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Modulation of Endosomal Cholesteryl Ester Metabolism by Membrane Cholesterol*

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Cells acquire cholesterol in part by endocytosis of cholesteryl ester containing lipoproteins. In endosomes and lysosomes cholesteryl ester is hydrolyzed by acidic cholesteryl ester hydrolase producing cholesterol and fatty acids. Under certain pathological conditions, however, such as in atherosclerosis, excessive levels of cholesteryl ester accumulate in lysosomes for reasons that are poorly understood. Here, we have studied endosomal and lysosomal cholesteryl ester metabolism in cultured mouse macrophages and with cell-free extracts. We show that net hydrolysis of cholesteryl ester is coupled to the transfer of cholesterol to membranes. When membrane cholesterol levels are low, absorption of cholesterol effectively drives cholesteryl ester hydrolysis. When cholesterol levels in acceptor membranes approach saturation or when cholesterol export is blocked, cholesterol is re-esterified in endosomes. These results reveal a new facet of cellular cholesterol homeostasis and provide a potential explanation for cholesteryl ester accumulation in lysosomes of atherosclerotic cells.

One of the major hallmarks of atherosclerosis is the progressive accumulation of intracellular and extracellular cholesterol (1–3). In early stages of atherosclerosis excess cholesterol is mainly deposited as cholesteryl fatty acyl esters ("cholesteryl ester"). As atherosclerosis progresses, both cholesteryl ester and cholesterol accumulate (4). Advanced lesions are characterized by the formation of extracellular lipid cores that consist of cholesteryl ester, crystals of cholesterol monohydrate, and debris (5–7). Intracellularly, cholesterol builds up as a component of membranes and as oily droplets of cholesteryl ester that form in the cytoplasm and inside lysosomes (4, 5). At the late stages of disease progression, lysosomes become the major site of lipid accumulation (4, 8).

The mechanisms that are responsible for the formation of cholesteryl esters in atheromatous cells have only partly been resolved. It is clear that cholesteryl ester in cytoplasmic lipid droplets is produced by ER[1]-localized acyl-CoA:cholesterol acyltransferases (ACATs) that are allosterically activated by cholesterol (9, 10). The aberrant accumulation of cholesteryl ester in lysosomes, however, remains to be explained (8).

Cholesterol in atherosclerotic lesions is predominantly derived from serum lipoproteins such as low density lipoprotein (LDL) (11). LDL consists of a hydrophobic core containing mainly cholesteryl ester and triglycerides surrounded by a lipid monolayer and apolipoprotein B100 (12). LDL and its atherogenic derivatives are recognized by cell surface receptors, internalized, and transferred to the endosomal system. Cholesteryl ester and triglycerides are then hydrolyzed by acidic cholesteryl ester hydrolase (aCEH), generating cholesterol and fatty acids (11, 13, 14).

It has previously been proposed that lysosomal cholesteryl ester accumulation in atheromatous cells might result from a deficiency of aCEH (5, 15). Most subsequent studies on aCEH activity in atherosclerotic biopsies have found, however, that aCEH levels were unchanged or increased (14). Other hypotheses that have been proposed to explain the build-up of cholesteryl ester in lysosomes include esterification of cholesterol by a local enzyme (16), saturation of aCEH with excessive substrate (15), impaired delivery of aCEH to substrate-carrying endosomes (17), and transport of ACAT-derived cholesteryl ester to lysosomes. Direct evidence in favor of any of these models, however, has not yet been available.

To further address this issue we have studied the metabolism of cholesteryl ester and triglycerides in mouse macrophages *in vivo* and with intact endosomes *in vitro*. We show that net hydrolysis of cholesteryl ester depends on the ability of membranes to absorb cholesterol. When membrane cholesterol approaches saturation, as it does in atherosclerosis, cholesterol is re-esterified in endosomes. Endosomal esterification occurs independently of canonical ACATs. Endosomal hydrolysis of triglycerides was not affected by cholesterol, indicating that activity and transport of aCEH remained unchanged. These findings reveal a feedback connection between cellular cholesterol balance and endosomal cholesterol dispensation and provide a potential explanation for why cholesterol-saturated cells in atherosclerotic lesions accumulate cholesteryl ester in lysosomes.

EXPERIMENTAL PROCEDURES

Materials

We obtained egg yolk phosphatidylcholine (P-2772), dioleoylphosphatidylserine (P-1060), dicetyl phosphate, trioleoylglycerol (T-9275), cholesteryl oleate, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide dihydrate, the sodium salt of adenosine 5′-monophosphate (A-1752), imipramine (I-0899), and progesterone (P-8783) from Sigma; U18666A from Biomol; 25-hydroxycholesterol from Steraloids; pepstatin A,

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[1] The abbreviations used are: ER, endoplasmic reticulum; ACAT, acyl-coenzyme A:cholesterol acyltransferase; aCEH, acidic cholesteryl ester hydrolase; LDL, low density lipoprotein; AcLDL, acetylated LDL; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PVT, polyvinyltoluene; β-hex, β-hexosaminidase; CCR, cytochrome *c* reductase.

leupeptin and the sodium salt of ATP from Roche Applied Science; [1,2,6,7-^{3}H]cholesteryl oleate ([^{3}H]cholesteryl oleate, NET-746), glyceryl tri-[9,10-^{3}H]oleate ([^{3}H]trioleoylglycerol, NET-431), and [1,2-^{3}H]cholesterol ([^{3}H]cholesterol, NET-139) from PerkinElmer Life Sciences; wheat germ agglutinin-conjugated polyvinyltoluene (PVT) beads (RPNQ 0001) from Amersham Biosciences; FBS from Invitrogen; and other cell culture reagents from Mediatech. The ACAT inhibitor CI-976 was a gift from Pfizer Global Research and Development. The Rab5 antibody used in this study was a kind gift from A. Wandinger-Ness, University of New Mexico School of Medicine, Albuquerque, NM. Lipoprotein-deficient FBS (LPDS) (18), LDL (18), and acetylated LDL (19) were prepared as described. Anionic liposomes were prepared as described (20) and contained 1 mM phosphatidylcholine, 1 mM phosphatidylserine, 0.2 mM dicetyl phosphate plus neutral lipids as follows: liposomes prepared with [^{3}H]cholesteryl oleate contained 4 μM [^{3}H]cholesteryl oleate, 6 μM non-radioactive cholesteryl oleate, and 1 μM non-radioactive trioleoylglycerol; liposomes prepared with [^{3}H]trioleoylglycerol contained 1 μM [^{3}H]trioleoylglycerol plus 10 μM non-radioactive cholesteryl oleate; liposomes prepared with [^{3}H]cholesterol contained 1.3 mM phosphatidylcholine, 1.2 mM phosphatidylserine plus 4 μM [^{3}H]cholesterol.

Cell Culture

J774 mouse macrophages were grown in monolayer culture at 37 °C in an atmosphere of 8–9% CO_2 in medium A (a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FBS. SRD-4 and 25RA Chinese hamster ovary cells were maintained at 37 °C in an atmosphere of 8–9% CO_2 in medium A supplemented with 5% FBS and 0.1 μg/ml of 25-hydroxycholesterol.

Buffers

PBS refers to phosphate-buffered saline, pH 7.4 (catalog number 21-040-CM, Mediatech). Buffer A contains 5 mM sodium phosphate, pH 7.5, and 250 mM sucrose. Buffer B contains 50 mM Hepes, pH 7.5, 125 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, plus protease inhibitors (10 μg/ml leupeptin and 5 μg/ml pepstatin A). Buffer C contains 10 mM Hepes, pH 7.5, 10 mM KCl, and 1 mM $MgCl_2$ plus protease inhibitors. Buffer D refers to 10 mM Hepes, pH 7.4, plus protease inhibitors. Buffer E contains 20 mM sodium citrate, pH 4.5, 125 mM KCl, 5 mM $MgCl_2$, and 2 mM EDTA plus protease inhibitors. Buffer F contains 100 mM Tris-HCl, pH 7.5, or 20 mM sodium citrate, pH 4.5, 12.5 mg/ml fatty acid-free bovine serum albumin, and 100 μM oleoyl-CoA plus protease inhibitors.

Cell Fractionations

Protocol 1—Three confluent 10-cm dishes of J774 cells were scraped into PBS and centrifuged for 5 min at 1000 × g. Cells were resuspended in 2 ml of buffer C, incubated for 15 min on ice, homogenized using a bent 22-gauge needle attached to a 5-ml syringe, and centrifuged for 5 min at 1000 × *g*. The resulting post-nuclear supernatant was mixed with 4 ml of buffer B supplemented with 62% sucrose, overlaid with a 6-ml linear gradient of 15–35% sucrose in buffer B and centrifuged at 280,000 × g for 3.5 h at 4 °C in a Beckman SW 40Ti rotor.

Protocol 2—Following *in vitro* reactions with membrane-covered PVT beads, samples were supplemented with 250 mg of sucrose plus 100 μl of 1 M sodium carbonate, pH 11, and vortexed to dissolve the sucrose. The mixture was then overlaid successively with 0.5 ml of 20% sucrose and 0.5 ml of 5% sucrose and centrifuged at 87,000 × *g* for 30 min at 4 °C in a Beckman TLA 100.4 rotor. The bead-containing fraction was collected from the 20%/5% sucrose interface.

Protocol 3—J774 cells were harvested, and a post-nuclear supernatant was prepared as described under "Protocol 1." The post-nuclear supernatant was then subjected to 30% Percoll gradient centrifugation as described previously (21).

Enzyme Assays

The activities of α-mannosidase II, β-hexosaminidase (β-hex) (22), 5′-nucleotidase (23), and NADPH-cytochrome *c* reductase (CCR) (24) were determined as described in the indicated references.

Preparation of Plasma Membrane-covered PVT Beads

Two confluent 10-cm dishes of J774 cells were incubated for 45 min on ice in the presence of 3 ml of PBS containing 5 mg/ml wheat germ agglutinin PVT beads. Cells were then washed 3 times with ice-cold PBS and scraped into 0.5 ml of ice-cold buffer D. Cells were vortexed for 15 s and disrupted for 10 s with a model W-375 Ultrasonic sonicator. Homogenates (1 ml) then received an equal volume of 60% sucrose in buffer B. The samples were overlaid with a 10-ml linear gradient of 0–30% sucrose in buffer B and centrifuged at 280,000 × *g* for 30 min in a Beckman SW 40Ti rotor. Plasma membrane acceptor beads were harvested from the gradient and quantified by an A_{600} measurement using bead suspensions of known concentrations for calibration. Protein and phospholipids were quantified as described below.

Preparation of Phospholipid-covered PVT Beads

150 μl of 7% (w/v) wheat germ agglutinin PVT beads in water were mixed with 300 μl of hexane. Phosphatidylcholine (105 μg) and phosphatidylserine (113 μg) were added and the organic solvent dried under a stream of nitrogen gas. In some cases cholesterol was added at concentrations indicated in the figure legends. Beads were washed three times with cold PBS and resuspended in 250 μl of cold buffer E. Beads were quantified as described above. Protein and phospholipid were measured as described below.

In Vitro Acidic Hydrolysis and Esterification Assays

Unless otherwise stated, each reaction contained, in a final volume of 1 ml 250 mM sucrose, 125 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, J774 endosomes (35–75 μg of protein), and 20 mM sodium citrate (pH 4.5 reactions) or 50 mM Hepes-KOH (pH 7.5 control reactions). Reactions were carried out in siliconized 1.5-ml microcentrifuge tubes and terminated by transfer of tubes to ice. Immediately thereafter, samples were extracted twice with chloroform: methanol (95:5) and subjected to thin layer chromatography.

ACAT Assay

One confluent 10-cm dish of J774 cells was incubated for 6 h in medium A supplemented with 10% LPDS, liposomes (containing [^{3}H]cholesterol; 10 μCi/ml medium), 5 μg/ml 25-hydroxycholesterol, and 10 μM CI-976. Cells were then harvested, homogenized with a bent 22-gauge needle, and centrifuged at 1000 × *g* for 5 min. The resulting post-nuclear supernatant was centrifuged at 16,000 × *g* for 10 min. The membrane pellet was resuspended in 300 μl buffer F, pH 7.5, without oleoyl-CoA. ACAT activity was assayed *in vitro* as described (25).

Lipid and Protein Analyses

To prepare whole-cell lipid extracts, cells were washed with PBS and lysed in 0.5% Nonidet P-40 plus protease inhibitors. Lipids were extracted with chloroform:methanol (95:5) and analyzed by thin layer chromatography.

To quantify tritiated cholesteryl ester and triglycerides, lipids were separated on silica gel G plates (Analtech) using hexane:ethyl ether: acetic acid (70:30:1) as the mobile phase. Plates were calibrated with authentic standards, and lanes were split into 16 fractions and analyzed by liquid scintillation counting.

To quantify [^{3}H]cholesterol and [^{3}H]cholesteryl ester, lipids were separated on silica gel LK5D plates (Whatman, 150-Å pores) using hexane:ethyl acetate (90:10) as the mobile phase. Lanes were split into eight fractions, scraped, and analyzed by liquid scintillation counting. Total phospholipids were measured as described (26). Cholesterol was measured using an Amplex Red cholesterol assay kit (Molecular Probes). Protein was quantified using a bicinchoninic acid kit or with Coomassie Plus reagent (Pierce) using bovine serum albumin as a standard.

Immunoblot Analysis

Samples were separated by 12% SDS-PAGE, transferred to Hybond C-extra nitrocellulose (Amersham Biosciences) and blotted with anti-Rab5 antibody (1:3,000). Blots were then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:40,000; Jackson ImmunoResearch Laboratories), developed with Supersignal West Pico substrate (Pierce), and exposed to Kodak X-Omat Blue XB-1 film (PerkinElmer Life Sciences) at room temperature.

RESULTS

Reconstitution of Endosomal Cholesteryl Ester Hydrolysis in Vitro—To understand why atherosclerotic foam cells accumulate cholesteryl ester in lysosomes we started by setting up a system to study endosomal cholesteryl ester metabolism *in vitro*. The assay, described in the legend to Fig. 1 and in Table I, is based on the use of separately prepared cell fractions enriched for endosomes and plasma membrane, respectively. Endosomes were prepared from mouse J774 macrophages

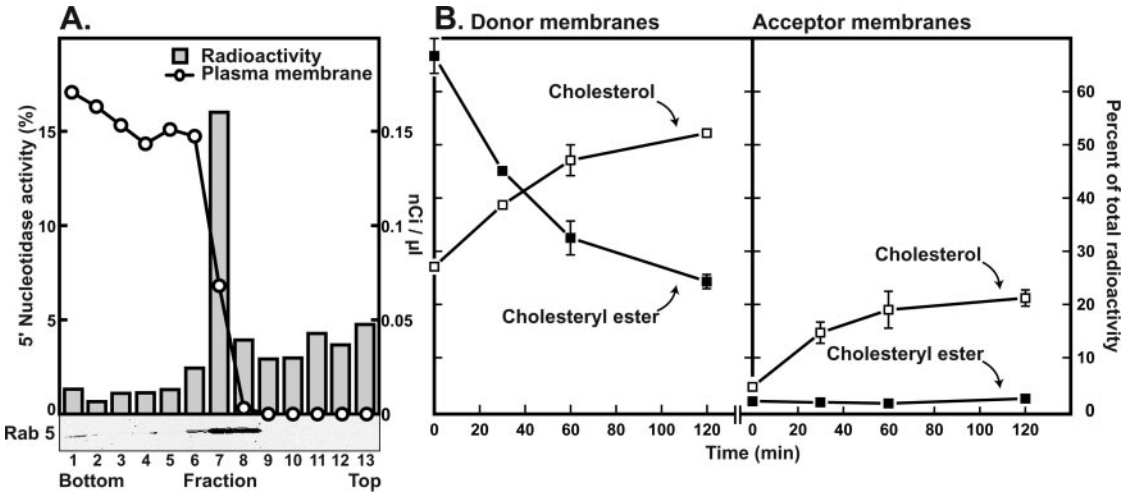

FIG. 1. **Reconstitution of cholesteryl ester hydrolysis in endosomes *in vitro*.** *A*, three confluent dishes of J774 mouse macrophages were incubated for 4 h at 15 °C in medium A supplemented with 10% LPDS plus liposomes (containing non-radioactive trioleoylglycerol and [^{3}H]cholesteryl oleate; 8 μCi/ml medium). Cells were fractionated by sucrose gradient centrifugation as described under "Experimental Procedures" (see "Protocol 1"). 13 fractions were collected and each analyzed for the activities of several markers. *A*, *bars* indicate total radioactivity and *circles* indicate the activity of 5′nucleotidase (plasma membrane). The activity of NADPH-cytochrome *c* reductase (ER) peaked in *fraction 1*, and the activity of β-hexosaminidase (endo/lysosomes) peaked in *fraction 7* (profiles not shown). The sucrose concentration in *fraction 7* was 35%. In this and subsequent fractionation experiments, the activity of the plasma membrane marker 5′-nucleotidase in *fraction 7* varied from 2–11% with a mean of 8%. Aliquots of fractions were also subjected to SDS-PAGE and blotted with an antibody against Rab5 (*lower panel*). *B*, J774 cells were labeled and fractionated as described for *A*. Plasma membrane fractions were prepared as described under "Experimental Procedures." Aliquots of endosomes (donors) containing non-radioactive trioleoylglycerol and [^{3}H]cholesteryl oleate (gradient fraction 7; 45 μg of protein/reaction) were incubated with plasma membrane fractions (acceptors, 20 μg of phospholipid) and 10 μM CI-976 at 37 °C for the indicated time. Donor and acceptor fractions were then separated on sucrose step gradients as described under "Experimental Procedures" (see "Protocol 2"). [^{3}H]Cholesterol (*open squares*) and [^{3}H]cholesteryl ester (*filled squares*) were quantified as described under "Experimental Procedures." Data indicate the amount of radioactivity for each lipid divided by the total radioactivity in donor plus acceptor membranes. *Error bars* denote S.D. (n = 2).

TABLE I
Characterization of plasma membrane-covered PVT beads

Post-nuclear supernatants and plasma membrane-covered PVT beads were prepared from J774 cells as described under "Experimental Procedures." Aliquots were assayed for the activities of the indicated enzymes. 5′-Nucleotidase (plasma membrane) and cytochrome c reductase (ER) activities are expressed as optical density/mg of protein/h. α-Mannosidase II (Golgi) and β-hexosaminidase (endosomes/lysosomes) activities are expressed as artificial fluorescence units/mg of protein/h. Values indicate mean ± S.D. (n = 3).

Enzyme marker	Post-nuclear supernatants	Membrane-covered beads
5′-Nucleotidase	0.72 ± 0.08	7.2 ± 1.3
NADPH-cytochrome c reductase	9.6 ± 1.3	1.1 ± 0.6
α-Mannosidase II	36.4 ± 3.6	2.5 ± 1.5
β-Hexosaminidase	834 ± 27	143 ± 23

grown in the presence of liposomes containing [^{3}H]cholesteryl oleate plus non-radioactive trioleoylglycerol at 15 °C. Previous studies have shown that at this temperature, endocytosed lipoproteins accumulate in early endosomes and cholesteryl ester is hydrolyzed only to a small degree (27). Similarly, we found that cholesteryl oleate delivered to J774 cells in liposomes for 4 h at 15 °C is hydrolyzed only by ~15% (data not shown). After sucrose gradient centrifugation, the bulk of the remaining esters can be collected from a sharp band that cofractionates with the endosomal marker Rab5, well separated from the plasma membrane marker 5′-nucleotidase (Fig. 1*A*). By analyzing the sedimentability of the soluble endosomal enzyme β-hexosaminidase we estimated that more than 90% of endosomal vesicles obtained by this method were intact and remained so during 2-h incubations at 37 °C (data not shown). Plasma membrane was purified by attachment to lectin-conjugated latex beads and equilibrium centrifugation and contained only small amounts of other organelles (Table I).

To determine whether cholesteryl ester hydrolysis can be reconstituted with this material *in vitro*, [^{3}H]cholesteryl oleate-containing endosomes ("donors") were mixed with plasma membrane-covered beads ("acceptors") and incubated at pH 4.5. Subsequently, donor and acceptor membranes from each reaction were separated again by floating the plasma membrane-covered beads on sucrose gradients. Examination of the radioactive lipids showed a 64% reduction of [^{3}H]cholesteryl oleate levels and a concomitant increase of [^{3}H]cholesterol (Fig. 1*B*). Significantly, 29% of the [^{3}H]cholesterol that was produced during 2-h incubations, but only trace amounts of [^{3}H]cholesteryl oleate, were transferred to acceptor membranes (Fig. 1*B*, *right panel*). These results demonstrate that the *in vitro* system effectively reconstitutes hydrolysis of cholesteryl ester and that it allows for selective release of cholesterol. Hydrolysis was maximal at pH 4.5, and no activity was found with endosomes prepared from aCEH-deficient fibroblasts, indicating that hydrolysis under our assay conditions was catalyzed by aCEH (data not shown).

Increased Endosomal Cholesteryl Ester Hydrolysis in the Presence of Plasma Membrane—In previous studies with cultured cells it has been estimated that about 70% of cholesterol produced by endosomal hydrolysis of cholesteryl ester is transferred to the plasma membrane (28, 29). Using our cell-free system we asked, therefore, whether the presence of plasma membrane fractions affects the degree and the kinetics of cholesteryl ester hydrolysis. When labeled endosomes were incubated without acceptors for 2 h, [^{3}H]cholesteryl oleate was reduced by 40% (Fig. 2*A*). Upon addition of increasing amounts of plasma membrane [^{3}H]cholesteryl oleate levels further decreased in a dose-dependent manner. Similar results were obtained with beads that had been covered with artificially prepared phospholipid membranes whereas naked beads were without effect (data not shown). Time curves for hydrolysis are shown in Fig. 2, *B* and *C*. In the presence of plasma membrane, hydrolysis proceeded three times faster during the first 30 min of the reaction as compared with controls (Fig. 2*B*). After 2 h, 40% of [^{3}H]cholesteryl oleate was hydrolyzed in the absence of

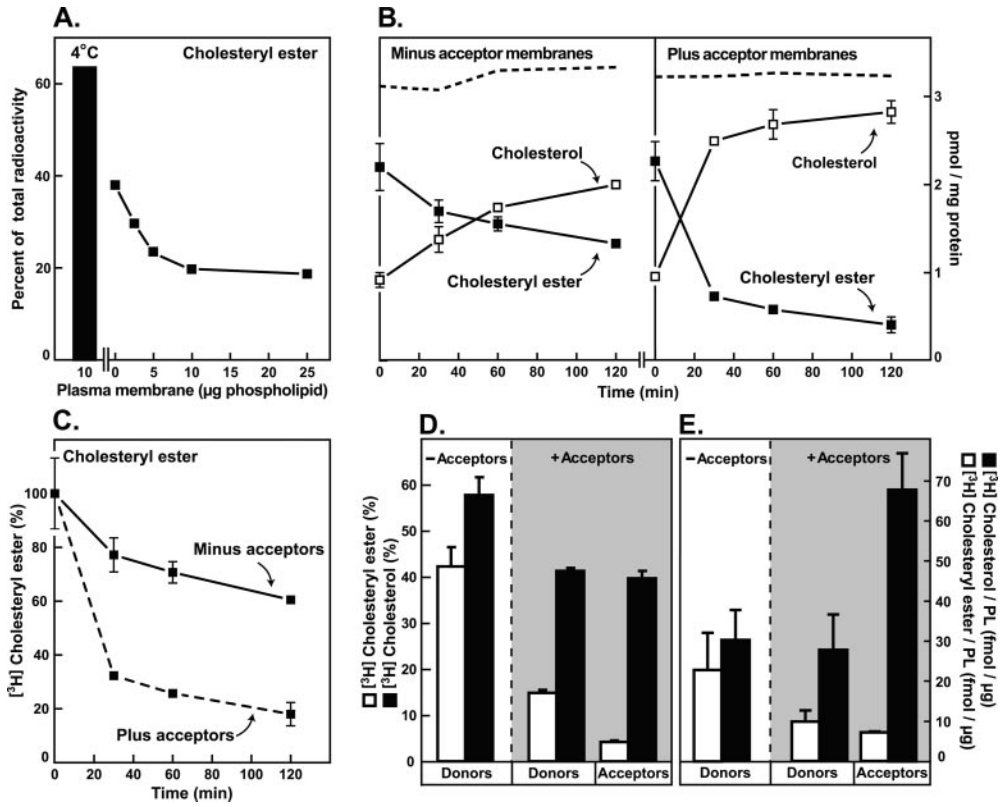

FIG. 2. **Acceptor membranes promote cholesteryl ester hydrolysis *in vitro*.** *A*, [^{3}H]cholesteryl oleate-containing donor endosomes and plasma membrane acceptors were prepared as described in the legend to Fig. 1*B*. Aliquots of endosomes (36 μg of protein/reaction) were mixed with 10 μM CI-976 plus the indicated amount of plasma membrane-covered beads and incubated on ice (*bar*) or at 37 °C (*curve*) for 2 h. [^{3}H]Cholesteryl esters are expressed as a percentage of the total radioactivity in each sample. *B*, [^{3}H]cholesteryl oleate-containing endosomes and plasma membrane acceptors were prepared as described above. Aliquots of endosomes (35 μg of protein/reaction) were mixed without (*left panel*) or with (*right panel*) plasma membrane-covered beads (30 μg of phospholipid) and incubated with 10 μM CI-976. [^{3}H]Cholesterol (*open squares*) and [^{3}H]cholesteryl ester (*filled squares*) were determined as described above. *Dashed lines* indicate the sum of [^{3}H]cholesterol plus [^{3}H]cholesteryl ester. The rate of [^{3}H]cholesteryl oleate hydrolysis during the first 30 min was 16.8 ± 13.1 fmol/mg/min in the absence of acceptors and 51.1 ± 8.3 fmol/mg/min in the presence of acceptors. *C*, the [^{3}H]cholesteryl ester data from *B* are re-plotted as a percentage of the [^{3}H]cholesteryl ester concentration at time 0. *D* and *E*, aliquots of [^{3}H]cholesteryl oleate-containing endosomes (45 μg of protein/reaction) were incubated plus or minus phospholipid acceptors (20 μg of phospholipid) at 37 °C for 2 h. Donor and acceptor fractions from each reaction were then separated on sucrose step gradients as in Fig. 1*B*. [^{3}H]Cholesterol, [^{3}H]cholesteryl ester, and phospholipids were quantified as described under "Experimental Procedures." *D*, data indicate the amount of [^{3}H]cholesterol (*filled bars*) and [^{3}H]cholesteryl ester (*open bars*) as a percentage of the total radioactivity in each set of reactions. *E*, data indicate the amount of [^{3}H]cholesterol (*filled bars*) and [^{3}H]cholesteryl ester (*open bars*) divided by phospholipids. *Error bars* indicate S.D. (n = 2; where not visible, *error bars* are smaller than symbols).

acceptors, while 82% was hydrolyzed in their presence (Fig. 2*C*).

The observation that synthetic phospholipid membranes stimulate endosomal cholesteryl ester hydrolysis suggests that the effect seen with plasma membrane fractions also results from the presence of phospholipids rather than addition of a neutral hydrolase or other protein factors. Further support for this conclusion comes from experiments showing that (i) hydrolysis was significantly more efficient at pH 4.5 than at pH 7.5 and (ii) at pH 7.5 plasma membrane fractions exhibited no hydrolytic activity toward cholesteryl ester in liposomes, while hydrolysis at this pH was efficient when microsomes were used (data not shown).

In summary, the results in Fig. 2 demonstrate that supplementary membranes promote net hydrolysis of cholesteryl ester. Since cholesterol is only poorly soluble in water (30) and a large fraction is selectively transferred to acceptors (Fig. 1*C*) the simplest explanation for this effect is that membranes promote hydrolysis by removing cholesterol from the endosomal "equilibrium" of cholesteryl ester metabolism (31). As one corollary of this model, the ratio of cholesterol to phospholipids in endosomal donors would be expected to remain constant. To test this prediction, [^{3}H]cholesteryl oleate-containing endosome fractions were incubated in the absence or presence of phospholipid-covered beads. Subsequently, the reaction mixtures were again separated to quantify [^{3}H]cholesterol, [^{3}H]cholesteryl ester, and phospholipids on donors and acceptors. Although the total amount of [^{3}H]cholesterol produced was significantly higher in the presence than in the absence of acceptors (Fig. 2*D*), the [^{3}H]cholesterol-to-phospholipid ratio in the two populations of donor membranes remained in fact the same (Fig. 2*E*). The cholesterol difference was found quantitatively in the acceptor fraction, supporting the notion that the degree of net cholesteryl ester hydrolysis depends on the transfer of cholesterol to phospholipid membranes.

Modulation of Net Cholesteryl Ester Hydrolysis by Membrane Cholesterol Concentration—Studies with purified lipids *in vitro* have shown that the capacity of phospholipid bilayers for cholesterol becomes saturated at about 50 mol %. Above that concentration, crystals of cholesterol monohydrate begin to form and cholesterol saturation is thought to be responsible for cholesterol precipitation in advanced atherosclerotic lesions (6). The plasma membrane fractions used in our experiments contained ~30% cholesterol with respect to phospholipids (Table II), which explains why these membranes are still efficient at absorbing cholesterol (Fig. 1*C*). To determine whether the cholesterol concentration in acceptor membranes affects the endosomal metabolism of cholesteryl ester, [^{3}H]cholesteryl

TABLE II
Quantification of cellular lipids following AcLDL treatment

On day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 3.5×10^6 cells per well. On day 1, cells were grown at 37 °C for 4 h in medium A containing 10% LPDS and 10 μM CI-976 in the presence of the indicated concentration of AcLDL. Cellular cholesterol, phospholipids, and proteins were quantified as described under "Experimental Procedures." To measure plasma membrane lipids following AcLDL treatment, J774 cells were set up in 6-cm dishes in medium A plus 10% FBS at 3×10^6 cells per dish. On day 1, cells were grown at 37 °C for 4 h in medium A containing 10% LPDS and 10 μM CI-976 plus or minus 50 μg/ml AcLDL as indicated. Plasma membrane-covered PVT beads were isolated from treated cells as described under "Experimental Procedures," and cholesterol, phospholipids, and proteins were quantified as described above.

	AcLDL	CI-976	Cholesterol	Cholesterol/(cholesterol + phospholipids)
	μg/ml		*μg/mg protein*	*mol %*
Total cell extracts:				
	0	−	9.9 ± 0.3	29.3 ± 1.3
		+	9.7 ± 0.6	28.8 ± 0.1
	5	−	12.5 ± 0.4	32.2 ± 1.4
		+	12.3 ± 0.3	30.9 ± 1.2
	15	−	13.3 ± 0.3	34.4 ± 0.2
		+	14.5 ± 0.8	35.4 ± 1.9
	50	−	17.6 ± 0.3	39.0 ± 0.003
		+	20.4 ± 1.7	42.5 ± 2.1
Plasma membrane:				
	0	+	104.4 ± 1.3	26.2 ± 0.7
	50	+	210.7 ± 7.2	42.5 ± 2.7

oleate-containing endosomes were incubated with membranes containing varying concentrations of cholesterol. Compared with endosomes incubated alone (Fig. 3*A*, *Reaction 2*), addition of cholesterol-free membranes more than doubled the amount of [^{3}H]cholesteryl ester hydrolyzed (*Reaction 3*). When membranes with increasing amounts of cholesterol were added, net hydrolysis decreased in a concentration-dependent manner (*Reactions 4–6*). At the saturating concentration of 50 mol % cholesterol (6), [^{3}H]cholesteryl ester levels remained similar to those in samples without acceptors (compare *Reactions 2* and *6*). These results demonstrate that saturation with cholesterol renders membranes ineffective at stimulating endosomal cholesteryl ester hydrolysis *in vitro*.

To study the effect of increased membrane cholesterol on endosomal cholesteryl ester metabolism *in vivo*, J774 cells were grown for 4 h with increasing concentrations of acetylated LDL (AcLDL). As a consequence, the molar free cholesterol concentration with respect to phospholipids rose from 29% in controls to 39% in cells grown with 50 μg/ml of AcLDL and to 43% in cells grown with AcLDL plus the ACAT inhibitor CI-976 (Table II) (32). Similar results were obtained with isolated plasma membrane fractions (Table II).

In Fig. 3*B* J774 cells were loaded with cholesterol by incubation with AcLDL and CI-976 as described above. The cells were then washed and received [^{3}H]cholesteryl oleate in liposomes for 3 h at 15 °C. Preincubation with AcLDL had no effect on the uptake of liposomes (data not shown). But when cells were subsequently washed and moved to 37 °C, significantly more [^{3}H]cholesteryl oleate was found in cells that had been pre-loaded with AcLDL as compared with controls (Fig. 3*B*). The increased cholesteryl ester levels found in response to AcLDL are unlikely to result from the action of ACAT as all incubations were performed in the presence of ACAT inhibitor. The drug effectively blocks ACAT both in cells and membrane extracts (Figs. 5 and 6, see below).

The results in Fig. 3, *A* and *B*, indicate that increased cholesterol concentrations in membranes reduce net hydrolysis of cholesteryl ester. To explain this effect, we next tested whether cholesterol loading might cause inhibition of aCEH. To address this possibility, we took advantage of the fact that aCEH is the sole enzyme responsible for endosomal hydrolysis of both cholesteryl ester and triglycerides (14). If cholesterol affected the activity of aCEH, hydrolysis of liposome-derived triglycerides should also be reduced. However, preincubation of J774 cells with AcLDL had no effect on hydrolysis of [^{3}H]trioleoylglycerol (Fig. 3*C*). As aCEH is thought to be monomeric with a single substrate-binding site (14, 33), these data do not support the hypothesis that cholesterol modulates the activity of aCEH. They also rule out the possibility that high cholesterol levels might interfere with the movement of substrates to more acidic or aCEH-rich vesicles or that AcLDL loading might have overwhelmed the enzyme with excessive amounts of substrate. In summary, the data in Fig. 3 demonstrate that saturation of membranes with cholesterol reduces net hydrolysis of cholesteryl ester, apparently without affecting the activity of aCEH.

Cholesteryl Ester Synthesis in Endosomes—The next set of experiments was designed to test whether cholesterol-loaded cells synthesize cholesteryl ester in endosomes and lysosomes. In Fig. 4 endosomes were prepared from J774 cells that had been grown with liposomes containing [^{3}H]trioleoylglycerol and unlabeled cholesteryl oleate at 15 °C. Aliquots were then incubated *in vitro* at pH 4.5 in the presence of ACAT inhibitor. Similar to the results with living cells, no significant difference in [^{3}H]trioleoylglycerol hydrolysis was detectable between samples incubated in the absence or presence of phospholipids (Fig. 4, *A* and *B*, *upper panels*) or plasma membrane (data not shown), confirming that the activity of aCEH remained unchanged.

To determine whether some of the liberated [^{3}H]fatty acids had been esterified with cholesterol, the same samples were analyzed for the presence of cholesteryl [^{3}H]oleate. In this case, the outcome was substantially different. The endosomal fractions incorporated [^{3}H]oleate into cholesteryl ester in the absence but not in the presence of acceptor membranes (Fig. 4*A*, *lower panel*). Cholesteryl [^{3}H]oleate was also synthesized when membranes containing increasing concentrations of cholesterol were added (Fig. 4*B*, *lower panel*, *Reactions 4–6*). At saturating levels of cholesterol, cholesteryl [^{3}H]oleate increased to similar levels as in samples containing endosomes alone (compare *Reactions 2* and *6*). These results indicate that a lack of cholesterol-absorbing membranes causes cholesterol re-esterification in endosomes *in vitro*.

Neutral ACAT enzymes can be ruled out as catalysts in the esterification reactions as the experiments in Fig. 4, *A* and *B*, were performed at pH 4.5 in the presence of an ACAT inhibitor, conditions that effectively block microsomal cholesterol esterification activity even in extracts from cells that had been pre-

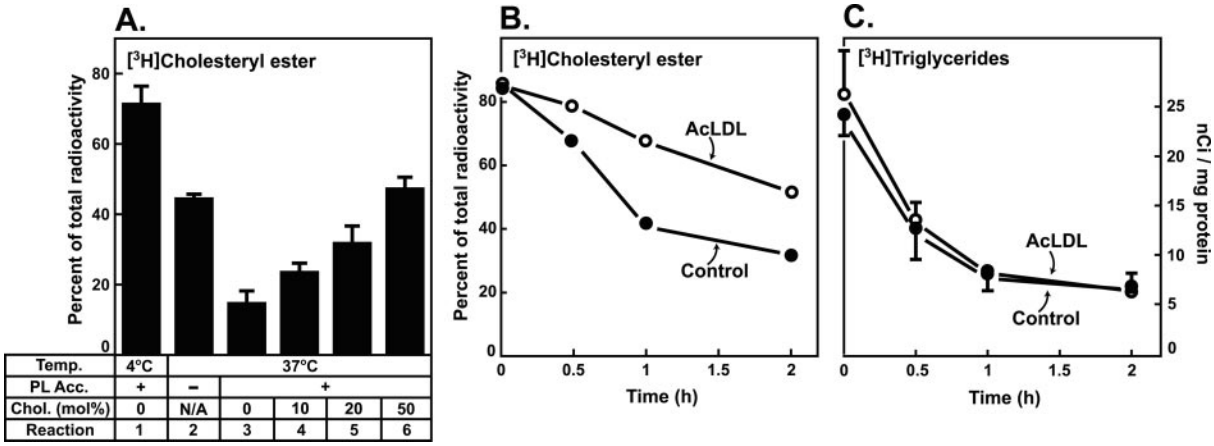

FIG. 3. **Modulation of cholesteryl ester hydrolysis by membrane cholesterol (*Chol.*) concentration.** *A*, J774 endosomes containing [^{3}H]cholesteryl oleate plus non-radioactive trioleoylglycerol were prepared as described in the legend to Fig. 1*B*. PVT beads were covered with synthetic membranes containing increasing concentrations of cholesterol (acceptors) as described under "Experimental Procedures." Acceptor lipids contained the indicated molar concentration of cholesterol plus a 1:1 mixture of phosphatidylserine and phosphatidylcholine. Prior to *in vitro* reactions, donors (55 μg of protein/reaction) were mixed with (*Reactions 1* and *3–6*) or without (*Reaction 2*) phospholipid acceptor membranes (*PL Acc.*, 48 nmol of total lipids/reaction). Reactions were incubated for 2 h on ice (*Reaction 1*) or at 37 °C (*Reactions 2–6*). [^{3}H]Cholesterol and [^{3}H]cholesteryl esters were analyzed as described under "Experimental Procedures." Results are plotted as the amount of [^{3}H]cholesteryl ester divided by the sum of [^{3}H]cholesteryl ester plus [^{3}H]cholesterol. *N*/*A*, not applicable. *B* and *C*, on day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 3.5×10^6 cells per well. On day 1, cells were grown at 37 °C for 3 h in medium A containing 10% LPDS and 10 μM CI-976 plus (*open circles*) or minus (*filled circles*) 50 μg/ml AcLDL. Cells were washed and switched to medium A supplemented with 10% LPDS, 10 μM CI-976 and liposomes containing either non-radioactive trioleoylglycerol and [^{3}H]cholesteryl oleate (*B*) or non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol (*C*) and incubated at 15 °C for 2 h. Cells were washed and chased at 37 °C in medium A containing 0.2% bovine serum albumin and 10 μM CI-976 for the indicated time. Proteins, [^{3}H]cholesteryl oleate, and [^{3}H]trioleoylglycerol were quantified as described under "Experimental Procedures." *B*, results are plotted as the amount of [^{3}H]cholesteryl ester in cells divided by the sum of [^{3}H]cholesteryl ester plus [^{3}H]cholesterol in cells and medium. *A* and *B*, *error bars* indicate S.D. ($n = 2$; where not visible, *error bars* are smaller than symbols).

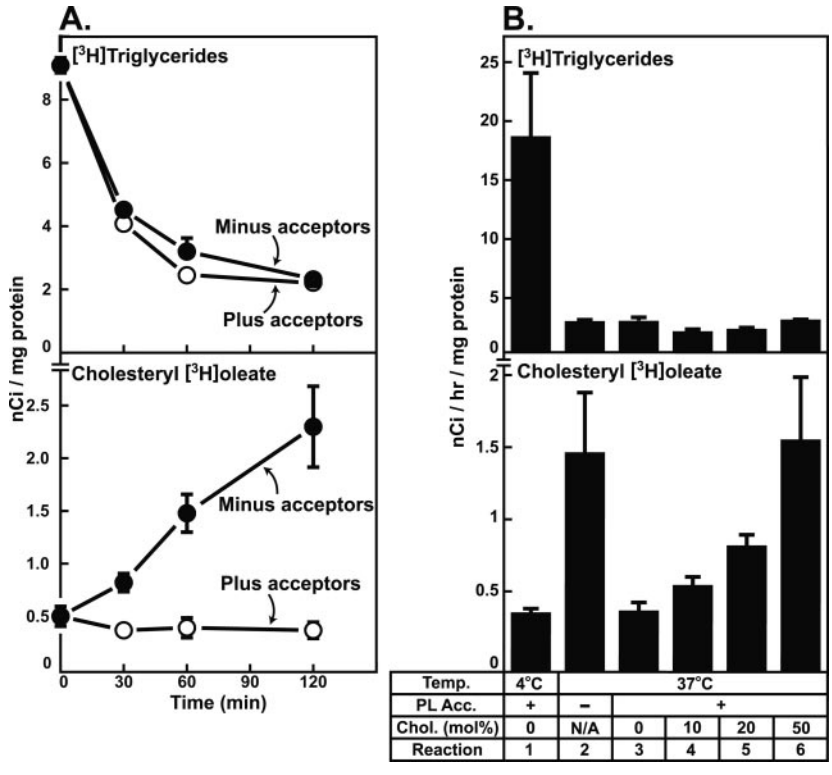

FIG. 4. **Cholesteryl ester synthesis in isolated endosomes *in vitro*.** *A*, J774 endosomes were isolated as described in the legend to Fig. 1*B* except that liposomes contained non-radioactive cholesteryl oleate and [^{3}H]trioleoylglycerol (4 μCi/ml medium). Phospholipid-covered PVT beads (*acceptors*) were prepared as described under "Experimental Procedures." Aliquots of endosomes (63 μg of protein/reaction) were mixed with (*open circles*) or without (*filled circles*) phospholipid-covered beads (25 μg of phospholipid/reaction) and incubated in the presence of 10 μM CI-976 at 37 °C for the indicated time. *B*, J774 endosomes containing [^{3}H]trioleoylglycerol plus non-radioactive cholesteryl oleate were prepared as described above. PVT beads were covered with synthetic membranes containing increasing concentrations of cholesterol (*Chol.*) as in Fig. 3*A*. Prior to *in vitro* reactions, endosomes (45 μg of protein/reaction) were mixed with (*Reactions 1* and *3–6*) or without (*Reaction 2*) phospholipid acceptor membranes (*PL Acc.*, 48 nmol of total lipids/reaction). Reactions were incubated for 2 h on ice (*Reaction 1*) or at 37 °C (*Reactions 2–6*). *A* and *B*, lipids were extracted, and [^{3}H]triglycerides (*upper panels*) and cholesteryl [^{3}H]oleate (*lower panels*) were determined as described under "Experimental Procedures."

treated with 25-hydroxycholesterol (Fig. 5*A*), a potent activator of ACAT (10).

Contrary to results obtained with microsomes (25, 34–36), addition of 25-hydroxycholesterol to endosomes *in vitro* at pH 4.5 or pH 7.5 had no effect on cholesterol esterification (data not shown).

Endosomal triglyceride hydrolysis and cholesterol esterification exhibit a clear maximum at pH 4.5 (Fig. 5*B*). The peak at pH 4.5 for cholesteryl [^{3}H]oleate synthesis can in part be explained by the fact that aCEH-catalyzed hydrolysis is a prerequisite for esterification. However, lowering the pH from 7.5 to 4.5 increased triglyceride hydrolysis 3-fold, while esterification was increased 22-fold, indicating that pH affects cholesteryl ester formation synergistically. Taken together, these results support the conclusion that cholesteryl ester synthesis during the *in vitro* reactions is catalyzed by an acidic endosomal enzyme.

In Fig. 4*B* endosomal cholesteryl ester synthesis was increased in the presence of membranes containing high concen-

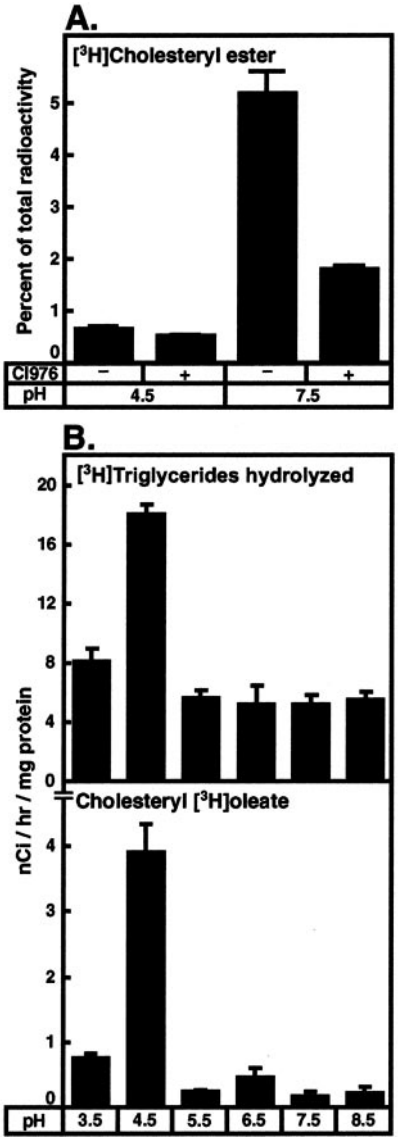

FIG. 5. **Cholesteryl ester synthesis in isolated endosomes *in vitro* is independent of ACAT.** *A*, J774 mouse macrophages were incubated for 6 h at 37 °C in medium A supplemented with the following: 10% LPDS plus liposomes (containing [^{3}H]cholesterol; 10 μCi/ml medium), 5 μg/ml 25-hydroxycholesterol, and 10 μM CI-976. Total membranes were then isolated and incubated *in vitro* plus or minus 10 μM CI-976 at the indicated pH as detailed under "Experimental Procedures." *B*, J774 endosomes were prepared as in Fig. 1*B*, except that liposomes contained [^{3}H]trioleoylglycerol plus non-radioactive cholesteryl oleate. Aliquots (65 μg of protein/reaction) were incubated with 10 μM CI-976 without acceptors at the indicated pH. *Upper panel*, data indicate the amount of [^{3}H]triglycerides in samples that had been incubated on ice minus the amount of [^{3}H]triglycerides in samples that had been incubated at 37 °C. *Lower panel*, data indicate the amount of cholesteryl [^{3}H]oleate produced at 37 °C corrected for the amount of cholesteryl [^{3}H]oleate produced in reactions that had been incubated on ice. *Error bars* denote S.D. (n = 3).

trations of cholesterol. Since saturation of cellular membranes with cholesterol is a hallmark of macrophages in atherosclerotic lesions, we next performed a set of experiments to test whether cholesterol loading leads to lysosomal cholesteryl ester synthesis *in vivo*. First, J774 cells were grown for 3 h in the absence or presence of 50 μg/ml AcLDL and then received liposomes containing [^{3}H]trioleoylglycerol and unlabeled cholesteryl oleate for another 4 h (Fig. 6*A*). In the absence of ACAT inhibitor, pretreatment with AcLDL increased cellular cholesteryl [^{3}H]oleate levels about 2-fold. In the presence of AcLDL plus ACAT inhibitor, cholesteryl [^{3}H]oleate levels were increased 40-fold compared with samples with ACAT inhibitor alone. Varying the AcLDL concentration indicated that ACAT-independent cholesteryl [^{3}H]oleate synthesis was increased at AcLDL concentrations as low as 5 μg/ml (Fig. 6*B*).

To determine the subcellular localization of the cholesteryl [^{3}H]oleate that accumulates in the presence of ACAT inhibitor, J774 cells were grown plus or minus AcLDL and then received liposomes containing [^{3}H]trioleoylglycerol and cholesteryl oleate as described above. ACAT inhibitor was present throughout the experiment. Cell homogenates were then subjected to sucrose gradient centrifugation and fractions were analyzed for organelle markers and radiolabeled lipids. Due to the presence of the ACAT inhibitor, only minimal quantities of cholesteryl [^{3}H]oleate were seen in the ER fractions under all conditions (Fig. 6*C*, *upper panel*). In lysosomes from cells grown with AcLDL, cholesteryl [^{3}H]oleate levels were 14-fold higher compared with the same fraction from control samples.

The results in Fig. 6 show that elevated cholesterol levels can promote cholesteryl ester synthesis in lysosomes of living cells, which mirrors the results we obtained with endosomes and cholesterol-saturated membranes *in vitro* (Fig. 4*B*).

Inhibition of Cholesterol Transport Promotes Cholesteryl Ester Synthesis in Lysosomes in Vivo—The results in Figs. 3–6 show that endosomes esterify cholesterol when phospholipid acceptors are absent or when available membranes are saturated with cholesterol. These data support the conclusion that endosomal cholesteryl ester metabolism is coupled to the transfer of cholesterol to phospholipids. As another verification of this concept *in vivo*, we tested whether cholesteryl ester levels are increased when cholesterol export from the endosomal system is blocked pharmacologically.

In Fig. 7*A* J774 macrophages were initially starved for cholesterol and then loaded with [^{3}H]cholesteryl oleate plus unlabeled trioleoylglycerol at 15 °C to accumulate the esters in endosomes. The cells were then shifted to 37 °C in the absence or presence of the cholesterol transport inhibitor progesterone (37, 38). At 3–10 μg/ml progesterone, cellular [^{3}H]cholesteryl ester levels were 50–75% higher as compared with controls (*upper panel*).

To rule out that progesterone treatment increased cholesteryl ester levels by reducing the activity of aCEH, cells were loaded with [^{3}H]trioleoylglycerol plus non-radioactive cholesteryl oleate and then incubated with progesterone as described above. As shown in Fig. 7*B* (*upper panel*), [^{3}H]triglyceride levels remained similar in the absence and presence of progesterone confirming that aCEH is unaffected by this drug. To determine whether cholesterol was re-esterified in the presence of progesterone, the same extracts were then analyzed for the presence of radiolabeled cholesteryl ester. In response to progesterone, cholesteryl [^{3}H]oleate indeed increased in a dose-dependent manner (Fig. 7*B*, *lower panel*). Taken together, the results in Fig. 7 are consistent with the conclusion that cholesterol export from the endosomal system is necessary for complete hydrolysis of cholesteryl ester. When export is blocked, cholesterol is actively re-esterified.

Both experiments in Fig. 7 were performed in the absence of an ACAT inhibitor as several studies have shown that progesterone blocks transport of cholesterol to the ER (37). To further verify whether cholesteryl ester synthesis can occur in endosomes we performed a series of experiments that are shown in Fig. 8. First, J774 cells were allowed to endocytose [^{3}H]trioleoylglycerol together with non-radioactive cholesteryl oleate for 4 h. Some cells also received CI-976, progesterone, U18666A, or imipramine. U18666A and imipramine, similar to progesterone, block cholesterol release from lysosomes (37, 39, 40). In the absence of cholesterol transport inhibitors, cho-

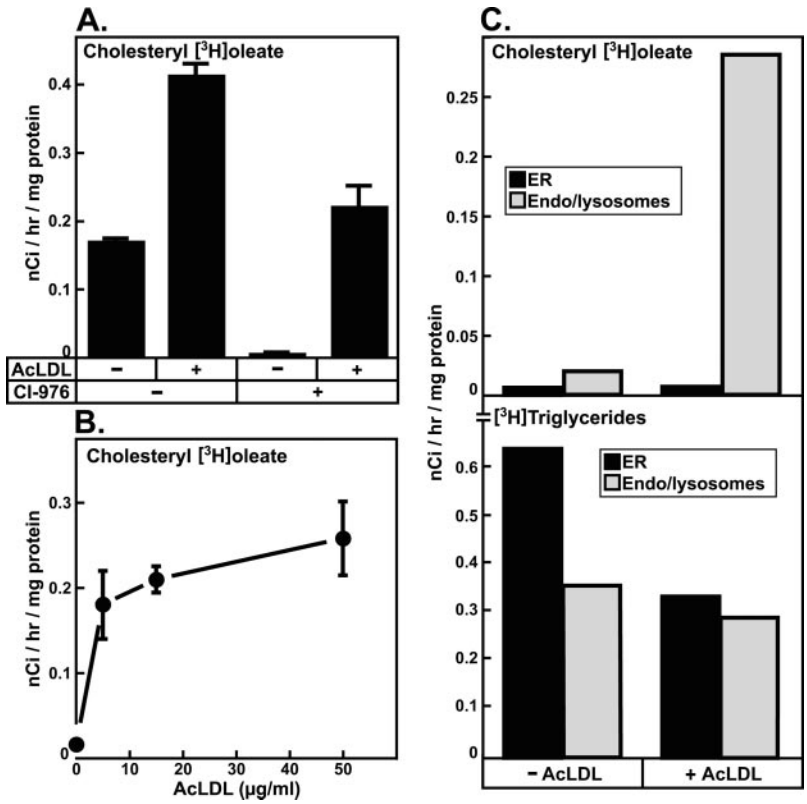

FIG. 6. **Cholesterol loading promotes cholesteryl ester synthesis in endo/lysosomes *in vivo*.** All incubations were at 37 °C. *A*, on day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 2.5×10^6 cells per well. On day 2, cells were grown for 3 h in medium A containing 10% LPDS plus or minus 10 μM CI-976 and 50 μg/ml AcLDL as indicated. Cells were washed to remove AcLDL and incubated for 4 h in medium A supplemented with 10% LPDS and liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml) plus or minus 10 μM CI-976 as indicated. Proteins and cholesteryl [^{3}H]oleate were determined as described under "Experimental Procedures." *Error bars* indicate S.D. ($n = 2$). *B*, on day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 2.5×10^6 cells per well. On day 2, cells were grown for 3 h in medium A containing 10% LPDS plus 10 μM CI-976 and the indicated concentration of AcLDL. Cells were washed to remove AcLDL and switched to 10% LPDS, 10 μM CI-976, and liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml) for 4 h. Proteins and cholesteryl [^{3}H]oleate were determined as described above. *Error bars* indicate S.D. ($n = 3$). *C*, J774 cells were grown for 3 h in medium A supplemented with 10% LPDS and 10 μM CI-976 plus or minus 50 μg/ml of AcLDL as indicated. Cells were washed to remove AcLDL and switched for 4 h to medium A supplemented with 10% LPDS, 10 μM CI-976, and liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml). Cells were then fractionated as described under "Experimental Procedures" (see "Protocol 3"). Fractions were analyzed for organelle markers. β-Hex (*Endo/lysosomes*) activity peaked in fraction 2 (containing 28% of the total β-hex activity and 0% of total NADPH-CCR (ER) activity), and CCR activity peaked in fractions 9–10 (containing 93% of total the CCR activity and 12% of total β-hex activity). Pools were analyzed for protein concentration and lipids as described above.

lesteryl [^{3}H]oleate was produced and this was largely blocked by the ACAT inhibitor CI-976 (Fig. 8*A*, *upper panel*). In the presence of progesterone, U18666A, or imipramine, however, cholesteryl [^{3}H]oleate levels remained high even in the presence of CI-976.

As a genetic approach to addressing a potential involvement of ACAT, we performed experiments with control 25RA and ACAT-deficient SRD-4 Chinese hamster ovary cells (41–43). Here again, the absence of ACAT did not prevent U18666A-induced cholesteryl [^{3}H]oleate synthesis (Fig. 8*B*).

Inspection of [^{3}H]triglycerides in Fig. 8, *A* and *B*, indicates that the cholesterol transport inhibitors did not interfere with aCEH activity *per se* (*lower panels*). Inhibition of cholesteryl ester synthesis, however, consistently led to a compensatory increase of [^{3}H]triglyceride levels.

To determine the subcellular localization of the cholesteryl [^{3}H]oleate that accumulates upon inhibition of cholesterol transport, J774 cells were incubated with [^{3}H]trioleoylglycerol and non-radioactive cholesteryl oleate plus or minus U18666A and ACAT inhibitor as described above. Cells were then fractionated by density gradient centrifugation to separate lysosomes from ER and cytosolic lipid droplets. In homogenates from control-treated cells, most of the cholesteryl [^{3}H]oleate was found in cytosolic lipid droplets and a sizable amount was found in the ER (Fig. 8*C*, *upper panel*). In the presence of ACAT inhibitor, accumulation of cholesteryl [^{3}H]oleate in lipid droplets and in the ER was blocked as expected (10, 44). Addition of U18666A also prevented accumulation of cholesteryl [^{3}H]oleate in lipid droplets and in the ER but led to a 10–18-fold increase of cholesteryl [^{3}H]oleate levels in lysosomes. In summary, the data in Figs. 7 and 8 demonstrate that pharmacological inhibition of cholesterol transport leads to ACAT-independent cholesterol esterification in lysosomes.

In Fig. 9 we asked whether lysosomal accumulation of cholesteryl ester in response to inhibition of cholesterol transport is reversible. J774 cells were allowed to accumulate cholesteryl [^{3}H]oleate in lysosomes after growth with non-radioactive cholesteryl oleate, [^{3}H]trioleoylglycerol, ACAT inhibitor, and imipramine. When imipramine was subsequently washed out, hydrolysis resumed and cholesteryl [^{3}H]oleate levels decreased by 96% after 4 h (Fig. 9, *upper panel*). In cells that continued to grow in the presence of imipramine, cholesteryl [^{3}H]oleate levels remained high. [^{3}H]Triglycerides decreased with similar kinetics with or without imipramine, indicating that the intrinsic activity of aCEH remained unchanged (Fig. 9, *lower panel*). In addition to demonstrating that cholesteryl [^{3}H]oleate accumulation in response to imipramine is reversible, these results provide an illustration for how cholesterol release from lysosomes promotes net hydrolysis of cholesteryl ester.

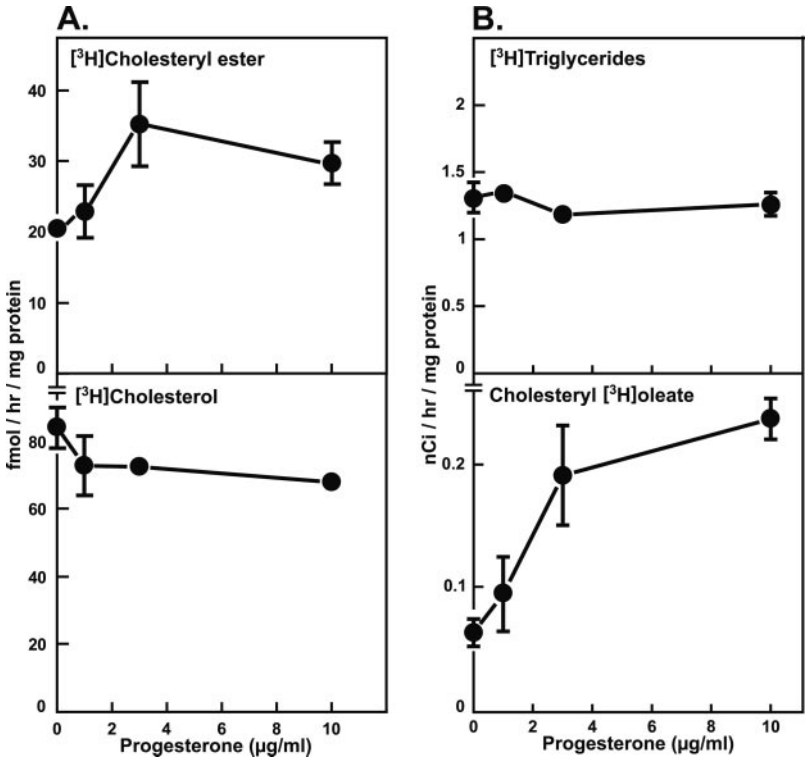

FIG. 7. **Inhibition of cholesterol export from lysosomes reduces net cholesteryl ester hydrolysis *in vivo*.** On day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 3.5×10^6 cells per well. On day 1, cells were depleted of cholesterol by incubation in 10% LPDS plus 20 μM lovastatin, 50 μM mevalonic acid, and 1% hydroxypropyl-β-cyclodextrin for 2 h at 37 °C. Cells were then washed and switched to 10% LPDS plus liposomes containing either non-radioactive trioleoylglycerol and [^{3}H]cholesteryl oleate (*A*) or non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol (*B*) and incubated for 3 h at 15 °C. Cells were then washed and incubated in 10% LPDS in the presence of the indicated concentration of progesterone at 15 °C for 0.5 h. Subsequently, dishes were moved to 37 °C for an additional 4 h without change of medium. Proteins and lipids were analyzed as described under "Experimental Procedures." *Error bars* indicate S.D. ($n = 3$).

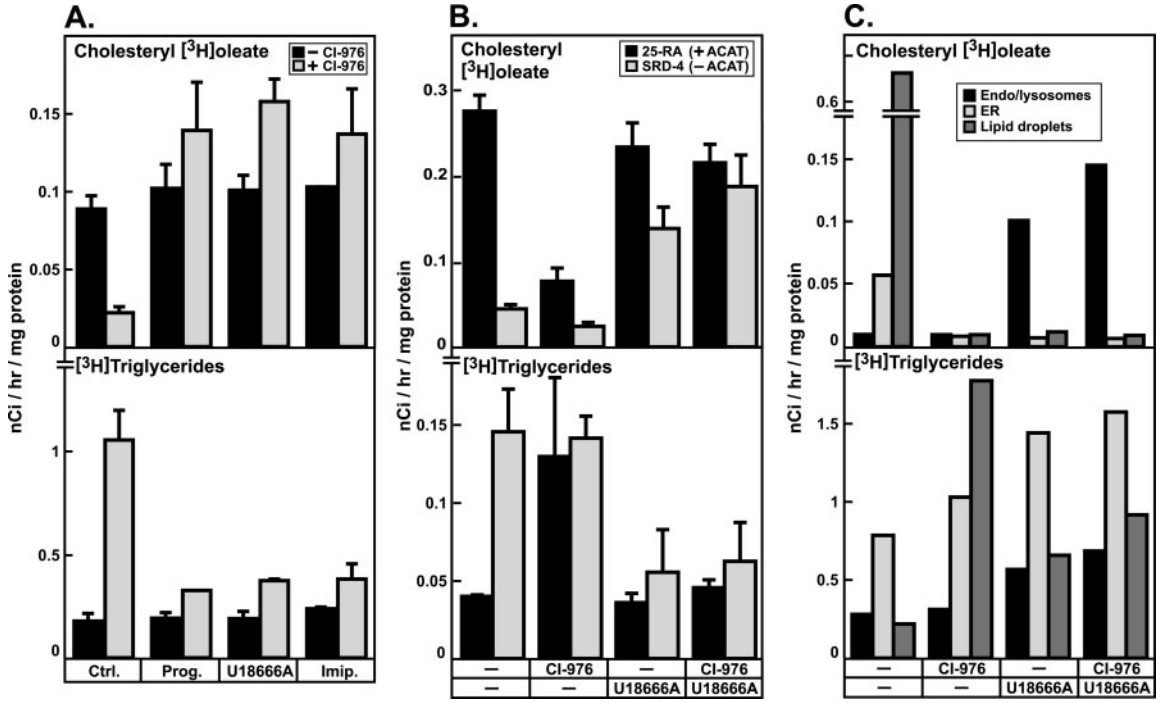

FIG. 8. **Inhibition of cholesterol export leads to cholesteryl ester synthesis in endo/lysosomes *in vivo*.** All incubations were at 37 °C. *A*, on day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 2.5×10^6 cells per well. On day 2, cells were switched for 4 h to medium A supplemented with 10% LPDS plus liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml medium) in the absence or presence of 10 μg/ml progesterone, 2 μg/ml U18666A, 1.6 μg/ml imipramine, and 10 μM CI-976 as indicated. Proteins and lipids were analyzed as described under "Experimental Procedures." *B*, on day 0, 25RA- and ACAT-deficient SRD-4 cells were set up in 6-well plates in medium A containing 5% FBS at 5×10^6 cells per well. On day 1, cells were switched to 5% LPDS. On day 2, cells were incubated with 5 μg/ml LDL (reconstituted with non-radioactive cholesteryl oleate and [^{3}H]trioleoylglycerol; 34 nCi/μg protein) in the absence or presence of 2 μg/ml U18666A and 10 μM CI-976 as indicated. Cells were grown for 4 h, and proteins and lipids were analyzed as described above. *C*, J774 cells were set up as described for *A* and incubated in medium A supplemented with 10% LPDS, liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml medium) plus or minus 2 μg/ml U18666A and 10 μM CI-976 as indicated. After 4 h at 37 °C, cell homogenates were fractionated as described under "Experimental Procedures" (see "Protocol 3"). Fractions were analyzed for organelle markers as described in the legend to Fig. 6. β-Hex peaked in fractions 1 and 2 (containing 64% of the total β-hex activity and 17% of total CCR activity), CCR peaked in fractions 6–9 (containing 45% of total the CCR activity and 13% of total β-hex activity), and lipid droplets were in fractions 10–12 (containing 5% of total β-hex activity and 20% of CCR activity). The indicated pools were analyzed for protein concentration and lipids as described above. *Upper panels*, [^{3}H]cholesteryl oleate; *lower panels*, [^{3}H]triglycerides. *Error bars* indicate S.D. ($n = 3$).

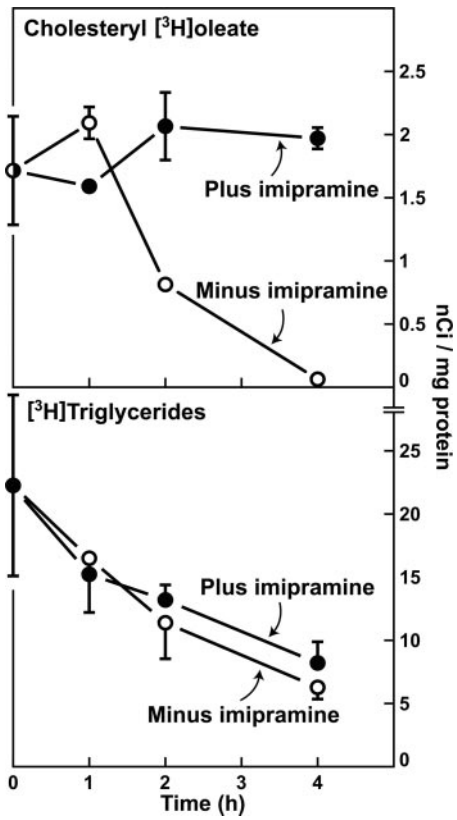

FIG. 9. **Release of cholesterol export block leads to cholesteryl ester hydrolysis in endo/lysosomes *in vivo*.** On day 0, J774 cells were set up in 6-well plates in medium A plus 10% FBS at 2.5×10^6 cells per well. On day 2, all wells were switched to medium A containing 10% LPDS, liposomes (containing non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol; 1 μCi/ml medium), 1.6 μg/ml imipramine, and 10 μM CI-976. Cells were incubated for 4 h at 37 °C, washed, and re-fed with medium A containing 10% LPDS and 10 μM CI-976 plus (*filled circles*) or minus (*open circles*) 1.6 μg/ml imipramine. At the indicated time, cells were harvested, and proteins and lipids were analyzed as described above. *Upper panel*, [^{3}H]cholesteryl oleate; *lower panel*, [^{3}H]triglycerides. *Error bars* indicate S.D. (n = 3).

DISCUSSION

The current data support a model in which endosomal cholesteryl ester hydrolysis proceeds in two steps. Following transfer to the endocytic system, cholesteryl ester is initially hydrolyzed only to a point at which proximal endosomal membranes are saturated with cholesterol. At that stage, net cholesteryl ester hydrolysis stalls as cholesterol is re-esterified in endosomes. In a second step, transfer of cholesterol to extra-endosomal membranes drives cholesteryl ester hydrolysis to completion or to a stage at which these membranes, too, are saturated with cholesterol.

In support of this scheme, we found that isolated endosomes hydrolyzed cholesteryl ester only to a limited extent, whereas addition of cholesterol-poor membranes allowed selective cholesterol release and promoted cholesteryl ester reduction (Figs. 1 and 2). Equivalent results were obtained in living cells; upon pharmacological inhibition of cholesterol export from lysosomes, cholesteryl ester levels remained high but then declined when the transport block was removed (Figs. 7 and 9).

Our studies indicate that, under physiological conditions, net hydrolysis of cholesteryl ester is determined by the cholesterol concentration in acceptor membranes. Overloading cells with cholesterol in the absence of serum and in the presence of an ACAT inhibitor reduced net hydrolysis of cholesteryl ester. Similarly, experiments with cell-free extracts demonstrated that endosomal cholesteryl ester levels remain high in the presence of membranes already saturated with cholesterol (Fig. 3).

A number of papers reported increased cell death in macrophages exposed to modified lipoproteins for prolonged periods of time (45–47). Under the conditions used here, however, no increased apoptosis was detected as judged by caspase activation (data not shown).

The conclusion that endosomes esterify cholesterol under conditions of limited cholesterol release is based on experiments in which cells were loaded with non-radioactive cholesteryl oleate plus [^{3}H]trioleoylglycerol. If cholesteryl oleate was re-synthesized in endosomes, we reasoned, hydrolysis of [^{3}H]trioleoylglycerol should cause the occasional incorporation of a tritiated fatty acid molecule to produce cholesteryl [^{3}H]oleate. Cholesteryl [^{3}H]oleate was in fact produced in endosomes *in vitro* when net cholesterol release was limited due to lack of acceptors or because acceptor membranes were saturated with cholesterol (Figs. 4 and 5). Saturation of membranes with cholesterol also led to a build-up of lysosomal cholesteryl [^{3}H]oleate *in vivo* (Fig. 6). Whether cholesteryl ester sequestration under these conditions results from changes in cholesterol trafficking or whether the lysosomal cholesterol pool remains in dynamic equilibrium with other membranes remains to be determined.

Increased amounts of cholesteryl [^{3}H]oleate were synthesized in lysosomes of cells that had been grown in the presence of drugs that block the export of cholesterol from lysosomes, supporting the notion that endosomal cholesteryl ester metabolism is coupled to cholesterol release (Figs. 7–9). The current results on the effects of progesterone, U18666A, and imipramine might seem paradoxical in light of the fact that these drugs were described as cholesterol transport inhibitors based in part on their ability to cause the accumulation of unesterified cholesterol in lysosomes (37). In these studies, cells were exposed to inhibitors for one to several days, while our experiments were terminated after 4 h. We noted that the synthesis of cholesteryl [^{3}H]oleate in the presence of cholesterol transport inhibitors started to diminish after about 8 h (data not shown), possibly because endosomes start to accumulate phospholipids, increasing their capacity for unesterified cholesterol.

The identity of the enzyme that is responsible for the endosomal acidic esterification activity we observed is currently unknown. The most well understood cellular cholesterol-esterifying enzyme is ACAT, which uses cholesterol and acyl-CoA as substrates. Two main isoforms have been described, ACAT1 and ACAT2 (34, 48). In addition, a novel splice variant of human ACAT1 has recently been described whose biochemical characteristics have not yet been reported (49). However, several lines of evidence support the conclusion that the cholesteryl [^{3}H]oleate synthesis we observed under the above-described conditions was not catalyzed by canonical ACAT. (i) In living cells, synthesis continued in ACAT-deficient cells and in the presence of an ACAT inhibitor (Figs. 6 and 8); (ii) the pH optimum for cholesteryl [^{3}H]oleate synthesis by endosomal fractions *in vitro* was 4.5 (Fig. 5), whereas the pH optimum for ACATs is above neutral (50); (iii) cholesteryl [^{3}H]oleate produced *in vivo* co-fractionated with endosomal/lysosomal vesicles (Figs. 6 and 8), whereas ACAT resides predominantly in the ER and its products are released either into the cytoplasm or the ER lumen (44, 51–53); (iv) unlike ACAT (25, 34–36), the acidic esterification activity was not stimulated by 25-hydroxycholesterol (data not shown).

The only acidic cholesterol-esterifying enzyme that has been described is aCEH, which in reversal of its well known hydrolytic activity can synthesize cholesteryl ester from cholesterol

and fatty acids (14). Previous studies with aCEH-deficient cell extracts have indicated that aCEH is the only acidic cholesterol-esterifying enzyme in human fibroblasts (54). However, based on the current data, the existence of another acidic cholesterol-esterifying enzyme in macrophages or other cell types cannot be ruled out.

Effectively, the dependence of endosomal cholesteryl ester hydrolysis on the cellular cholesterol content can be regarded as another safeguard protecting cells against the potentially toxic effects of excess lipoprotein-derived cholesterol (47). In the sequence of feedback responses that follows the cellular uptake of cholesteryl ester, endosomal cholesteryl ester retention is an early effect that may diminish over time as other mechanisms of cholesterol reduction take over (10, 55, 56). However, cholesteryl ester does persist in lysosomes under certain conditions such as in atherosclerotic foam cells containing large quantities of cholesterol. Based on the current results, we propose that atheromatous cells accumulate cholesteryl ester in lysosomes due to local re-esterification of cholesterol.

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REFERENCES

1. Stary, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 1512–1531
2. Stary, H. C., Chandler, A. B., Glagov, S., Guyton, J. R., Insull, W., Jr., Rosenfeld, M. E., Schaffer, S. A., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1994) *Arterioscler. Thromb.* **14,** 840–856
3. Stary, H. C., Blankenhorn, D. H., Chandler, A. B., Glagov, S., Insull, W., Jr., Richardson, M., Rosenfeld, M. E., Schaffer, S. A., Schwartz, C. J., Wagner, W. D., *et al.* (1992) *Circulation* **85,** 391–405
4. Johnson, W. J., Warner, G. J., Yancey, P. G., and Rothblat, G. H. (1996) *Subcell. Biochem.* **27,** 239–293
5. de Duve, C. (1974) *Acta Cardiol. Suppl.* **20,** 9–25
6. Small, D. M. (1988) *Arteriosclerosis* **8,** 103–129
7. Tabas, I. (2002) *J. Clin. Invest.* **110,** 905–911
8. Jerome, W. G., and Yancey, P. G. (2003) *Microsc. Microanal.* **9,** 54–67
9. Buhman, K. K., Chen, H. C., and Farese, R. V., Jr. (2001) *J. Biol. Chem.* **276,** 40369–40372
10. Chang, T. Y., Chang, C. C., and Cheng, D. (1997) *Annu. Rev. Biochem.* **66,** 613–638
11. Glass, C. K., and Witztum, J. L. (2001) *Cell* **104,** 503–516
12. Jonas, A. (2002) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J. E., eds) 4th Ed., pp. 483–504, Elsevier Science Publishers B. V., Amsterdam
13. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232,** 34–47
14. Assmann, G., and Seedorf, U. (1995) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2563–2587, McGraw-Hill, Inc., New York
15. Goldfischer, S., Schiller, B., and Wolinsky, H. (1975) *Am. J. Pathol.* **78,** 497–504
16. Nilsson, A., Norden, H., and Wilhelmsson, L. (1973) *Biochim. Biophys. Acta* **296,** 593–603
17. Jerome, W. G., Cash, C., Webber, R., Horton, R., and Yancey, P. G. (1998) *J. Lipid Res.* **39,** 1362–1371
18. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol.* **98,** 241–260
19. Basu, S. K., Goldstein, J. L., Anderson, G. W., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3178–3182
20. Aikawa, K., Furuchi, T., Fujimoto, Y., Arai, H., and Inoue, K. (1994) *Biochim. Biophys. Acta* **1213,** 127–134
21. Lange, Y., Ye, J., and Chin, J. (1997) *J. Biol. Chem.* **272,** 17018–17022
22. Storrie, B., and Madden, E. A. (1990) *Methods Enzymol.* **182,** 203–225
23. Liscum, L., Ruggiero, R. M., and Faust, J. R. (1989) *J. Cell Biol.* **108,** 1625–1636
24. Sillence, D. J., and Downes, C. P. (1993) *Biochem. J.* **290,** 381–387
25. Cheng, D., Chang, C. C., Qu, X., and Chang, T. Y. (1995) *J. Biol. Chem.* **270,** 685–695
26. Zhou, X., and Arthur, G. (1992) *J. Lipid Res.* **33,** 1233–1236
27. Brown, M. S., and Goldstein, J. L. (1976) *Cell* **9,** 663–674
28. Underwood, K. W., Jacobs, N. L., Howley, A., and Liscum, L. (1998) *J. Biol. Chem.* **273,** 4266–4274
29. Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) *J. Biol. Chem.* **271,** 21604–21613
30. Merck Index. (2001) in *An encyclopedia of chemicals, drugs, and biologicals* (Budavari, S., ed) 13th Ed., John Wiley & Sons, Whitehouse Station, NJ
31. Le Chatelier, H. L. (1888) *Annales des Mines* **13,** 157–182
32. Bocan, T. M., Mueller, S. B., Uhlendorf, P. D., Newton, R. S., and Krause, B. R. (1991) *Arterioscler. Thromb.* **11,** 1830–1843
33. Roussel, A., Canaan, S., Egloff, M. P., Riviere, M., Dupuis, L., Verger, R., and Cambillau, C. (1999) *J. Biol. Chem.* **274,** 16995–17002
34. Cases, S., Novak, S., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Welch, C. B., Lusis, A. J., Spencer, T. A., Krause, B. R., Erickson, S. K., and Farese, R. V., Jr. (1998) *J. Biol. Chem.* **273,** 26755–26764
35. Erickson, S. K., Shrewsbury, M. A., Brooks, C., and Meyer, D. J. (1980) *J. Lipid Res.* **21,** 930–941
36. Lichtenstein, A. H., and Brecher, P. (1980) *J. Biol. Chem.* **255,** 9098–9104
37. Liscum, L., and Munn, N. J. (1999) *Biochim. Biophys. Acta* **1438,** 19–37
38. Butler, J. D., Blanchette-Mackie, J., Goldin, E., O'Neill, R. R., Carstea, G., Roff, C. F., Patterson, M. C., Patel, S., Comly, M. E., Cooney, A., Vanier, M. T., Brady, R. O., and Pentchev, P. G. (1992) *J. Biol. Chem.* **267,** 23797–23805
39. Liscum, L., and Faust, J. R. (1989) *J. Biol. Chem.* **264,** 11796–11806
40. Rodriguez-Lafrasse, C., Rousson, R., Bonnet, J., Pentchev, P. G., Louisot, P., and Vanier, M. T. (1990) *Biochim. Biophys. Acta* **1043,** 123–128
41. Chang, T. Y., and Limanek, J. S. (1980) *J. Biol. Chem.* **255,** 7787–7795
42. Nohturfft, A., Hua, X., Brown, M. S., and Goldstein, J. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13709–13714
43. Metherall, J. E., Ridgway, N. D., Dawson, P. A., Goldstein, J. L., and Brown, M. S. (1991) *J. Biol. Chem.* **266,** 12734–12740
44. Khelef, N., Soe, T. T., Quehenberger, O., Beatini, N., Tabas, I., and Maxfield, F. R. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 1769–1776
45. Yao, P. M., and Tabas, I. (2001) *J. Biol. Chem.* **276,** 42468–42476
46. Tabas, I., Marathe, S., Keesler, G. A., Beatini, N., and Shiratori, Y. (1996) *J. Biol. Chem.* **271,** 22773–22781
47. Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) *Nat. Cell Biol.* **5,** 781–792
48. Chang, C. C., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) *J. Biol. Chem.* **268,** 20747–20755
49. Yang, L., Lee, O., Chen, J., Chang, C. C., Zhou, P., Wang, Z. Z., Ma, H. H., Sha, H. F., Feng, J. X., Wang, Y., Yang, X. Y., Wang, L., Dong, R., Ornvold, K., Li, B. L., and Chang, T. Y. (2004) *J. Biol. Chem.* **279,** 46253–46262
50. Kinnunen, P. M., DeMichele, A., and Lange, L. G. (1988) *Biochemistry* (*Mosc.*) **27,** 7344–7350
51. Balasubramaniam, S., Venkatesan, S., Mitropoulos, K. A., and Peters, T. J. (1978) *Biochem. J.* **174,** 863–872
52. Khelef, N., Buton, X., Beatini, N., Wang, H., Meiner, V., Chang, T. Y., Farese, R. V., Jr., Maxfield, F. R., and Tabas, I. (1998) *J. Biol. Chem.* **273,** 11218–11224
53. Sakashita, N., Miyazaki, A., Takeya, M., Horiuchi, S., Chang, C. C., Chang, T. Y., and Takahashi, K. (2000) *Am. J. Pathol.* **156,** 227–236
54. Slotte, J. P. (1987) *Biochim. Biophys. Acta* **917,** 231–237
55. Repa, J. J., and Mangelsdorf, D. J. (2002) *Nat. Med.* **8,** 1243–1248
56. Tall, A. R. (1998) *Eur. Heart J.* **19,** Suppl. A, A31–A35