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Suppression of Macrophage Eicosanoid Synthesis by Atherogenic Lipoproteins Is Profoundly Affected by Cholesterol-Fatty Acyl Esterification and the Niemann-Pick C Pathway of Lipid Trafficking*

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Atheroma macrophages internalize large quantities of lipoprotein-derived lipids. While most emphasis has been placed on cholesterol, lipoprotein-derived fatty acids may also play important roles in lesional macrophage biology. Little is known, however, about the trafficking or metabolism of these fatty acids. In this study, we first show that the cholesterol-fatty acyl esterification reaction, catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT), competes for the incorporation of lipoprotein-derived fatty acids into cellular phospholipids. Furthermore, conditions that inhibit trafficking of cholesterol from late endosomes/lysosomes to the endoplasmic reticulum (ER), such as the amphipathic amine U18666A and the *Npc1*+/− mutation, also inhibit incorporation of lipoprotein-derived fatty acids into phospholipids. The biological relevance of these findings was investigated by studying the suppression of agonist-induced prostaglandin E_2 (PGE_2) and leukotriene $C_4/D_4/E_4$ production during lipoprotein uptake by macrophages, which has been postulated to involve enrichment of cellular phospholipids with non-arachidonic fatty acids (NAAFAs). We found that eicosanoid suppression was markedly enhanced when ACAT was inhibited and prevented when late endosomal/lysosomal lipid trafficking was blocked. Moreover, PGE_2 suppression depended entirely on acetyl-LDL-derived NAAFAs, not on acetyl-LDL-cholesterol, and was not due to decreased $cPLA_2$ activity *per se*. These data support the following model: lipoprotein-derived NAAFAs traffic via the NPC1 pathway from late endosomes/lysosomes to a critical pool of phospholipids. In competing reactions, these NAAFAs can be either esterified to cholesterol or incorporated into phospholipids, resulting in suppression of eicosanoid biosynthesis. In view of recent evidence suggesting dysfunctional cholesterol esterification in late lesional macrophages, these data predict that such cells would have highly suppressed eicosanoid synthesis, thus affecting eicosanoid-mediated cell signaling in advanced atherosclerosis.

Macrophages are a prominent feature of atherosclerotic lesions and play critical roles in both lesion initiation and progression (1–3). During atherogenesis, macrophages internalize atherogenic lipoproteins in the arterial subendothelium via receptor-mediated endocytosis and phagocytosis (4, 5). In late endosomes/lysosomes or phagosomes, hydrolases release both cholesterol and fatty acids from these lipoproteins. Free cholesterol is exported from these degradative organelles by a vesicular transport process that requires the NPC1[1] protein and that is sensitive to a class of amphipathic amines, such as U18666A (6). An important fate of this exported cholesterol is its esterification to fatty acids in the ER by ACAT (4). This reaction is particularly critical during the early stages of atherogenesis, because early lesional macrophages accumulate large amounts of ACAT-derived cholesteryl esters ("foam cells") (5).

While the fate of lipoprotein-cholesterol has been widely studied, much less is known about the fate of lipoprotein-derived fatty acids. The majority of released fatty acids traffic to the plasma membrane for efflux. Of those retained, most are incorporated into phospholipids (7, 8). In contrast to the situation with lipoprotein-derived cholesterol, however, the mechanisms and consequences of lipoprotein-derived fatty acid transport and metabolism are poorly understood.

Our laboratory became interested in this topic in the context of our studies on lipid metabolism relevant to late lesional macrophages. In advanced atherosclerotic lesions, macrophages accumulate large amounts of unesterified, or "free," cholesterol (FC) (3), and recent *in vivo* data from our laboratory has provided evidence that this event is due to defective ACAT-mediated cholesterol-fatty acyl esterification (9). The consequences of FC accumulation are profound, including triggering of an ER stress pathway and apoptosis (3, 10). However, a pool of lipoprotein-derived fatty acids should also accumulate as a consequence of defective cholesterol-fatty acyl esterification, and we wondered what the consequences of this would be.

To explore this question, we took advantage of a model in which lipoprotein-derived fatty acids affect eicosanoid biosynthesis. In particular, investigators have observed that foam cell formation is associated with decreased eicosanoid synthesis, although the mechanism remains controversial (11, 12, 33). In

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[1] The abbreviations used are: NPC, Niemann-Pick C; AA, arachidonic acid; ACAT, acyl-CoA:cholesterol acyltransferase; $cPLA_2$, cytosolic phospholipase A_2; ER, endoplasmic reticulum; FC, free cholesterol; LDL, low-density lipoprotein; LT, leukotriene; NAAFA, non-arachidonic fatty acid; PGE_2, prostaglandin E_2; rAcLDL, reconstituted acetyl-LDL; FBS, fetal bovine serum; BSA, bovine serum albumin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

this report, we explore the effects of altered lipid trafficking and cholesterol-fatty acyl esterification on lipoprotein-fatty acid incorporation into cellular phospholipids, $cPLA_2$-induced fatty acid release, and eicosanoid synthesis in macrophages. We provide evidence for a model in which lipoprotein-derived non-arachidonic acid fatty acids (NAAFAs) traffic via the NPC1 pathway from late endosomes/lysosomes to a critical pool of phospholipids. In competing reactions, these NAAFAs can be either esterified to cholesterol or incorporated into phospholipids, resulting in suppression of eicosanoid biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Falcon tissue culture plastic ware was purchased from Fisher Scientific. Tissue culture reagents were from Invitrogen Life Technologies, Inc. Fetal Bovine Serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). 3-[Decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide (compound 58035) (14), an inhibitor of acyl-CoA:cholesterol *O*-acyltransferase, was generously provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ); a 10 mg/ml stock solution was prepared in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration in both treated and control cells was 0.05%. U18666A (3-β-[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) was from Biomol (Plymouth Meeting, PA). Cholesteryl-[1-^{14}C]oleate was purchased from Amersham Biosciences, and phosphatidylcholine-1-stearoyl-2-[^{3}H]arachidonyl was from New England Nuclear. Dioleoylglycerol and *sn*-2 arachidonyl PC were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Human GST-tagged recombinant $cPLA_2\alpha$ was produced in Sf9 cells using a baculovirus transfection system and was purified by anti-GST affinity chromatography. All other chemicals and reagents were obtained from Sigma Chemical Co.

Harvesting, Culturing, and Incubations of Mouse Peritoneal Macrophages—The mice used in this study were wild-type C57BL6/J (Jackson Laboratories), C57BL6/J in which a floxed macrophage CTP:phosphocholine cytidylyltransferase α gene was silenced by LysMCre (15), and Nctr-*npc*1^N heterozygous/C57BL6/J. The *npc*1^N mutation was detected by PCR analysis of tail DNA using PCR primers mp25–8f (GGTGCTGGACAGCCAAGTA) and mp25-INTR3 (GATGGTCTGTTCTCCCATG) as described by Loftus *et al.* (16). Macrophages were harvested from the peritoneum of the mice 3 days after the intraperitoneal injection of 40 μg of concanavalin A in 0.5 ml of phosphate-buffered saline and then cultured as described previously (17). On the day of the experiment, the cells were incubated for 5 h in 1% FBS in Dulbecco's modified Eagle's medium (w/v) containing 100 μg of acetyl-LDL/ml and 10 μg of compound 58035/ml, either in combination or separately as described previously (18).

Preparation of Membranes and Cytosol—The details of this procedure are described elsewhere (19). Briefly, murine peritoneal macrophages were plated to confluency on a 6-well dish. After treatment, the monolayers were washed twice with 1 ml of ice-cold phosphate-buffered saline. Cells were scraped in 200 μl of 10 mM HEPES, 10% glycerol, 0.34 M sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, pH 7.4. Cell suspensions were then sonicated at 4 °C using twenty 0.5-s bursts at setting no. 1 on a Branson 450 sonicator equipped with a tapered microtip. The homogenate was centrifuged at 2000 × *g* for 5 min at 4 °C to remove whole cells, and then the supernatant fraction was centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet resulting from this centrifugation was used as a source of cellular membranes, and the supernatant fraction was used as a source of cytosol. The cytosol was used immediately for the $cPLA_2$ activity assay. For immunoblotting, the cytosol was stored at −20 °C and thawed once.

Lipoproteins—LDL (*d*, 1.020–1.063 g/ml) from fresh human plasma were isolated by preparative ultracentrifugation (20), and acetyl-LDL was prepared by reaction with acetic anhydride as described previously (21). Acetyl-LDL was reconstituted with defined neutral lipids as described by Krieger (27). Briefly, 1.9 mg of acetyl-LDL and 25 mg of potato starch were frozen and lyophilized in a 15-ml glass tube. The endogenous core lipids were then extracted three times with 5 ml of heptane, and then 6 mg of the desired neutral lipid core (dissolved in 200 μl of heptane) was added to the tube. For cholesteryl-[^{14}C]oleate, 2500 cpm/nmol neutral lipid was added. This mixture was dried, and the sample was resuspended in 1 ml of 10 mM Tricine, pH 8.4. This suspension was incubated for 10–24 h. The solubilized reconstituted lipoprotein was separated from the bulk of the starch and excess lipid by low-speed centrifugation (2000 rpm). The specimen was further clarified by two additional centrifugations at 10,000 rpm for 10 min and stored under argon at 4 °C.

Arachidonic Acid Release and Eicosanoid Production—Confluent mouse peritoneal macrophages were grown on 24-well plates and incubated overnight in Dulbecco's modified Eagle's medium containing 10% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml glutamine, and 0.5 μCi/ml [^{3}H]arachidonic acid. Cells were washed three times with phosphate-buffered saline, incubated with lipoproteins, and then stimulated with media containing 0.2% BSA and 20 μM A23187. After 5 min, the media were withdrawn and centrifuged for 5 min at 14,000 rpm in a microcentrifuge to remove cellular debris, and the radioactivity was quantified by liquid scintillation counting. The cells were dissolved in 1 ml of 0.1 N NaOH at room temperature for 5 h. A 100-μl aliquot of the cell lysate was counted, and the percent efflux was calculated as [(media cpm)/(cell + media cpm)] × 100. Background release from unstimulated cells (2–3%) was subtracted from each experimental point. The amount of PGE_2 and LT-C_4/D_4/E_4 in the media was determined using displacement enzyme-linked immunosorbent assay kits from Amersham Biosciences, Inc.

$cPLA_2$ Immunoblotting—Laemmli electrophoresis sample buffer (5×) was added to 20 μg of cytosol. This mixture was boiled for 10 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. After electrotransfer to a 0.22-μm nitrocellulose membrane, the blot was incubated overnight at 4 °C with rabbit anti-$cPLA_2$ serum (1:2500) (23). The protein bands were detected with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and ECL (Amersham Biosciences). The membrane was then reprobed with an anti-β-actin monoclonal antibody to control for possible differences in loading.

$cPLA_2$ Activity Assay—$cPLA_2$ activity in macrophage cytosol fractions was assayed using a liposomal substrate as described by de Carvalho *et al.* (19). Briefly, 30 μM unlabeled *sn*-2 arachidonyl PC, and 30 μM dioleoylglycerol, and phosphatidylcholine-1-stearoyl-2-[^{3}H]arachidonyl (100,000 cpm/assay) were dried under nitrogen and then resuspended in 150 mM NaCl, 1 mg/ml BSA, with or without 1 mM $CaCl_2$. The assay tubes were incubated at 37 °C in a shaking water bath for 5 min, and the reaction was started by the addition of 20 μg cytosol (50 μl final assay volume). The mixture was incubated for an additional 10 min, and then the reaction was stopped by the addition of 2.5 ml of Dole reagent (isopropyl alcohol/heptane/1 N H_2SO_4; 300:75:15) plus 20 μg of oleic acid carrier. To extract the lipids, 1.5 ml of heptane and 1 ml of water were added, followed by vortexing and centrifugation. The upper phase was passed over a 3-ml silica column (Supelco Cupelclean LC-Si SPE), and the released [^{3}H]arachidonic acid was eluted with 2 ml of chloroform. The chloroform was evaporated, and the radioactivity was counted using liquid scintillation. For the experiments using $cPLA_2\alpha$, unlabeled *sn*-2 linoleoyl phosphatidylcholine was added to create liposomes with varying *sn*-2 arachidonoyl percentages. These liposomes were then incubated with $cPLA_2$ for 1 min, and the release of [^{3}H]AA was assayed as above. The cpm data were converted to mass data based upon the specific activity of [^{3}H]AA in each liposome preparation.

Fatty Acid Analysis—Macrophage lipids were extracted with 0.5 ml of hexane/isopropyl alcohol (3:2, v/v) for two 30-min periods. The phospholipids were isolated by thin-layer chromatography on soft-layer adsorbosil using a solvent system of hexane/ethyl ether/glacial acidic acid (80:20:1, v/v/v). The phospholipid spot was scraped, and methyl ester derivatives of the phospholipid fatty acids were prepared by adding 1.5 ml of 7% acetyl chloride/dry methanol to the silica. After boiling for 60 min, the released fatty acyl methyl esters were extracted with 100 μl of hexane and 5 ml of 6% K_2CO_3. After vortexing and centrifugation, the fatty acyl methyl esters in the upper phase were analyzed by gas-liquid chromatography as described previously (24).

Whole Cell Cholesterol Esterification Assay—Macrophages were incubated for 5 h in Dulbecco's modified Eagle's medium, 1% FBS containing 0.1 mM [^{14}C]oleate complexed with albumin and 100 μg/ml AcLDL. Then, the cells were washed two times with ice-cold PBS, and cellular lipids were extracted twice with 0.5 ml of hexane/isopropyl alcohol (3:2, v/v) for 30 min at room temperature. Whole cell cholesterol esterification activity was assayed by determining the cellular content of cholesteryl-[^{14}C]oleate by thin-layer chromatography (25). The lipid-extracted cellular material was dissolved in 1 ml of 0.1 N NaOH, and aliquots were assayed for protein by the method of Lowry *et al.* (26).

Trafficking of Lipoprotein-derived Fatty Acids to Phospholipids—Mouse peritoneal macrophages were grown to confluency on 24-well plates. The cells were incubated for 5 h in Dulbecco's modified Eagle's medium, 1% FBS containing 100 μg/ml reconstituted acetyl-LDL containing a neutral lipid core of cholesteryl-[^{14}C]oleate. Various pharmacological agents were added as described under "Results." At the end of the 5-h incubation, cellular lipids were extracted in hexane/isopropyl alcohol (3:2) as described above. The phospholipids were isolated by TLC using 80:20:1 (v/v) hexane/diethyl ether/glacial acetic acid. The

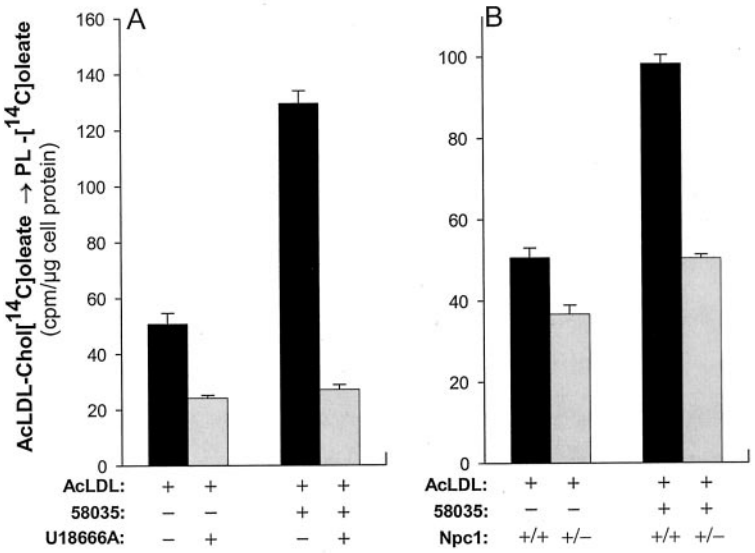

FIG. 1. **Effect of inhibition of ACAT and of late endosomal/lysosomal trafficking on the incorporation of lipoprotein-derived fatty acids into cellular phospholipids.** *A*, macrophages were incubated for 5 h in media containing 100 μg/ml acetyl-LDL reconstituted with cholesteryl-[^{14}C]oleate in the absence or presence of 10 μg/ml 58035 or 1 μM U18666A. *B*, macrophages from *Npc1*+/+ (*black bars*) and *Npc1*+/− (*gray bars*) mice were incubated for 5 h with 100 μg/ml acetyl-LDL reconstituted with cholesteryl-[^{14}C]oleate in the absence or presence of 10 μg/ml 58035. Cellular phospholipids were isolated and assayed for [^{14}C]oleate incorporation.

phospholipid spots were scraped, and [^{14}C]oleate incorporation was determined by liquid scintillation counting.

Statistics—Values are given as means ± S.E. (n = 3); absent error bars in the bar graphs signify S.E. values smaller than the graphic symbols.

RESULTS

Incorporation of Lipoprotein-derived Fatty Acids into Cellular Phospholipids Is Enhanced by ACAT Inhibition and Is Dependent on a U18666A-senstitive, NPC1-dependent Trafficking Pathway—The incorporation of lipoprotein-derived fatty acids into cellular phospholipids can have important biological consequences, yet little is known about the how such events are regulated. Our first goal was to determine how cholesterol-fatty acyl esterification, a potentially competing reaction for fatty acid incorporation into phospholipids, affects this process. To evaluate the incorporation of lipoprotein-derived fatty acids into cellular phospholipids, the neutral lipid core of acetyl-LDL was reconstituted with cholesteryl-[^{14}C]oleate using the procedure of Krieger (27). These lipoproteins were then incubated with mouse peritoneal macrophages for 5 h. Cellular lipids were extracted, and phospholipids were isolated by thin-layer chromatography to determine formation of phospholipid-[^{14}C]oleate. To determine how cholesterol-fatty acyl esterification might affect this process, the ACAT inhibitor 58035 was included in some of the incubations. As shown by the *black bars* in Fig. 1*A*, inhibition of esterification led to a substantial 2.6-fold increase in the incorporation of lipoprotein-derived fatty acids into cellular phospholipids. These data suggest cholesterol-fatty acyl esterification uses a pool of lipoprotein-derived fatty acids that would otherwise be incorporated into cellular phospholipids. Thus, when ACAT is active, lipoprotein-derived fatty acid incorporation into phospholipids is limited by the competing cholesterol esterification reaction.

Previous work from our laboratory has shown that incubation of macrophages with acetyl-LDL plus 58035 induces *de novo* phospholipid biosynthesis via activation of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase α (CTα) (18, 28). Thus, the increased incorporation of fatty acids into phospholipids displayed in Fig. 1*A* might result from an overall increase in *de novo* phospholipid biosynthesis. To test this possibility, CTα-deficient macrophages, which have markedly diminished *de novo* phospholipid biosynthesis in response to acetyl-LDL and 58035 (15), were incubated with cholesteryl-[^{14}C]oleate-labeled acetyl-LDL and 58035 and compared with wild-type macrophages in their incorporation of lipoprotein fatty acids into cellular phospholipids. We found that the CTα-deficient macrophages showed the same increase in phospholipid [^{14}C]oleate incorporation as wild-type macrophages (1.6-fold increase in macrophages lacking CTα *versus* 1.7-fold for macrophages with functional CTα). These data suggest that lipoprotein-derived fatty acids, when not utilized for the competing cholesterol esterification reaction, are incorporated into cellular phospholipids via transesterification rather than by an increase in *de novo* phospholipid biosynthesis.

Recent evidence suggests that lipoprotein-derived cholesterol-fatty acyl esters are hydrolyzed in an endosomal compartment and then transferred to late endosomes/lysosomes, where the liberated cholesterol is trafficked to peripheral cellular sites via an NPC1-dependent pathway (29). To determine if the trafficking of acetyl-LDL-derived [^{14}C]oleate to cellular phospholipids shares properties with the trafficking of lipoprotein-derived cholesterol, we determined the susceptibility of phospholipid-[^{14}C]oleate formation to the cholesterol trafficking inhibitor, U18666A. U18666A is an amphipathic amine that blocks the NPC1-dependent pathway of late endosomal/lysosomal cholesterol transport in cells (30). As shown by the *gray bars* in Fig. 1*A*, U18666A markedly diminished the incorporation of lipoprotein-derived [^{14}C]oleate into phospholipids, an effect particularly apparent in the ACAT-inhibited macrophages. These data imply that lipoprotein-derived fatty acids traffic via an U18666A-inhibitable pathway from late endosomes/lysosomes to cellular phospholipids.

To further support a role for late endosomal/lysosomal trafficking, we employed macrophages with a heterozygous mutation in *Npc1*, which have a partial defect in intracellular cholesterol trafficking (10, 31). These *Npc1*+/− mice, unlike *Npc1*−/− mice, have a normal lifespan and thus are more amenable for the isolation of peritoneal macrophages. Because of the partial defect in cholesterol trafficking, we predicted that these macrophages would have a partial defect in the trafficking of lipoprotein-derived fatty acids. To test this idea, peritoneal macrophages from *Npc1*+/+ and *Npc1*± mice were assayed for their ability to incorporate acetyl-LDL-derived [^{14}C]oleate into phospholipids. As predicted, [^{14}C]oleate incorporation into phospholipids was partially suppressed in *Npc1*+/− macrophages, which was most apparent in ACAT-

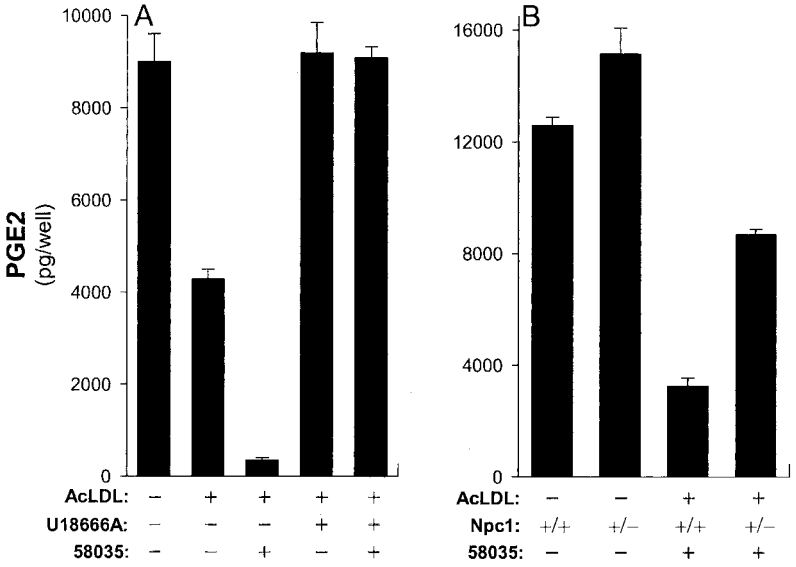

FIG. 2. **Effect of inhibition of ACAT and of late endosomal/lysosomal trafficking on A23187-induced PGE_2 production.** *A*, macrophages were incubated for 5 h in media alone or containing 100 μg/ml acetyl-LDL in the absence or presence of 10 μg/ml 58035 or 1 μM U18666A. *B*, macrophages from *Npc1*+/+ and *Npc1*+/− mice were incubated for 5 h in media alone or media containing 100 μg/ml acetyl-LDL plus 10 μg/ml 58035. The cells were then incubated for 5 min with 20 μM A23187, and the amount of PGE_2 in the media was quantified by ELISA.

inhibited cells (Fig. 1*B*). Thus, the NPC1 pathway is not only involved in late endosomal/lysosomal cholesterol trafficking (32) but also in the trafficking of lipoprotein-derived fatty acids.

Suppression of A23187-induced Eicosanoid Secretion Is Enhanced by ACAT Inhibition and is Dependent on a U18666A-senstitive, NPC1-dependent Trafficking Pathway—We hypothesized that the effects of cholesterol-fatty acyl esterification and late endosomal/lysosomal trafficking on lipoprotein-derived fatty acid incorporation into cellular phospholipid would have important biological effects on cells that internalize lipoproteins. To test this idea, we first used a model in which incubation of macrophages with foam cell-inducing lipoproteins like acetyl-LDL causes a defect in agonist-induced AA release and PGE_2 production (11, 12, 33). Despite the commonality of this observation, there has been confusion about mechanism. Field and coworkers (33) concluded that cholesterol-loading of macrophages leads to a defect in $cPLA_2$ activation. Pollaud *et al.* (12) hypothesized that stimulation of ACAT was necessary for the suppression in PGE_2 synthesis by sequestering AA. Arai *et al.* (11) demonstrated an inverse correlation with percent AA composition of cellular phospholipids, which is consistent with a mechanism in which enrichment of cellular phospholipids with lipoprotein-derived non-AA fatty acids suppresses PGE_2 synthesis.

To address these uncertainties in the context of our new findings on lipoprotein-fatty acid trafficking and metabolism, we first assayed PGE_2 secretion in macrophages incubated with acetyl-LDL in the absence or presence of 58035 and U18666A. In particular, we hypothesized that if lipoprotein-derived fatty acid incorporation into cellular phospholipids was important in suppressing PGE_2 production, then ACAT inhibition should enhance the suppression and U18666A should prevent the suppression. Thus, macrophages were incubated for 5 h with 100 μg/ml acetyl-LDL alone or in addition to 10 μg/ml 58035, 1 μM U18666A, or both compounds. PGE_2 secretion was then stimulated for 5 min with 20 μM A23187, a calcium ionophore that stimulates $cPLA_2$-mediated free arachidonic acid release and PGE_2 secretion. As shown in Fig. 2*A*, incubation with acetyl-LDL alone caused a ~50% decrease in A23187-induced PGE_2 release, consistent with the previous reports mentioned above. Remarkably, inclusion of the ACAT inhibitor almost completely abolished PGE_2 secretion, and U18666A fully restored PGE_2 release even in the presence of the ACAT inhibitor. Inclusion of the ACAT inhibitor alone did not diminish PGE_2 synthesis (data not shown). The effect of the

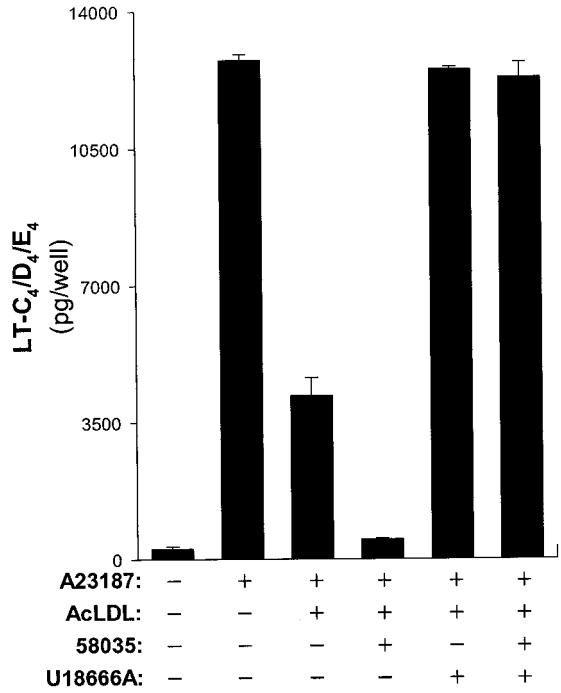

FIG. 3. **Effect of inhibition of ACAT and of late endosomal/lysosomal trafficking on A23187-induced LT-C_4/D_4/E_4 production.** Macrophages were incubated for 5 h in media alone or containing 100 μg/ml acetyl-LDL in the absence or presence of 10 μg/ml 58035 or 1 μM U18666A. The cells were then incubated for 5 min with 20 μM A23187 or buffer control, and the amount of LT-C_4/D_4/E_4 in the media was quantified by ELISA.

Npc1+/− mutation is shown in Fig. 2*B*. Consistent with the data in Fig. 1*B*, the suppression of PGE_2 production in macrophages incubated with acetyl-LDL plus the ACAT inhibitor is partially yet substantially prevented in *Npc1*+/− macrophages. These data support the hypothesis the suppression of PGE_2 production in macrophages by atherogenic lipoproteins involves late endosomal/lysosomal trafficking of lipoprotein-derived fatty acids to cellular phospholipids and incorporation into these phospholipids by a reaction that is competed by cholesterol-fatty acyl esterification.

To broaden the scope of these findings, we explored the effects of acetyl-LDL loading and inhibition of cholesterol-fatty acyl esterification and lipid trafficking on the production of

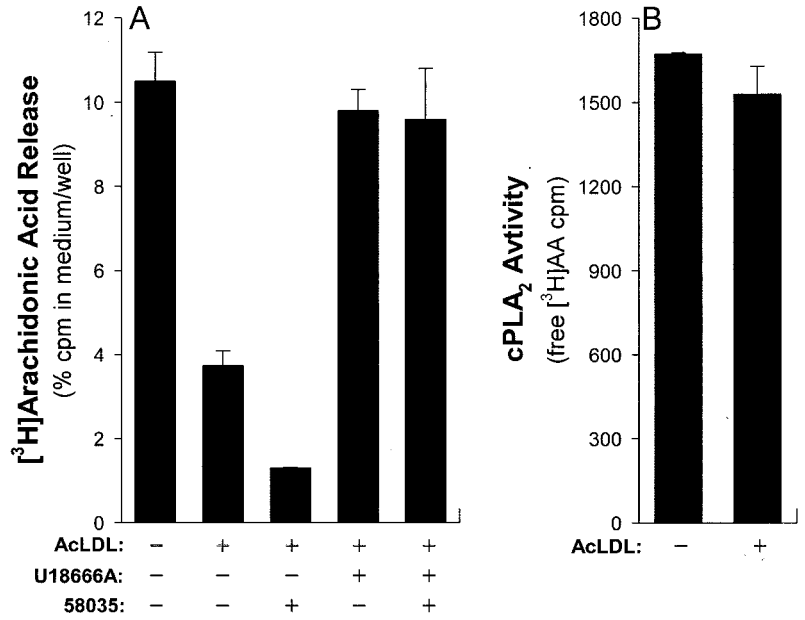

FIG. 4. **Effect of inhibition of ACAT and of late endosomal/lysosomal trafficking on A23187-induced AA release from cellular lipids.** *A*, macrophages prelabeled for 24 h with [^{3}H]AA were incubated for 5 h in media alone or containing 100 μg/ml acetyl-LDL in the absence or presence of 10 μg/ml 58035 or 1 μM U18666A. The cells were then incubated for 5 min in media containing 0.2% BSA and 20 μM A23187, and the cells and media were assayed for free [^{3}H]AA. The data are expressed as percent [^{3}H]AA cpm in the media. *B*, cytosol (20 μg of protein) harvested from macrophages incubated for 5 h in media alone or containing 100 μg/ml acetyl-LDL was assayed for $cPLA_2$ activity.

another macrophage eicosanoid, leukotriene $C_4/D_4/E_4$ (LT-C_4/D_4/E_4). As shown in Fig. 3, macrophages produced large quantities of LT-$C_4/D_4/E_4$ in response to A23187, and this was partially suppressed by incubation with acetyl-LDL. Most importantly, this suppression was markedly enhanced when cholesterol-fatty acyl esterification was inhibited by 58035 and completely prevented when late endosomal/lysosomal lipid trafficking was inhibited by U18666A. Thus, the model proposed above is applicable to the synthesis of both of the major classes of macrophage eicosanoids.

The Effects of Cholesterol-Fatty Acyl Esterification and Late Endosomal Lipid Trafficking on Lipoprotein-mediated Eicosanoid Suppression Occur at the Level of AA Release from Phospholipids but Does Not Involve Direct Inhibition of $cPLA_2$ Activity—The commonality of the results with both prostaglandins and leukotrienes suggested that cholesterol-fatty acyl esterification and late endosomal/lysosomal lipid trafficking were affecting eicosanoid production proximal to the late stage biosynthetic reactions of any individual class of eicosanoids. In this context, we considered a model in which these processes were occurring at the level of $cPLA_2$-mediated AA release from cellular phospholipids. To directly test this point, we assayed AA release by measuring A23187-induced efflux of cellular AA to BSA-containing medium. Macrophage AA pools were labeled by overnight incubation with media containing 0.5 μCi of [^{3}H]arachidonic acid, and the cells were then either left untreated or incubated for 5 h in media containing 100 μg/ml acetyl-LDL in the absence or presence of 58035 or U18666A. cPLA2-mediated hydrolysis of AA from phospholipids was stimulated by a 5-min incubation in media containing 20 μM A23187 and 0.2% BSA, and the [^{3}H]arachidonic acid content of the media and cells was determined. The results are shown in Fig. 4*A*. Similar to the case with PGE_2 release, acetyl-LDL alone caused a decrease in AA release of ~50%. Inclusion of the ACAT inhibitor further inhibited AA release to ~80%, while inclusion of U18666A completely abolished the suppression of AA release. Thus, the effects of blocking cholesterol esterification and late endosomal/lysosomal lipid trafficking on eicosanoid production are paralleled by effects on AA release, further indicating that the effects are proximal to the eicosanoid synthesizing enzymes.

The defect in phospholipid-AA release could be due to alterations in the phospholipids themselves or to direct suppression of $cPLA_2$ protein or activity. We therefore assayed $cPLA_2$ protein and activity in cytosolic fractions from untreated and lipoprotein-treated macrophages. We found that that $cPLA_2$ protein levels by immunoblot analysis (data not shown) and, most importantly, $cPLA_2$ activity in untreated and acetyl-LDL-loaded macrophages were very similar (Fig. 4*B*). Furthermore, calcium-dependent binding of $cPLA_2$ to cellular membranes, an important step in phospholipase activity in stimulated cells, was also not altered by acetyl-LDL loading (data not shown). These data support a model in which cholesterol-fatty acyl esterification and late endosomal/lysosomal lipid trafficking affect lipoprotein-mediated eicosanoid suppression by altering the phospholipid substrates for $cPLA_2$.

Lipoprotein-mediated PGE_2 Suppression in ACAT-inhibited Macrophages Depends on Lipoprotein-derived Non-arachidonic Acid Fatty Acids, Not Cholesterol—A plausible explanation for how cholesterol-fatty acyl esterification affects lipoprotein-mediated eicosanoid suppression is by alterations in enrichment of cellular $cPLA_2$-substrate phospholipids with NAAFAs. An alternative explanation, particularly in the context of the late endosomal/lysosomal trafficking data, is that excess cellular free cholesterol inhibits $cPLA_2$-mediated AA release. Indeed, FC loading of macrophages has profound effects on the function of the ER (10), which is a major site of $cPLA_2$-mediated hydrolysis of AA-containing phospholipids (34).

To identify the component of acetyl-LDL responsible for the suppression of PGE_2 production in ACAT-inhibited macrophages, we reconstituted acetyl-LDL with one of four neutral lipid cores: cholesteryl linoleate (rAcLDL-Chol-18:2), cholesteryl arachidonate (rAcLDL-Chol-20:4), trilinolein (rAcLDL-Tri-18:2) or triarachidonate (rAcLDL-Tri-20:4). Macrophages were incubated for 5 h with 100 μg/ml of the various rAcLDLs in the presence of 10 μg/ml 58035, and the cells were then stimulated for 5 min with 20 μM A23187 and assayed for PGE_2 production. As shown in the first three *bars* in Fig. 5, incubation with rAcLDL-Chol-18:2 led to a ~50% decrease in PGE_2 production, while rAcLDL-Chol-20:4 caused no suppression. This difference was observed despite the similar ability of both particles to deliver cholesterol to cells as determined by a [^{14}C]oleate whole-cell cholesterol esterification assay: cholesteryl–[^{14}C]oleate formation (cpm/μg cell protein ± S.E.) was 106.2 ± 12.3 for rAcLDL-Chol-18:2 and 131.0 ± 4.2 for rAcLDL-Chol-20:4. Moreover, rAcLDL-tri-18:2, a particle es-

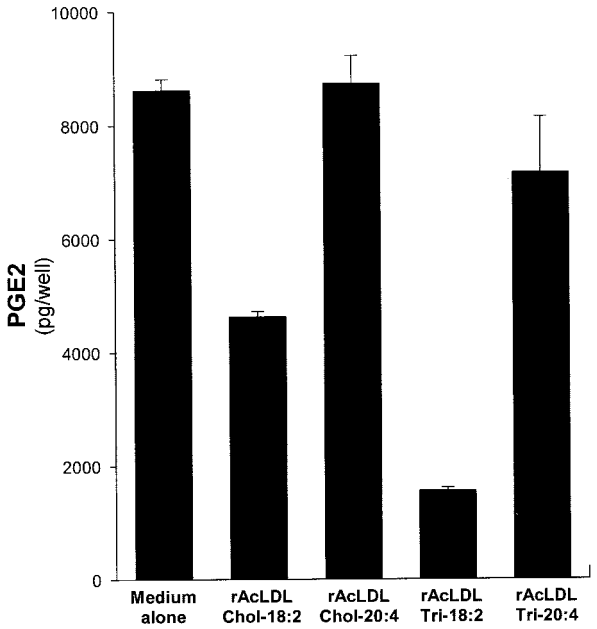

FIG. 5. **Effect of varying lipoprotein neutral lipid core composition on PGE_2 secretion in lipoprotein-loaded, ACAT-inhibited macrophages.** Macrophages were incubated for 5 h in media alone or media containing 100 μg/ml of reconstituted acetyl-LDL plus 10 μg/ml 58035. The acetyl-LDL with reconstituted with cholesteryl linoleate (rAcLDL-Chol-18:2), cholesteryl arachidonate (rAcLDL-Chol-20:4), trilinolein (rAcLDL-tri-18:2), and triarachidonate (rAcLDL-tri-20:4). The cells were then incubated for 5 min with 20 μM A23187, and the amount of PGE_2 in the media was quantified by ELISA.

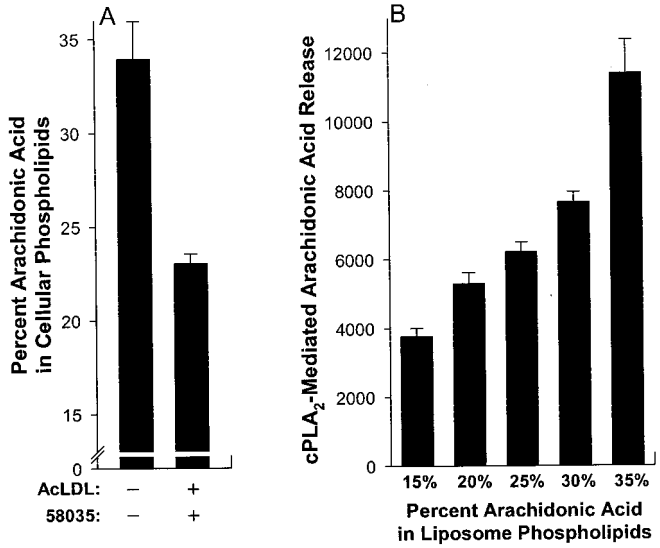

FIG. 6. **Effect of varying phospholipid AA:NAAFA ratio on $cPLA_2$-mediated AA hydrolysis.** *A*, macrophages were incubated for 5 h in media alone or media containing 100 μg/ml of acetyl-LDL plus 10 μg/ml 58035. Cellular phospholipids were isolated and assayed for the mass of individual fatty acid species. The data are expressed as percent AA in cellular phospholipids. *B*, purified recombinant $cPLA_2$ (100 ng) was added to phosphatidylcholine liposomes with varying percentages of *sn*-2 arachidonoyl *versus* *sn*-2 linoleoyl, and the release of AA was determined.

sentially free of cholesterol, was able to completely abolish PGE_2 secretion, while rAcLDL-tri-20:4 had relatively little effect (last two *bars* in Fig. 5).[2] These data show that the suppression of PGE_2 synthesis in lipoprotein-loaded, ACAT-inhibited macrophages is independent of cholesterol but requires lipoprotein-derived NAAFAs.

To test the principle that enrichment of phospholipids with NAAFAs leads to less $cPLA_2$-mediated release of AA, we first measured the change in phospholipid composition in control macrophages and macrophages incubated with acetyl-LDL plus 58035 and then determined whether this degree of change could effect $cPLA_2$-mediated AA release in an *in vitro* system. As shown in Fig. 6*A*, the percent AA in cellular phospholipids was decreased from 34 to 23% by incubation of macrophages with acetyl-LDL plus the ACAT inhibitor. Using an *in vitro* assay, a similar change in percent AA in liposomal phospholipids led to an ~2-fold decrease in $cPLA_2$-mediated AA release (Fig. 6*B*). Although the actual change in percent AA in the subpopulation of cellular phospholipids that is the substrate pool of $cPLA_2$ would be difficult to determine, these data demonstrate that enrichment of phospholipids with NAAFAs can lead to decreased $cPLA_2$-mediated AA release.

DISCUSSION

The data in this report support the following model of lipoprotein-derived fatty acid trafficking and metabolism in the context of prostanoid biosynthesis (Fig. 7): in non-loaded macrophages (Fig. 7*A*), endogenous phospholipids containing AA in the *sn*-2 position supply ample substrate for $cPLA_2$-induced eicosanoid synthesis. With lipoprotein loading and full ACAT activity (Fig. 7*B*), most of the lipoprotein-derived fatty acids and cholesterol that are delivered to the ER are esterified by ACAT, but a portion of the fatty acids are incorporated into phospholipids. In the case of human LDL, which is the source of acetyl-LDL used in this study, most of these fatty acids are NAAFAs, and so the AA:NAAFA ratio in these phospholipids is decreased. This leads to a decreased release of AA upon activation of $cPLA_2$ and thus a lower production of eicosanoids. When ACAT activity is compromised (Fig. 7*C*), more of the lipoprotein-derived NAAFAs are available for incorporation into phospholipids, thus exacerbating the suppression of eicosanoid production. If lipoprotein-derived NAAFAs are prevented from trafficking to the site of phospholipid biosynthesis (Fig. 7*D*), cellular phospholipids remain relatively enriched in AA, and agonist-induced eicosanoid production remains high.

There are three important implications of these findings. First, this study provides new information about the trafficking and metabolism of lipoprotein-derived fatty acids. In particular, we show that the fatty acids destined for incorporation into a $cPLA_2$ substrate pool of phospholipids use a late endosomal/lysosomal trafficking pathway that appears to be the same as that used by lipoprotein-derived cholesterol. The role of NPC1 in lipoprotein-derived fatty acid transport is of particular interest. NPC1 is a glycoprotein that resides in the membranes of late endosomes/lysosomes (35). Initial interest in NPC1 stemmed from the observation that cells lacking this protein accumulate large amounts of cholesterol within their endosomal-lysosomal system (32, 36). For this reason, and because the protein contains a putative sterol-sensing domain (35), the cholesterol transport function of NPC1 has been widely studied. However, mammalian cells with the *Npc1*−/− mutation accumulate a variety of lipids, notably gangliosides (37–40), and our findings provide evidence that NPC1 can play a role in the incorporation of lipoprotein-derived fatty acids into cellular

[2] The enhanced inhibition of PGE_2 production seen with rAcLDL-Tri-18:2 in comparison with rAcLDL-Chol-18:2 was not associated with an increased incorporation of rAcLDL-Tri-18:2-derived fatty acids into cellular phospholipids (data not shown). Therefore, it is possible that fatty acids derived from lipoprotein-triglycerides might incorporate preferentially into a subset of phospholipids critically important in eicosanoid synthesis. Indeed, others have observed that the mode of delivery of fatty acids to cells can influence fatty acid trafficking and metabolism (59).

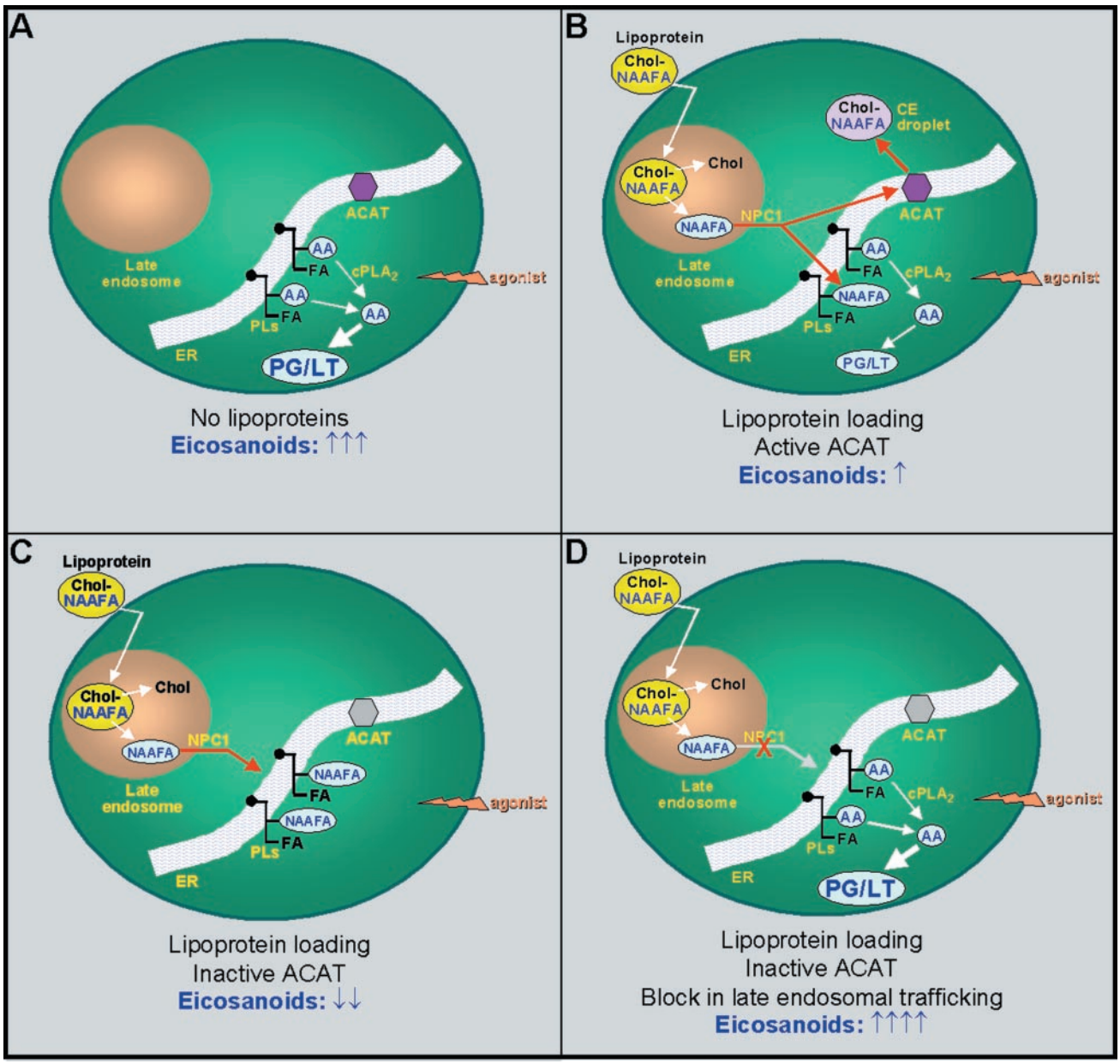

FIG. 7. **Model of how lipoproteins, cholesterol esterification, and late endosomal/lysosomal trafficking affect eicosanoid synthesis in macrophages.** *A*, when $cPLA_2$ activity is stimulated in the absence of prior lipoprotein loading, cellular phospholipids (*PLs*) containing AA in the *sn*-2 position contribute to robust prostaglandin and leukotriene (*PG/LT*) synthesis. *B*, with lipoprotein loading, some of the cellular phospholipids are remodeled with lipoprotein-derived NAAFAs in the *sn*-2 position, thus limiting the amount of eicosanoids synthesized in response to agonists. This remodeling is limited because a substantial portion of the lipoprotein-derived NAAFAs are esterified to cholesterol by ACAT. *C*, when ACAT is inhibited, a greater portion of lipoprotein-derived NAAFAs are incorporated into phospholipids, because the NAAFAs are not utilized in the cholesterol esterification reaction. Thus, eicosanoid synthesis is maximally suppressed. *D*, when late endosomal/lysosomal lipid trafficking is inhibited, lipoprotein-derived NAAFAs cannot traffic to the site of phospholipid synthesis, presumably the ER. Thus, phospholipids are not remodeled with NAAFAs, and eicosanoid synthesis is not suppressed, even in the face of ACAT inhibition.

phospholipids, at least in the case of lipoprotein-loaded macrophages.

Moreover, we show that the ACAT-mediated cholesterol-fatty acyl esterification reaction competes for the pool of lipoprotein-derived fatty acids that otherwise would be incorporated into cellular phospholipids. In an analogous study, we showed that the ACAT reaction also competes for an important pool of cholesterol, namely, that used in sterol-mediated down-regulation of the LDL receptor and hydroxymethylglutaryl-coenzyme A reductase (41). Of interest, both of the reactions competed for by ACAT-fatty acid incorporation into phospholipids and cholesterol-mediated regulation occur in the ER (42, 43). Thus, the focality of these reactions may be important in their utilization of common substrate pools.

Second, the study helps resolve a controversy in the literature related to the mechanism by which PGE_2 synthesis is suppressed during macrophage foam cell formation. The idea that there is direct inhibition of $cPLA_2$ activity, as proposed by Mathur *et al.* (33), is clearly not supported by the data in this report. Another study proposed that acetyl-LDL caused suppression of PGE_2 synthesis by stimulating ACAT activity and thus sequestering cellular AA into cholesteryl esters (12). The ACAT inhibitor data presented here indicate that cholesterol esterification is not necessary for this effect. Finally, other investigators showed an inverse correlation between PGE_2 production and lipoprotein-derived NAAFA content of macrophage phospholipids (11), but definitive proof of a direct causal relationship was not provided. In this report, the results of exper-

iments using reconstituted acetyl-LDL (Fig. 5), liposomes of various NAAFA:AA ratios (Fig. 6), and manipulations of lipoprotein-derived fatty acid trafficking and metabolism (Figs. 1–4) provide direct evidence for the hypothesis that incorporation of lipoprotein-derived NAAFAs into cellular phospholipids directly causes the suppression of $cPLA_2$-mediated AA release, leading to decreased production of both PGE_2 and LT-C_4/D_4/E_4. Of interest, previous work has shown that incubation of other cell types with lipoproteins that are enriched in AA can actually enhance eicosanoid biosynthesis (44, 45). These data are consistent with the overall model outlined in this paper (Fig. 7) and are consistent with our finding that AA-rich lipoproteins failed to suppress eicosanoid production (Fig. 5). However, unlike the studies cited above, AA-rich lipoproteins in A23187-stimulated macrophages did not enhance PGE_2 production above the medium-alone control (Fig. 5).

Regarding the mechanism of suppression of $cPLA_2$-mediated AA release, there may be two related processes working in concert. In the simplest scenario, the *sn*-2 arachidonoyl substrate pool for $cPLA_2$ would be rate-limiting when the enzyme encounters phospholipids that are relatively enriched in NAAFAs *versus* AA. An additional possible mechanism is based on previous work showing the translocation of $cPLA_2$ from the cytosol to ER/Golgi membranes, which is the essential first step in $cPLA_2$ action, is insensitive to the identity of the acyl chain in the *sn*-2 position of membranes phospholipids (46). Fatty acid release by $cPLA_2$, however, is strongly dependent on the identity of the *sn*-2 fatty acid, with a 20–40-fold preference of arachidonyl over stearoyl (47). Thus, it is possible that membranes enriched in NAAFAs trap limiting amounts of $cPLA_2$ on non-substrate phospholipids.

Third, the findings in this report may have direct relevance to eicosanoid signaling in advanced atherosclerotic lesions. In light of the evidence for lipoprotein loading and dysfunctional cholesterol-fatty acyl esterification in advanced lesional macrophages (9), we propose that these macrophages might have highly suppressed eicosanoid production. The net effect of this suppression would depend upon the relative pathobiological roles of macrophage-derived PGE_2, LT-C_4/D_4/E_4, and perhaps other AA-derived bioactive molecules. On the one hand, macrophage-derived leukotrienes may be atherogenic (48), although little is known about the specific effects of these molecules on atherosclerotic processes (49). On the other hand, the effects of PGE_2 on atherosclerotic processes have been widely studied, and the findings suggest an overall protective role. For example, PGE_2 has been shown to inhibit T-cell proliferation (50); selectively suppress the expression of several pro-atherogenic chemokines in activated macrophage via interaction with the EP4 receptor (51); enhance platelet aggregation at low concentrations through the EP3 receptor but inhibit platelet aggregation at high concentrations through activation of the prostacyclin receptor (52); and inhibit IL-1-induced SMC proliferation (53). Moreover, another AA-derived product in macrophages, 15-hydroxyeicosatetraenoic acid (15-HETE), is a ligand for PPAR-γ, and thus would also be expected confer an overall protective effect on atherosclerosis (54, 55). Therefore, if suppression of PGE_2 and possibly 15-HETE are dominant effects in advanced lesional macrophages, lesion development and lesional complications might be exacerbated. Of interest in this regard, macrophages with defective cholesterol esterification also accumulate FC, which induces cholesterol efflux defects, ER dysfunction, apoptosis, and lesional necrosis (9, 10, 31, 56, 57). What is remarkable about these detrimental consequences of macrophage ACAT dysfunction is that all are ameliorated by interrupting the NPC1-dependent late endosomal/lysosomal trafficking pathway (data here and in Refs. 9, 10, 31, 58). Indeed, late lesional complications are significantly prevented in *Npc1*+/− mice (9), suggesting that partial interruption of the NPC1-dependent late endosomal/lysosomal trafficking pathway may be the basis of a novel plaque-stabilizing therapeutic strategy.

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REFERENCES

1. Ross, R. (1995) *Annu. Rev. Physiol.* **57,** 791–804
2. Libby, P. and Clinton, S. K. (1993) *Curr. Opin. Lipidol.* **4,** 355–363
3. Tabas, I. (2002) *J. Clin. Invest.* **110,** 905–911
4. Brown, M. S., and Goldstein, J. L. (1983) *Annu. Rev. Biochem.* **52,** 223–261
5. Tabas, I. (2000) *Biochim. Biophys. Acta* **1529,** 164–174
6. Liscum, L., and Munn, N. J. (1999) *Biochim. Biophys. Acta* **1438,** 19–37
7. Groener, J. E., Bax, W., and Poorthuis, B. J. (1996) *J. Lipid Res.* **37,** 2271–2279
8. Li, Q., Fuhrman, B., and Aviram, M. (1995) *Isr. J. Med. Sci.* **31,** 474–478
9. Feng, B., Zhang, D., Kuriakose, G., Devlin, C. M., Kockx, M., and Tabas, I. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10423–10428
10. Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) *Nat. Cell Biol.* **5,** 781–792
11. Arai, H., Nagano, Y., Narumiya, S., and Kita, T. (1992) *J. Biochem.* (*Tokyo*) **112,** 482–487
12. Pollaud, C., Krause, S., Lepert, J. C., Orfila, C., Seguelas, M., Festal, D., Decerprit, J., and Pipy, B. (1995) *Biochim. Biophys. Acta* **1259,** 211–219
13. Deleted in proof
14. Ross, A. C., Go, K. J., Heider, J. G., and Rothblat, G. H. (1984) *J. Biol. Chem.* **259,** 815–819
15. Zhang, D., Tang, W., Yao, P. M., Yang, C., Xie, B., Jackowski, S., and Tabas, I. (2000) *J. Biol. Chem.* **275,** 35368–35376
16. Loftus, S. K., Morris, J. A., Carstea, E. D., Gu, J. Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) *Science* **277,** 232–235
17. Tang, W., Walsh, A., and Tabas, I. (1999) *Biochim. Biophys. Acta* **1437,** 301–316
18. Shiratori, Y., Okwu, A. K., and Tabas, I. (1994) *J. Biol. Chem.* **269,** 11337–11348
19. de Carvalho, M. G., Garritano, J., and Leslie, C. C. (1995) *J. Biol. Chem.* **270,** 20439–20446
20. Havel, R. J., Eder, H., and Bragdon, J. (1955) *J. Clin. Invest.* **34,** 1345–1353
21. Basu, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3178–3182
22. Deleted in proof
23. Leslie, C. C. (1991) *J. Biol. Chem.* **266,** 11366–11371
24. Kuwae, T., Schmid, P. C., Johnson, S. B., and Schmid, H. H. (1990) *J. Biol. Chem.* **265,** 5002–5007
25. Tabas, I., Boykow, G. C., and Tall, A. R. (1987) *J. Clin. Invest.* **79,** 418–426
26. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
27. Krieger, M. (1986) *Methods Enzymol.* **128,** 608–613
28. Shiratori, Y., Houweling, M., Zha, X., and Tabas, I. (1995) *J. Biol. Chem.* **270,** 29894–29903
29. Sugii, S., Reid, P. C., Ohgami, N., Du, H., and Chang, T. Y. (2003) *J. Biol. Chem.* **278,** 27180–27189
30. Liscum, L. (1990) *Biochim. Biophys. Acta* **1045,** 40–48
31. Feng, B., and Tabas, I. (2002) *J. Biol. Chem.* **277,** 43271–43280
32. Liscum, L., and Klansek, J. J. (1998) *Curr. Opin. Lipidol.* **9,** 131–135
33. Mathur, S. N., Albright, E., and Field, F. J. (1989) *J. Lipid Res.* **30,** 1385–1395
34. Gijon, M. A., and Leslie, C. C. (1999) *J. Leukoc. Biol.* **65,** 330–336
35. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymeropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., Tagle, D. A., *et al.* (1997) *Science* **277,** 228–231
36. Pentchev, P. G., Vanier, M. T., Suzuki, K., and Patterson, M. C. (1995) in *Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2625–2639, McGraw-Hill, New York
37. Sugimoto, Y., Ninomiya, H., Ohsaki, Y., Higaki, K., Davies, J. P., Ioannou, Y. A., and Ohno, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12391–12396
38. Vanier, M. T. (1999) *Neurochem. Res.* **24,** 481–489
39. Harzer, K., and Kustermann-Kuhn, B. (2001) *Clin. Chim. Acta* **305,** 65–73
40. Harzer, K., Massenkeil, G., and Frohlich, E. (2003) *FEBS Lett.* **537,** 177–181
41. Tabas, I., Weiland, D. A., and Tall, A. R. (1986) *J. Biol. Chem.* **261,** 3147–3155
42. Starodub, O., Jolly, C. A., Atshaves, B. P., Roths, J. B., Murphy, E. J., Kier, A. B., and Schroeder, F. (2000) *Am. J. Physiol. Cell. Physiol.* **279,** C1259-C1269
43. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *Cold Spring Harb. Symp. Quant. Biol.* **67,** 491–498
44. Pomerantz, K. B., Tall, A. R., Feinmark, S. J., and Cannon, P. J. (1984) *Circ. Res.* **54,** 554–565
45. Salbach, P. B., Specht, E., von Hodenberg, E., Kossmann, J., Janssen-Timmen, U., Schneider, W. J., Hugger, P., King, W. C., Glomset, J. A., and Habenicht, A. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2439–2443
46. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) *Cell* **65,** 1043–1051
47. Diez, E., Louis-Flamberg, P., Hall, R. H., and Mayer, R. J. (1992) *J. Biol. Chem.* **267,** 18342–18348

48. Mehrabian, M., Allayee, H., Wong, J., Shi, W., Wang, X. P., Shaposhnik, Z., Funk, C. D., Lusis, A. J., and Shih, W. (2002) *Circ. Res.* **91,** 120–126
49. Tabas, I. (2002) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J., eds) pp. 573–597, Elsevier, Amsterdam
50. Goodwin, J. S., Bankhurst, A. D., and Messner, R. P. (1977) *J. Exp. Med.* **146,** 1719–1734
51. Takayama, K., Garcia-Cardena, G., Sukhova, G. K., Comander, J., Gimbrone, M. A., Jr., and Libby, P. (2002) *J. Biol. Chem.* **277,** 44147–44154
52. Fabre, J. E., Nguyen, M., Athirakul, K., Coggins, K., McNeish, J. D., Austin, S., Parise, L. K., FitzGerald, G. A., Coffman, T. M., and Koller, B. H. (2001) *J. Clin. Investig.* **107,** 603–610
53. Libby, P., Warner, S. J., and Friedman, G. B. (1988) *J. Clin. Investig.* **81,** 487–498
54. Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999) *Nature* **400,** 378–382
55. Duval, C., Chinetti, G., Trottein, F., Fruchart, J. C., and Staels, B. (2002) *Trends Mol. Med.* **8,** 422–430
56. Warner, G. J., Stoudt, G., Bamberger, M., Johnson, W. J., and Rothblat, G. H. (1995) *J. Biol. Chem.* **270,** 5772–5778
57. Yao, P. M., and Tabas, I. (2000) *J. Biol. Chem.* **275,** 23807–23813
58. Kellner-Weibel, G., Jerome, W. G., Small, D. M., Warner, G. J., Stoltenborg, J. K., Kearney, M. A., Corjay, M. H., Phillips, M. C., and Rothblat, G. H. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 423–431
59. Teruya, J., Cluette-Brown, J., Szczepiorkowski, Z. M., and Laposata, M. (1995) *J. Lipid Res.* **36,** 266–276