Regulation of the threshold for lipoprotein-induced acyl-CoA:cholesterol *O*-acyltransferase stimulation in macrophages by cellular sphingomyelin content

Anselm K. Okwu, Xiang-Xi Xu,[1] Yoshimune Shiratori, and Ira Tabas[2]

Departments of Medicine and Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032

Abstract Macrophage acyl-CoA:cholesterol *O*-acyltransferase (ACAT), a key enzyme in atheroma foam cell formation, is stimulated by lipoproteins only after a "threshold" amount of cholesterol has accumulated in the cell. The present study explores the hypothesis that cellular sphingomyelin, by increasing the capacity of the cell to accommodate excess cholesterol, can influence the threshold of ACAT stimulation by lipoproteins. When the sphingomyelin content of macrophages was increased by either incubation with exogenous sphingomyelin or ceramide (a stimulator of endogenous sphingomyelin synthesis), the ability of acetyl-LDL to stimulate whole-cell ACAT activity was substantially reduced despite similar lipoprotein uptake and total cholesterol accumulation as in control cells. When the sphingomyelin content of macrophages was decreased by sphingomyelinase treatment, the ability of acetyl-LDL to stimulate whole-cell ACAT activity was enhanced despite no change in lipoprotein uptake. Importantly, microsomes isolated from control, sphingomyelin-, or sphingomyelinase-treated macrophages showed no difference in ACAT activity when assayed in vitro in the presence of exogenous cholesterol, suggesting that these treatments affected cholesterol trafficking. Lastly, a corollary of the hypothesis, that cells might adapt to a large increase in free cholesterol by increasing their sphingomyelin content, was supported by showing that the sphingomyelin content of macrophages increased 2.6-fold when the cells were induced to accumulate free cholesterol by incubation with acetyl-LDL plus an ACAT inhibitor. Thus, the sphingomyelin content of macrophages can influence the threshold at which ACAT is stimulated by lipoprotein delivery of cholesterol, and the cholesterol content of macrophages can influence the sphingomyelin content of the cell. These findings are consistent with a model in which cellular sphingomyelin plays an important role in accommodating pools of cellular cholesterol that result from the uptake of atherogenic lipoproteins by macrophages.—**Okwu, A. K., X-X. Xu, Y. Shiratori, and I. Tabas.** Regulation of the threshold for lipoprotein-induced acyl-CoA:cholesterol *O*-acyltransferase stimulation in macrophages by cellular sphingomyelin content. *J. Lipid Res.* 1994. **35:** 644–655.

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Cholesteryl ester (CE)-loaded macrophages, or foam cells, are prominent features of atherosclerotic lesions (1–3). CE accumulation in these cells is thought to occur as a result of the endocytosis of native or modified lipoproteins followed by the intracellular esterification of cholesterol by the microsomal enzyme, acyl-coenzyme A:cholesterol *O*-acyl transferase (ACAT) (4, 5). Thus, understanding the regulation of this key enzyme represents a major goal in the areas of both atherogenesis and intracellular cholesterol metabolism. Direct assays for the amount of the ACAT enzyme are not yet available. Thus, it is not definitively known whether or not the enzyme molecule itself is induced when lipoproteins are internalized by cells. Nonetheless, data from protein synthesis inhibition experiments as well as from experiments in which microsomal ACAT was reconstituted into cholesterol-containing liposomes have strongly suggested that a major means of ACAT regulation is related to the amount of cholesterol that comes into contact with ACAT (6–8), which is thought to reside in the endoplasmic reticulum (9). The cholesterol may stimulate ACAT by substrate provision and/or allosteric activation (5, 10).

Given the importance of cholesterol accessibility to ACAT in the regulation of this key enzyme, a major goal of our and other laboratories has been to explore cellular factors that influence this process. Previous work from our laboratory and others has indicated that after lipoprotein-CE is hydrolyzed in lysosomes or other degradative organelles, the newly released lipoprotein-derived cholesterol rapidly mixes with cellular cholesterol; this mixture of endogenous cellular and lipoprotein-cholesterol is then esterified by ACAT (11–14). Short-term timecourse studies

Abbreviations: ACAT, acyl-CoA:cholesterol *O*-acyltransferase; BSA, bovine serum albumin; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; TLC, thin-layer chromatography.

[1]Present address: Department of Biochemistry, St. Jude Children's Hospital, Memphis, TN 38105.

[2]To whom correspondence should be addressed.

from our laboratory have indicated that there is a variable lag period between the onset of lipoprotein degradation and the stimulation of ACAT (14). The duration of this lag period depends upon the rate of net (i.e., influx minus efflux) cholesterol accumulation in the cells (14). Our interpretation of these data is that ACAT is stimulated after the capacity (or "threshold") of cellular membranes to accommodate additional cholesterol is exceeded (14). After threshold is reached, the cholesterol then comes into contact with ACAT, and enzyme activity increases.

The goal of the present study was to elucidate cellular properties that might influence the threshold level at which atherogenic lipoproteins stimulate ACAT in macrophages. Previous work by others has shown that sphingomyelin can interact strongly with cholesterol in both artificial and biological membranes (15–19). In addition, the sphingomyelin content of cells has been shown to influence the intracellular trafficking of endogenous cellular cholesterol (20–23). In this context, the present study explores the hypothesis that macrophage sphingomyelin content can influence the threshold of ACAT stimulation by lipoproteins by affecting the capacity of macrophages to accommodate the excess cholesterol that results from lipoprotein uptake. This capacity, in turn, would determine whether ACAT threshold was relatively high or low.

The data herein show that when the sphingomyelin content of various macrophage cell types was increased or decreased, the threshold level of whole-cell ACAT stimulation by acetyl-LDL was affected in the direction predicted by the hypothesis. Importantly, cellular sphingomyelin content did not alter microsomal ACAT activity when assayed in the presence of exogenous cholesterol, suggesting no direct effect on the ACAT enzyme but rather an effect on cholesterol trafficking. Lastly, the cellular sphingomyelin content of macrophages could be increased by inducing free cholesterol loading of the macrophages, suggesting that cells might adapt to an increase in free cholesterol by increasing their sphingomyelin content. These findings are consistent with a model in which cellular sphingomyelin plays a key physiological role in accommodating pools of cellular cholesterol resulting from the uptake of atherogenic lipoproteins by macrophages.

METHODS

Materials

Sphingomyelinase (from *Bacillus cereus*) was purchased from Boehringer Mannheim. Egg phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, palmitic acid, oleic acid, and bovine brain ceramides were from Sigma. Tissue culture media and reagents were obtained from Gibco Laboratories. Fetal calf serum and iron-supplemented bovine calf serum were from Hyclone Laboratories (Logan, UT). Lipoprotein-deficient serum (LPDS) was prepared from fetal calf serum by preparative ultracentrifugation (density, 1.21 g/ml) (24). [^{14}C]oleate (58 mCi/mmol), [^{3}H]oleate (5 Ci/mmol), [^{3}H]palmitate (30 Ci/mmol), and Na^{125}I were from DuPont-New England Nuclear (Boston, MA). [^{3}H]oleoyl-CoA was synthesized from [^{3}H]oleate as previously described (25). Compound 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide, generously provided by Dr. John Heider of Sandoz (East Hanover, NJ) was prepared in a dimethyl sulfoxide stock solution of 5 mg/ml; cells not receiving 58035 received an equivalent volume (0.1%) of dimethyl sulfoxide. Organic solvents were purchased from Fisher Scientific Company. All other reagents and chemicals were from Sigma.

Cell culture

All cells were maintained in a 37°C tissue culture incubator with a 5% CO_2 atmosphere, using Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 units/ml), streptomycin (100 μg/ml), and freshly added glutamine (2 mM). J774.A1 macrophages (from the American Type Culture Collection) were maintained in spinner culture in DMEM with 10% heat-inactivated (56°C for 30 min) bovine calf serum, with addition of fresh medium every 2 days. J774.A2 (26, 27) cells were grown in monolayer in DMEM/10% fetal calf serum and harvested using a rubber policeman. On the day prior to experiments, the J774 macrophages (either A1 or A2) were plated to 90% confluence on 12-, 24-, or 100-mm culture dishes. After 1 h for adherence, the medium was changed to DMEM/10% LPDS. Mouse peritoneal macrophages were obtained from 25–35 g female ICR mice (28), which had been injected intraperitoneally with 1 ml sterile thioglycollate broth 4 days prior to cell harvesting. After plating the cells in DMEM/10% fetal calf serum for 1 h, the monolayers were washed with warm PBS and were maintained in DMEM/10% LPDS for 1–2 days prior to experiments.

Lipoproteins

LDL (d 1.020–1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation (24). Acetyl-LDL was prepared by acetylation of LDL with acetic anhydride (29) and labeled with ^{125}I using the iodine monochloride method (30).

Lipoprotein degradation and whole-cell ACAT assays

All incubations with cells were carried out in DMEM/0.2% BSA. ^{125}I-labeled lipoprotein degradation and whole-cell ACAT assays were performed as previously described (30). For the whole-cell ACAT assays, [^{14}C]oleate was added to the cells for 3 h. As the $t_{1/2}$ of intracellular CE is, even under optimal conditions of cellular cholesterol efflux, 12–24 h (31), the 3-h incubation with

[^{14}C]oleate is an accurate measure of cellular ACAT activity. Degradation of the ^{125}I-labeled LDL in control wells without cells was typically less than 5% of cellular degradation. In some experiments, an aliquot of the cellular lipid extract was used to measure free cellular cholesterol by gas chromatography (8) (using β-sitosterol as an internal standard) prior to the ACAT assay. After lipid extraction, the cell protein was dissolved in 0.1 N NaOH and an aliquot was used for protein measurement according to the method of Lowry et al. (32).

Phospholipid assay

Cellular lipids (extracted twice with hexane–isopropanol 3:2) were separated by thin-layer chromatography (TLC) in a solvent system of chloroform–methanol–acetic acid–H_2O 50:25:8:4. Individual phospholipids were visualized by iodine staining and compared to phospholipid standards. Each spot was scraped, extracted, and assayed for phosphate content by the method of Bartlett (33). In the ceramide preincubation experiment (Fig. 3, below), there was a potential problem in the ability to separate newly synthesized sphingomyelin and phosphatidylcholine by TLC. Thus, in this experiment, the cellular lipids were treated with base to selectively hydrolyze the non-sphingolipid phospholipids, including phosphatidylcholine (34). For this procedure, 0.8 ml 0.125 N KOH in methanol was added to 0.2 ml cellular lipid extract in chloroform, and the solution was incubated for 1 h at 37°C. After addition of 0.11 ml 1 N HCl to neutralize the solution, the lipids were re-extracted and separated by TLC. As expected, the phosphatidylcholine and phosphatidylethanolamine spots were absent from the TLC plates under these alkaline-treatment conditions.

Phospholipid enrichment of the cells

Stock solutions of phospholipids in ethanol were injected into DMEM/0.2% BSA to prepare a 1 mM phospholipid solution containing 0.1% ethanol. The mixtures were sonicated for 20 5-sec bursts at 4°C under argon atmosphere using a tapered microtip on a Branson 185 sonicator (setting 3) to obtain a stable suspension. Ceramide ± phosphatidylcholine (1:1 mole ratio) in chloroform were dried under nitrogen in glass test tubes. DMEM/0.2% BSA was added to the dried lipid(s) so that the final concentration of each of the lipids was 1 mM. The mixture was sonicated as above to obtain a near clear suspension. The lipid suspensions were used for the experiments on the day of their preparation.

Microsomal ACAT assay

ACAT activity in microsomes isolated from macrophages was determined as described (25) with the following modifications. Monolayers of macrophages in 100-mm tissue culture dishes were washed with ice-cold PBS, and the cells were scraped into homogenization buffer (250 mM sucrose, 1 mM EDTA, 3 mM Tris-HCl, pH 7.4, containing 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.01 mM pepstatin A, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.002 mM leupeptin, 0.01 mM iodoacetamide, 50 μg antipain/ml, and 1 μg aprotinin/ml). The cellular suspension was disrupted by sonication at 4°C (two 30-sec bursts) using a tapered microtip on a Branson 185 sonicator. All additional manipulations were conducted at 4°C except where indicated. Nuclei and other cell debris were pelleted by centrifugation at 1000 *g* for 10 min, and the resulting postnuclear supernate was further centrifuged at 100,000 *g* for 1 h. The microsomal pellet was resuspended in ACAT buffer (100 mM potassium phosphate, pH 7.4, containing 2 mM dithiothreitol) using a 2-ml Teflon/glass homogenizer (Thomas Scientific, Swedesboro, NJ). Aliquots of microsomes and ACAT buffer were preincubated for 15 min at 37°C in the presence, unless indicated otherwise, of 300 μM cholesterol (from a 15.6 mM stock in acetone); then BSA and [^{3}]oleoyl-CoA (200 cpm/pmol) were added to give a final concentration of 50 μM for both compounds. The mixture (200μl) was incubated for 15 min at 37°C, and then 2 ml chloroform–methanol 2:1 (v/v) containing 20 μg unlabeled cholesteryl ester carrier was added to stop the reaction. [^{3}H]CE in the chloroform–methanol extract was resolved by TLC as described (25).

Statistics

Unless otherwise indicated, results are given as means ± SD (n = 3), and absent error bars in these figures signify SD values smaller than the graphic symbols. The Student's *t*-test (two-tailed unless otherwise indicated) was used to determine whether differences between pairs of values were statistically different ($P < 0.05$).

RESULTS

Analysis of threshold values for lipoprotein-induced ACAT stimulation in three different types of mouse macrophages

Three types of mouse macrophages, mouse peritoneal macrophages and two variants (27) of the J774 macrophage cell line (J774.A1 and J774.A2), were incubated with increasing amounts of ^{125}I-labeled acetyl-LDL, and both ^{125}I-labeled acetyl-LDL degradation and whole-cell ACAT activity were measured (**Fig. 1**). From the degradation data and the protein:cholesterol ratio of ^{125}I-labeled acetyl-LDL, an estimate of lipoprotein-cholesterol delivery to the cell was made (14). The data in Fig. 1 show that, in mouse peritoneal macrophages, ACAT was not substantially stimulated until lipoprotein-cholesterol delivery was > 10 nmol/mg per 3 h. In contrast, ACAT was stimulated in J774.A2 cells when cholesterol delivery was ≤ 1.7 nmol/mg per 3 h. The corresponding threshold

value in the J774.A1 macrophages was intermediate between those for the other two cell types → 5 nmol/mg per 3 h. Note that at the higher concentrations of lipoprotein-cholesterol delivery, there is little difference in ACAT activity among the three cell types. These differences in threshold values for ACAT stimulation could not be explained by differences in acetyl-LDL-mediated cellular cholesterol efflux among the three cell types: after a 4-h incubation with 10 μg/ml acetyl-LDL, the cellular cholesterol efflux percentages in mouse peritoneal, J774.A1, and J774.A2 macrophages (which were prelabeled for 24 h with [^{3}H]cholesterol as described (14)) were 11.7%, 8.2%, and 12.1%, respectively. Furthermore, the differences were not due to a relatively higher baseline cholesterol content in the J774.A2 cells (cholesterol contents of LPDS-incubated mouse peritoneal, J774.A1, and J774.A2 macrophages were 53.4, 46.8, and 29.6 nmol/mg cell protein, respectively). Rather, the data indicate that stimulation of ACAT activity in the three macrophage cell types by acetyl-LDL occurs at different levels of lipoprotein-cholesterol delivery to the cells. In the experiments described below, these differences among the macrophage cell types were utilized to help explore the regulation of ACAT threshold.

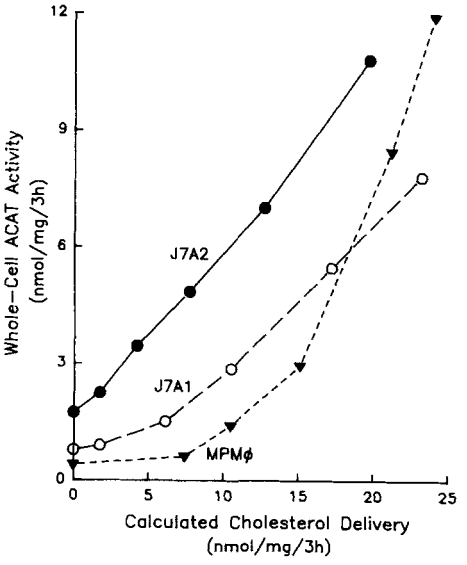

Fig. 1. Dependence of whole-cell ACAT activity on lipoprotein cholesterol delivery in mouse peritoneal, J774.A1, and J774.A2 macrophages. Monolayers of mouse peritoneal (*MPMφ*), J774.A1 (*J7A1*), and J774.A2 (*J7A2*) macrophages were incubated in DMEM/0.2% BSA containing ^{125}I-labeled acetyl-LDL (0, 2, 5, 10, 15, 20 μg/ml) and [^{14}C]oleate (0.1 mM) for 3 h. Whole-cell ACAT activity and ^{125}I-labeled acetyl-LDL degradation were assayed and expressed as per mg cell protein; the degradation data, together with the protein:cholesterol ratio of the acetyl-LDL, were used to calculate lipoprotein-cholesterol delivery to the cells (14). The data points are averages of duplicate values which varied less than 10%.

Acetyl-LDL-stimulated ACAT activity in sphingomyelin-enriched J774.A2 macrophages

To begin to test the idea that cellular sphingomyelin content can affect the threshold level at which atherogenic lipoproteins stimulate ACAT in macrophages, J774.A2 cells were preincubated in the absence or presence of sonicated dispersions of sphingomyelin, as well as phosphatidylethanolamine and phosphatidylcholine, and then assayed for phospholipid subclass content and whole-cell ACAT activity (**Fig. 2**). Note, as shown above, that J774.A2 macrophages normally have a relatively low threshold for ACAT stimulation by lipoproteins, and these cells also have a relatively low basal sphingomyelin content (9.3 ± 0.3 nmol sphingomyelin/mg cell protein vs. 35.0 ± 2.0 nmol/mg for mouse peritoneal macrophages). The inset in Fig. 2A shows that sphingomyelin preincubation led to a 4.5-fold increase in the sphingomyelin content of the cells, phosphatidylethanolamine preincubation led to a 2.4-fold increase in phosphatidylethanolamine, and phosphatidylcholine preincubation led to a 25% increase in phosphatidylcholine content ($P < 0.025$). None of the phospholipid preincubations, prior to the addition of the acetyl-LDL, affected the unesterified cholesterol content of the cells (data not shown). The whole-cell ACAT data in Fig. 2A show that ACAT stimulation by acetyl-LDL in J774.A2 macrophages was inhibited, in a dose-response manner, by sphingomyelin preincubation. In repeat experiments, the magnitude of the effect was sometimes not as great as that shown in Fig. 1A (below), but whole-cell ACAT activity was always inhibited by at least 50% in sphingomyelin-treated cells. Sphingomyelin preincubation did not affect the incorporation of [^{14}C]oleate into mono-, di-, or triglycerides, indicating that inhibition of ACAT cannot be explained by either dilution of [^{14}C]oleate with unlabeled, sphingomyelin-derived fatty acids or by depletion of [^{14}C]oleate by other biosynthetic pathways. Furthermore, sphingomyelin preincubation did not affect the efflux of cellular cholesterol under the conditions of the experiment. ACAT was also inhibited by preincubation with the highest concentration of phosphatidylethanolamine, although the extent of inhibition was less than that seen with sphingomyelin. Phosphatidylcholine preincubation, which increased the phospholipid content of the cells much less than the other two treatments (above), had little effect on acetyl-LDL stimulation of ACAT. Thus, stimulation of ACAT by acetyl-LDL in J774.A2 macrophages was inhibited by treatments that increased the cells' phospholipid content; the inhibition was particularly marked when the sphingomyelin content of the cells was increased, and therefore further efforts were focused on this phospholipid subclass.

To explore the effect of sphingomyelin enrichment on J774.A2 ACAT activity in more detail, cells preincubated in the absence or presence of sphingomyelin were incubated with increasing concentrations of ^{125}I-labeled acetyl-LDL. Both whole-cell ACAT activity and ^{125}I-

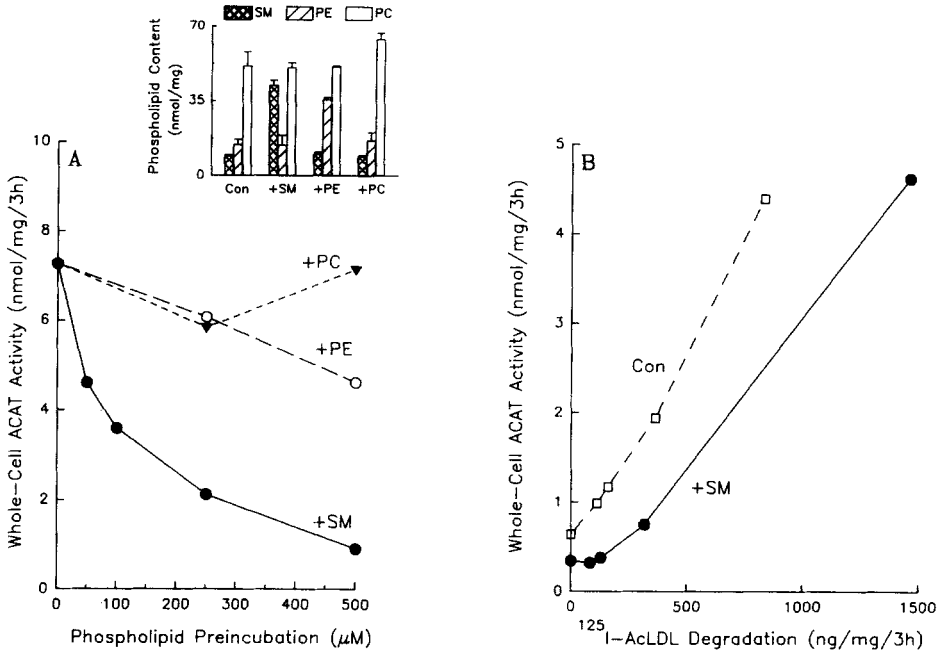

Fig. 2. Effect of phospholipid enrichment on ^{125}I-labeled acetyl-LDL degradation and acetyl-LDL-induced whole-cell ACAT activity in J774.A2 macrophages. Fig. 2A: J774.A2 cells were incubated for 3 h at 37°C in DMEM/0.2% BSA in the absence (control, Con) or presence of sonicated dispersions of sphingomyelin (+SM, closed circles), phosphatidylethanolamine (+PE, open circles), or phosphatidylcholine (+PC, closed triangles) at the indicated phospholipid concentrations. After washing three times with warm medium, the cells were incubated with 5 μg/ml ^{125}I-labeled acetyl-LDL and [^{14}C]oleate (0.1 mM) for 3 h and assayed for ^{125}I-labeled acetyl-LDL degradation and whole-cell ACAT activity. ^{125}I-labeled acetyl-LDL degradation was not altered (< 5% variation) by preincubation of the cells with any of the phospholipids. Inset: Parallel sets of monolayers as those described above (Con, +SM, +PE, +PC) were assayed for cellular content of sphingomyelin (SM, cross-hatched bars), phosphatidylethanolamine (PE, diagonal-hatched bars), and phosphatidylcholine (PC, open bars) after preincubation with or without 250 μM phospholipid. Fig. 2B: J774.A2 macrophages, preincubated for 3 h in the absence (open squares) or presence (closed circles) of 500 μM sphingomyelin, were incubated with various concentrations of ^{125}I-labeled acetyl-LDL plus [^{14}C]oleate and assayed for ^{125}I-labeled acetyl-LDL degradation (abscissa) and whole-cell ACAT activity. The values shown in Fig. 2A and 2B are averages of duplicate values which varied less than 10%.

labeled acetyl-LDL degradation were measured so that ACAT stimulation could be determined at equivalent levels of lipoprotein degradation (and thus equivalent levels of lipoprotein-cholesterol delivery (14)). Sphingomyelin preincubation did not affect the rate or extent of acetyl-LDL-CE lysosomal hydrolysis (data not shown). The data in Fig. 2B show that at several different levels of ^{125}I-labeled acetyl-LDL degradation, whole-cell ACAT activity was substantially inhibited in cells that had been enriched in sphingomyelin. Importantly, the data also show that whereas untreated J774.A2 cells have no apparent threshold for ACAT stimulation (see also Fig. 1), sphingomyelin-treated cells show a "lag" preceding ACAT stimulation that is indicative of a threshold effect.

To determine the effect of increasing endogenously synthesized sphingomyelin on ACAT stimulation by lipoproteins, cellular sphingomyelin biosynthesis was stimulated by preincubating macrophages with the sphingomyelin precursor, ceramide (35, 36). These experiments utilized J774.A1 macrophages, which like J774.A2 macrophages, have a lower threshold for ACAT stimulation by lipoproteins and a lower basal sphingomyelin content (16.8 ± 2.1 nmol sphingomyelin/mg cell protein) than mouse peritoneal macrophages (above). The data in **Fig. 3A** show that this method led to a 42% increase in the sphingomyelin content of these cells. Concomitantly, whole-cell ACAT activity induced by a low concentration of acetyl-LDL was decreased by ~50% in the ceramide-treated cells despite equal amounts of ^{125}I-labeled acetyl-LDL degradation in the control and treated cells. As a negative control, triglyceride synthesis (i.e., [^{14}C]oleate incorporation into cellular triglycerides) was not affected by ceramide preincubation. That the ACAT-inhibitory effect of ceramide was, in fact, due to enhancement of cellular sphingomyelin content rather than to some other effect of ceramide was supported by the finding that sphingomyelinase treatment of the ceramide-preincubated cells completely negated the ability of ceramide to inhibit ACAT in acetyl-LDL-incubated macrophages (see below). Thus, a modest increase in endogenously synthesized sphin-

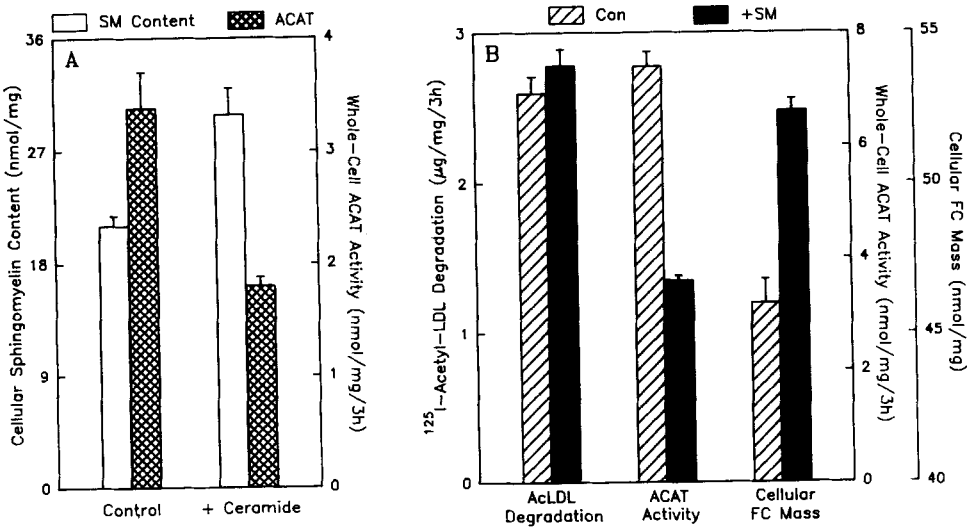

Fig. 3. Effect of ceramide preincubation on sphingomyelin content and acetyl-LDL-induced whole-cell ACAT activity in J774.A1 macrophages (Fig. 3A) and effect of sphingomyelin preincubation on acetyl-LDL-induced free cholesterol accumulation in J774.A2 macrophages (Fig. 3B). Fig. 3A: J774.A1 macrophages were preincubated for 4 h in DMEM/0.2% BSA alone (Control) or containing 0.2 mM ceramide (+Ceramide). One set of cells (open bars) was assayed for sphingomyelin (SM) content by the alkaline-treatment method (see Methods). A parallel set of cells was incubated with ^{125}I-labeled acetyl-LDL (5 μg/ml) and [^{14}C]oleate (0.1 mM) for 3 h and assayed for whole-cell ACAT activity (cross-hatched bars). The amount of ^{125}I-labeled acetyl-LDL degraded for both the control and ceramide-treated cells was 2.3 μg/mg cell protein/3 h. Fig. 3B: J774.A2 macrophages were preincubated for 2 h in the absence (diagonal-hatched bars, Con) or presence (solid bars, +SM) of sphingomyelin (200 μM) as described in the legend to Fig. 2. The cells were then incubated with ^{125}I-labeled acetyl-LDL (5 μg/ml) and [^{14}C]oleate (0.1 mM) for 3 h and assayed for ^{125}I-labeled acetyl-LDL degradation, whole-cell ACAT activity, and cellular free cholesterol (FC) mass.

gomyelin content is associated with a decrease in the ability of acetyl-LDL to stimulate ACAT in these cells.

If cells enriched in sphingomyelin sequester cholesterol away from ACAT (above), the unesterified cholesterol content should be higher in sphingomyelin-enriched macrophages. To test this point, control and sphingomyelin-treated J774.A2 macrophages were incubated with ^{125}I-labeled acetyl-LDL plus [^{14}C]oleate, and lipoprotein degradation, whole-cell ACAT activity, and cellular free cholesterol mass were measured (Fig. 3B). Despite equivalent lipoprotein degradation, there was approximately 4 nmol/mg less ACAT-derived CE in sphingomyelin-enriched macrophages compared with control cells; the free cholesterol content of the sphingomyelin-enriched macrophages was increased by a similar amount. This finding is consistent with the idea that when lipoprotein-cholesterol is delivered to sphingomyelin-enriched macrophages, free cholesterol is sequestered away from ACAT.

The possibility that sphingomyelin treatment of J774.A2 macrophages directly inhibited the ACAT enzyme instead of cholesterol trafficking to ACAT was next investigated. In one experiment, microsomes from control macrophages were preincubated in vitro in the absence or presence of 250 μM sphingomyelin for 15 min at 37°C, and then ACAT was assayed for an additional 30 min in the absence or presence of the sphingomyelin, respectively. This direct sphingomyelin treatment of the microsomes had no effect on subsequent ACAT activity (data not shown). To examine this issue in more detail, microsomes were isolated from control and sphingomyelin-treated J774.A2 macrophages, and ACAT activity in these microsomes was assayed in vitro in the presence of excess exogenous cholesterol. Under these conditions, any influence of cholesterol trafficking that might have existed in the intact living cell should be markedly diminished (6, 28). The data in **Fig. 4** show that microsomes isolated from J774.A2 macrophages enriched in sphingomyelin had ACAT activity identical to microsomes from control cells, in marked contrast to the whole-cell ACAT activity data shown previously (Figs. 2 and 3). Importantly, when the microsomal ACAT activity was assayed in the absence of exogenously added cholesterol (Fig. 4, inset), ACAT activity was approximately one-third lower in microsomes isolated from sphingomyelin-treated cells. These data support the hypothesis that the mechanism of inhibition of acetyl-LDL-induced ACAT activity by sphingomyelin is due to alteration of trafficking of cellular cholesterol in intact macrophages or of endogenous cholesterol in isolated microsomes, rather than by a direct inhibition of the ACAT enzyme itself.

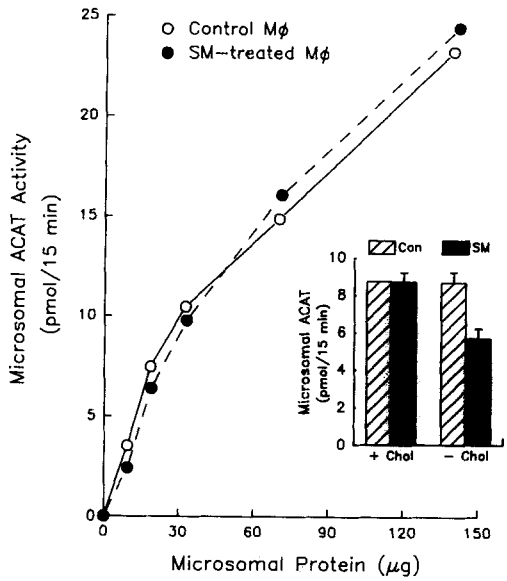

Fig. 4. In vitro ACAT activity in microsomes isolated from control and sphingomyelin-treated J774.A2 macrophages. J774.A2 macrophages were preincubated with 300 μM cholesterol in the absence (closed circles) or presence (open circles) of 250 μM sphingomyelin and then incubated for an additional 3 h with 10 μg acetyl-LDL/ml as described in the legend to Fig. 2. Microsomes were prepared from these cells and then assayed for ACAT activity. The values shown are averages of duplicate values which varied less than 10%. Inset: Microsomes (100 μg) from control (Con, hatched bars) and sphingomyelin-treated (SM, solid bars) J774.A2 macrophages were assayed for ACAT activity either in the presence of exogenous cholesterol as above (+Chol) or in the absence of exogenous cholesterol (−Chol).

Acetyl-LDL-stimulated ACAT activity in sphingomyelinase-treated mouse peritoneal macrophages

To further test the hypothesis that cellular sphingomyelin content can influence the threshold for ACAT stimulation by lipoproteins, mouse peritoneal macrophages were preincubated with sphingomyelinase to decrease their sphingomyelin content. This cell type normally has a relatively high threshold for ACAT stimulation by lipoproteins (Fig. 1) and a relatively high sphingomyelin content (above). The inset in **Fig. 5** shows that sphingomyelinase treatment led to ~50% decrease in sphingomyelin content with only a slight decrease in phosphatidylethanolamine content and a slight increase in phosphatidylcholine content. The data in Fig. 5A show that sphingomyelinase treatment had absolutely no effect on the intracellular degradation of ^{125}I-labeled acetyl-LDL.[3] In other experiments not shown, there were also no differences in cellular cholesterol efflux under the conditions of this experiment, in agreement with the data of Stein et al. (23). In contrast, as shown by the data in Fig. 5B, sphingomyelinase treatment had a substantial effect on the timecourse of whole-cell ACAT stimulation by acetyl-LDL. Whereas control macrophages showed the typical lag period characteristic of this high-threshold cell, the curve for the sphingomyelinase-treated cells showed a much shorter lag period. In fact, this ACAT curve from sphingomyelinase-treated mouse peritoneal macrophages is reminiscent of the ACAT curve from J774 macrophages (see Fig. 1), which naturally have relatively low sphingomyelin content. Also note that by 10 h of incubation, the percent difference in whole-cell ACAT activity between the control and sphingomyelinase-treated cells was less than at 3–8 h of incubation, which is consistent with the idea that ACAT threshold, rather than the ACAT enzyme itself, was affected by the sphingomyelinase treatment.

To further support this conclusion, microsomes isolated from cells treated with acetyl-LDL alone or acetyl-LDL plus sphingomyelinase were assayed for ACAT activity (Fig. 5C). In contrast to the whole-cell ACAT data, sphingomyelinase treatment of the cells had no effect on microsomal ACAT activity. In other experiments not shown here, microsomes from control macrophages were pre-incubated in vitro in the absence or presence of sphingomyelinase, and then ACAT was assayed, also in the absence or presence of the sphingomyelinase. This direct sphingomyelinase treatment of the microsomes had no effect on subsequent ACAT activity (data not shown). These data, together with the previous data from sphingomyelin-enriched J774 macrophages, support the hypothesis that the sphingomyelin content of macrophages, most likely by affecting intracellular cholesterol trafficking, can strongly influence the threshold level at which atherogenic lipoproteins stimulate macrophage ACAT.

Relationship between contents of free cholesterol and sphingomyelin in macrophages

If cellular sphingomyelin can accommodate excess free cholesterol resulting from lipoprotein uptake in macrophages, cellular sphingomyelin content might adaptively increase in response to a free cholesterol load in these cells. To directly test this idea, J774.A1 macrophages were incubated for 2 days in the absence or presence of a relatively high concentration of acetyl-LDL or acetyl-LDL plus the ACAT inhibitor, compound 58035 (37). As shown by the cross-hatched bars in **Fig. 6,** this prolonged incubation with acetyl-LDL led to a 3-fold increase in macrophage free cholesterol content, and incubation with acetyl-LDL plus the ACAT inhibitor led to 8.6-fold increase in cellular free cholesterol. Under these two conditions, the cellular sphingomyelin content increased 1.8-fold and 2.6-fold, respectively. Incubation with compound 58035 alone had no effect on either macrophage free cholesterol or sphingomyelin content. Thus, excess

[3]Whereas incubation of native LDL with sphimgomyelinase causes LDL self-aggregation (and increased uptake by macrophages), incubation of acetyl-LDL with sphingomyelinase does not cause acetyl-LDL self-aggregation (56).

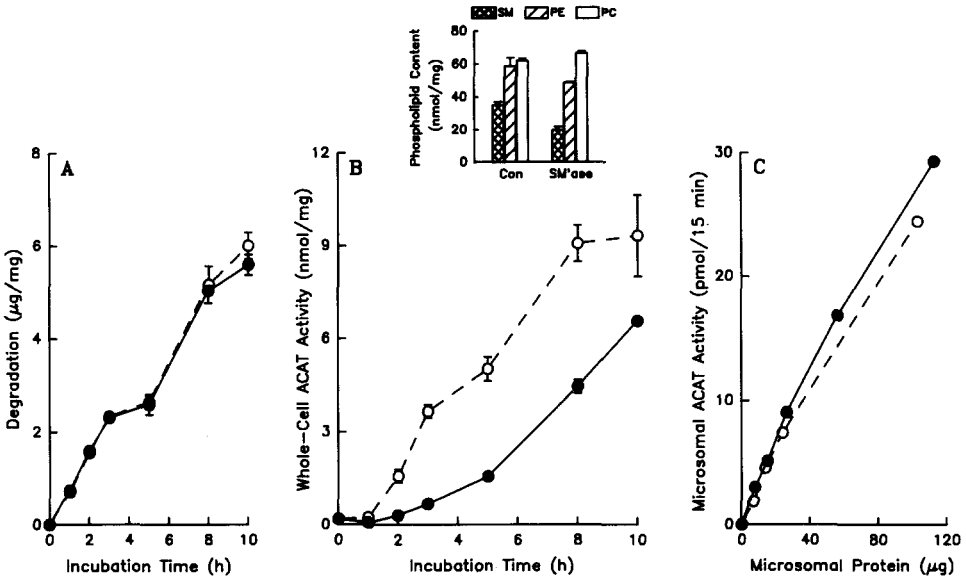

Fig. 5. Effect of sphingomyelinase treatment of mouse peritoneal macrophages on cellular phospholipid content, ^{125}I-labeled acetyl-LDL degradation, whole-cell acetyl-LDL-induced ACAT activity, and microsomal ACAT activity. Mouse peritoneal macrophages were incubated for 1 h at 37°C in DMEM/0.2% BSA alone (Con) or containing 50 mU sphingomyelinase/ml (SM'ase). One set of cells was then assayed for cellular content of sphingomyelin (SM, cross-hatched bars), phosphatidylethanolamine (PE, diagonal-hatched bars), and phosphatidylcholine (PC, open bars) (inset). Other sets of cells were subsequently incubated at 37°C for the indicated times in DMEM/BSA/0.1 mM [^{14}C]oleate containing 2 μg ^{125}I-labeled acetyl-LDL/ml (closed circles) or ^{125}I-labeled acetyl-LDL plus 50 mU sphingomyelinase/ml (open circles), and ^{125}I-labeled acetyl-LDL degradation (Fig. 5A) and whole-cell ACAT activity (Fig. 5B) were determined. Fig. 5C: Microsomes were isolated from mouse peritoneal macrophages incubated with acetyl-LDL alone (closed circles) or acetyl-LDL plus sphingomyelinase (open circles) and then assayed for in vitro ACAT activity. The values in Fig. 5C are averages of duplicate values which varied by less than 10%.

free cholesterol resulting from prolonged lipoprotein uptake can influence the sphingomyelin content of the cell.

DISCUSSION

The stimulation of ACAT in macrophages by endocytosed atherogenic lipoproteins is a key event leading to macrophage foam cell formation (4, 5). The overall goal of the present project was to elucidate cellular properties that influence lipoprotein-induced activation of ACAT in macrophages. Previous work from other laboratories, as well as our own, has shown the following: *a)* lipoprotein-cholesterol rapidly mixes with cellular cholesterol prior to ACAT activation (11–14); *b)* lipoproteins stimulate ACAT only after cellular cholesterol has been increased above a certain threshold level (14); *c)* sphingomyelin interacts avidly with cholesterol in membranes (15–19); and *d)* the sphingomyelin content of cells can influence intracellular trafficking of endogenous cellular cholesterol (20–22). Based upon these findings, the following hypothesis was explored: cellular sphingomyelin can influence the threshold of ACAT stimulation by lipoproteins by affecting the capacity of macrophages to accommodate the excess cholesterol that results from lipoprotein uptake. Our data show that the threshold at which ACAT is stimulated

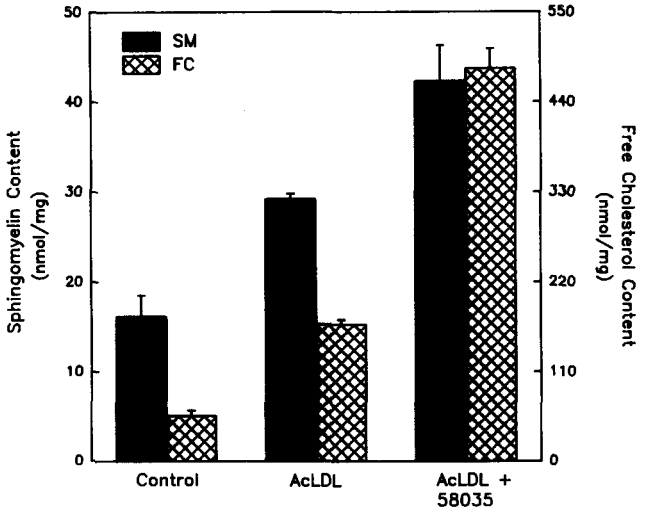

Fig. 6. Sphingomyelin and free cholesterol contents of J774.A1 macrophages subjected to prolonged incubation with acetyl-LDL ± an ACAT inhibitor. J774.A1 macrophages were incubated for 2 days in DMEM/10% LPDS alone (Control) or containing 25 μg acetyl-LDL/ml (AcLDL) or acetyl-LDL plus 5 μg 58035/ml (AcLDL + 58035). The medium was changed every 12 h. Lipid extracts of the cells were then assayed for sphingomyelin (SM, solid bars) and free cholesterol (FC, cross-hatched bars) mass. The SM and FC values for cells incubated with 58035 alone were not significantly different from those of the control cells.

by lipoproteins in macrophages can be altered by changing the sphingomyelin content of the cells, most likely by influencing intracellular cholesterol trafficking (see below). Furthermore, the data show that excess free cholesterol resulting from prolonged lipoprotein uptake can influence the sphingomyelin content of the cell. These findings are consistent with a model in which cellular sphingomyelin plays a key role in accommodating pools of cellular cholesterol resulting from the uptake of atherogenic lipoproteins by macrophages.

In order for a lipoprotein to stimulate ACAT, there must be lipoprotein endocytosis, lipoprotein-CE hydrolysis, cholesterol trafficking to ACAT, and an active ACAT enzyme. The effects of sphingomyelin and sphingomyelinase on acetyl-LDL-stimulated ACAT activity in macrophages reported in this paper are most consistent with the idea that these treatments affect intracellular cholesterol trafficking. Under the conditions of our experiments, the treatments had no effect on lipoprotein uptake or lysosomal hydrolysis. Regarding effects on the ACAT enzyme itself, it would, in theory, be possible for sphingomyelin or one of its cellular metabolites to be directly inhibitory or stimulatory. For instance, ceramide and sphingosine, which can affect the phosphorylation state of certain proteins (38, 39), might directly affect ACAT (10). Several points, however, strongly suggest that neither sphingomyelin nor its metabolites directly affects ACAT. First, in vitro treatment of microsomes from control cells with either sphingomyelin or sphingomyelinase had no effect on microsomal ACAT activity (see text). Second, and most significant, microsomes isolated from macrophages treated with sphingomyelin or sphingomyelinase had identical ACAT activity, when assayed in the presence of excess exogenous cholesterol, to those from control cells (Figs. 4 and 5C). Importantly, however, we found that microsomes from sphingomyelin-enriched cells, when assayed in vitro in the absence of exogenous cholesterol, had less ACAT activity than microsomes from control cells (Fig. 4, inset). One interpretation of these data is that the membranes containing ACAT or a critical ACAT substrate pool in the microsomal preparation from sphingomyelin-treated cells are relatively cholesterol-poor, since less cholesterol was transferred to these membranes in the cell. Alternatively, or in addition, the microsomal preparation probably consists of potential donor membranes (e.g., plasma membrane) that are rich in cholesterol, but these membranes transfer their cholesterol to ACAT poorly in vitro as the membranes are also rich in sphingomyelin.

Regarding the possibility of ceramide- or sphingosine-induced phosphorylation changes in ACAT, there is now increasing evidence that phosphorylation/dephosphorylation events do not affect ACAT activity (40). In fact, recent sequence analysis of human ACAT revealed that the enzyme does not appear to contain any of the usual protein kinase A or C recognition sites (41). In agreement with these findings, we found that 100 nM okadaic acid, which inhibits protein phosphatases 1 and 2A (42), including ceramide-induced protein phosphatase activity (43), did not inhibit the effects of either sphingomyelin or sphingomyelinase on acetyl-LDL-induced ACAT stimulation in macrophages (A. K. Okwu and I. Tabas, unpublished observations). Furthermore, 1 μM staurosporine, which, like sphingosine and lysosphingolipids (38, 39), inhibits protein kinase C, did not mimic any of the effects of either sphingomyelin or sphingomyelinase on ACAT threshold (A. K. Okwu and I. Tabas, unpublished observations). All of these data together, therefore, strongly suggest that sphingomyelin and sphingomyelinase treatment of macrophages affects ACAT threshold by influencing cholesterol delivery to ACAT and not by directly affecting the ACAT enzyme itself.

The most likely scheme to explain the effect of cellular sphingomyelin content on ACAT threshold is that sphingomyelin-enriched membranes, by physico-chemical interactions, sequester cholesterol away from ACAT. This scheme is mechanistically simple and takes into account the known interactions between cholesterol and sphingomyelin (15–19) and the known influence of sphingomyelin of the trafficking of endogenous cellular cholesterol (20–22). Our data do not definitively rule out the possibility, however, that sphingomyelin or a sphingomyelin metabolite (cf. refs. 44 and 45) might affect some other aspect of intracellular cholesterol trafficking (e.g., inhibiting the function of a possible cholesterol transport protein). Whatever mechanism is involved in the ability of sphingomyelin to disrupt intracellular cholesterol trafficking to ACAT, it should theoretically be possible to isolate and identify the cellular membrane(s) where the unesterified cholesterol accumulates in sphingomyelin-enriched macrophages. Attempts at using subcellular fractionation to address this point have not yet been successful, perhaps because of cholesterol diffusion during the fractionation procedure or perhaps because the accumulated cholesterol is distributed throughout several cellular membranes.

The cellular phospholipid examined in most detail in this report was sphingomyelin. Other cellular phospholipid subclasses and total phospholipid content, however, may also influence ACAT threshold in macrophages. For example, enrichment of cellular phosphatidylethanolamine content led to some degree of ACAT inhibition (Fig. 2A). Along these lines, it is possible that one of the factors responsible for the ACAT differences among the three macrophage cells types used in this project is the basal phospholipid content and makeup of the cells. For instance, mouse peritoneal and J774 macrophages differ in their basal sphingomyelin, phosphatidylethanolamine, and total phospholipid contents (insets to Figs. 2 and 5), and changing these con-

tents, especially sphingomyelin, influences their ACAT threshold (above). The origin of the ACAT differences may be complex and multifactorial, however, and other factors not related to cellular phospholipids may also have a role in the ACAT threshold differences.

The data from others cited above as well as those presented here suggest that sphingomyelin plays an important role in interacting with endogenous cellular cholesterol and excess cholesterol resulting from the cellular uptake of lipoproteins. Based upon this concept, the hypothesis that sphingomyelin content might increase adaptively in response to a free cholesterol load was tested. The data shown in Fig. 6 are consistent with this idea. Clearly, ACAT-mediated esterification is a major adaptive response to excess free cholesterol (5). Under the conditions of the experiment in Fig. 6, however, there was a very large influx of lipoprotein cholesterol, and cellular free cholesterol increased. Concomitant with this increase in cellular free cholesterol content, cellular sphingomyelin content also increased. In the presence of an ACAT inhibitor, the cholesterol content rose further, and the sphingomyelin increase was, accordingly, more marked (Fig. 6). Whether the mechanism of the increase in sphingomyelin content involves an increase in sphingomyelin biosynthesis (cf. ref. 46) and/or a decrease in sphingomyelin degradation is currently being investigated by our laboratory.

The data in Fig. 6 indicate that the rise in cellular sphingomyelin content could directly interact with only ~10–20% of the excess free cholesterol. The rest of the free cholesterol is probably accommodated by other phospholipids, as the phosphatidylcholine content of FC-loaded macrophages increases by ~60–130 nmol/mg, depending upon the conditions of the experiment (57). Phosphatidylethanolamine and phosphatidylserine contents also increase in these cells (57). In fact, consistent with others' data (47, 48), we have found that these cells have intracellular membrane whorls (57). Therefore, the most likely scenario is that all of the increased phospholipids contribute to the cells' ability to accommodate the excess free cholesterol. Although the contribution by the sphingomyelin in the free cholesterol-loaded cells may seem relatively small, this sphingomyelin, depending upon its intracellular location, may lead to increased sequestration of a particularly important pool of cholesterol.

The findings that cellular sphingomyelin content can influence the stimulation of ACAT by internalized lipoproteins and that macrophage free cholesterol loading leads to an increase in cellular sphingomyelin content may have relevance to the physiology of atheroma foam cells. A rate-limiting enzyme of sphingomyelin biosynthesis is induced in atheroma (49), and there is a progressive rise in the sphingomyelin content of the lesions (50), perhaps related to the increase in free cholesterol content in macrophages of advanced lesions (51–54). It may be that sphingomyelin and ACAT work together to protect arterial wall macrophages, which are exposed to high levels of atherogenic lipoproteins, from toxic levels of free cholesterol and cholesterol crystal formation: ACAT esterifies (and thus detoxifies (5)) free cholesterol, and sphingomyelin, by sequestering at least some excess cholesterol away from ACAT, may help prevent or retard the esterifying enzyme from becoming saturated. The disruption of this proposed protective function of sphingomyelin, for instance by an increase in sphingomyelinase activity (known to be present in advanced atherosclerotic lesions (55)), might help contribute to cholesterol toxicity, cholesterol crystal formation (54), macrophage necrosis, and progression to more complicated lesions.

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