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 amphoteric 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 E2 (PGE2) and leukotriene C4/D4/E4 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, PGE2 suppression depended entirely on acetyl-LDL-derived NAAFAs, not on acetyl-LDL-cholesterol, and was not due to decreased cPLA2 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 NPC11 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
this report, we explore the effects of altered lipid trafficking and cholesterol-fatty acid esterification on lipoprotein-fatty acid incorporation into cellular phospholipids, cPLA₂-induced fatty acid release, and eicosanoid synthesis in macrophages. We provide evidence for a model in which lipoprotein-derived non-arachidonic acid fatty acids (NAFAAs) traffic via the NPC1 pathway from late endosomes/lysosomes to a critical pool of phospholipids. In competing reactions, these NAFAAs 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-Dicydimeethylamino-14-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 stock of compound 58035 was prepared in 7% acetyl chloride/dry methanol to the silica. 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 + medin cpm]) / 100. Back- ground release from unstimulated cells (2-3% of total activity) was subtracted from each experimental point. The amount of PGE₂, and LT-C/D₂/E₂ in the media was determined using displacement enzyme-linked immunosorbent assay kits from Amersham Biosciences, Inc.

**cPLA₂ Immunoblotting**—Laemmli electrophoresis sample buffer (6X) 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 electrophoresis to a 0.2μm nitrocellulose membrane, the blot was incubated overnight at 4°C with rabbit anti-cPLA₂ 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-beta-actin monoclonal antibody to confirm equal protein loading.

**cPLA₂ Activity Assay**—cPLA₂ activity in macrophase cytosol fractions was assayed using a liposomal substrate as described by de Carvalho et al. (19). Briefly, 30 μl unlabeled sn-2 arachidonyl PC, and 30 μl dioleoylglycerol, and phosphatidylcholine-1-stearoyl-2-[3H]arachidonyl were dried under nitrogen and then re-suspended in 150 μl of NaCl, 1 mg/ml BSA, with or without 1 μM CaCl₂. 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 of 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 (isopropl alcohol/heptane/1 N H₂SO₄; 300:75:15) plus 20 μl of water. The mixture was vortexed and centrifuged. The upper phase was passed over a 3-ml silica column (Supelco Cupelclean LC-Si SPE), and the released [3H]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 cPLA₂, unlabeled sn-2 linoleoyl phosphatidylcholine was added to create liposomes with varying sn-2 arachidonoyl percentages. These liposomes were then incubated with cPLA₂ for 1 min, and the release of [3H]AA was measured as above. The cpm data were converted to mass data based upon the specific activity of [3H]AA in each liposome preparation.

**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). Acetylated-LDL was reconstituted with defined neutral lipids as described by Krieger (27). Briefly, 1.9 mg of acetyl-LDL and 25 mg of phosphatidylcholine were dissolved in a 15-ml glass tube using chloroform (2:1). The endogenous core lipids were then extracted three times with 5 ml of chloroform, and then 6 mg of the desired neutral lipids (dissolved in 200 μl of heptane) was added to the tube. For cholesteryl-[14C]oleate, 2500 cpm/nmol neutral lipid was added. This mixture was dried, and the sample was resuspended in 1 ml of 10 μl Tricine, pH 8.4. This suspension was used as the final liposomal material for use in 10–20 μl of liposomes. The liposome samples were then dispersed in 30–100 μl with 100 mg/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/isopropl 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-[14C]oleate by thin-layer 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 cholesteryl-[14C]oleate 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/isopropl 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-[14C]oleate by thin-layer chromatography (25). The liposomes were dissolved in 5 ml of heptane/isopropl alcohol (3:2, v/v) for 15 min at room temperature, and aliquots were assayed for protein by the method of Lowry et al. (26).
phospholipid spots were scraped, and \[^{14}C\]oleate incorporation was determined by liquid scintillation counting.

**RESULTS**

**Incorporation of Lipoprotein-derived Fatty Acids into Cellular Phospholipids Is Enhanced by ACAT Inhibition and Is Dependent on a U18666A-sensitive, 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. 1A, 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 \(a\) (CT\(a\)) (18, 28). Thus, the increased incorporation of fatty acids into phospholipids observed in Fig. 1A might result from an overall increase in de novo phospholipid biosynthesis. To test this possibility, CT\(a\)-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 CT\(a\)-deficient macrophages showed the same increase in phospholipid-[\(^{14}C\)]oleate incorporation as wild-type macrophages (1.6-fold increase in macrophages lacking CT\(a\) versus 1.7-fold for macrophages with functional CT\(a\)). These data suggest that lipoprotein-derived fatty acids, when not utilized for the competing cholesterol esterification reaction, are incorporated into cellular phospholipids via transacylation 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. 1A, 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-
inhibited cells (Fig. 1B). 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-sensitive, 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 PGE2 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 cPLA2 activation. Pollaud et al. (12) hypothesized that stimulation of ACAT was necessary for the suppression in PGE2 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 PGE2 synthesis.

To address these uncertainties in the context of our new findings on lipoprotein-fatty acid trafficking and metabolism, we first assayed PGE2 secretion in macrophages incubated with acetyl-LDL 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 PGE2 in the media was quantified by ELISA.

Fig. 2. Effect of inhibition of ACAT and of late endosomal/lysosomal trafficking on A23187-induced PGE2 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 g/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 PGE2 in the media was quantified by ELISA.

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 eicosanoids. We first assayed LT-C4/D4/E4 production in macrophages incubated with acetyl-LDL alone or in addition to 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-C4/D4/E4 in the media was quantified by ELISA.

Npc1+/− mutation is shown in Fig. 2B. Consistent with the data in Fig. 1B, the suppression of PGE2 production in macrophages incubated with acetyl-LDL plus the ACAT inhibitor is partially yet substantially prevented in Npc1+/− macrophages. These data support the hypothesis that suppression of PGE2 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.

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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 [3H]EAA 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 [3H]AA. The data are expressed as percent [3H]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 cPLA2 activity.

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 cPLA2 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 cPLA2-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 [3H]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 [3H]arachidonic acid content of the cells and media was determined. The results are shown in Fig. 4A. Similar to the case with PGE2 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 cPLA2 protein or activity. We therefore assayed cPLA2 protein and activity in cytosolic fractions from untreated and lipoprotein-treated macrophages. We found that that cPLA2 protein levels by immunoblot analysis (data not shown) and, most importantly, cPLA2 activity in untreated and acetyl-LDL-loaded macrophages were very similar (Fig. 4B). Furthermore, calcium-dependent binding of cPLA2 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 cPLA2.

Lipoprotein-mediated PGE2 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 cPLA2-substrate phospholipids with NAAFAAs. An alternative explanation, particularly in the context of the late endosomal/lysosomal trafficking data, is that excess cellular free cholesterol inhibits cPLA2-mediated AA release. Indeed, FC loading of macrophages has profound effects on the function of the ER (10), which is a major site of cPLA2-mediated hydrolysis of AA-containing phospholipids (34).

To identify the component of acetyl-LDL responsible for the suppression of PGE2 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 (tAcLDL-Tri-18:2) or triarachidonate (tAcLDL-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 PGE2 production. As shown in the first three bars in Fig. 5, incubation with rAcLDL-Chol-18:2 led to a ~50% decrease in PGE2 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 [14C]oleate whole-cell cholesterol esterification assay; cholesteryl-[14C]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-
mentally free of cholesterol, was able to completely abolish PGE₂ secretion, while rAcLDL-tri-20:4 had relatively little effect (last two bars in Fig. 5). These data show that the suppression of PGE₂ 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 cPLA₂-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 cPLA₂-mediated AA release in an in vitro system. As shown in Fig. 6A, 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 cPLA₂-mediated AA release (Fig. 6B). Although the actual change in percent AA in the subpopulation of cellular phospholipids that is the substrate pool of cPLA₂ would be difficult to determine, these data demonstrate that enrichment of phospholipids with NAAFAs can lead to decreased cPLA₂-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 prostaglandin biosynthesis (Fig. 7): in non-loaded macrophages (Fig. 7A), endogenous phospholipids containing AA in the sn-2 position supply ample substrate for cPLA₂-induced eicosanoid synthesis. With lipoprotein loading and full ACAT activity (Fig. 7B), 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 cPLA₂ and thus a lower production of eicosanoids. When ACAT activity is compromised (Fig. 7C), 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. 7D), 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 cPLA₂ 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...
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 NAFA/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 NAFAAs into cellular phospholipids directly causes the suppression of cPLA₂-mediated AA release, leading to decreased production of both PGE₂ and LT-C₄/D₄/E₄. Of interest, previous work has shown that incubation of other cell types with lipoproteins that are enriched in AA 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 PGE₂ production above the medium-alone control (Fig. 5).

Regarding the mechanism of suppression of cPLA₂-mediated AA release, there may be two related processes working in concert. In the simplest scenario, the sn-2 arachidonoyl substrate pool for cPLA₂ would be rate-limiting when the enzyme encounters phospholipids that are relatively enriched in NAFAAs versus AA. An additional possible mechanism is based on previous work showing the translocation of cPLA₂ from the cytosol to ER/Golgi membranes, which is the essential first step in cPLA₂ action, is insensitive to the identity of the acyl chain in the sn-2 position of membranes phospholipids (46). Fatty acid release by cPLA₂ however, is strongly dependent on the identity of the sn-2 fatty acid, with a 20–40-fold preference of arachidonoyl over stearoyl (47). Thus, it is possible that membrane enriched in NAFAAs trap limiting amounts of cPLA₂ 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 lipidprotein 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 macrophase-derived PGE₂, LT-C₄/D₄/E₄, and perhaps other AA-derived bioactive molecules. On the one hand, macrophase-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 PGE₂ on atherosclerotic processes have been widely studied, and the findings suggest an overall protective role. For example, PGE₂ has been shown to inhibit T-cell proliferation (49), example, PGE₂ has been shown to inhibit T-cell proliferation (49), 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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