Effect and Cellular Site of Action of Cysteine Protease Inhibitors on the Cholesterol Esterification Pathway in Macrophages and Chinese Hamster Ovary Cells†

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ABSTRACT: Stimulation of intracellular cholesterol esterification, which is catalyzed by the enzyme acyl-CoA:cholesterol *O*-acyltransferase (ACAT), by atherogenic lipoproteins in macrophages is a key step in the development of atheroma foam cells. Since other aspects of intracellular cholesterol metabolism involve proteolytic reactions, we looked for evidence of intracellular proteolysis in the stimulation of the cholesterol esterification pathway. When macrophages and CHO cells were incubated with the cysteine protease inhibitor *N*-acetylleucylleucylnorleucinal (ALLN), the ability of β-very-low-density lipoprotein (β-VLDL) and free cholesterol-rich liposomes to stimulate cholesterol esterification was inhibited by 60−90%. Epoxysuccinylleucylamido-3−methylbutane ethyl ester (EST), a cysteine protease inhibitor structurally different from ALLN, also inhibited β-VLDL-induced cholesterol esterification in CHO cells. The inhibitory effect of the protease inhibitors could not be explained by decreased net expansion of cellular cholesterol pools, inhibition of lipoprotein cholesteryl ester hydrolysis, or blockage of cholesterol trafficking through the lysosomal pathway. Furthermore, stimulation of cholesterol esterification by 25-hydroxycholesterol and sphingomyelinase was not inhibited by ALLN, indicating that ALLN is not acting as a direct ACAT inhibitor in the cells, and suggesting that the ALLN effect is specific for methods of stimulating cholesterol esterification that expand cellular cholesterol pools. Previous studies have shown that inhibition of protein synthesis (*e.g.*, by cycloheximide) stimulates cholesterol esterification in macrophages and CHO cells, suggesting the presence of a short-lived protein inhibitor of cholesterol esterification. Herein, we show that, when added after cycloheximide, ALLN does not inhibit cycloheximide-induced cholesterol esterification in either cell type. The data in this report are consistent with a novel model in which a proteolytic reaction mediates the stimulation of cholesterol esterification specifically by expanded cellular cholesterol pools. The apparent protease-dependent step is not dependent upon lysosomal trafficking of cholesterol and is proximal to the ACAT enzyme itself; it may function by cleaving an endogenous inhibitor of the interaction of expanded cellular cholesterol pools with ACAT.

Cholesteryl ester (CE)[1]-loaded macrophages, or foam cells, are prominent features of atherosclerotic lesions (Gerrity, 1991; Faggioto et al., 1984; Schaffner et al., 1980) and may play a major role in the clinical progression of these lesions (Libby & Clinton, 1993; Fuster et al., 1992). Macrophages become CE-loaded through a series of events involving the cellular uptake of atherogenic lipoproteins followed by intracellular esterification of cholesterol by the microsomal enzyme acyl-coenzyme A:cholesterol *O*-acyltransferase (ACAT) (Brown & Goldstein, 1983). The results of several studies have suggested the following scenario (Tabas, 1995): lipoprotein−cholesterol, which is released in lysosomes (Goldstein et al., 1985) or other cellular sites (Tabas et al., 1990, 1991; Myers et al., 1993), first rapidly mixes with plasma membrane cholesterol (Brasaemle & Attie, 1990). Then, when a certain threshold level of cholesterol is reached (Xu & Tabas, 1991), a mixture of endogenous cellular cholesterol and lipoprotein−cholesterol is esterified by ACAT (Brown et al., 1975a; Tabas et al., 1988; Lange et al., 1993; Nagy & Freeman, 1990).

The mechanism whereby the ACAT enzyme, recently cloned by Chang and co-workers (Chang et al., 1993), is acutely stimulated by lipoproteins in cultured cells is known to be posttranslational (Tabas & Boykow, 1987; Chang et al., 1986; Chang & Chang, 1986; Wang et al., 1994a), but the nature of the critical posttranslational event has not yet been elucidated (Tabas, 1995). Although recent data have suggested that ACAT can be regulated by cholesterol-mediated allosteric activation (Cheng et al., 1995), there is substantial experimental evidence that other levels of regulation also exist and are important (Tabas, 1995). One such

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[1] Abbreviations: ACAT, acyl-coenzyme A:cholesterol *O*-acyltransferase; ALLN, *N*-acetylleucylleucylnorleucinal; BSA, bovine serum albumin; CE, cholesteryl ester; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EST, epoxysuccinylleucylamido-3-methylbutane ethyl ester; FC-rich, free cholesterol-rich; GC, gas−liquid chromatography; LPDS, lipoprotein-deficient serum; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; β-VLDL, β-very-low-density lipoprotein.

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body of evidence has come from studies in macrophages and CHO cells in which protein synthesis has been inhibited by cycloheximide or puromycin, or mRNA transcription has been inhibited by actinomycin D. Under these conditions, cholesterol esterification is stimulated by levels of cellular cholesterol expansion that otherwise would be too low to stimulate this process (Tabas & Boykow, 1987; Chang et al., 1986; Chang & Chang, 1986). These data suggested the presence of a short-lived endogenous protein inhibitor of the cholesterol esterification pathway that is somehow removed or degraded when the cells are loaded with cholesterol (Tabas & Boykow, 1987; Chang et al., 1986; Chang & Chang, 1986).

Cholesterol esterification can also be stimulated by certain inducers that do not expand cellular cholesterol pools. These inducers include certain hydroxysterols such as 25-hydroxycholesterol, which may somehow activate the ACAT enzyme (Brown et al., 1975b), and the enzyme sphingomyelinase, which may stimulate the "release" of cholesterol from the plasma membrane (Slotte & Bierman, 1988). In the case of 25-hydroxycholesterol, most of the sterol esterified by ACAT is endogenous cellular cholesterol (Tabas et al., 1988), and in the case of sphingomyelinase, the esterified cholesterol is derived entirely from endogenous pools (Slotte & Bierman, 1988). Whether the mechanism involved in the stimulation of cholesterol esterification by agents that expand cellular cholesterol pools is the same as that for agents that do not expand cellular cholesterol is not known.

In contrast to the uncertainties surrounding ACAT stimulation, the molecular mechanisms involved in the sterol-mediated regulation of other cellular metabolic pathways have been well-characterized. Studies have shown that 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) activity, the enzyme catalyzing the rate-limiting step in endogenous cholesterol synthesis, is down-regulated in response to increases in cellular cholesterol (Goldstein & Brown, 1990). One of the mechanisms involves the direct proteolytic degradation of the HMG-CoA reductase molecule by an endogenous protease whose activity is blocked by the cysteine protease inhibitor *N*-acetylleucylleucylnorleucinal (ALLN) (Inoue et al., 1991). Transcription of the genes for the LDL receptor and 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) is also down-regulated in the presence of high cellular free cholesterol (Brown & Goldstein, 1986). These structural genes are under the control of an enhancer element, sterol regulatory element 1 (SRE-1), whose activity depends upon the binding of a specific DNA-binding protein called the sterol regulatory element-binding protein (SREBP-1) (Briggs et al., 1993). Recent studies have shown that sterols block the proteolytic maturation of SREBP-1 to its active form and, therefore, block the transcription of genes whose function favors an increase in intracellular cholesterol levels (Wang et al., 1994b). Furthermore, data from the same study indicate that the mature SREBP-1 itself is degraded by an endogenous protease whose activity is blocked by ALLN (Wang et al., 1994b).

Given the role of proteases in general, and of ALLN-sensitive proteases in particular, in the regulation of molecules involved in intracellular cholesterol metabolism, we decided to explore the possible role of a protease in lipoprotein-induced cholesterol esterification in both macrophages and CHO cells. Herein, we show that cysteine protease inhibitors are able to block the ability of atherogenic lipoproteins and free cholesterol-rich liposomes (FC-rich liposomes) to stimulate cholesterol esterification in macrophages and CHO cells. Cysteine protease inhibitors, however, do not block the stimulation of cholesterol esterification induced by 25-hydroxycholesterol or sphingomyelinase. Furthermore, when added after cycloheximide, ALLN does not inhibit cycloheximide-induced cholesterol esterification. These data are consistent with a novel model in which a proteolytic reaction in macrophages and CHO cells mediates the stimulation of cholesterol esterification by expanded cellular cholesterol pools, perhaps by cleaving an endogenous inhibitor of the interaction of these expanded pools with ACAT.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media and reagents were purchased from Gibco Laboratories, tissue culture plates were from Corning, and fetal bovine serum was from Hyclone Laboratories, 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-^{3}H]Cholesteryl linoleate (73.0 Ci/mmol) and [1-^{14}C]oleic acid (50 mCi/mmol) were obtained from Du Pont-New England Nuclear. Cholesterol and cholesteryl oleate were purchased from Steraloids, Inc. (Wilton, NH). Oleic acid, fatty acid-free bovine serum albumin, filipin, sphingomyelinase (*Bacillus cereus*), imipramine, all protease inhibitors, and other reagents were from Sigma. Organic solvents were from Fisher. Compound 58035 [3-(decyldimethylsilyl)-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide] was kindly provided by Dr. John Heider of Sandoz, Inc. (East Hanover, NJ). Stock solutions (10 mg/mL) were prepared in DMSO. Compound U18666A [3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one] was the generous gift of Dr. Laura Liscum of Tufts University (Boston MA). Stock solutions (1 mM) were prepared in ethanol.

Cells. Resident mouse peritoneal macrophages were harvested from female ICR mice (25−35 g) as previously described (Tabas et al., 1987). Approximately 2.5×10^6 cells were plated on 25-mm dishes in DMEM and 10% fetal bovine serum. After 1 h of adherence, the cells were washed three times with PBS and then incubated in DMEM/10% LPDS containing penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (200 mM). The cell monolayers were maintained for 2 days in DMEM/LPDS (with daily changes of medium) in a 37 °C CO_2 tissue culture incubator. For the fluorescence microscopy experiments, macrophages were plated on 10-mm polylysine-coated coverslip bottom dishes as described previously (Tabas et al., 1990). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Ham's F-12 medium containing 10% fetal calf serum (FCS). The cells were trypsinized and plated 2 days prior to each experiment in full medium and then switched to Ham's F-12/10% LPDS approximately 15 h before each experiment; cell confluency on the day of each experiment was 80−90%.

Lipoproteins. Lipoproteins were isolated by preparative ultracentrifugation (Havel et al., 1955) as follows: LDL (density = 1.020−1.063 g/mL) from fresh human plasma and β-VLDL (density < 1.006 g/mL) from the plasma of cholesterol-fed male New Zealand white rabbits [4−8 weeks

on a diet of Purina rabbit chow supplemented with 0.2% cholesterol and 10% soybean oil (w/w)]. Acetyl-LDL was prepared by acetylation of LDL with acetic anhydride as described previously (Goldstein et al., 1979). By using a modification of a method described previously (Tabas et al., 1987), β-VLDL was labeled with [^{3}H]CE by the transfer of [^{3}H]CE from LDL reconstituted with [^{3}H]CE (Krieger et al., 1978) using purified cholesteryl ester transfer protein (a gift from Drs. Paul Kussie and Alan Tall, Columbia University). The specific activity was 320 cpm/ng protein. All lipoproteins were stored under argon at 4 °C and were used within 4 weeks of preparation.

Free Cholesterol-Rich Liposomes. Phosphatidylcholine (1.2 mg) and 1.4 mg of cholesterol were added in chloroform to a sonication vial. The vial was placed under a N_2 stream to evaporate the chloroform and then lyophilized for 1 h to remove residual organic solvent. After lyophilization, 1 mL of PBS was added to the dried lipid, and the mixture was sonicated for 1 h under nitrogen and in an ice bath using a Branson 450 sonifier. The sonicate was centrifuged twice at 5000*g* to remove the titanium shed from the sonication probe and then passed through a 0.45 μM Millipore filter for sterilization. Liposomes were added to cells at a final concentration of 150 μg of cholesterol/mL.

Incubations with Drugs and Compounds. All protease inhibitors and imipramine were added from 100× stock solutions in PBS, ethanol or DMSO; control dishes received an equal concentration (1%) of ethanol or DMSO alone. Cycloheximide was added from a 1000× stock solution in PBS. 25-Hydroxycholesterol was added from a 1000× stock solution in ethanol; control dishes received an equal concentration (0.1%) of ethanol alone.

Whole-Cell Cholesterol Esterification Assays. After the preincubations described in the text and figure legends, the macrophages or CHO cells were washed with PBS and incubated for the indicated times with the indicated lipoprotein plus [^{14}C]oleate (final concentration = 50 μM) complexed with albumin in medium indicated in the figure legends. After the incubations, the cells were washed two times with cold PBS, and the cell monolayers were extracted twice with 1 mL of hexane/isopropyl alcohol (3:2, v/v) for 1 h at room temperature. Whole-cell cholesterol esterification activity was assayed by determining the cellular content of cholesteryl [^{14}C]oleate, which was separated by TLC and quantified by liquid scintillation counting as previously described (Tabas et al., 1987). In the experiments where measured, as indicated in the figure legends, [^{14}C]oleate incorporation into cellular triglyceride and phospholipids was also determined by TLC. The cell monolayers remaining after lipid extraction were dissolved in 1 mL of 0.1 N NaOH, and aliquots were assayed for protein by the method of Lowry et al. (1951).

Cellular Lipid Analysis. Cell monolayers were extracted twice with 1 mL of hexane/isopropyl alcohol (3:2, v/v) for 1 h at room temperature. The cell extracts were dried under nitrogen and then analyzed by TLC or GC as described previously (Tabas & Boykow, 1987). β-Sitosterol was included in the extracts as an internal standard for GC.

[^{3}H]CE−β-VLDL Intracellular CE Hydrolysis Assay. CHO cells were incubated with [^{3}H]CE−β-VLDL plus [^{14}C]-oleate. The cellular contents of free [^{3}H]cholesterol and [^{3}H]-CE (and [^{14}C]CE to assay cholesterol esterification) were determined by TLC of the cellular lipids as described earlier.

Filipin Staining of Intracellular Free Cholesterol. The method of Blanchette-Mackie et al. (1988) was followed. Briefly, a 0.05 mg/mL filipin solution was made from a 5 mg/mL stock solution (in DMSO) by dilution with 10% FCS in PBS. Macrophages were plated on polylysine-coated coverslip bottom dishes and preincubated for 2 days in DMEM/10% LPDS. After the incubations described in the legend to Figure 3, the cells were rinsed with PBS and fixed with 3% paraformaldehyde in PBS for 1 h at room temperature. The monolayers were then rinsed three times with PBS (5 min each) and incubated with 1.5 mg of glycine/mL of PBS for 10 min at room temperature. The monolayers were incubated with the 0.05 mg/mL filipin solution for 2 h at room temperature and then rinsed three times with PBS. Fluorescence images were obtained by using a Leitz Diavert microscope system with a 63×, NA 1.4 objective and recorded with a cooled, charge-coupled device (CCD) camera (Photometrics CH 250, Tucson, AZ). A UV filter set (340−380-nm excitation and 400-nm dichroic and 430-nm long-pass filters) was used to observe filipin fluorescence. The images were printed on a Tektronix Phaser II SDX printer using Adobe Photoshop.

Statistics. Unless otherwise indicated, results are given as means ± SD ($n = 3$). Absent error bars signify SD values smaller than the graphics symbol.

RESULTS

Cysteine Protease Inhibitors N-Acetylleucylleucylnorleucinal (ALLN) and EST Inhibit β-VLDL-Induced Cholesterol Esterification in CHO Cells. CHO cells were preincubated for 6 h in medium (Ham's F-12/10% LPDS) alone or in medium containing 100 μg/mL cysteine protease inhibitor ALLN. These conditions are similar to those used by Simoni and co-workers (Inoue et al., 1991) to show the inhibition of HMG-CoA reductase degradation by ALLN. Furthermore, by using the assay of Kato et al. (1989), we documented that ALLN inhibited intracellular proteolysis by over 2-fold (from a basal rate of 18.8% of total cellular protein degraded over 4 h in the absence of ALLN to 7.9%/4 h in the presence of ALLN). The cells were then incubated for 3 h with β-VLDL plus [^{14}C]oleate, also in the absence or presence of ALLN, in order to measure β-VLDL-induced cholesterol esterification. The control cells (which were treated with solvent alone) and the ALLN-treated cells appeared to be similar by phase microscopy and had similar protein contents. The data in Figure 1 (first and second bars) show that treatment with ALLN led to a much lower level of β-VLDL-induced cholesterol esterification than with control cells. In contrast, ALLN did not inhibit the incorporation of [^{14}C]oleate into phospholipids and triglycerides under these conditions ([^{14}C]oleate incorporation into phospholipids was 17.5 ± 1.48 and 18.75 ± 0.87 in the absence and presence of ALLN, respectively; [^{14}C]oleate incorporation into triglycerides was 29.0 ± 0.76 and 25.35 ± 1.24 in the absence and presence of ALLN, respectively). These acylation data indicate that the decreased β-VLDL-induced cholesterol esterification in ALLN-treated cells was not due to general cell toxicity, inhibition of [^{14}C]oleate uptake by the cells, or [^{14}C]oleate substrate dilution.

Other protease inhibitors were also tested. CHO cells were incubated for 15 h with 100 μg/mL phosphoramidon (a metalloprotease inhibitor), bestatin (an aminoprotease inhibi-

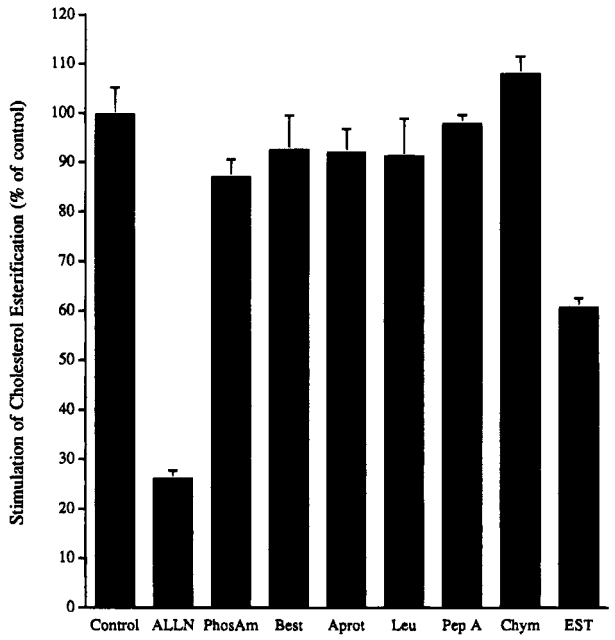

FIGURE 1: Effect of protease inhibitors on β-VLDL-induced cholesterol esterification in CHO cells. CHO cells were preincubated in Ham's F-12 medium, 10% LPDS containing 1% solvent (Control) or ALLN, phosphoramidon (PhosAm), bestatin (Best), aprotinin (Aprot), leupeptin (Leu), pepstatin A (PepA), chymostatin (Chym), or EST. The preincubation times were 15 h for all inhibitors except ALLN (6 h) and EST (4 h); all protease inhibitors were at a final concentration of 100 μg/mL except EST (50 μg/mL). After preincubation with proteases, the cells were incubated for 3 h with Ham's F-12 medium, 0.2% BSA containing 5 μg β-VLDL/mL, 0.1 mM [^{14}C]oleate, and either solvent or protease inhibitor as before. The cells were then assayed for whole-cell cholesterol esterification activity. The data are expressed as percent stimulation of cholesterol esterification compared with cholesterol esterification in cells incubated with β-VLDL alone, which was 11.7 ± 0.2 nmol/mg cell protein/3 h. Cholesterol esterification in cells incubated without β-VLDL was 1.1 ± 0.02 nmol/mg protein/3 h.

tor), aprotinin (a serine protease inhibitor), leupeptin (a serine and thiol protease inhibitor), pepstatin A (an aspartic protease inhibitor), or chymostatin (a chymotrypsin inhibitor) and then assayed for cholesterol esterification as before. Despite the 15-h preincubation, none of these other inhibitors significantly inhibited β-VLDL-induced cholesterol esterification (Figure 1). Cells were also preincubated for 4 h with 50 μg/mL EST, a cell-permeable cysteine protease inhibitor structurally different from ALLN (Mehdi, 1991). Inhibition of cholesterol esterification, while not as great as that seen with ALLN, was clearly evident (Figure 1, last bar). These data indicate either that the inhibitory effect is specific for cysteine protease inhibitors or that the other inhibitors are unable to enter the cells or a critical intracellular site (*cf.* Inoue et al., 1991).

To more precisely define the inhibition of β-VLDL-induced cholesterol esterification by ALLN, it was necessary to measure cholesterol esterification under conditions of equal β-VLDL-induced increments in free cholesterol in treated and untreated cells (Table 1). Control and ALLN-treated CHO cells were incubated with β-VLDL plus [^{14}C]oleate, and a parallel set of cells was incubated in the presence of the ACAT inhibitor, compound 58035. In the ACAT-inhibited cells, the increment in free cholesterol represents the net amount of β-VLDL-derived cholesterol that has accumulated intracellularly (*cf.* Tabas et al., 1994). In these experiments, it was necessary to use a higher concentration of β-VLDL in the ALLN-treated cells (10 μg/mL) than in control cells (2 μg/mL) in order to match increments in free cholesterol. The fact that a higher concentration of β-VLDL was needed in the presence of ALLN indicates that endocytosis and/or lipoprotein catabolism (*e.g.*, protein degradation, CE hydrolysis) is partially inhibited by ALLN. Nonetheless, the data in Table 1 show that, under conditions where free cholesterol increments are matched, cholesterol esterification was inhibited by more than 2-fold in ALLN-treated cells. Similar results were obtained when, through the use of [^{3}H]CE-labeled β-VLDL, the cholesterol esterification data were matched for equal β-VLDL−[^{3}H]CE hydrolysis (data not shown). Furthermore, EST, unlike ALLN, did not inhibit the uptake and/or catabolism of β-VLDL (data not shown), and yet still caused substantial inhibition of cholesterol esterification (Figure 1).

ALLN Inhibits β-VLDL-Induced Cholesterol Esterification in Mouse Peritoneal Macrophages. Monolayers of resident mouse peritoneal macrophages, which were cultured for 2 days in the absence of lipoproteins, were preincubated for 3 h in the absence or presence of ALLN at a concentration of 50 μg/mL. The cells were then incubated for 3 h with β-VLDL plus [^{14}C]oleate, also in the absence or presence of ALLN, and assayed for free cholesterol mass and cholesteryl [^{14}C]oleate. The data in Figure 2A show that ALLN markedly inhibits β-VLDL-induced cholesterol esterification, even when comparing cells treated with 10 μg of β-VLDL/mL in the presence of ALLN to cells treated with 1 μg β-VLDL/mL in the absence of ALLN. The data in Figure 2B clearly show that, at β-VLDL concentrations above 2 μg/mL, cellular cholesterol pools were adequately expanded in the presence of ALLN. Thus, the cholesterol esterification:cellular cholesterol increment ratio (at 1 μg of β-VLDL/mL in control cells and 2 μg β-VLDL/mL in ALLN-treated cells) was inhibited 18-fold by ALLN. These data show that, in macrophages, even more so than in CHO cells, ALLN treatment leads to a marked inhibition of β-VLDL-induced cholesterol esterification.

Site in the Cholesterol Esterification Pathway of ALLN-Mediated Inhibition. To more precisely define the site of ALLN-mediated inhibition of cholesterol esterification, we designed several experiments to determine whether ALLN acted by nonspecifically blocking cholesterol trafficking or by directly inhibiting the ACAT reaction in the cells (*cf.* Lange & Steck, 1994; Roff et al., 1991). In addition, we sought to determine whether ALLN-mediated inhibition was specific for stimulators of cholesterol esterification that expanded cellular cholesterol pools (see introduction).

To determine whether ALLN acted by blocking the export of lipoprotein-derived free cholesterol from lysosomes (*cf.* Pentchev et al., 1985; Liscum et al., 1989; Liscum & Faust, 1989; Roff et al., 1991), macrophages were incubated in the absence or presence of β-VLDL, ALLN, or both compounds together. On the basis of the results of the cholesterol mass experiment in Figure 2B, cells treated with ALLN were incubated with 2 μg of β-VLDL/mL and control cells were incubated with 1 μg of β-VLDL/mL. The cells were then fixed, stained with filipin to visualize intracellular accumulations of free cholesterol (Blanchette-Mackie et al., 1988), and viewed by fluorescence microscopy (Figure 3). Compared with cells incubated without lipoproteins (Figure 3A,C), those incubated with β-VLDL showed a slightly

Table 1: Effect of ALLN on β-VLDL-Induced Cholesterol Esterification in CHO Cells at Equivalent Increments in Cellular Free Cholesterol Content[a]

incubation conditions	parameter assayed	
	increment in cellular FC [nmol (mg of cell protein)$^{-1}$ (3 h)$^{-1}$]	stimulation of cholesterol esterification [nmol (mg of cell protein)$^{-1}$ (3 h^{-1})]
β-VLDL	31.0 ± 2.0	6.5 ± 0.3
β-VLDL + ALLN	30.7 ± 3.5	3.1 ± 0.3

[a] 15 h before the addition of β-VLDL, CHO cells were incubated in Ham's F-12 medium/10% LPDS; 6 h before β-VLDL was added, the cells were incubated with the same medium in the absence or presence of 100 μg of ALLN/mL. At this point, the cells were 80−90% confluent. The cells were then incubated for 3 h with medium containing 50 μM [^{14}C]oleate plus β-VLDL, in the absence or presence of ALLN. The concentrations of β-VLDL used were 2 μg/mL for control cells and 10 μg/mL for the ALLN-treated cells. The cells were then assayed for free cholesterol (FC) mass and cholesteryl [^{14}C]oleate content (cholesterol esterification activity). The increment in cellular FC data were calculated as follows: (FC content of a parallel set of cells incubated with β-VLDL in the presence of the ACAT inhibitor 58035) minus (FC content of cells incubated without β-VLDL) (*cf.* Xu & Tabas, 1991). The absolute cellular FC mass values for the untreated and ALLN-treated cells incubated without β-VLDL were 48.3 ± 0.52 and 50.9 ± 0.63 nmol/mg cell protein, respectively. The cholesterol esterification data are expressed as the stimulation of cholesterol esterification compared with cholesterol esterification in cells incubated without β-VLDL, which was 0.94 ± 0.14 and 0.56 ± 0.06 nmol/mg protein/3 h in untreated and ALLN-treated cells, respectively.

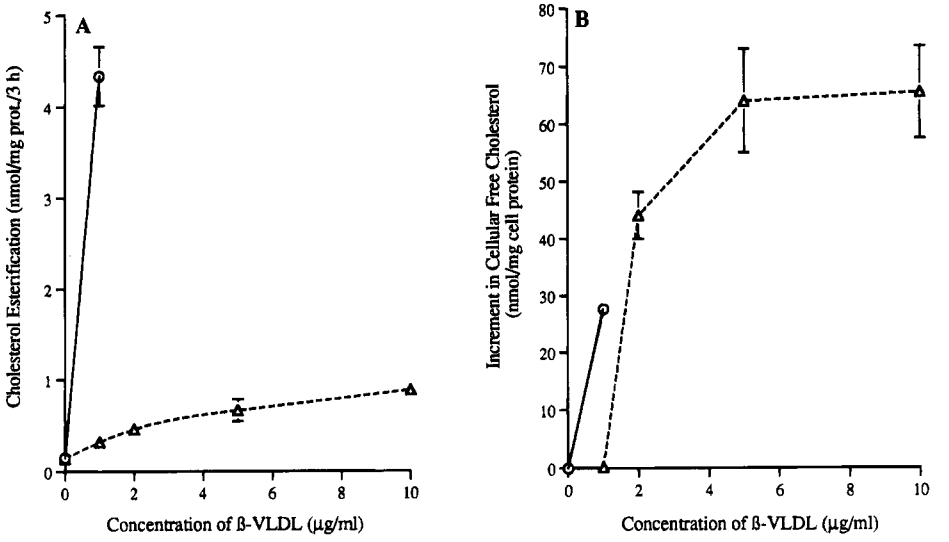

FIGURE 2: Effect of ALLN on β-VLDL-induced cholesterol esterification in mouse peritoneal macrophages at equivalent increments in cellular free cholesterol content. Macrophages were preincubated for 3 h with DMEM/0.2% BSA, in the absence (solid line) or presence (dashed line) of 50 μg of ALLN/mL. The cells were then incubated for 3 h with DMEM/0.2% BSA, also in the absence or presence of ALLN, containing 50 μM [^{14}C]oleate and the indicated concentrations of β-VLDL. At the end of the 3-h incubation, all cells were incubated for 15 min with DMEM/0.2% BSA (to allow the internalization of any residual surface-bound lipoprotein). The cells were then assayed for cholesteryl [^{14}C]oleate content (cholesterol esterification activity; Panel A) and increment in free cholesterol (FC) mass compared with cells incubated without β-VLDL; the absolute cellular cholesterol mass values for the untreated and ALLN-treated cells incubated without β-VLDL were 76.9 ± 5.0 and 85.7 ± 4.7 nmol/mg cell protein, respectively.

increased level of filipin fluorescence (Figure 3B,D). This finding is consistent with the expansion of cellular free cholesterol by β-VLDL in either the absence or presence of ALLN (see Figure 2). The absence of bright filipin staining, however, indicated that the cholesterol had been widely distributed to cellular membranes, since bright staining is seen only when cholesterol is concentrated in cellular membranes (Blanchette-Mackie et al., 1988). To compare these patterns with one in which lysosomal trapping of free cholesterol is known to occur, macrophages were incubated with 1 μg of β-VLDL/mL plus compound U18666A, which blocks the exit of lipoprotein-derived cholesterol out of lysosomes (Liscum & Faust, 1989). In contrast to the pattern seen with β-VLDL, in either the absence or presence of ALLN, the macrophages incubated with β-VLDL plus U18666A demonstrated very bright punctate fluorescence (Figure 2E), which is indicative of a high concentration of free cholesterol in cellular organelles, presumably lysosomes (Blanchette-Mackie et al., 1988). These data indicate that, unlike compound U18666A, ALLN does not block the exit of β-VLDL-derived free cholesterol out of lysosomes.

Although ALLN does not cause the accumulation of free cholesterol in lysosomes, it may disrupt some other component of the transfer of lysosomal cholesterol to cellular membranes. Therefore, we tested the effect of ALLN on the stimulation of cholesterol esterification by FC-rich liposomes, which are not internalized by receptor-mediated endocytosis, to determine whether the ALLN effect is independent of lysosomal routing of free cholesterol. CHO cells and mouse peritoneal macrophages were preincubated in the absence or presence of ALLN and then incubated for 4 h with either β-VLDL or FC-rich liposomes, also in the absence or presence of ALLN. The data in Table 2A show that ALLN inhibited FC-rich liposome-induced cholesterol esterification equally as well as β-VLDL-induced cholesterol esterification in both CHO cells and mouse peritoneal macrophages. In additional experiments using [^{3}H]cholesterol-labeled FC-rich liposomes, ALLN was found not to block the cellular uptake of liposomal cholesterol, but was found to inhibit the intracellular esterification of liposomal-

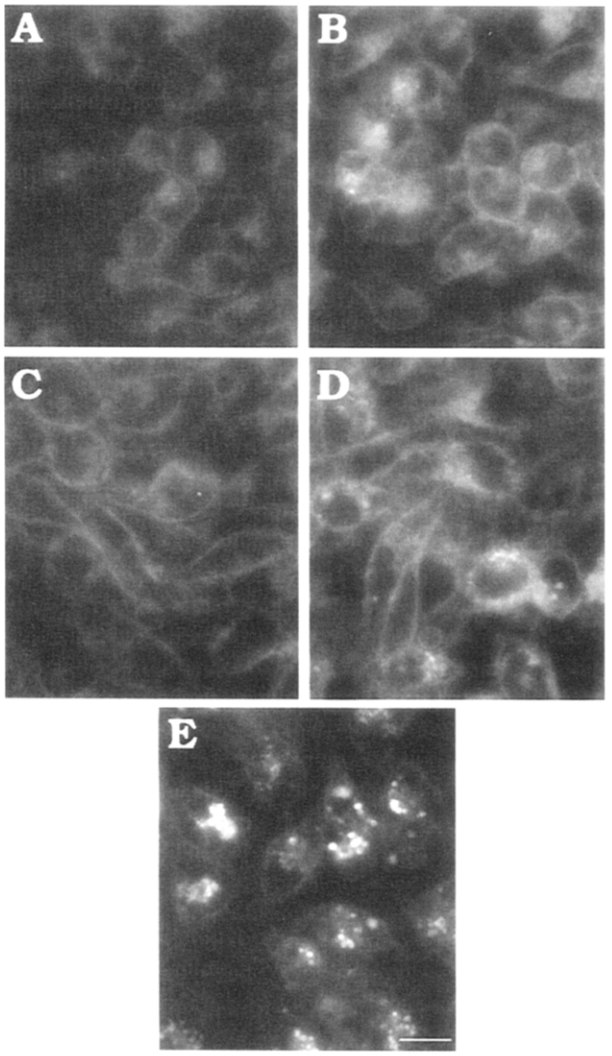

FIGURE 3: Fluorescence microscopy of macrophages stained for free cholesterol by filipin: effects of incubation with β-VLDL, ALLN, and compound U18666A. The macrophages were incubated as follows: (A) 6 h with DMEM, 0.2% BSA, and 0.5% ETOH; (B) the same as the cells in panel A but with 1 μg of β-VLDL/mL added to the incubation for the last 3 h; (C) 6 h with DMEM, 0.2% BSA, and 50 μg of ALLN/mL; (D) the same as the cells in panel C but with 2 μg of β-VLDL/mL added to the incubation for the last 3 h; (E) preincubated for 30 min with medium containing 1 μM U18666A and then incubated for 3 h with medium containing 1 μM U18666A plus 1 μg of β-VLDL/mL. The cells were then fixed, stained with filipin, and viewed by fluorescence microscopy. Bar, 10 μM.

derived cholesterol by 80%. These data suggest that ALLN-mediated inhibition of cholesterol esterification may be independent of lysosomal trafficking of cholesterol.

In order to conclude from these data that ALLN-mediated inhibition of cholesterol esterification is not dependent upon lysosomal cholesterol pathways, it was necessary to show that the stimulation of cholesterol esterification by FC-rich liposomes does not, in fact, involve the trafficking of cholesterol through lysosomes. This goal was accomplished by using imipramine, which blocks the transport of lysosomal, but not plasma membrane, free cholesterol (Harmala et al., 1994). CHO cells were preincubated for 2 h in the absence or presence of 20 μM imipramine and then incubated for 4 h with either β-VLDL, 25-hydroxycholesterol, or FC-rich liposomes, also in the absence or presence of imipramine. The data in Table 2B show that imipramine markedly inhibits β-VLDL-induced cholesterol esterification, a process dependent on lysosomal cholesterol transport, but does not inhibit 25-hydroxycholesterol-induced cholesterol esterification, a process not dependent on the transport of cholesterol from lysosomes (Tabas et al., 1988). Thus, confirming previous reports, imipramine inhibits cholesterol esterification only when the lysosomal pathway is involved (Rodriguez-Lafrasse et al., 1990; Harmala et al., 1994). With this tool in hand, we tested the effect of imipramine on FC-rich liposome-induced cholesterol esterification. The data in Table 2B clearly show no inhibition in this case, indicating that the stimulation of cholesterol esterification by FC-rich liposomes does not require the lysosomal trafficking of cholesterol. The fact that cholesterol esterification by these liposomes is inhibited by ALLN therefore indicates that the ALLN-mediated blockage of cholesterol esterification must be related to the expansion of cellular cholesterol pools, but is not dependent upon lysosomal trafficking of cholesterol.

Next, we sought to determine whether ALLN was non-specifically blocking the transfer of cholesterol from the plasma membrane to ACAT or was a direct inhibitor of the ACAT reaction in the cells. To explore these points, we tested the effect of ALLN on inducers of cholesterol esterification that, in contrast to lipoproteins and FC-rich liposomes, do not require the expansion of cellular free cholesterol pools. Two such cholesterol esterification inducers are 25-hydroxycholesterol (Brown et al., 1975b; this work) and the enzyme sphingomyelinase (Slotte & Bierman, 1988). CHO cells were incubated with either 5 μg of β-VLDL/mL, 0.5 μg 25-hydroxycholesterol/mL, or 50 munits of SMase/mL in the absence or presence of ALLN and then assayed for [^{14}C]oleate incorporation into CE (Figure 4). In preliminary experiments using [^{3}H]-25-hydroxycholesterol, the cellular uptake of the sterol was shown not to be affected by ALLN. As before, β-VLDL-induced cholesterol esterification was markedly inhibited by ALLN (Figure 4A). In contrast, ALLN had little inhibitory effect on 25-hydroxycholesterol-induced and SMase-induced cholesterol esterification (Figure 4B,C, respectively). Similar results were obtained in mouse peritoneal macrophages (data not shown). These data indicate three important points. First, ALLN does not act as a direct inhibitor of the ACAT enzymatic reaction when added to intact, living cells. Second, ALLN is not a general inhibitor of cellular cholesterol transport to ACAT, since most of the sterol esterified in 25-hydroxycholesterol-treated cells, and all of the sterol in SMase-treated cells, is cellular cholesterol (Tabas et al., 1988; Slotte & Bierman, 1988). Third, ALLN-mediated inhibition of cholesterol esterification appears to be specifically related to the expansion of cellular cholesterol pools.

Effects of ALLN on Cycloheximide-Induced Cholesterol Esterification in Macrophages and CHO Cells. Previous studies have shown that the incubation of mouse peritoneal macrophages with LDL plus cycloheximide, puromycin, or actinomycin D results in greater cholesterol esterification than treatment with LDL alone in macrophages (Tabas & Boykow, 1987) and that cycloheximide stimulates cholesterol esterification by endogenously synthesized cholesterol in CHO cells (Chang & Chang, 1986). Although the mechanism by which protein synthesis inhibition stimulates cholesterol esterification is unknown, the data are consistent with the presence of a short-lived, endogenous protein inhibitor that acts at some site along the cholesterol esterification pathway (Tabas & Boykow, 1987; Chang et al., 1986; Chang

Table 2: Evidence That ALLN-Mediated Inhibition of Cholesterol Esterification Is Not Dependent upon Lysosomal Cholesterol Trafficking

incubation conditions	cell type	stimulation of cholesterol esterification [nmol (mg of cell protein)$^{-1}$ (3 h)$^{-1}$]
A. ALLN Blocks FC-Rich Liposome-Induced Cholesterol Esterification in CHO Cells and Macrophages[a]		
β-VLDL	CHO cell	10.1 ± 0.14
β-VLDL + ALLN	CHO cell	4.8 ± 0.4
FC liposomes	CHO cell	1.0 ± 0.1
FC liposomes + ALLN	CHO cell	0.3 ± 0.1
β-VLDL	macrophage	3.3 ± 0.4
β-VLDL + ALLN	macrophage	1.1 ± 0.1
FC liposomes	macrophage	1.1 ± 0.2
FC liposomes + ALLN	macrophage	0.1 ± 0.02
B. FC-Rich Liposome-Induced Cholesterol Esterification Does Not Involve Lysosomal Cholesterol Trafficking[b]		
β-VLDL	CHO cell	7.2 ± 0.6
β-VLDL + imipramine	CHO cell	2.5 ± 0.1
25-hydroxycholesterol	CHO cell	3.3 ± 0.2
25-hydroxycholesterol + imipramine	CHO cell	3.2 ± 0.3
FC liposomes	CHO cell	3.3 ± 0.1
FC liposomes + imipramine	CHO cell	3.2 ± 0.3

[a] CHO cells were preincubated for 6 h with Ham's F-12 medium/0.2 BSA in the absence or presence of 100 μg of ALLN/mL and then incubated for 4 h with medium, also in the absence or presence of ALLN, containing 50 μM [^{14}C]oleate plus β-VLDL or FC-rich liposomes at a concentration of 150 μg of cholesterol/mL; the concentrations of β-VLDL used were 2 μg/mL for control cells and 10 μg/mL for ALLN-treated cells. Mouse peritoneal macrophages were preincubated for 3 h with DMEM/0.2% BSA in the absence or presence of 50 μg of ALLN/mL and then incubated for 4 h with medium, also in the absence or presence of ALLN, containing 50 μM [^{14}C]oleate plus β-VLDL or liposomes at a concentration of 150 μg of cholesterol/mL; the concentrations of β-VLDL used were 1 μg/mL for control cells and 2 μg/mL for ALLN-treated cells. The data are expressed as the stimulation of cholesterol esterification over that seen in cells incubated with only medium or medium plus ALLN, which was 1.1 ± 0.06 and 0.54 ± 0.03 nmol/mg protein/4 h, respectively, in CHO cells and 0.21 ± 0.02 and 0.17 ± 0.04 nmol/mg protein/4 h, respectively, in macrophages. [b] CHO cells were preincubated for 2 h with Ham's F-12 medium/0.2% BSA in the absence or presence of 20 μM imipramine and then incubated for 4 h with medium, also in the absence or presence of imipramine, containing 50 μM [^{14}C]oleate plus 2 μg of β-VLDL/mL, 0.5 μg of 25-hydroxycholesterol/mL, or liposomes at a concentration of 150 μg of cholesterol/mL. The data are expressed as the stimulation of cholesterol esterification over that seen in cells incubated with only medium or medium plus imipramine, which were 1.7 ± 0.24 and 1.90 ± 0.10 nmol/mg protein/4 h, respectively.

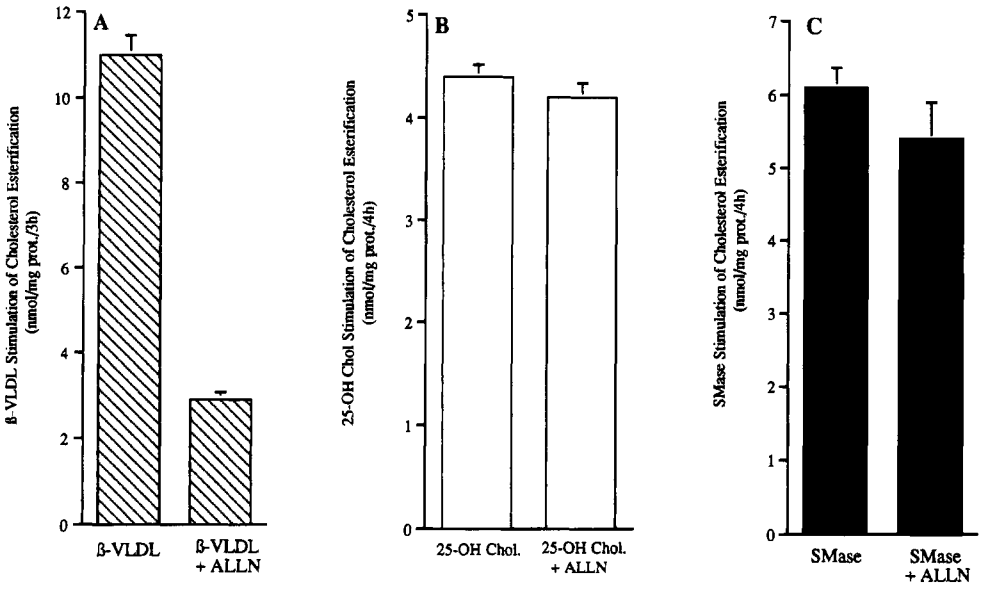

FIGURE 4: Effects of ALLN on 25-hydroxycholesterol-induced and SMase-induced cholesterol esterification. CHO cells were preincubated for 6 h with Ham's F-12 medium and 0.2% BSA, in the absence or presence of 100 μg of ALLN/mL. The cells were then incubated with medium, also in the absence or presence of ALLN, containing [^{14}C]oleate plus 5 μg of β-VLDL/mL (A), 0.5 μg of 25-hydroxycholesterol/mL (B), or 50 munits of SMase/mL (C). The incubation time was 3 h for the β-VLDL experiment and 4 h for the 25-hydroxycholesterol and SMase experiment. The data in panels A−C are expressed as the stimulation of cholesterol esterification compared with cholesterol esterification in cells incubated without β-VLDL, which was 1.39 ± 0.03 and 0.52 ± 0.11 nmol/mg protein/3 h in untreated and ALLN-treated cells, respectively.

& Chang, 1986). Since one possible mechanism of the protease inhibitor effect is via blockage of a protease that degrades an endogenous protein inhibitor of cholesterol esterification, we decided to explore the relationship between the effects of protein synthesis inhibition and protease inhibition on cholesterol esterification.

We first asked whether the stimulation of cholesterol esterification by cycloheximide could be blocked by *prior* treatment of the cells with ALLN. If an ALLN-sensitive protease is responsible for the normal turnover of the putative short-lived inhibitor, then prior treatment of the cells with ALLN should result in persistent inhibitor levels, despite subsequent blockage of inhibitor synthesis by cycloheximide. To test this point, mouse peritoneal macrophages were incubated for 5 h with 200 μg of LDL/mL in the absence or presence of 10 μM cycloheximide, exactly as described

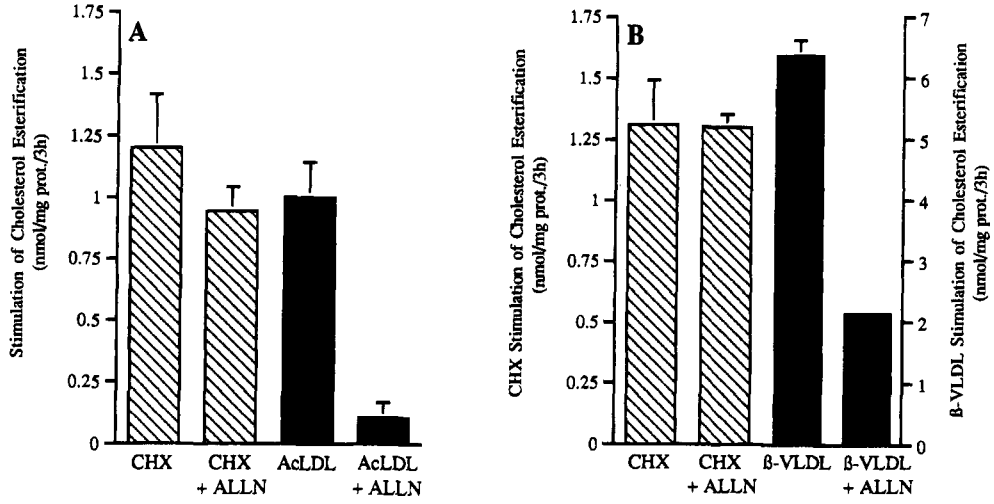

FIGURE 5: Effect of ALLN on cycloheximide-induced cholesterol esterification in macrophages and CHO cells. In panel A, one set of macrophages (hatched bars) was incubated for 3 h with DMEM/0.2% BSA in the absence or presence of 2 μM cycloheximide (CHX) followed by a 3-h incubation in DMEM and 0.2% BSA ± 50 μg of ALLN/mL, also in the absence or presence of cycloheximide. Acetyl-LDL was then added to all cells (to provide a source of cholesterol) at concentrations of 5 μg/mL for untreated and 10 μg/mL for ALLN-treated cells, and the cells were incubated for an additional 2 h; 50 μM [^{14}C]oleate was added during the last hour to assay whole-cell cholesterol esterification. Different concentrations of acetyl-LDL were used for the untreated and ALLN-treated cells because preliminary experiments indicated that these concentrations led to equal cholesterol delivery to the cells (data not shown). A second set of macrophages (solid bars) was incubated the same as those cells in Figure 2, with the exception that acetyl-LDL, instead of β-VLDL, was used at concentrations of 5 and 10 μg/mL in untreated and ALLN-treated cells, respectively; these cells were a positive control for ALLN-mediated inhibition of lipoprotein-induced cholesterol esterification. The data for the cycloheximide experiment (hatched bars) represent the stimulation of cholesterol esterification compared with cells treated without cycloheximide (0.69 ± 0.03 and 0.166 ± 0.03 nmol/mg protein/h in untreated and ALLN-treated cells, respectively). The data for the second experiment (solid bars) represent the stimulation of cholesterol esterification compared with cholesterol esterification in cells not incubated with acetyl-LDL (0.36 ± 0.05 and 0.41 ± 0.06 nmol/mg protein/3 h in untreated and ALLN-treated cells, respectively). In panel B, one set of CHO cells (hatched bars) was incubated for 8 h with Ham's F-12/10% LPDS to up-regulate endogenous cholesterol synthesis. The cells were then incubated for 3 h with Ham's F-12/1% LPDS, in the absence or presence of 850 μM cycloheximide, followed by a 6-h incubation with medium ± 100 μg of ALLN/mL, also in the absence or presence of cycloheximide. [^{14}C]oleate (50 μM) was then added and the cells were incubated for 1 h to assay cholesterol esterification. A second set of CHO cells (solid bars) was incubated the same as the cells in Table 1; these cells were a positive control for ALLN-mediated inhibition of lipoprotein-induced cholesterol esterification. The data for the cycloheximide experiment (hatched bars) represent the stimulation of cholesterol esterification compared with control cells not treated with cycloheximide (1.01 ± 0.10 and 0.33 ± 0.04 nmol/mg protein/h in untreated and ALLN-treated cells, respectively). The data for the second experiment (solid bars) represent the stimulation of cholesterol esterification compared with cells not incubated with β-VLDL (2.0 ± 0.15 nmol/mg protein/3 h).

previously (Tabas & Boykow, 1987). In the absence of cycloheximide, cholesterol esterification activity was only 1.1 ± 0.1 nmol/mg/h, whereas in the presence of cycloheximide, cholesterol esterification activity was 4.3 ± 0.4 nmol/mg/h. This 4-fold stimulation of cholesterol esterification activity by cycloheximide is similar to that reported previously (Tabas & Boykow, 1987). In a third set of cells, the macrophages were preincubated for 3 h with 50 μg of ALLN/mL *prior* to the addition of LDL plus cycloheximide. In these cells, cholesterol esterification activity was only 0.5 ± 0.1 nmol/mg/h. These data, although open to several interpretations, are consistent with the idea that an ALLN-sensitive protease is responsible for the normal turnover of the putative short-lived cholesterol esterification inhibitor.

We next performed the reverse of the experiment described earlier, namely, adding ALLN *after* cycloheximide. If the mechanism of inhibition of lipoprotein-induced cholesterol esterification by ALLN is via its ability to block the turnover of the putative cholesterol esterification inhibitor, then ALLN should not inhibit cycloheximide-induced cholesterol esterification. In other words, if the putative inhibitor is already absent (due to prior treatment with cycloheximide in the absence of ALLN), then subsequent treatment with ALLN should have little or no effect. To test this prediction, one set of macrophages was preincubated for 3 h in the presence or absence of 2 μM cycloheximide and then *subsequently* incubated for 3 h in the absence or presence of 50 μg of ALLN/mL, also in the absence or presence of cycloheximide. The cells were then incubated for 2 h with acetyl-LDL [to provide a source of cholesterol; see Tabas and Boykow (1987)], also in the absence or presence of cycloheximide and/or ALLN, and assayed for whole-cell cholesterol esterification activity (Figure 5A, hatched bars). Cycloheximide stimulated cholesterol esterification (*cf.* Tabas & Boykow, 1987), but ALLN caused only a slight inhibition of this stimulated activity. In contrast, when macrophages were incubated under our standard conditions (3-h preincubation in the absence or presence of ALLN, followed by a 3-h incubation with acetyl-LDL also in the absence or presence of ALLN), the stimulated cholesterol esterification activity was markedly inhibited by ALLN (Figure 5A, solid bars).

A similar experiment was conducted in CHO cells that were plated and grown for 2 days in medium supplemented with 10% FCS and then incubated in medium supplemented with 10% LPDS for 8 h to increase endogenous cholesterol synthesis (Chang & Chang, 1986). The cells were then incubated for 3 h in the absence or presence of 850 μM cycloheximide [the concentration used by Chang and Chang (1986)] and for 7 h in the absence or presence of 100 μg of ALLN/mL, also in the absence or presence of cycloheximide. Whole-cell cholesterol esterification activity was then assayed (Figure 5B, hatched bars). As reported previously (Chang & Chang, 1986), cycloheximide stimulated cholesterol esterification but, similar to the situation with macrophages,

the stimulated activity was not inhibited by ALLN. CHO cells incubated under our standard conditions (6-h preincubation in the absence or presence of ALLN followed by a 3-h incubation with β-VLDL ± ALLN) showed clear evidence of the inhibition of cholesterol esterification activity by ALLN (Figure 5B, solid bars). In summary, in both macrophages and CHO cells, ALLN inhibits lipoprotein-induced cholesterol esterification, but not cycloheximide-induced cholesterol esterification (when ALLN is added *after* cycloheximide). These data, together with those earlier in which ALLN was added prior to cycloheximide, are compatible with a model in which ALLN blocks lipoprotein-induced cholesterol esterification by inhibiting a cellular protease that degrades a short-lived, endogenous protein inhibitor of the cholesterol esterification pathway.

DISCUSSION

The uptake and storage of lipoprotein-derived cholesterol by macrophages and the eventual development of foam cells in atherosclerotic lesions involve the binding and endocytosis of atherogenic lipoproteins by macrophages followed by the stimulation of intracellular cholesterol esterification (Brown & Goldstein, 1983). The mechanisms regulating these processes are complex, and the details have yet to be elucidated (Tabas, 1995). The data in this report provide evidence that the activity of an endogenous cellular protease is important for cholesterol esterification induced by the expansion of cellular cholesterol pools in both macrophages and a nonmacrophage cell type, CHO cells (Figure 6).

What might be the mechanism whereby intracellular proteolysis leads to the stimulation of cholesterol esterification? There are many sites along the cholesterol esterification pathway that, in theory, could be involved, including lipoprotein endocytosis, lysosomal CE hydrolysis, exit of cholesterol from lysosomes, trafficking of cholesterol from lysosomes to an ACAT substrate pool [*e.g.*, the plasma membrane (Brasaemle & Attie, 1990; Brown et al., 1975a; Tabas et al., 1988; Lange et al., 1993; Nagy & Freeman, 1990)], trafficking of cholesterol to ACAT, and the ACAT enzyme itself. The data in this report clearly indicate that the site where ALLN works is proximal to direct inhibition of the ACAT enzyme (Figures 4 and 5), but is not due to the blockage of cholesterol exit from lysosomes (Figure 3) and is not dependent upon the lysosomal trafficking of cholesterol (Table 2). Thus, protease inhibition appears to block the interaction of a nonlysosomal ACAT substrate pool with ACAT. There is an added level of specificity to this process, however, since the effect is seen only when cellular cholesterol pools are expanded (Table 2A and Figure 4); ALLN clearly is not acting as a general inhibitor of cholesterol trafficking to ACAT (*cf.* Lange & Steck, 1994; Roff et al., 1991). These data suggest the possibility that, when cellular cholesterol pools are expanded, there is induction of a protease-mediated signal that leads to the esterification of these pools (Figure 6). We considered the possibility that this signal may involve the induction of intracellular actin polymerization, since disruption of the actin cytoskeleton leads to the inhibition of lipoprotein-induced cholesterol esterification in macrophages (Tabas et al., 1994). In contrast to the effect of cysteine protease inhibitors, however, actin disruption does not affect cholesterol esterification in CHO cells (Tabas et al., 1994), and phalloidin staining experiments have revealed an apparently intact actin cytoskeleton in ALLN-treated macrophages (unpublished data).

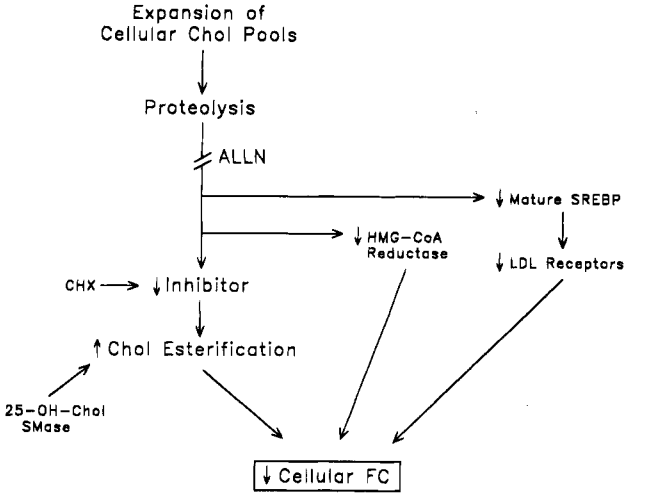

FIGURE 6: Hypothetical scheme of how expanded cholesterol pools could lead to the induction of cholesterol esterification via a proteolysis-mediated step and how intracellular proteolysis could lead to an integrated system for preventing the overexpansion of cellular free cholesterol. We hypothesize that when cellular cholesterol (Chol) pools are expanded, an intracellular proteolytic reaction (or reactions) is induced. With respect to cholesterol esterification, we propose that this proteolytic event destroys an intracellular protein that inhibits the interaction of the expanded cholesterol pools with ACAT, leading to increased cholesterol esterification. Inhibition of the synthesis of this putative inhibitor by cylcoheximide (CHX) would have a similar effect, thus explaining the ability of cycloheximide to stimulate cholesterol esterification in an ALLN-resistant manner. Treatment of cells with 25-hydroxycholesterol (25-OH-Chol) or exogenous sphingomyelinase (SMase) also stimulates cholesterol esterification, but without expanding cellular cholesterol pools and without involving the proposed proteolysis/inhibitor pathway. See text for details. Intracellular proteolysis is also known to lead to the destruction of the mature form of SREBP, which results in decreased transcription of LDL receptors (Wang et al., 1994b) and of HMG-CoA reductase, which results in decreased endogenous cholesterol synthesis (Inoue et al., 1991). Thus, the proposed role of proteolysis in inducing cholesterol esterification and the known roles in affecting SREBP and HMG-CoA reductase together would form an integrated system to limit the expansion of cellular free cholesterol (FC) to potentially toxic levels.

Interestingly, Liscum and co-workers (Dahl et al., 1994) have isolated a CHO cell mutant that shows poor stimulation of cholesterol esterification when incubated with LDL, but shows nearly normal stimulation of cholesterol esterification when incubated with 25-hydroxycholesterol. Furthermore, this mutant's phenotype cannot be attributed to a block in LDL−CE hydrolysis or a block in cholesterol transport from the lysosome to the plasma membrane, which is similar to the situation reported here with protease inhibitor-treated cells. The presence of this mutant provides strong genetic evidence for a specific regulatory molecule of lipoprotein-induced cholesterol esterification that acts extralysosomally but proximal to the ACAT enzyme itself.

In general, proteases can be involved in signaling either by proteolytically activating an important molecule in the signaling pathway or by degrading an endogenous inhibitor of that molecule (*e.g., cf.* Machleidt et al., 1994; Vu et al., 1991). Our laboratory has shown previously that mouse peritoneal macrophages treated with cycloheximide, puromycin, or actinomycin D plus LDL have a significantly

higher level of cholesterol esterification than cells treated with LDL alone (Tabas & Boykow, 1987). Similar results were obtained when acetyl-LDL [under "sub-threshold" conditions; see Xu and Tabas (1991)] was used as the source of cholesterol (Figure 5A, first hatched bar). Likewise, Chang and Chang (1986) have shown that cycloheximide treatment of lipid-starved CHO cells (which accumulate endogenously synthesized cholesterol) stimulates cholesterol esterification. These data implicate the presence of a short-lived endogenous protein inhibitor of cholesterol esterification in these two cell types (Tabas & Boykow, 1987; Chang et al., 1986; Chang & Chang, 1986). If the inhibition of lipoprotein-induced cholesterol esterification by ALLN is due to blockage of the degradation of this putative inhibitor, then, when added after cycloheximide, ALLN should not inhibit cycloheximide-induced cholesterol esterification. The data in Figure 5 of this report clearly indicate that subsequent addition of ALLN does not inhibit cycloheximide-induced cholesterol esterification in either macrophages or CHO cells. These data, together with those described in the preceding paragraph, are consistent with a model in which the expansion of cellular cholesterol pools activates a protease, which in turn cleaves an endogenous protein inhibitor of the interaction of this cholesterol with ACAT (Figure 6). Until the molecules involved in this process are identified, however, other models involving proteases must also be considered.

The findings and ideas put forth in this study fit well into an overall scheme involving ALLN-sensitive proteolytic signaling in intracellular cholesterol homeostasis (Figure 6). ALLN-sensitive proteolysis has been implicated in the down-regulation of HMG-CoA reductase (Inoue et al., 1991), which is a rate-limiting enzyme in cellular cholesterol biosynthesis, and in the destruction of the active form of SREBP-1 (Wang et al., 1994b), which is necessary for the expression of LDL receptor and HMG-CoA synthase. In both circumstances, therefore, ALLN-sensitive proteolysis would prevent cellular free cholesterol levels from becoming too high. According to our findings, ALLN-sensitive proteolysis is also important in the stimulation of cholesterol esterification by lipoproteins (or FC-rich liposomes), which is another important mechanism for handling excess cellular free cholesterol. Thus, considering all three processes together, the cell would possess a unified mechanism for protecting itself against potentially toxic levels of free cholesterol (Figure 6).

In summary, we have shown that the treatment of macrophages and CHO cells with inhibitors of cysteine proteases leads to the inhibition of cholesterol esterification induced by atherogenic lipoproteins or FC-rich liposomes, but not by 25-hydroxycholesterol or sphingomyelinase. The effect cannot be explained by general inhibitory effects on cholesterol trafficking or by direct inhibition of the ACAT enzyme in these cells. Rather, the data suggest that an endogenous protease plays an important role in cholesterol esterification stimulated by the expansion of cellular cholesterol pools, perhaps by cleaving an endogenous inhibitor of the interaction of these expanded pools with ACAT (Figure 6). Given the fact that ALLN-sensitive proteolysis can lead to the down-regulation of HMG-CoA reductase, HMG-CoA synthase, and the LDL receptor, these data may suggest a common mechanism to protect the cell against high levels of free cholesterol (Figure 6). Further testing of our ideas should provide important information regarding the cholesterol esterification pathway in particular and cellular cholesterol homeostasis in general.

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