The Distal Pathway of Lipoprotein-induced Cholesterol Esterification, but Not Sphingomyelinase-induced Cholesterol Esterification, Is Energy-dependent*

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The stimulation of the intracellular cholesterol esterification pathway by atherogenic lipoproteins in macrophages is a key step in the development of atheroma foam cells. The esterification pathway can also be stimulated by hydrolysis of cell-surface sphingomyelin by the enzyme sphingomyelinase (SMase). In both cases, intracellular cholesterol transport to the cholesterol esterifying enzyme, acyl-CoA:cholesterol O-acyltransferase (ACAT), is thought to be critical, although the mechanism of cholesterol transport is not known. In this report, we explore two fundamental properties of the cholesterol esterification pathway, namely its dependence on energy and the effect of other treatments that block membrane vesicle trafficking. After the atherogenic lipoprotein, b-very low density lipoprotein (b-VLDL), was internalized by macrophages and hydrolyzed in lysosomes, the cells were depleted of energy by treatment with sodium azide and 2-deoxyglucose or by permeabilization. Under these conditions, which allowed equal b-VLDL-cholesterol ester hydrolysis, cholesterol esterification was markedly decreased in the energy-depleted cells. This effect was not due to blockage of lysosomal cholesterol export. In the permeabilized cell system, energy repletion restored b-VLDL-induced cholesterol esterification. Remarkably, stimulation of cholesterol esterification by SMase was not inhibited by energy depletion. Energy depletion also inhibited b-VLDL-induced, but not SMase-induced, cholesterol esterification in Chinese hamster ovary cells. Similar experiments were carried out using N-ethylmaleimide, low potassium medium, or inhibitors of phosphatidylinositol 3-kinase, each of which blocks intracellular membrane vesicle trafficking. These treatments also inhibited b-VLDL-induced, but not SMase-induced, cholesterol esterification. Finally, we show here that SMase treatment of cells leads to an increase in plasma membrane vesiculation that is relatively resistant to energy depletion. In summary, the stimulation of cholesterol esterification by lipoproteins, but not by SMase, is energy-dependent, N-ethylmaleimide-sensitive, and blocked by both low potassium and phosphatidylinositol 3-kinase inhibitors. The affected step or steps are distal to cholesterol export from lysosomes and not due to direct inhibition of the ACAT enzyme. Thus, the mechanisms involved in lipoprotein-induced versus SMase-induced cholesterol esterification are different, perhaps due to the involvement of energy-dependent vesicular cholesterol transport in the lipoprotein pathway and a novel, energy-independent vesicular transport mechanism in the SMase pathway.

Cholesteryl ester (CE)b-filled macrophages, or foam cells, are prominent features of atherosclerotic lesions (1–3) and play important roles in atherogenesis (4) and lesion complications (5, 6). Macrophages become loaded with CE by the uptake of atherogenic lipoproteins followed by intracellular cholesterol esterification catalyzed by the enzyme ACAT (7). Our knowledge of the cholesterol esterification pathway to date can be summarized as follows (8). Following lipoprotein uptake, lipoprotein-associated free cholesterol and cholesterol derived by lysosomal hydrolysis of lipoprotein-CE are distributed to cellular cholesterol pools. These expanded cholesterol pools appear to be in or associated with the plasma membrane. After a threshold increment in cellular cholesterol mass has been reached, mixed pools of cellular and lipoprotein-cholesterol and cholesterol derived by lysosomal hydrolysis of lipoprotein-CE are distributed to cellular cholesterol pools. These expanded cholesterol pools appear to be in or associated with the plasma membrane. After a threshold increment in cellular cholesterol mass has been reached, mixed pools of cellular and lipoprotein-cholesterol come into contact with ACAT and are esterified. The mechanism and regulation of ACAT stimulation by cellular cholesterol loading are poorly understood, but increases in ACAT mRNA or protein have not been shown not to be the predominant mechanism (9, 10).

Two sequential intracellular cholesterol transport pathways appear to be critically involved in cholesterol esterification. The first pathway, the “proximal” pathway, results in cholesterol delivery to the plasma membrane or a plasma membrane–associated pool (8). This occurs via lysosomal–to-plasma membrane cholesterol transport (11) and possibly by direct delivery of lipoprotein cholesterol to the plasma membrane following entry of the lipoproteins in cell-surface invaginations (12–14). A report examining CHO cells has shown lysosomal–to-plasma membrane cholesterol transport to be energy-independent (15), but, in macrophages, lysosomal and/or endosomal acidification, an energy-dependent process, may be necessary for this process (16). Lysosomal–to-plasma membrane cholesterol transport is not dependent upon an intact cellular cytoskeleton (15). A second pathway, the “distal” pathway, involves plasma membrane–to-ACAT cholesterol transport (17, 18). ACAT is thought to be primarily localized to the endoplasmic reticulum (10), but other intracellular sites, including association with the plasma membrane, have not been ruled out (cf. Ref. 8). The distal

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1 The abbreviations used are ACAT, acyl-coenzyme A:cholesterol acyltransferase; BSA, bovine serum albumin; CE, cholesteryl ester; CHO, Chinese hamster ovary; LPDS, lipoprotein-deficient serum; NEM, N-ethylmaleimide; NSF, NEM-sensitive factor; PBS, phosphate-buffered saline; SMase, sphingomyelinase; b-VLDL, b-very low-density lipoprotein.
pathway is dependent upon the action of cofilin in macrophages, but not in CHO cells (19); a role for intermediate filaments in an adrenal cell line has also been reported (20). Furthermore, there is evidence that intracellular actin cytoskeletal protease activity, perhaps by cleaving an endogenous inhibitor of this pathway (21, 22), plays an important role in plasma membrane-to-ACAT cholesterol transport (23).

Interestingly, treatment of cells with the enzyme sphingomyelinase (SMase) can lead to the stimulation of cholesterol esterification without expanding cellular cholesterol pools (24). SMase treatment causes esterification of endogenous plasma membrane cholesterol (24), and thus one might expect similar mechanisms of cholesterol transport to ACAT as that seen in the distal pathway of lipoprotein-induced cholesterol esterification. The mechanism of SMase-induced esterification, however, is not known (25). Recent work in our laboratory has shown that SMase-induced cholesterol esterification, unlike lipoprotein-induced cholesterol esterification, does not depend upon an intact actin cytoskeleton in macrophages (19) and is not affected by cytoskeletal protease inhibitors (23).

With this background, it is clear that several fundamental properties of the cholesterol esterification pathway in cells remain to be elucidated. Salient among these properties is whether the lipoprotein-induced distal cholesterol transport pathway and/or the SMase pathway are energy-dependent, as would be expected, for example, if cholesterol transport to ACAT involved vesicular transport (26–32). The absence of previous reports on this fundamental property of the distal pathway of cholesterol esterification is likely due to methodological problems (see "Results"). In this report, we have devised several strategies to overcome these problems, and we herein show that in both macrophages and CHO cells, the distal cholesterol esterification pathway induced by lipoproteins, but not that induced by SMase, is energy-dependent. In addition, three other treatments that block intracellular vesicular processes have similar effects as energy depletion. Finally, we show here that SMase treatment of cells leads to an increase in plasma membrane vesiculation that is relatively resistant to energy depletion. Thus, the mechanisms involved in lipoprotein-induced versus SMase-induced cholesterol esterification are different, perhaps due to the involvement of energy-dependent vesicular cholesterol transport in the lipoprotein pathway and a novel, energy-independent vesicular transport mechanism in the SMase pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture media and reagents were purchased from Life Technologies, tissue culture plates were from Corning, and defined fetal bovine serum was from Hydrolife, Inc. (Logan, UT). Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of the fetal bovine serum to obtain the d > 1.21 g/ml fraction. [1,2,6,7-3H]Cholesteryl linoleate (73 Ci/mmol) and [9,10-3H]oleic acid (92 Ci/mmol) were obtained from DuPont NEN. [3H]Joleoyl-CoA was synthesized by the method of Kawaguchi et al. (33) and purified by preparative acid precipitation. The [3H]Joleoyl-CoA had a specific activity of 583 cpm/pmol and was 94% pure by TLC. Cholesterol and cholesteryl oleate were purchased from Steraloids, Inc. (Wilton, NH). Sodium azide, 2-deoxyglucose, N-ethylmaleimide, fatty acid-free bovine serum albumin, filipin, Wortmannin, progesterone, and other reagents were from Sigma. Wortmannin was prepared as a 1-mM stock solution in DMEM, 0.2% BSA. NEM was added from a 100 mM stock solutions in DMEM, 0.2% BSA. Potassium depletion was carried out using low potassium medium (142 mM NaCl, 3.6 mM CaCl2, 20 mM HEPES, 0.34 mM KH2PO4, 0.35 mM KHPO4, 0.81 mM MgCl2, 0.2% BSA) (35).

**Measurement of Cellular ATP Levels**—Cellular ATP levels were measured using the Sigma ATP bioluminescent assay kit.

**Cell Permeabilization**—Macrophages that were incubated in the absence or presence of β-VLDL for up to 17°C to allow internalization but not hydrolysis of the β-VLDL were washed twice with sterile PBS, incubated for 1 h at 17°C in the absence of β-VLDL, and then incubated with 2 units of streptolysin-O/ml of PBS for 5 min at 37°C (36). The cells were then washed for 30 min at 4°C in buffer 1 (137 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM NaPO4, 0.2% BSA, 25 mM Tris, pH 7.4). Cholesterol esterification activity was then assayed with [3H]Joleoyl-CoA (final concentration = 50 μM) in buffer 1 as described above.

**Whole-cell Cholesterol Esterification Assay**—Cells were incubated in the absence or presence of β-VLDL for 6 h at 17°C to allow internalization but not hydrolysis of the β-VLDL. After the preincubations described in the text and figure legends, the cells were washed with PBS and incubated for the indicated times with [3H]Joleoyl-CoA (final concentration = 50 μM). Lipid extracts were prepared as described previously (22); cellular DNA content was determined by the method of Labarca and Paigen (37). For certain other experiments, cholesterol esterification was assayed by quantifying the esterification of endogenous, labeled cellular cholesterol. For these experiments, the cells were prelabeled with [3H]cholesterol as described previously (37). [3H]Joleoyl-CoA (final concentration = 50 μM) in buffer 1 as described above was incubated with [3H]Joleoyl-CoA A (final concentration = 50 μM) in buffer 1 as described above.

**Whole-cell Cholesterol Esterification Assay**—Cells were incubated in the absence or presence of β-VLDL for 6 h at 17°C to allow internalization but not hydrolysis of the β-VLDL. After the preincubations described in the text and figure legends, the cells were washed with PBS and incubated for the indicated times with [3H]Joleoyl-CoA A (final concentration = 50 μM) in buffer 1 as described above.

**Cholesterol Esterification**—Whole-cell cholesterol esterification was assayed by quantifying the esterification of endogenous, labeled cellular cholesterol. For these experiments, the cells were prelabeled with [3H]cholesterol as described previously (37). [3H]Joleoyl-CoA (final concentration = 50 μM) in buffer 1 as described above was incubated with [3H]Joleoyl-CoA A (final concentration = 50 μM) in buffer 1 as described above.

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**Fluorescence Microscopy**—Filipin fluorescence was observed using a Leitz Diavert microscope (63× N.A. 1.4 objective) and recorded with a cooled charge-coupled device. A UV-filter set (340–380-nm excitation, 430-nm long pass filters) was used to observe filipin fluorescence. For the fluorescein-dextran experiment shown in Fig. 7, fluorescence images were obtained using a Leitz Diavert microscope (63× N.A. 1.4 objective) and recorded with a cooled charge-coupled device.
were obtained with a laser scanning confocal microscope (MRC 600; Bio-Rad Microscience, Cambridge, MA) on an inverted microscope (Axiovert; Zeiss, Oberkochen, Germany) using a 63 × 0.14 NA 1.4 Zeiss Plan-Apo infinity-corrected objective. The illumination sources were the 488- and 514-nm lines from a 25-milliwatt argon laser. The fluorescein-dextran was visualized with a 488-nm band-pass excitation filter, a 510-nm dichroic mirror, and a 515-nm long pass emission filter. All images were printed on a Tektronix Phaser II SDX printer using Adobe Photoshop.

Statistics—Unless indicated otherwise, results are given as means ± S.D. (n = 3). Absent error bars signify standard deviation values smaller than the graphics symbol.

RESULTS

Energy Depletion Inhibits β-VLDL-Induced Stimulation of Cholesterol Esterification in Macrophages and CHO Cells—Our initial goal was to assess the effect of energy depletion on those steps of the β-VLDL-induced cholesterol esterification pathway that are distal to endocytosis and lysosomal β-VLDL-CE hydrolysis. To accomplish this goal, it was necessary to overcome three problems. First, whole-cell cholesterol esterification assays are typically conducted by incubating the cells with labeled fatty acid, which is then converted by an energy-dependent intracellular reaction to the ACAT substrate, fatty acyl-CoA (39). In preliminary experiments, however, we found that exogenously added [3H]oleoyl-CoA was incorporated into cellular cholesteryl esters to the same extent as [3H]oleate in J 774 macrophages. This process did not involve hydrolysis and re-esterification of [3H]oleate (see below). Thus, we were able to study the effect of energy depletion on the cholesterol esterification pathway without the complication of inhibited fatty acyl-CoA synthesis. Second, as expected (28–31), energy depletion blocked β-VLDL endocytosis. This problem was solved by incubating the cells with β-VLDL at 17°C, which allows internalization but not lysosomal delivery (40), prior to the addition of energy poisons. In our experimental system, we verified this point by showing lack of substantial lysosomal β-VLDL-CE hydrolysis at 17°C; when J 774 macrophages were incubated with [3H]CE-labeled β-VLDL for 6 h at 17°C, only 7.4% of the [3H]CE was hydrolyzed to [3H]cholesterol. When the cells were then incubated for an additional 3 h at 37°C, 72% of the β-VLDL-CE was hydrolyzed. Third, in preliminary experiments we found that energy depletion blocked β-VLDL-CE hydrolysis. Therefore, after the initial 17°C internalization period, we incubated the cells an additional 1 h at 37°C prior to the addition of energy poisons, which resulted in 61% hydrolysis of β-VLDL-CE.

Using this experimental strategy, the effect of energy depletion on β-VLDL-induced cholesterol esterification was determined (Fig. 1). The data show that under conditions of equal β-VLDL-CE hydrolysis (inset), β-VLDL-induced cholesterol esterification was markedly inhibited by energy depletion. Similar results were obtained using an assay in which the cells were prelabeled with [3H]cholesterol, and the formation of [3H]cholesteryl esters was determined (data not shown). Dose experiments with the energy poisons showed a slight but significant inhibitory effect at 25 mM sodium azide and 25 mM 2-deoxyglucose, and a maximal effect at 100 mM of both compounds. At 100 mM of both compounds, fluid phase endocytosis, as measured by fluorescein-dextran uptake, was completely inhibited within 5 min. In addition, the cellular ATP concentration was decreased by ~98% compared with untreated cells. The inhibition of cholesterol esterification in energy-depleted cells was not due to increased cellular cholesterol efflux, which was not affected by energy depletion (11.9 ± 1.0% of cellular cholesterol efflux in control cells versus 11.4 ± 1.6% in energy-depleted cells).

Treatments of cells with the enzyme sphingomyelinase (SMase) induces esterification of cellular cholesterol like β-VLDL but without expanding cellular cholesterol pools (24); the mechanism of this effect of SMase is not known. To determine the effect of energy depletion on SMase-induced cholesterol esterification, macrophages were incubated exactly as in the β-VLDL experiment, including the preincubation at 17°C (above), but with SMase added during the 3-h 37°C incubation period. Surprisingly, SMase-induced cholesterol esterification was found to be stimulated under conditions of energy depletion (Fig. 2A). Similar results were found using the [3H]cholesterol assay instead of the [3H]oleoyl-CoA assay (Fig. 2B). These SMase data reveal two important points. First, energy depletion appears to specifically inhibit a process involved in lipoprotein-induced cholesterol esterification. Second, energy depletion does not simply inhibit the ACAT enzyme or re-esterification of oleate to oleoyl-CoA (see above).

To explore these effects using an alternative strategy, we utilized permeabilized cells, which are energy-depleted due to loss of ATP unless an ATP-regenerating system is included in
the system (cf. Ref. 36). For \( \beta \)-VLDL-induced esterification (A), macrophages were incubated with \( \beta \)-VLDL for 6 h at 17°C, incubated for 1 h at 17°C in the absence of \( \beta \)-VLDL to allow internalization of residual cell-surface-bound \( \beta \)-VLDL, and then permeabilized at 37°C using streptolysin-O. Cholesterol esterification was assayed for 3 h in the absence or presence of an ATP-regenerating system using the \([^{3}H]\)oleoyl-CoA assay. For SMase-induced esterification (B), the 7 h-17°C incubation was done in the absence of \( \beta \)-VLDL, and SMase was included during the 3-h esterification assay. The data shown are calculated from subtracting basal cholesterol esterification values (i.e. in the absence of \( \beta \)-VLDL or SMase), which were 1.13 ± 0.2 (+ATP) and 1.45 ± 0.2 (–ATP) pmol/3 h/mg DNA.

We next explored the effect of energy depletion on cholesterol esterification in CHO cells. These cells were subjected to all of the preliminary experiments and controls described above for the J 774 macrophages; in particular, CHO cells were also able to utilize exogenously added \([^{3}H]\)oleoyl-CoA as substrate for the intracellular cholesterol esterification reaction. CHO cells were more sensitive to the energy inhibitors, so that virtually complete depletion of ATP and inhibition of fluid phase endocytosis was seen at 15 mM sodium azide and 15 mM 2-deoxyglucose. At these concentrations of inhibitors, \( \beta \)-VLDL-induced cholesterol esterification was 39 ± 13.7 pmol/3 h/mg DNA, whereas the value in uninhibited cells was 153 ± 11.0 (74% inhibition). The SMase experiment was done using 5 mM inhibitors, since cell toxicity occurred with SMase plus 15 mM inhibitors. At 5 mM inhibitors, \( \beta \)-VLDL-induced cholesterol esterification was inhibited by 30%, whereas SMase-induced esterification was not inhibited at all (255 ± 19 versus 242 ± 16 pmol/3 h/mg DNA, minus and plus energy inhibitors, respectively (difference not statistically significant)). Thus, similar to the situation with J 774 macrophages, energy depletion inhibited \( \beta \)-VLDL-induced, but not SMase-induced, cholesterol esterification in CHO cells.

In summary, \( \beta \)-VLDL-induced cholesterol esterification is an energy-dependent process whereas SMase-induced cholesterol esterification is not. The energy-dependent step or steps in \( \beta \)-VLDL-induced esterification are distal to lysosomal \( \beta \)-VLDL-CE hydrolysis. The significance of the increase in SMase-induced esterification in energy-depleted J 774 cells remains to be explored (see below and "Discussion").

Effect of Other Inhibitors of Membrane Vesicle Trafficking on the Cholesterol Esterification Pathway in Macrophages—If the effect of energy depletion on \( \beta \)-VLDL-induced cholesterol esterification is due to inhibition of membrane vesicle trafficking (eg. inhibition of vesicular cholesterol transport), then other inhibitors of membrane vesicle trafficking should have similar effects as energy depletion. One such inhibitor is the protein alkylating agent N-ethylmaleimide (NEM); the mechanism appears to involve inhibition of fusion of carrier vesicles with

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**FIG. 2. Effect of energy depletion on SMase-induced cholesterol esterification in macrophages.** A, macrophages were cultured for 2 days in DMEM, 10% LPDS, then rinsed and incubated at 17°C with DMEM, 0.2% BSA for 7 h. The cells were then rinsed with PBS and assayed for cholesterol esterification by incubating for 3 h at 37°C with DMEM, 0.2% BSA containing 50 \( \mu \)M \([^{3}H]\)oleoyl-CoA in the absence or presence of 50 milliunits of SMase/ml. Some of the cells were exposed to 100 mM 2-deoxyglucose plus 100 mM sodium azide (+ 2-DG + NaN) during the 3-h assay. B, macrophages were cultured for 1 day in DMEM, 10% LPDS followed by 8 h in DMEM, 10% LPDS containing \([^{3}H]\)cholesterol-PC liposomes and then 16 h in DMEM, 10% LPDS alone. The cells were then rinsed, incubated at 17°C for 7 h with DMEM, 0.2% BSA, and finally incubated for 3 h at 37°C with DMEM, 0.2% BSA containing 50 \( \mu \)M unlabelled oleoyl-CoA ± SMase and ± energy poisons as above. Cellular \([^{3}H]\)CE cpm were then determined.

**FIG. 3. Effect of energy depletion on \( \beta \)-VLDL-induced and SMase-induced cholesterol esterification in permeabilized macrophages.** A, macrophages were incubated in the absence or presence of \( \beta \)-VLDL for 6 h at 17°C, washed twice with PBS, incubated for 1 h at 17°C in the absence of \( \beta \)-VLDL, and then incubated with 2 units of streptolysin O/ml in PBS for 5 min at 37°C. The cells were then incubated for 30 min at 4°C in buffer 1 (see "Experimental Procedures") containing 0.2% BSA alone (–ATP) or containing an ATP-regenerating system (+ATP). The ATP-regenerating system consisted of 1 mM ATP, 8 mM creatine phosphate, and 40 units of creatine phosphokinase/ml. Cholesterol esterification activity was then assayed at 37°C for 3 h in the same buffer containing \([^{3}H]\)oleoyl-CoA. B, macrophages were incubated as above except \( \beta \)-VLDL was not present in the 7-h, 17°C incubation, and the 3-h, 37°C incubation was done in the absence or presence of 50 milliunits of SMase. The data shown are calculated from subtracting basal cholesterol esterification values (i.e. in the absence of \( \beta \)-VLDL or SMase), which were 1.13 ± 0.2 (+ATP) and 1.45 ± 0.2 (–ATP) pmol/3 h/mg DNA.

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target membranes (41), as well as inhibition of other vesicular transport processes (42). Using a similar strategy as with the energy depletion experiments, J 774 macrophages were preincubated with β-VLDL at 17 °C and then assayed for cholesterol esterification in the absence or presence of NEM. The data in Fig. 4A show that NEM completely inhibited β-VLDL-induced stimulation of cholesterol esterification. Cells incubated with SMase after the NEM treatment, however, exhibited the same degree of stimulation of cholesterol esterification as in untreated cells (Fig. 4B).

Another method of inhibiting plasma membrane vesicle trafficking is by incubating cells in low potassium medium, which inhibits clathrin-coated pit formation (35) and, under certain circumstances, non-coated pit formation and endocytosis as well (43). This latter point was demonstrated in our system by showing complete inhibition of fluorescein-dextran uptake in macrophages incubated in low potassium medium. To test the effect of potassium depletion on cholesterol esterification, macrophages were incubated in the presence or absence of β-VLDL at 17 °C. The cells were then assayed for cholesterol esterification in normal or low potassium medium. The data in Fig. 5A show that potassium depletion markedly inhibited β-VLDL-induced cholesterol esterification. Sphingomyelinase-induced cholesterol esterification was not affected by this treatment (Fig. 5B).

A third method of disrupting membrane vesicle trafficking, albeit by a mechanism that is not precisely known (44, 45), is by treatment of cells with inhibitors of phosphatidylinositol 3-kinase. One such inhibitor is the cell-permeant fungal metabolite wortmannin (45). Preliminary experiments revealed that treatment of J 774 macrophages with 25 nm wortmannin partially slowed fluorescein-dextran uptake without signs of cytotoxicity, consistent with the data of Li et al. (45); concentrations of wortmannin up to 250 nm did not further block fluorescein-dextran uptake, and concentrations above this level resulted in cell toxicity. Thus, unlike the other inhibitors used above, wortmannin did not completely block membrane vesicle trafficking. Nonetheless, using the same experimental protocol as described above for the other inhibitors, we found that 25 nm wortmannin treatment inhibited β-VLDL-induced cholesterol esterification by 35.3 ± 4.1%, whereas SMase-induced esterification was stimulated by 21.9 ± 7.3%. Another phosphatidylinositol 3-kinase inhibitor is compound LY294002 (2-(4-morpholinoyl)-8-phenyl-4H-1-benzopyran-4-one) (46). Using our standard protocol, we found that this compound inhibited β-VLDL-induced cholesterol esterification by 32.0 ± 1.1%; SMase-induced esterification was not inhibited at all by this compound.

In summary, four cellular perturbations that are known to inhibit membrane vesicular trafficking, namely energy depletion, NEM treatment, incubation in low potassium medium, and treatment with inhibitors of phosphatidylinositol 3-kinase, inhibit β-VLDL-induced cholesterol esterification but not SMase-induced esterification.

Energy Depletion Does Not Affect Cholesterol Export from Lysosomes—The most straightforward interpretation of the above data is that β-VLDL-induced cholesterol esterification involves membrane vesiculation, for example vesicular cholesterol transport, whereas SMase-induced esterification does not. The two major cholesterol transport pathways involved in lipoprotein-induced cholesterol esterification are lysosomal-to-plasma membrane (proximal pathway) and plasma membrane-to-ACAT (distal pathway) (see Introduction). Inhibition of the distal pathway is difficult to assess by morphological means, since the actual amount of cholesterol delivered to ACAT is a small percentage of total plasma membrane cholesterol (cf. Ref. 25). Inhibition of lysosomal cholesterol export, however, can be readily assessed by using a filipin-staining technique (cf. Ref. 38). For this purpose, macrophages were incubated in lipoprotein-deficient medium or in medium containing β-VLDL. In the absence or presence of sodium azide plus 2-deoxyglucose. The cells were then fixed, stained with filipin to visualize intracellular accumulations of free cholesterol (38), and viewed by fluorescence microscopy. Compared with cells incubated without lipoproteins (Fig. 6A), those incubated with β-VLDL showed increased filipin fluorescence, particularly in the plasma membrane (Fig. 6B). The pattern and intensity of fluorescence in the energy-depleted cells incubated with β-VLDL (Fig. 6C) were very similar to those seen in cells incubated with β-VLDL alone. To compare these patterns with one in which lysosomal trapping of free cholesterol is known to occur, macrophages were incubated with β-VLDL plus compound U18666A, which blocks the exit of lipoprotein-derived cholesterol from lysosomes (47). In contrast to the pattern seen with the other treatments, macrophages incubated with β-VLDL plus U18666A demonstrated very bright punctate perinuclear fluorescence without plasma membrane staining (Fig. 6D), indicative of a high concentration of free cholesterol in lysosomes and blockage of transport to the plasma membrane. These data indicate that energy depletion does not substantially block the proximal pathway of β-VLDL-induced cholesterol esterification (i.e. lysosome-to-plasma membrane cholesterol transport).
SMase Stimulates Vesiculation of the Plasma Membrane in a Relatively Energy-independent Manner—One possible explanation for the differential effects of energy depletion, NEM, low potassium, and phosphatidylinositol 3-kinase inhibitors on β-VLDL-induced versus SMase-induced cholesterol esterification is that the former process involves vesicular cholesterol transport whereas the latter utilizes a protein-mediated cholesterol transport process. This explanation appeared unlikely to us, however, since SMase was still able to induce cholesterol esterification in permeabilized cells (Fig. 3), which are depleted of most of their soluble proteins (see Ref. 36 and data not shown). An alternative explanation, supported by the data in Fig. 7, is that SMase-induced esterification involves an energy-independent membrane vesiculation process. In this experiment, J 774 macrophages were incubated for 30 min with fluorescein-labeled dextran in the absence or presence of SMase, under control or energy-depleted conditions, and then viewed by confocal fluorescence microscopy (Fig. 7). Compared with the intensity of the fluorescein-dextran-labeled membrane vesicles in the control cells (A), there appeared to be an increase in fluorescence intensity in the SMase-treated cells (B). This impression was confirmed by quantification of fluorescence power of three different fields of these cells; the values for the untreated and SMase-treated cells were 2.23 ± 0.32 and 3.93 ±
0.45 \times 10^6 \text{ units per cell, respectively (80% increase). Remarkably, whereas fluorescein-dextran uptake was completely inhibited by energy depletion in the cells not treated with SMase (C), there was still considerable dextran uptake in energy-depleted, SMase-treated cells (D). Moreover, the pattern of fluorescence was distinct; the labeled vesicles were localized in proximity to the plasma membrane. Importantly, fluorescein quenching experiments using nonpermeable trypan blue (see Ref. 14) indicated that the labeled vesicles in these cells were sealed intracellular compartments. Longer incubation times (up to 3 h) and pulse-chase experiments in energy-depleted cells revealed continued localization of the vesicles in the cell periphery; when these cells were placed in fresh glucose-containing medium without energy inhibitors, however, most of the fluorescence moved toward the center of the cell, similar to the pattern seen in A. In summary, SMase treatment of J 774 macrophages stimulates the uptake of fluorescein-dextran; remarkably, fluorescein-dextran uptake is still quite marked, although morphologically distinct, even when the cells are depleted of energy. It is possible, therefore, that the stimulation of cholesterol esterification in SMase-treated cells, which is resistant to cellular energy depletion, is related to this novel membrane vesiculation process.

**DISCUSSION**

Lipoprotein-induced cholesterol esterification in macrophages is a prominent and important event during atherosclerosis (1–6). SMase-induced esterification of macrophages and other cells may also be physiologically important, since many cells are known to respond to cytokines by a burst of cellular sphingomyelin hydrolysis (48, 49). In fact, Chatterjee and colleagues (50) have specifically shown that one such cytokine, namely tumor necrosis factor-α, leads to stimulation of intracellular cholesterol esterification. Although the SMases involved in this pathway have not been definitively elucidated (see Ref. 51), our recent discovery that macrophages and other cell types secrete a soluble Zn2+-stimulated SMase may be potentially relevant to the findings reported herein. In any case, studies comparing lipoprotein-induced versus SMase-induced cholesterol esterification are likely to give insight into important mechanistic aspects of this intracellular reaction. In this report, we show that the distal pathway of the β-VLDL-induced cholesterol esterification in both macrophages and CHO cells is energy-dependent, while that involved in SMase-induced esterification is not. These findings raise two important questions, what role or roles does energy play in the lipoprotein pathway, and what are the mechanistic implications of the lack of energy dependence in the SMase pathway?

The filipin data in Fig. 6 and the fact that the ACAT enzyme itself is not inhibited by energy depletion (Fig. 2) indicate that the energy-dependent step or steps are in the distal pathway of lipoprotein-induced cholesterol esterification, most likely in the delivery of cholesterol from the plasma membrane (or from a plasma membrane-associated ACAT substrate pool) to ACAT. A prominent effect of cellular ATP depletion is inhibition of membrane vesiculation, such as occurs in receptor-mediated endocytosis occurring through clathrin-coated pits as well as noncoated pit-mediated fluid phase endocytosis (28–32). In addition, three other treatments that inhibit membrane vesicle trafficking, namely NEM (41, 42), potassium depletion (35, 43, 52), and phosphatidylinositol 3-kinase inhibitors (44, 45), were found to have similar effects as ATP depletion on β-VLDL-induced cholesterol esterification. Thus, although each of these four treatments have other effects on cells, our findings are most consistent with the idea that membrane vesicle trafficking, such as vesicle-mediated transport of cholesterol, is important in the distal pathway of lipoprotein-induced cholesterol esterification. In this regard, the findings of Kaplan and Simoni (53) and Lange and colleagues (27) regarding vesicular transport of cholesterol between the endoplasmic reticulum and plasma membrane are entirely consistent with our conclusion.

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2 X. Zha, P. J. Skiba, I. Tabas, and F. R. Maxfield, submitted for publication.

3 S. L. Schissel, E. H. Schuchman, K. J. Williams, and I. Tabas, submitted for publication.
The data in this report and those from our previous work reveal interesting differences between the distal lipoprotein-induced cholesterol esterification pathway and SMase-induced cholesterol esterification, including differences in susceptibility to energy depletion, to inhibitors of membrane vesicle trafficking, to disruption of the actin cytoskeleton (see Ref. (9)), and to intracellular protease inhibition (23). We have no evidence that SMase-induced esterification involves a different esterifying enzyme; for example, both lipoprotein-induced and SMase-induced esterification are blocked by the ACAT inhibitor, compound 58035.4 Also, we consider it unlikely that either a protein carrier mechanism or ceramide-induced signal transduction (48, 49) plays an important role in SMase-induced cholesterol esterification (see Fig. 3 and Refs. 54, 55, 4 respectively). Rather, our novel finding herein that SMase treatment itself stimulates plasma membrane vesiculation in a relatively energy-resistant manner suggests a specific hypothesis to explain our biochemical data. In particular, we propose that the vesicles formed by SMase treatment, perhaps as a result of ceramide-mediated rearrangement of plasma membrane lipids (see Ref. 56), deliver cholesterol to an intracellular site or sites that are optimal for ACAT stimulation. These vesicles would be expected to be excellent cholesterol donors, since they are depleted of sphingomyelin (cf. Refs. 57–61). Interestingly, in energy-depleted cells, peripheral vesicles predominate, perhaps due to inhibition of centripetal transport of the vesicles by cytoplasmic, ATP-requiring motors (62, 63). Since cholesterol esterification is actually greater in these cells than in energy-replete cells (Fig 2), a site for cholesterol delivery in the vicinity of the plasma membrane by SMase-induced vesiculation may be particularly optimal for ACAT stimulation. Although there are other possible explanations for our data, our hypothesis is consistent with previous findings from our laboratory showing that lipoprotein internalization via plasma membrane invaginations is associated with marked stimulation of cholesterol esterification in macrophages (12–14); these findings, like our current data, could be explained by delivery of cholesterol from a plasma membrane-associated site to ACAT (8, 14). The exact nature of this site, and how it leads to the stimulation of ACAT, remains to be elucidated. Consistent with the data of Harmala et al. (64), we found that U18666A and progestrone (65) inhibit both distal lipoprotein-induced cholesterol esterification and SMase-induced esterification; this finding may indicate that a common mechanism is involved in the final transfer of cholesterol from both the SMase-induced vesicles and the vesicles that are presumably involved in lipoprotein-induced esterification to ACAT.

How do the findings in this report relate to other properties of the cholesterol esterification pathways in macrophages and CHO cells? As mentioned above, marked disruption of the actin cytoskeleton by cytochalasin D results in the inhibition of lipoprotein-induced, but not SMase-induced, cholesterol esterification in macrophages (see Ref. 19).4 The mechanism of the effect of the four treatments reported in this paper, however, cannot be explained by effects on actin. Phalloidin-staining experiments revealed that actin was not grossly disrupted by these treatments in comparison with cytochalasin D. Most importantly, energy depletion inhibited cholesterol esterification in CHO cells, which are resistant to the effects of actin disruption on lipoprotein-mediated cholesterol esterification (19). A cellular treatment that does mimic the effects of the four inhibitors on cholesterol esterification in both macrophages and CHO cells is treatment with cell-permeable cysteine protease inhibitors (23). These protease inhibitor data and other

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4 P. J. Skiba and I. Tabas, unpublished data.
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Lipoprotein- vs. Sphingomyelinase-induced Cholesterol Esterification