells were seeded into 12-well plates (15,000 cells/well) in H-5% NCS. On day 2, cells were refed H-5% LPDS. On day 3, cells were fed H-5% LPDS/mev containing 20 μg/ml [3H]CL-LDL and the indicated concentrations of U18666A and imipramine. After 2 h at 37 °C, cells were cholesterol oxidase-treated, and the conversion of LDL [3H]cholesterol to [3H]cholestenone was quantified. Hepatoma cells were subjected to the identical experiment in parallel using the appropriate Dubecco's modified Eagle's medium-based media. Statistical comparisons were made using a Student's t test, unpaired Student's t test (GraphPad Software In-Stat, GraphPad Software version 2.02).

Comparison of Slote and Langer Methods—On day 0, CHO cells were seeded into 100-mm dishes (1000 cells/dish) containing coverslips in H-5% NCS. On day 2, cells were refed H-5% NCS with 1 μCi/dish [3H]cholesterol. On day 3, cells were refed H-5% NCS. On day 4, cells were subjected to no treatment or to cholesterol oxidase treatment as described (2). Coverslips were removed and processed for filipin fluorescence microscopy (9). Dishes were washed with PBS, and lipids were extracted with hexane/isopropyl alcohol (3:2). [3H]Cholesterol and [3H]cholestenone were quantified (7) using one-fifth of the sample. Cholesterol and cholestenone mass were quantified by gas-liquid chromatography using a J & W Scientific DB-17 column (15 m, 0.53-m inner diameter) (Alltech Associates).

RESULTS

Cholesterol Transport from Lysosomes to ER

Amphotericin B Killing

On day 0, CHO cells were seeded in 96-well plates (5000 cells/well) in H-5% NCS. On day 1, cells were refed H-5% LPDS. On day 2, cells were refed H-5% NCS, H-5% LPDS, or H-5% LPDS/mev. After 8 h, cells were refed the same media with additions indicated in the Fig. 4 legend. On day 3, cells were incubated (3 x 5 min) in H-5% LPDS and then refed H-1% LPDS with or without 100 μg/ml amphotericin B. After 5 h, cells were washed with HBSS. Cell viability was assessed using a colorimetric 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (15, 16). Cells were incubated with 2.5 mg of MTT/ml of H-5% NCS for 2 h, after which the medium was removed and the reaction product was dissolved in Me₂SO. Plates were read using a Molecular Dynamics enzyme-linked immunosorbent assay reader at 560 nm.

Basal Esterification of Plasma Membrane Cholesterol

On day 0, CHO cells were seeded into 12-well plates (10,000 cells/well) in H-5% NCS. On day 2, cells were washed with HBSS and refed 0.5 ml of H-5% LPDS. Additions of 1 μM [3H]cholesterol in ethanol were made at staggered times. U18666A and imipramine were added 11.5 h before harvest. On day 4, cells were washed with TBS. Lipids were extracted with hexane/isopropyl alcohol (3:2), and a chromatography standard was added (50 μg of cholesterol, 30 μg of cholesteryl oleate, and 0.001 μCi of [14C]cholesteryl oleate). [3H]Cholesterol and [3H]cholesteryl oleate were separated by thin layer chromatography using toluene/ethyl acetate (2:1) and visualized with iodine. [3H]Cholesteryl oleate formation is expressed as a percentage of total cellular [3H]cholesterol and represents the average of triplicate values.

FITC-Dextran Uptake and Fluorescence Microscopy

On day 0, CHO cells were seeded into two-well chamber slides (Falcon 4102, 20,000 cells/well) in H-5% NCS. On day 2, cells were washed with HBSS and fed H-5% LPDS. On day 3, cells were incubated as described in the Fig. 6 legend and then washed with PBS and fixed with 3% paraformaldehyde for 30 min. Cells were washed with PBS and mounted. Fluorescence images were obtained using a Zeiss IM35 microscope (×40 objective) and photographed using Kodak Elite II 400 film.

Incorporation of LDL [3H]cholesterol and [14C]oleate into Cholesterol Esters

On day 0, CHO cells were seeded into six-well plates (25,000 cells/well) in H-5% NCS. On day 2, cells were washed with HBSS and fed H-5% LPDS. On day 4, cells were incubated as described in the Fig. 8 legend. After 8 h, cells were washed with TBS. Cells incubated with [3H]CL-LDL were harvested as described above. Cholesteryl [14C]oleate was isolated from cells incubated with [3H]cholesterol by thin layer chromatography using heptane/diethyl ether/glacial acetic acid (90:30:1) and visualized with iodine. Radioactivity was measured with liquid scintillation counting in ReadySafe. Protein was determined as described above. Cholesterol esterification is defined as pmol of cholesteryl [14C]oleate or [3H]cholesteryl oleate formed per μg of protein. Time 0 values were subtracted from those of later time points.

Hydrophobic Aminie Inhibition of LDL Cholesterol Transport to ACAT—Upon LDL uptake, the expanded cellular cholesterol pool activates ACAT. ACAT catalyzes the esterification of cellular cholesterol as well as LDL cholesterol, as measured by incorporation of [3H]oleic acid into cholesteryl [3H]oleate. LDL stimulation of ACAT is exquisitely sensitive to hydrophobic amine inhibition. This could be due to inhibition of an LDL-generated signal to activate ACAT or to inhibition of LDL cholesterol movement from lysosomes to the ER. To distinguish between these possibilities, we tested the effects of hydrophobic amines on cells incubated with [3H]CL-LDL and 25-HC. 25-HC activates ACAT independently of a signal from LDL, as this activation is not inhibited by U18666A at concentrations up to 50 μM (7, 9). [3H]CL-LDL is hydrolyzed in lysosomes to [3H]cholesterol. LDL [3H]cholesterol incorporation into [3H]cholesteryl oleate was quantified as a measure of LDL [3H]cholesterol transport from lysosomes to the ER.

When CHO cells were incubated for 6 h with [3H]CL-LDL in the absence of 25-HC, 7% of the LDL [3H]cholesterol had been...
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**Fig. 1. Effect of U18666A and imipramine on LDL [3H]cholesterol esterification.** CHO cells were grown as described under “Experimental Procedures.” Monolayers were incubated in H-5% LPDS/mev containing 20 μg/ml [3H]CL-LDL, in the absence (C) or presence of 1 μg/ml 25-HC (■), and U18666A (panel A) or imipramine (panel B) for 6 h at 37 °C. [3H]Cholesterol, [3H]cholesteryl linoleate, and [3H]cholesteryl oleate were analyzed as described under “Experimental Procedures.” Data points represent the [3H]cholesteryl oleate formed as a percentage of [3H]cholesterol plus [3H]cholesteryl oleate and are the average ± S.D. of triplicate wells, except for 0.18 μM and 0.4 μM in panel A, which represent the average of duplicate wells ± range of the mean.

transported to ACAT and incorporated into [3H]cholesteryl oleate (Fig. 1A). When ACAT was activated by inclusion of 25-HC, 21% of the LDL [3H]cholesterol was metabolized to [3H]cholesteryl oleate. U18666A caused a concentration-dependent inhibition of LDL [3H]cholesterol esterification; an IC50 of 0.09 μM was calculated by regression analysis. The mean ± S.D. IC50 for experiments was 0.14 ± 0.06 μM, which is similar to the IC50 for U18666A inhibition of LDL stimulation of cellular cholesterol esterification, 0.11 μM (7). The IC50 for U18666A inhibition of [3H]cholesterol oleate formation was the same in the absence and presence of 25-HC (data not shown).

In a separate experiment, imipramine inhibition of LDL [3H]cholesterol incorporation into [3H]cholesteryl oleate was measured in the presence of 25-HC. Imipramine caused a concentration-dependent inhibition of LDL [3H]cholesterol esterification, with an IC50 of 9.1 μM (Fig. 1B). The mean IC50 value for three experiments was 10.0 ± 2.8 μM, which again is very similar to the IC50 value for imipramine’s inhibition of LDL stimulation of cellular cholesterol esterification, 7.3 μM (7).

At these hydrophobic amine concentrations, LDL cholesterol is able to move freely from lysosomes to plasma membrane and from plasma membrane to ER. These inhibition results indicate that LDL cholesterol moves from lysosomes to ACAT via a pathway that is independent of the plasma membrane.

**Hydrophobic Amine Inhibition of LDL Cholesterol Transport to the Plasma Membrane**—In our previous study (7), the lysosome to plasma membrane cholesterol transport pathway was assessed by pulsing cells with [3H]CL-LDL and determining the amount of LDL [3H]cholesterol that is sensitive to exogenously added cholesterol oxidase (14), i.e. at the plasma membrane (2). However, our results were questioned by Lange et al. (17), who found that LDL [3H]cholesterol moves to a cholesterol-oxidase accessible pool, even in the presence of hydrophobic amines. They conclude that all LDL cholesterol is transported to the plasma membrane before moving to the cell interior, and that the hydrophobic amine inhibition of LDL cholesterol transport to ACAT is due solely to a block in plasma membrane-to-ER movement. This has led us to study the FUSAH rat hepatoma cells used by Lange and use an additional experimental approach to assess LDL cholesterol movement to plasma membrane.

CHO and hepatoma cells were incubated for 2 h with [3H]CL-LDL in the absence or presence of 1.5 μM U18666A or 100 μM imipramine. The amount of LDL-derived [3H]cholesterol sensitive to cholesterol oxidase treatment was determined using the method of Slotte et al. (14) (Fig. 2). In CHO cells, 48% of LDL [3H]cholesterol was oxidized to [3H]cholestene, as seen previously (7). U18666A and imipramine significantly inhibited LDL [3H]cholesterol movement to an oxidase-accessible pool (p = 0.01 and 0.02, respectively). In hepatoma cells, 65% of LDL [3H]cholesterol was oxidized in the absence of hydrophobic amines. U18666A caused significant inhibition of LDL [3H]cholesterol movement, while imipramine had an inhibitory effect that cannot be considered statistically significant.

Lange et al. (17) found no effect of U18666A and imipramine on accessibility of LDL cholesterol to cholesterol oxidase. We attribute this to their using a hepatoma cell line that is less sensitive to hydrophobic amine inhibition than CHO cells and to their using a cholesterol oxidation method that we find oxidizes intracellular pools of cholesterol. Fig. 3 shows fluorescence microscopy of filipin-stained CHO and FUSAH cells cultured in H-5% NCS. Filipin is a fluorescent polyene antibiotic that binds specifically to cholesterol and is used to detect cellular cholesterol pools (18). Untreated CHO cells exhibit filipin staining at the plasma membrane and in a punctate distribution, most likely representing endosomes and lysosomes (Fig. 3A). Each cell has one bright spot that we identified as the Golgi complex by co-localization with Bodipy FL C5-ceramide (Ref. 19 and data not shown).

Fig. 3B shows CHO cells after cholesterol oxidase treatment by the method of Slotte (14). Filipin fluorescence was no longer visible at the plasma membrane, but the intracellular staining was still present, as shown previously (20). This treatment oxidized 81% of cholesterol mass and 76% of [3H]cholesterol. Fig. 3C shows CHO cells after cholesterol oxidase treatment by the method of Lange (2). Some cells showed a diffuse fluorescence, but no distinct intracellular fluorescence was visible. This method oxidized 92.7% of cholesterol mass and 85.5% of [3H]cholesterol.

Our cholesterol oxidase experiments demonstrating that hy-
hydrophobic amines inhibit LDL cholesterol transport to the plasma membrane are in agreement with several other experimental approaches. We have now employed an amphotericin B test to demonstrate hydrophobic amine inhibition of LDL cholesterol transport to the plasma membrane. Amphotericin B is a polyene antibiotic that forms pores in cholesterol-rich membranes (21–23). Cells capable of transporting LDL cholesterol to the plasma membrane are lysed and killed by amphotericin B treatment, while cells with impaired transport of LDL cholesterol to the cell surface survive (24). In Fig. 4, we show that U18666A and imipramine prevented LDL cholesterol from causing amphotericin B-mediated cell killing.

CHO cells were incubated in various media for 16 h and treated with amphotericin B for 5 h, and then cell survival was evaluated using a colorimetric MTT assay. Cells cultured in H-5% LPDS/mev had a reduced plasma membrane cholesterol content because cholesterol synthesis is inhibited by mevinolin, and the media contain no lipoproteins. These cells survived amphotericin B treatment and exhibited high levels of MTT cleavage. LDL addition restored the plasma membrane cholesterol content and caused cells to be killed by amphotericin B (Fig. 4A). However, when LDL was added along with U18666A (Fig. 4B) or imipramine (Fig. 4C), cell killing was prevented. To prevent amphotericin B-mediated cell killing, hydrophobic amines had to be present throughout the LDL incubation. When cells were incubated with LDL for 16 h and then either U18666A or imipramine was added during the last hour of the incubation, cells were effectively killed by amphotericin B (data not shown). Our results indicate that, in the presence of hydrophobic amines, LDL cholesterol is not transported to the plasma membrane, i.e. not accessible to cholesterol oxidase or amphotericin B.

Hydrophobic Amine Inhibition of the Plasma Membrane to the ER Cholesterol Transport Pathway—Lange et al. (17) attribute hydrophobic amine inhibition of LDL cholesterol esterification to inhibition of the plasma membrane to the ER pathway. However, we found no effect of imipramine on this pathway. In our previous study (7), the plasma membrane to ER pathway was evaluated by treating [3H]cholesterol-labeled cells with sphingomyelinase (25). Digestion of plasma membranes sphingomyelin causes a redistribution of cholesterol within the plasma membrane and movement of 5–10% of plasma membrane cholesterol to the ER (26). The arrival of [3H]cholesterol in the ER was quantified by formation of [3H]cholesteryl esters. Recently, Skiba et al. (27) showed that sphingomyelinase treatment causes extensive plasma membrane vesiculation. We were concerned that sphingomyelinase treatment may not activate the normal mechanism of transport. Therefore, we measured hydrophobic amine inhibition of basal cholesterol movement from the plasma membrane to the ER.

CHO cells incubated for various times with [3H]cholesterol show a time-dependent incorporation of [3H]cholesterol into [3H]cholesteryl esters, reaching steady state at 10–12 h (Fig. 5A). U18666A inhibited the basal movement of plasma membrane cholesterol to ACAT with an IC50 of 0.6 μM (Fig. 5B), which is similar to the IC50 for U18666A inhibition of sphingomyelinase-stimulated esterification, 0.5 ± 0.1 μM (7). Imipramine consistently showed a very slight inhibition at 10 and 30 μM but no significant difference at 100 μM (Fig. 5C). This finding is consistent with imipramine’s lack of inhibition of sphingomyelinase stimulation of cholesterol esterification (7, 26). It underscores our assertion that imipramine inhibition of lysosome to ER cholesterol movement cannot be due to effects on plasma membrane-to-ER transport.

This result also indicates that [3H]cholesterol added to the medium in ethanol is transferred to the plasma membrane. If a substantial portion of the [3H]cholesterol had adhered to serum components and been delivered to lysosomes, we would have seen imipramine inhibition of [3H]cholesterol esterification.

**Effect of U18666A on Plasma Membrane Vesiculation Induced by Sphingomyelinase and Energy Poisons—**U18666A inhibits basal movement of plasma membrane cholesterol to the ER, as well as accelerated movement induced by plasma membrane sphingomyelin digestion (7, 26). One possible mechanism for U18666A action is inhibition of plasma membrane internalization. As stated above, Skiba et al. (27) showed that sphingomyelinase treatment induced plasma membrane internalization, which was viewed as punctate fluorescence throughout the cell by including FITC-dextran in the culture medium. Sphingomyelinase treatment in the presence of energy poisons caused the vesicles to remain in the cell periphery. However, energy poisons did not interfere with sphingomyelinase-induced delivery of plasma membrane cholesterol to ACAT. Since U18666A does inhibit sphingomyelinase-induced delivery of plasma membrane cholesterol to ACAT (7, 26), we wanted to determine if U18666A inhibits plasma membrane vesiculation or delivery of vesicles throughout the cell.

CHO cells were preincubated with and without U18666A and energy poisons for 10 min and then incubated with the same medium containing FITC-dextran, with or without sphingomyelinase, for 30 min before fixation. Fluorescence microscopy revealed punctate endocytic vesicles throughout control cells but not cells treated with energy poisons (data not shown), as seen by Skiba et al. (27). Sphingomyelinase treatment produced an increased number of vesicles with brighter fluorescent intensity (Fig. 6A). U18666A did not prevent FITC-dextran uptake (Fig. 6B). In the presence of energy poisons, the...
LDL and U18666A (panel B). After 16 h, monolayers in panel C mine (mutants 2-2 and 4-4) esterified considerably less, because the LDL-derived cholesterol was esterified in CHO cells. Ced-1 labeled with [3H]cholesterol and subjected to sphingomyelinase sphingomyelin degradation. Preventing plasma membrane internalization in response to distribution (Fig. 6). U18666A did not inhibit their formation or sphingomyelinase-induced vesicles remained at the cell periphery (Fig. 6C), and U18666A did not inhibit their formation or distribution (Fig. 6D). Therefore, U18666A does not act by preventing plasma membrane internalization in response to sphingomyelin degradation.

As a control, parallel cultures of the same experiment were labeled with [3H]cholesterol and subjected to sphingomyelinase treatment. Sphingomyelinase stimulated [3H]cholesterol conversion to [3H]choleryl esters, and U18666A inhibited the [3H]choleryl ester formation (data not shown).

The Lysosome-to-ER Pathway in Cholesterol Transport-defective CHO Cell Mutants—Additional evidence for a lysosome to ER cholesterol transport pathway comes from analysis of two complementation classes of cholesterol transport-defective CHO cells. The Ced-1 class exhibits a classical Niemann-Pick type C biochemical phenotype (24). Ced-1 mutants 2-2 and 4-4 show no LDL stimulation of cholesterol esterification, because efflux of LDL cholesterol from lysosomes is greatly impaired. Ced-2 mutant 3-6 shows LDL stimulation of cholesterol esterification that is reduced compared with control but that is clearly discernible (28). Mutant 3-6 displays normal movement of LDL cholesterol out of lysosomes but completely defective transport of cholesterol from the plasma membrane to ACAT (9). In the following experiment, we tested the hypothesis that the discernible LDL stimulation of ACAT in mutant 3-6 is due to an intact lysosome-to-ER cholesterol transport pathway.

Table I shows LDL [3H]cholesterol esterification of cells incubated for 6 h with [3H]CL-LDL in the absence and presence of 25-HC. In the absence of 25-HC stimulation of ACAT, 4.4% of LDL-derived cholesterol was esterified in CHO cells. Ced-1 mutants 2-2 and 4-4 esterified considerably less, because the LDL cholesterol is largely sequestered in lysosomes. Ced-2 mutant 3-6 esterified 3.1% of the LDL cholesterol despite the absence of a plasma membrane-to-ER cholesterol transport pathway. 25-HC activation of ACAT increased LDL cholesterol esterification in all cell lines. Near normal levels of LDL [3H]cholesterol esterification in mutant 3–6 indicates that LDL cholesterol must be transported from lysosomes to ER via a route that bypasses the plasma membrane.

Pharmacological Analysis of Lysosome-to-ER Cholesterol Transport—The mechanism by which LDL cholesterol is transported from lysosomes to ER was investigated. Table II shows the effects of agents that disrupt the cytoskeleton and acidic compartments and thus affect vesicular transport pathways. In these experiments, CHO cells were labeled for 2 h with [3H]CL-LDL in the presence of 100 μM imipramine, which allows uptake of [3H]CL-LDL but prevents movement of free [3H]cholesterol out of lysosomes. Loading lysosomes with [3H]CL-LDL was necessary, because many of the agents tested inhibit receptor-mediated endocytosis of LDL (4). Imipramine was washed out using medium containing various test compounds, after which the cells were incubated for 5 h.

Colchicine disruption of microtubules had no effect on LDL cholesterol movement to ACAT. Parallel cultures incubated with an anti-tubulin antibody and examined by indirect immunofluorescence showed observable changes in the microtubule network at 5 μM colchicine and dramatically altered appearance at 100 μM (data not shown). Nigericin, a potassium ionophore, consistently inhibited LDL cholesterol transport to ACAT. This inhibition is not due to action on the enzyme, since nigericin does not directly inhibit ACAT activity in cell homogenates (29).
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Fig. 6. Effect of U18666A on FITC-dextran uptake. CHO cells were grown as described under “Experimental Procedures.” Monolayers were preincubated for 10 min in H-BSA (panel A) or H-BSA containing either 1 μM U18666A (panel B), 100 mM 2-deoxyglucose, and 100 mM sodium azide (panel C) or 1 μM U18666A, 100 mM 2-deoxyglucose, and 100 mM sodium azide (panel D). Cells were then incubated in media containing the same compounds with 5 mg/ml FITC-dextran and 50 milliunits of sphingomyelinase for 30 min. Cells were processed for fluorescence microscopy as described under “Experimental Procedures.”

Table I

| Cell line       | [3H]Cholesteryl oleate formationa | %       |
|-----------------|----------------------------------|---------|
| CHO             | No addition                       | 4.36 ± 1.35 | 15.10 ± 3.14 |
|                 | 1 μg/ml 25-HC                     | 1.90 ± 1.09 | 5.41 ± 2.98  |
| Mutant 2–2/4–4  |                                  | 3.06 ± 2.49 | 10.21 ± 1.18 |
| Mutant 3–6      |                                  |          |             |

a The values below represent percentage of total cellular [3H]cholesterol.

Cytochalasin D at 10 and 50 μM disrupted actin filaments, as assessed by fluorescence microscopy after phalloidin staining (data not shown), and inhibited LDL cholesterol transport to ACAT by 36–40%. Monensin, a sodium ionophore, inhibited LDL cholesterol transport to the ER very effectively but does not directly inhibit ACAT activity in cell homogenates (29). The above results implicate the actin microfilament network and acidic compartments, such as transport vesicles and the trans-Golgi network, in LDL cholesterol transport to the ER.

Effect of Brefeldin A on the Lysosome to ER Pathway—To determine if LDL cholesterol is transported through the Golgi complex, we used brefeldin A, a fungal metabolite that prevents ER to Golgi vesicular transport (30). Retrograde transport still continues in brefeldin A-treated cells; thus, the cis and medial Golgi cisternae fuse with the ER. At 2 μg/ml, brefeldin A causes the Golgi to merge with the ER in CHO cells, as assessed by fluorescence microscopy with Bodipy FL C5-ceramide/BSA (Ref. 31 and data not shown).

Fig. 7 shows the effect of brefeldin A on [3H]CL-LDL uptake, hydrolysis, and LDL [3H]cholesterol esterification. Additions of brefeldin A were made at staggered times, and then [3H]CL-LDL and 25-HC were added after 1 h. In the absence of brefeldin A, we observed the expected time-dependent cell-association of [3H]CL, hydrolysis of [3H]CL to [3H]cholesterol, and esterification of LDL [3H]cholesterol. Brefeldin A severely inhibited [3H]CL-LDL uptake (Fig. 7A), consistent with its known effects on endocytosis and delivery of ligands to lysosomes (30); however, the residual [3H]CL internalized was efficiently hydrolyzed and esterified (Fig. 7, B and C). There was essentially no difference in the percentage of LDL [3H]cholesterol transported to ACAT and esterified in brefeldin A-treated cells for up to 4 h (Fig. 7D), suggesting that an intact Golgi apparatus is not necessary for cholesterol transport from lysosomes to ER.

At 6 h, there was a decrease in [3H]cholesterol esterification, but this could be due to secondary effects of the lengthy brefeldin A treatment on the membrane environment surrounding the enzyme. The actual effects of brefeldin A on cholesterol
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Fig. 7. Effect of brefeldin A (BFA) on LDL [3H]cholesterol esterification. CHO cells were grown as described under “Experimental Procedures.” Monolayers were preincubated for 1 h in H-5% LPDS/mev in the absence (□) or presence (●) of 2 μg/ml brefeldin A. Cells were then incubated in H-5% LPDS/mev containing 20 μg/ml [3H]CL-LDL, 1 μg/ml 25-HC in the absence (□) or presence (●) of 2 μg/ml brefeldin A. [3H]Cholesteryl linoleate (panel A), [3H]cholesterol (panel B), and [3H]cholesteryl oleate (panel C) were analyzed as described under “Experimental Procedures.” The data represent an average of triplicate wells ± S.D. Panel D represents the [3H]cholesteryl oleate formed as a percentage of [3H]cholesterol plus [3H]cholesteryl oleate.

Esterification is complex. As Golgi membranes merge with the ER, ACAT is activated 5-fold, as measured by [14C]oleate incorporation into cholesteryl [14C]esters (28). Here, we found that LDL cholesterol is efficiently transported to the plasma membrane but completely defective transport of plasma membrane cholesterol to ACAT (9, 28). However, brefeldin A also has the opposing effect of lowering ACAT activation by blunting LDL uptake. Only if brefeldin A treatment had dramatically inhibited LDL [3H]cholesterol transport and esterification could we conclude that an intact Golgi apparatus is required for proper cholesterol trafficking.

Time Course of Esterification of LDL [3H]Cholesterol versus Cellular Cholesterol—Our hypothesis is that while the bulk of LDL cholesterol leaving the lysosome is transported to the plasma membrane, a discrete portion is delivered to and activates ACAT independent of the plasma membrane. If this is true, then when [3H]CL-LDL is added simultaneously with [14C]oleate, the cholesteryl oleate formed should have a high [3H]/[14C] ratio as LDL [3H]cholesterol reaches ACAT. The ratio should then decline with time as cellular cholesterol is mobilized to ACAT. However, if LDL [3H]cholesterol is first transported to the plasma membrane, causing cellular cholesterol to be transported to ACAT, then the [3H]/[14C] ratio should start small and increase with time as LDL [3H]cholesterol arrives at ACAT from the plasma membrane.

Cells were labeled with staggered additions of either [3H]CL-LDL and 0.1 mM oleate-BSA, or LDL and 0.1 mM [14C]oleate-BSA. Fig. 8A shows the time-dependent formation of [3H]cholesteryl ester. Fig. 8B shows that formation of cholesteryl [14C]oleate exhibited lagged kinetics. This lag was not due to slow diffusion of [14C]oleate into the cell interior, since [14C]oleate incorporation into [14C]triglycerides was linear with time (data not shown). Fig. 8C shows the [3H]/[14C] ratio, which was high at early time points and then declined with time. This is consistent with a model in which LDL cholesterol reaches ACAT without first being transported to the plasma membrane and diluted by cellular cholesterol.

DISCUSSION

At steady state, 65–80% of cellular cholesterol is in the plasma membrane (3, 32). Cholesterol in the plasma membrane is not static, however; it constantly moves between the cell interior and surface (33). Plasma membrane cholesterol levels rise when LDL is internalized, as the LDL cholesterol is rapidly transported to the plasma membrane (6, 34). As the cholesterol pool expands, ACAT is activated in the ER, and excess cellular cholesterol is stored as cholesteryl esters (35).

How does the signal reach ACAT that LDL cholesterol is expanding the cellular cholesterol content? One possibility is that LDL cholesterol is efficiently transported to the plasma membrane. The rising plasma membrane cholesterol level increases cholesterol cycling into the cell interior, which activates ACAT. Another possibility is that LDL cholesterol is transported along multiple pathways.

Our hypothesis is that, while the bulk of LDL cholesterol is mobilized to the plasma membrane, a portion is delivered to the ER by a plasma membrane-independent route. Three lines of evidence from our studies support this model.

(i) Conditions of hydrophobic amine inhibition of cholesterol transport were defined in which LDL cholesterol freely moves from lysosomes to plasma membrane and plasma membrane cholesterol can freely move to ACAT in the ER. Yet under these conditions, LDL cholesterol does not activate ACAT and does not get esterified. Furthermore, LDL cholesterol is not esterified even if ACAT is independently activated by 25-HC. These results suggest that a cholesterol transport pathway exists that is exquisitely sensitive to hydrophobic amine inhibition.

(ii) Cholesterol transport mutant 3-6 exhibits normal movement of LDL cholesterol from lysosomes to plasma membrane but completely defective transport of plasma membrane cholesterol to ACAT (9, 28). Here, we found that LDL cholesterol transport from lysosomes to ACAT is normal in mutant 3-6, which suggests that the fraction of LDL cholesterol that is esterified does not traffic through the plasma membrane on its way to the ER. The portion transported to ACAT in mutant 3-6 is probably responsible for the detectable LDL stimulation of [3H]oleate incorporation into cholesteryl [14C]esters (28).

(iii) The kinetics of LDL cholesterol versus cellular cholesterol incorporation into cholesteryl esters was measured. If our hypothesis is correct, LDL [3H]cholesterol arrival at ACAT should activate [14C]oleate incorporation into cholesteryl esters. Thus, the [3H]/[14C] ratio should be high at early times and then decline. If LDL cholesterol was transported to the plasma membrane before moving to the ER, then we expected a low [3H]/[14C] ratio that increased with time. However, our results indicated that LDL cholesterol was not diluted by the plasma membrane cholesterol pool before reaching ACAT.

The existence of a lysosome-to-ER pathway of cholesterol transport was first proposed by Tabas (36). Evidence for such a pathway was previously presented by Underwood et al. (7) and Neufeld et al. (8). Neufeld et al. used 2-hydroxypropyl-β-cyclohex-2-enecyclodextrin (CD) to evaluate cholesterol transport pathways in cultured cells. CD is a cyclic oligomer of glucose that serves as a rapid, efficient extracellular acceptor of plasma membrane cholesterol (37). Cells were preloaded with LDL in the presence of progesterone and then incubated with CD in the absence of LDL and progesterone. [3H]Oleate incorporation into cholesteryl [3H]oleate was used as a measure of the arrival of LDL.
cholesterol in the ER. They found that CD blunted approximately 70% of the LDL stimulation of ACAT, which suggested that about 70% of the LDL cholesterol was transported to ACAT via the plasma membrane and that the residual LDL cholesterol was transported by a plasma membrane-independent pathway.

Evidence seemingly against a lysosome-to-ER pathway was presented by Lange et al. (17). In one experiment, hepatoma cells were incubated with [3H]CL-LDL, and the time course of re-esterification of LDL [3H]cholesterol was monitored along with its movement to a cholesterol oxidase-accessible pool. After 2 h, 78% of LDL [3H]cholesterol was found in a cholesterol oxidase-accessible pool, while the residual cholesterol was esterified by ACAT (8%) or in a cholesterol oxidase-inaccessible pathway (14%). These results are consistent with our model.

In a second experiment, the kinetics of LDL cholesterol and cellular cholesterol esterification were measured. Lange et al. (17) loaded endocytic compartments with [3H]CL-LDL at 18 °C while labeling the plasma membrane with [14C]cholesterol. Upon warming the cells, they observed that plasma membrane [14C]cholesterol incorporation into cholesteryl esters was linear with time. This is not surprising, given that FU5AH hepatoma cells have high constitutive cholesterol esterification activity (17). It is also not surprising that LDL [3H]cholesterol incorporation into cholesteryl esters showed a lag, since this reflects the need to hydrolyze [3H]cholesterol linoleate. Our experiments were performed with CHO cells cultured in lipoprotein-deficient serum, in which ACAT activity is at a low, basal level. Our data show that LDL [3H]cholesterol activates ACAT, which then stimulates esterification of cellular cholesterol.

In a third experiment, Lange et al. (17) found no effect of hydrophobic amines on LDL [3H]cholesterol accessibility to cholesterol oxidase. They attribute hydrophobic amine inhibition of LDL cholesterol esterification to a block in the plasma membrane-to-ER pathway. We and others (26) have found no significant effect of imipramine on a plasma membrane-to-ER pathway. Furthermore, we have used multiple experimental approaches to show that hydrophobic amines affect LDL cholesterol transport from lysosomes to the plasma membrane. In the presence of hydrophobic amines, LDL cholesterol is not accessible to cholesterol oxidase or amphotericin B. It is also not available for desorption to extracellular acceptors (38). By filipin staining and subcellular fractionation, it is sequestered within lysosomes (38, 39). We attribute the results of Lange et al. (17) results to cholesterol oxidase reaching an intracellular pool of cholesterol.

Our current study does not resolve whether the Golgi apparatus is involved in cholesterol movement. We added brefeldin A to the cultures 1 h prior to the addition of [3H]CL-LDL. By the time LDL [3H]cholesterol was leaving the lysosomes, the Golgi complex was disrupted (30). Yet esterification of LDL [3H]cholesterol proceeded normally for several hours. Therefore, an intact Golgi complex is not necessary for cholesterol transport from lysosomes to ER. However, we cannot conclude that the pathway is Golgi-independent. The lack of an observed brefeldin A effect on LDL cholesterol esterification could be due to a transport “vehicle,” which normally docks at the Golgi, being capable of finding its docking site at the merged Golgi/ER. This is consistent with the results of Neufeld et al. (8), who found little brefeldin A effect on LDL stimulation of [3H]oleate incorporation into cholesteryl [3H]oleate. However, this experiment was complicated by the effect of Golgi cholesterol arriving in the ER and stimulating ACAT. The most compelling evidence for Golgi involvement in cholesterol transport comes from filipin electron microscopy, showing cholesterol enrichment of Golgi cisternae following LDL uptake (40).

LDL cholesterol movement from lysosomes to ER is likely to be a vesicle-mediated event. First, transport was inhibited by monensin and nigericin, ionophores that neutralize acidic compartments such as endosomes and the trans-Golgi network. Second, actin filaments, but not microtubules, appear to play a role in cholesterol delivery to ACAT. Actin filaments were disrupted by cytochalasin D at 10 and 50 μM. Lower concentrations of cytochalasin D were ineffective in disrupting filaments, as assessed by fluorescence microscopy of phalloidin-stained cells. This finding is in contrast to that of Tabas et al. (41), who observed filament dissolution in CHO cells with 3 μM cytochalasin D, with no effect on β-very low density lipoprotein stimulation of cholesteryl [3H]oleate formation.

What is the mechanism by which hydrophobic amines inhibit cholesterol transport? Our work to date has eliminated mechanisms of U18666A action but has not provided positive evidence for a possible mechanism. U18666A does not act by preventing cholesterol desorption from membranes or by inhibiting P-glycoprotein activity (7). U18666A inhibited the basal movement of plasma membrane cholesterol to ACAT but did not block endocytosis of FITC-dextran. U18666A also inhibited the accelerated delivery of plasma membrane cholesterol to
ACAT that occurs with plasma membrane sphingomyelin digestion (7, 26); however, U18666A did not prevent the sphingomyelinase-induced plasma membrane vesiculation.

Evidence for a cholesterol transport pathway from lysosomes to the ER has implications for macrophages and atherosclerosis. If this pathway is necessary to activate ACAT, then drugs could be designed to selectively knock out this pathway, slowing down esterification and foam cell production in macrophages. Future work will focus on in vitro characterization of this pathway to determine proteins involved.

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Evidence for a Cholesterol Transport Pathway from Lysosomes to Endoplasmic Reticulum That Is Independent of the Plasma Membrane
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