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ABCA1-mediated Cholesterol Efflux Is Defective in Free Cholesterol-loaded Macrophages

MECHANISM INVOLVES ENHANCED ABCA1 DEGRADATION IN A PROCESS REQUIRING FULL NPC1 ACTIVITY*

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In advanced atherosclerosis, macrophage foam cells progressively accumulate large amounts of unesterified or "free" cholesterol (FC), a process that is thought to contribute to foam cell death and lesional necrosis. The cellular consequences of early FC accumulation, including those that lead to further FC accumulation, are poorly understood. In this context, we show that cholesterol and phospholipid efflux mediated by ABCA1, which is initially induced in the cholesterol-loaded macrophage, was inhibited by ~80% in pre-toxic FC-loaded macrophages. Cholesterol efflux to HDL_2, which is mediated by a non-ABCA1 pathway, was inhibited by only ~20% in FC-loaded macrophages. FC loading led to decreased levels of ABCA1 protein via increased degradation of ABCA1, and not by decreased transcription or translation of *AbcA1* mRNA. The decrease in ABCA1 protein occurred relatively early and was not prevented by caspase inhibitors, indicating that it was not a consequence of FC-induced apoptosis. However, inhibition of proteasomal function by lactacystin largely prevented the degradation of ABCA1. Importantly, the FC-induced decrease in ABCA1 function and protein was almost entirely prevented in macrophages that had partial deficiency of *npc1* or were exposed to nanomolar concentrations of U18666A, both of which lead to defective cholesterol trafficking to the endoplasmic reticulum, but leave trafficking to the plasma membrane largely intact. Thus, a relatively early event during FC loading of macrophages is increased degradation of ABCA1, which appears to require trafficking of cholesterol to a peripheral cellular site, but not bulk trafficking of excess cholesterol to the plasma membrane. These findings provide new insight into the post-translational regulation of ABCA1 and the pathobiology of the FC-loaded macrophage.

The hallmark of the early atherosclerotic lesion is the cholesteryl ester (CE)[1]-laden macrophage, or foam cell (1, 2). As lesions progress, however, there is a progressive increase in the unesterified or "free" cholesterol (FC) content of macrophages (3–6). Presumably, progressive lipid loading of macrophages leads to deficiencies in the cholesterol esterifying enzyme acyl-CoA:cholesterol acyltransferase (ACAT), cholesterol transport to ACAT, and/or cellular efflux of FC. A recent study suggested that increased activity of neutral CE hydrolase may also contribute to these events (7). Because the accumulation of large amounts of FC eventually leads to macrophage death (8, 9) and because macrophage death results in lesional necrosis (10, 11), the FC-loaded macrophage may be a critical turning point in atherosclerosis. In particular, lesional necrosis is a precipitating factor in plaque erosion and rupture, which in turn lead directly to acute thrombosis and acute vascular occlusion (12–15). Thus, FC-induced macrophage death may be one of the events leading to this fatal turn of events.

Recent *in vivo* studies have begun to provide support for components of this model. For example, our laboratory has shown that prevention of FC-induced death in a mouse model of atherosclerosis diminishes lesional necrosis (16), and another recent report has shown that accelerated macrophage FC accumulation *in vivo* results in atherosclerotic lesions with increased macrophage death (17). Importantly, this latter model also demonstrated increased lesion size *per se*, which raises the possibility that the FC-loaded macrophage has other attributes that affect lesion progression as well as lesional necrosis.

In this context, there has been increasing interest in understanding the biology of the FC-loaded macrophage. Cell culture studies have revealed a series of specific molecular events that occur as macrophages become progressively loaded with FC. Early adaptive processes include induction of phospholipid biosynthesis, which also occurs in lesional macrophages *in vivo*, and desaturation of the fatty acyl moieties of membrane phospholipids (18, 19). These events are adaptive in that they buffer the cell against the harmful effects of a high FC/phospholipid ratio in cellular membranes (9, 20). With continued FC loading, however, these adaptive mechanisms fail, and the cells develop the molecular and morphological properties of apoptosis and necrosis (8, 9). In particular, Fas ligand is activated; Bax levels increase; mitochondrial membrane potential drops; mitochondrial cytochrome *c* is released into the cytoplasm; and proximal and distal caspases are activated (21, 22). Cell death ensues, with the majority of macrophages showing the morphological signs of apoptosis initially, followed by typical necrotic properties after very long period of FC loading (8, 9). In atheroscle-

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[1] The abbreviations used are: CE, cholesteryl ester; FC, free cholesterol; ACAT, acyl-CoA:cholesterol acyltransferase; ALLN, *N*-acetyl-leucine-leucyl-norleucinal aldehyde; Z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; BAF, *t*-butoxycarbonyl-aspartyl(methoxy)-fluoromethyl ketone; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; RIPA, radioimmune precipitation assay; NPC, Niemann-Pick C.

rotic lesions, macrophages showing signs of apoptosis and necrosis are also observed (23).

The above-mentioned studies and observations suggest that interventions designed to diminish FC accumulation in lesional macrophages would be beneficial. Consistent with this idea, previous work has shown that promoting cellular cholesterol efflux during FC loading potently protects macrophages against FC-induced toxicity (8, 9). In this context, ABCA1 (ATP-binding cassette protein A1) may have a particularly important role. ABCA1 mediates cholesterol efflux from these cells in the presence of apolipoprotein acceptors like apoA-I (24–27). ABCA1 is regulated both at the transcriptional level, via liver X receptor (LXR)/retinoid X receptor-mediated induction of the *AbcA1* gene (24–27), and at the post-translational level, via changes in turnover of ABCA1 protein (28, 29). Humans with deficient ABCA1 are at increased risk for coronary heart disease (26, 30), and atherosclerosis-susceptible mice with selective absence of macrophage ABCA1 have markedly increased lesion size (31, 32). Thus, ABCA1-mediated cholesterol efflux is likely an important anti-atherosclerotic process that tends to prevent excess cholesterol accumulation in lesional macrophages.

Yet, as mentioned above, macrophages in advanced atherosclerotic lesions accumulate large amounts of FC, suggesting that one or more cholesterol efflux pathways become progressively dysfunctional. To explore this idea, the goal of this study was to assess cholesterol efflux in cultured macrophages at various stages of cholesterol loading. Although the *AbcA1* gene is transcriptionally induced when macrophages accumulate mostly CE and a limited amount of FC (33), we show here that an early consequence of FC accumulation is dysfunction of the ABCA1 cholesterol efflux pathway via induction of ABCA1 degradation. Interestingly, the accelerated turnover of ABCA1 protein induced by FC loading is almost completed abrogated by genetic and pharmacological manipulations that primarily block cholesterol trafficking to the endoplasmic reticulum. These findings provide new insight into the post-translational regulation of ABCA1 and suggest new ideas related to the pathobiology of the FC-loaded macrophage and the recently observed effects of macrophage FC accumulation on atherosclerotic lesion development *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media were from Invitrogen, and fetal bovine serum was from Hyclone Laboratories (Logan, UT). Tritium-labeled cholesterol and choline were from PerkinElmer Life Sciences. Concanavalin A, *N*-acetyl-leucine-leucyl-norleucinal aldehyde (ALLN), methyl-β-cyclodextrin, and imipramine were from Sigma. Compound 58035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]-propanamide (34), an inhibitor of ACAT, 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%. Glyburide, sodium orthovanadate, lactacystin, cycloheximide, and benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-fmk) were from Calbiochem. *t*-Butoxycarbonyl-aspartyl(methoxy)-fluoromethyl ketone (BAF) was obtained from Enzyme Systems Products (Livermore, CA). U18666A (3-β-[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) was from BIOMOL Research Labs Inc. ApoA-I was from BIODESIGN International (Saco, ME), and rabbit anti-ABCA1 serum was from Novus (Littleton, CO). Anti-β-actin antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG, and goat anti-mouse IgG were from Bio-Rad. Anti-β_1-integrin antiserum was a generous gift from Dr. Eugene Marcantonio (Department of Pathology, Columbia University). Low-density lipoprotein (LDL; *d* 1.020–1.063 g/ml) and HDL_2 (*d* 1.063–1.125 g/ml) from fresh human plasma were isolated by preparative ultracentrifugation as described (35). Acetyl-LDL was prepared by reaction with acetic anhydride (36) and labeled with [^{3}H]CE as described (37).

Harvesting and Culturing Mouse Peritoneal Macrophages—The mice used in this study were wild-type C57BL6/J and BALB/c, apoE knockout/C57BL6/J, apolipoprotein E knockout/C57BL6/J Nctr-*npc1*^{N} heterozygous, and BALB/cNctr-*npc1*^{N} heterozygous. The *npc1*^{N} mutation was detected by PCR analysis of tail DNA using PCR primers mp25-8F (GGTGCTGGACAGCCAAGTA) and mp25-INTR3 (GATGGTCTGT-TCTCCCATG) as described by Loftus *et al.* (38). The apoE knockout/C57 NPC1 heterozygous mice were produced by crossing BALB/cNctr-*npc1*^{N} mice (stock number 003092, Jackson Laboratory, Bar Harbor, ME) onto the apoE knockout/C57BL6/J background for five generations. Mice (6–10 weeks old) were injected with 0.5 ml of phosphate-buffered saline (PBS) containing 40 μg of concanavalin A intraperitoneally, and the macrophages were harvested 3 days later by peritoneal lavage. The harvested cells were plated in cell culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 20% L-cell-conditioned medium (39). The medium was replaced every 24 h until the macrophages were confluent, at which point they were incubated with 50–100 μg/ml acetyl-LDL in DMEM containing 0.2% bovine serum albumin (BSA) with or without 10 μg/ml compound 58035 and/or other inhibitors.

[^{3}H]Cholesterol Efflux Assay—First, acetyl-LDL (800 μg) was incubated with 10 μCi of [^{3}H]cholesterol for 30 min at 37 °C, and then 8 ml of DMEM and 0.2% BSA were added. The macrophages were incubated in this medium for 5 h, washed three times with PBS, and then incubated in DMEM and 0.2% BSA for 15 min at 37 °C. After washing with PBS, the macrophages were incubated in DMEM and 0.2% BSA containing 15 μg/ml apoA-I or 20 μg/ml HDL_2. At the indicated time points, 100 μl of the medium were removed and spun for 5 min at 14,000 rpm in a microcentrifuge to remove cellular debris, and the radioactivity in this fraction of the medium was quantified by liquid scintillation counting. After the last time point, the remainder of the medium was removed, and the cells were dissolved in 0.5 ml of 0.1 N NaOH containing 0.5% SDS (5 h at room temperature). A 100-μl aliquot of the cell lysate was counted, and the percent efflux was calculated as follows: ((media cpm)/(cell + media cpm)) × 100. Total protein in cell lysate was determined using the Bio-Rad DC protein assay kit. Note that there was no statistical difference in cellular cpm or protein between CE- and FC-loaded macrophages.

^{3}H-Labeled Phospholipid Efflux Assay—Macrophages were labeled with [^{3}H]choline (5 μCi/ml) in DMEM and 10% fetal bovine serum for 24 h. After washing three times with PBS, the macrophages were incubated with 100 μg/ml acetyl-LDL ± compound 58035 in DMEM and 0.2% BSA for 5 h. The cells were then incubated with 15 μg/ml apoA-I in DMEM and 0.2% BSA for the indicated time periods. [^{3}H]Choline-containing phospholipids in aliquots of the medium were extracted in chloroform/methanol (2:1, v/v), and those remaining in the cells in hexane/isopropyl alcohol (3:2, v/v); the radioactivity was measured by scintillation counting.

Whole-cell Cholesterol Esterification Assay—Macrophages were incubated in DMEM and 0.2% BSA containing 0.1 mM [^{14}C]oleate complexed with albumin and 3 μg/ml acetyl-LDL. At the indicated time points, the cells were washed two times with cold PBS, and the cell monolayers 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 (40). The cell monolayers were dissolved in 1 ml of 0.1 N NaOH, and aliquots were assayed for protein by the method of Lowry *et al.* (41).

Biotinylation of Cell-surface Proteins—Macrophage monolayers in 35-mm dishes were washed three times with ice-cold PBS and then incubated in ice-cold PBS containing 0.5 mg/ml sulfosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (Pierce) for 30 min at 4 °C. After washing five times with ice-cold PBS containing 20 mM Tris-HCl (pH 8.0), the cells were scrapped into PBS and pelleted by centrifugation. The pelleted macrophages were lysed in 50 μl of radioimmune precipitation assay (RIPA) buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 20 mM Tris, 150 mM NaCl, and 5 mM EDTA (pH 8)) containing 1 mM phenylmethylsulfonyl fluoride. Ten μl of the lysate were subjected directly to 4–20% gradient SDS-PAGE for determination of total ABCA1. The rest of the cell lysate was affinity-purified to isolate biotinylated proteins. Briefly, the cell lysates were diluted to 150 μl in RIPA buffer and incubated with 50 μl of immobilized streptavidin-agarose (Pierce), which was prewashed three times with RIPA buffer at 0 °C for 2 h with gentle shaking. The agarose was pelleted by centrifugation using a microcentrifuge at 5000 rpm for 2 min; the pellet was resuspended in 1 ml of RIPA buffer; and the process was repeated five times. The agarose was resuspended 30 μl of SDS-PAGE loading buffer containing 330 mM β-mercaptoethanol at 37 °C for 15 min and subjected to SDS-PAGE as described above. ABCA1 and β_1-integrin were detected by Western blotting using anti-ABCA1 and anti-β_1-integrin antisera.

The blots were reprobed with anti-β-actin antibody, which detected no actin signal, thus verifying that no cytosolic protein was biotinylated by the procedure.

Western Blot Analysis—Peritoneal macrophages were lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation at 3000 × *g* for 10 min at 4 °C. Supernatants (15–30 μg of protein) were electrophoresed on 4–20% gradient SDS-polyacrylamide gel and electrotransferred to 0.22-μm nitrocellulose membrane using a Bio-Rad mini-transfer tank. For Western blot detection of ABCA1, anti-ABCA1 antiserum was used. Signals were detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and ECL (Amersham Biosciences). The membranes were reprobed with anti-β-actin monoclonal antibody or anti-β_1-integrin antiserum for the proper internal control. The relative intensities of the bands were determined by densitometry.

Real-time Quantitative Reverse Transcription-PCR—The procedure used by Smith and colleagues (42) to measure *AbcA1* mRNA by quantitative PCR was used, with slight modifications. Monolayers of macrophages in 22-mm dishes were incubated for 5 h with 100 μg/ml acetyl-LDL in the absence or presence of 10 μg/ml compound 58035. After washing with cold PBS, the cells were lysed with 1 ml of TRIzol reagent to isolate total RNA. Five μg of total RNA were reversed-transcribed using Invitrogen Superscript II and poly(T) as the primer, and PCR was conducted using 62.5 ng of cDNA in the Mx4000TM Multiplex quantitative PCR system from Stratagene. The primers for the *AbcA1* gene were 5′-cctcagccatgacctgccttgtag-3′ and 5′-ccgaggaagacgtggacaccttc-3′. To control for input cDNA, a β-actin primer/probe set from PE Biosystems was used. The PCR products were checked by agarose gel electrophoresis to ensure that a single PCR product was obtained. A standard curve was obtained by plotting the cycle threshold *versus* the log of input cDNA, which was obtained from CE-loaded mouse peritoneal macrophages. Both the β-actin and *AbcA1* standard curves were linear between 31.25 and 250 ng of cDNA ($r^2 = 0.99$ for both). The PCRs were set up using SYBR green PCR core reagents from Applied Biosystems. The PCR was initiated at 95 °C for 10 min, followed by 45 cycles consisting of 95 °C for 0.5 min, 56 °C for 1.5 min, and 72 °C for 1.4 min. After obtaining real-time fluorescence measurements, cycle threshold values were determined. Using the standard curves in the linear range (*i.e.* exponential amplification phase), the quantities of *AbcA1* and β-actin mRNAs were calculated. The final data are expressed as the ratio of *AbcA1* to β-actin mRNA.

Statistics—Results are given as means ± S.E. ($n = 3$) unless otherwise noted. Absent error bars in the figures signify S.E. values smaller than the graphic symbols. For the quantitative PCR measurements, triplicate values were obtained, and there was <1% variation among these values.

RESULTS

FC Loading of Macrophages Leads to the Dysfunction of the ABCA1 Cholesterol Efflux Pathway

Whereas modest increases in cellular cholesterol increase expression of ABCA1 and thus increase apoA-I-mediated cholesterol efflux (33), we sought to determine the effect of more abundant FC loading, which occurs in macrophages in advanced atherosclerotic lesions (3–6). Thus, mouse peritoneal macrophages were incubated for 5 h with [^{3}H]cholesterol-labeled acetyl-LDL alone to effect predominantly CE loading or in the presence of the ACAT inhibitor compound 58035 to induce FC loading (21, 22). The cells were then chased for 2.5 h in medium containing apoA-I to measure ABCA1-mediated cholesterol efflux or containing HDL_2 to measure cholesterol efflux by other pathways (43). As shown in Fig. 1*A*, FC loading resulted in a marked reduction in cholesterol efflux to apoA-I. In contrast, cholesterol efflux to HDL_2 was only modestly reduced (Fig. 1*B*). The data in Fig. 1*B* demonstrate that FC loading did not substantially inhibit cholesterol trafficking to the plasma membrane, as might be expected in toxic cells. Indeed, although more prolonged FC loading eventually does cause cytotoxicity and death (21, 22), the macrophages at the end of this experiment appeared to be morphological healthy and excluded the cell-impermeant dye propidium iodide (data not shown). Most importantly, the HDL_2 data indicate that the defect in cholesterol efflux is specific to the ABCA1 pathway.

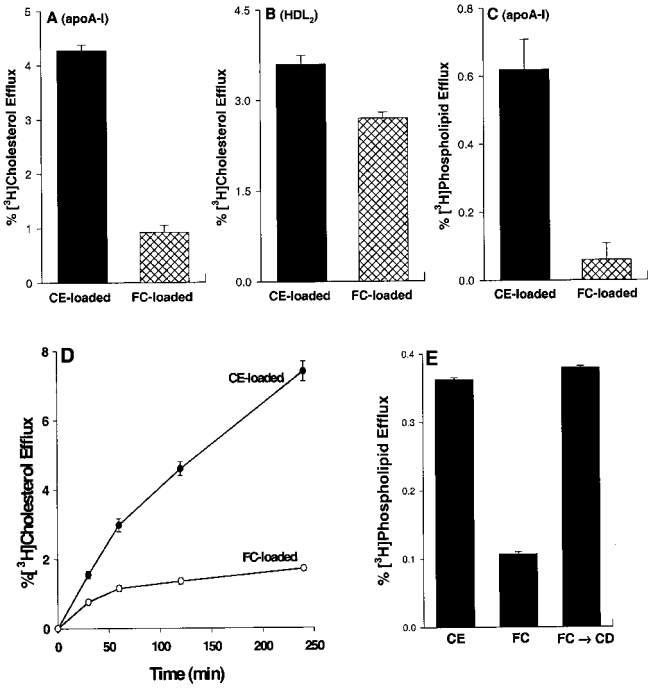

FIG. 1. **FC loading of macrophages results in dysfunction of the apoA-I/ABCA1 cholesterol and phospholipid efflux pathway.** *A* and *B*, peritoneal macrophages from C57 mice were incubated for 5 h with 100 μg/ml [^{3}H]cholesterol-labeled acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE-loaded*) or presence (*FC-loaded*) of 10 μg/ml compound 58035. The macrophages were then incubated for 2.5 h in DMEM and 0.2% BSA with or without compound 58035, respectively, containing 15 μg/ml apoA-I (*A*) or 20 μg/ml HDL_2 (*B*), and efflux of [^{3}H]cholesterol was measured. The data are expressed as the percentage of total cellular [^{3}H]cholesterol. Similar results were obtained when the data were expressed as cpm medium [^{3}H]cholesterol or ^{3}H-labeled phospholipids/mg of cellular proteins. For example, for *A*, these data were 13,124 and 4552 for CE- and FC-loaded cells, respectively. *C*, macrophages were labeled with [^{3}H]choline chloride for 24 h and then incubated exactly as described for *A*. After the 2.5-h incubation with apoA-I, efflux of ^{3}H-labeled phospholipids was measured; the data are expressed as the percentage of total cellular ^{3}H-labeled phospholipids. *D*, the experiment was conducted as described for *A*, except that the time of apoA-I incubation varied as indicated. *E*, the macrophages were labeled with [^{3}H]choline and incubated with acetyl-LDL with or without compound 58035 exactly as described for *C*. Aliquots of the FC-loaded cells were then incubated for 15 min at 37 °C in DMEM and 0.2% BSA in the absence or presence of 0.5 or 2% methyl-β-cyclodextrin (*CD*). This treatment removed ~30% of the total cellular cholesterol. All of the macrophages were then chased in medium containing 15 μg/ml apoA-I for 3.33 h, and efflux of ^{3}H-labeled phospholipids was measured as described under "Experimental Procedures."

Studies by Fielding and co-workers (44) Tall and co-workers (45) have suggested that ABCA1-mediated cholesterol efflux can be divided into two sequential steps. The first step is proposed to be phospholipid efflux to lipid-free apoA-I, and the second step is cholesterol efflux to these apoA-I·phospholipid complexes. According to this model, a defect in phospholipid efflux would indicate reduced ABCA1 transporter activity *per se*. To test this point, phosphatidylcholine efflux to apoA-I was measured for both FC- and CE-loaded macrophages. As shown in Fig. 1*C*, phosphatidylcholine efflux by FC-loaded cells was markedly less than that by CE-loaded cells.

The time course of cholesterol efflux to apoA-I in CE-loaded *versus* FC-loaded macrophages is shown in Fig. 1*D*. Of note, the decreased efflux in FC-loaded macrophages could be observed at the earliest time points after the initial 5-h incubation with [^{3}H]cholesterol-labeled acetyl-LDL plus the ACAT inhibitor. Finally, to prove that it was FC loading that caused the decrease in ABCA1 function, we tested the ability of FC removal to reverse the defect. For this purpose, FC-loaded macrophages

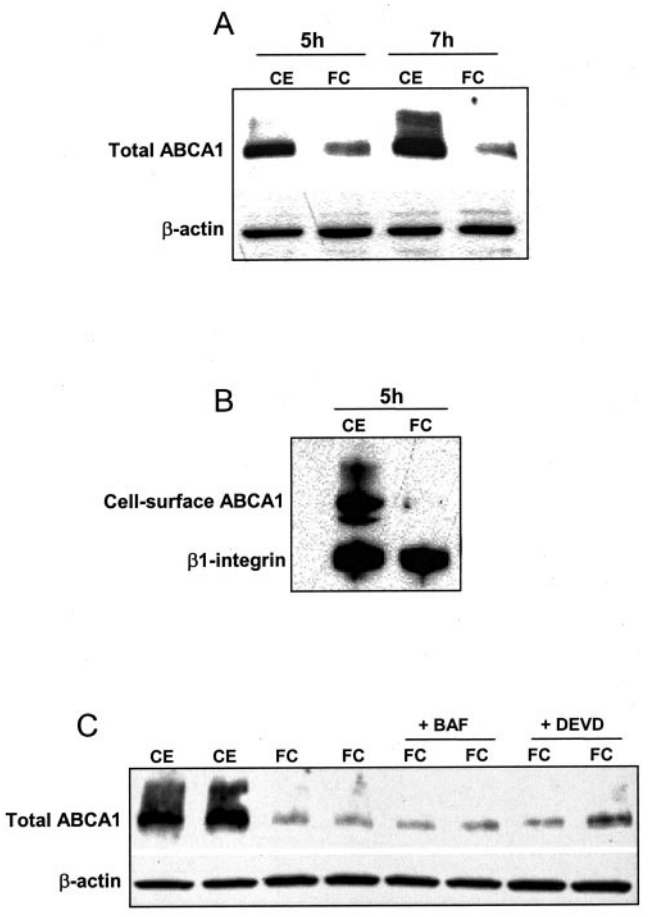

FIG. 2. **ABCA1 protein is decreased in FC-loaded macrophages.** Mouse peritoneal macrophages were incubated for 5 or 7 h with 100 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE*) or presence (*FC*) of compound 58035. Aliquots of total cell protein (*A*) or cell-surface protein (*B*) were then subjected to immunoblot analysis for ABCA1 and the standards β-actin and β_1-integrin. In *C*, a 5-h experiment similar to that in *A* was conducted in duplicate, except that some of the macrophages were incubated with the caspase inhibitor BAF (50 μM) or Z-DEVD-fmk (45 μM) during the period of FC loading.

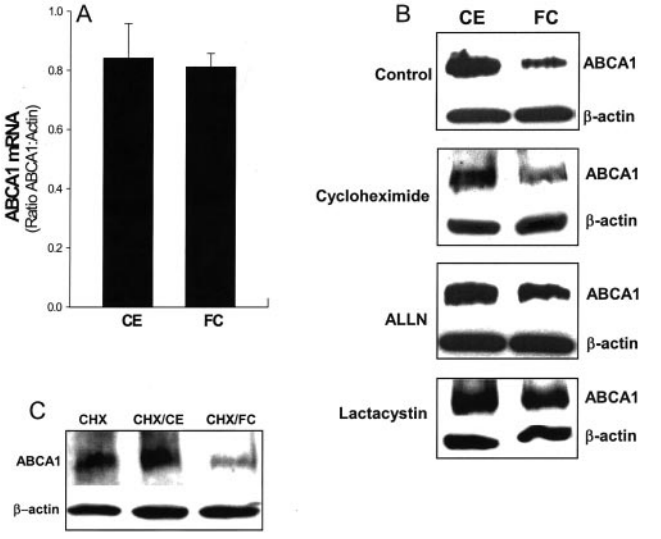

FIG. 3. **FC-loaded macrophages demonstrate enhanced degradation of ABCA1 protein.** *A*, mouse peritoneal macrophages were incubated for 5 h with 100 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE*) or presence (*FC*) of compound 58035. Total RNA was extracted from the cells, and the ratio of *AbcA1* to β-actin mRNA was determined by quantitative PCR. *B*, macrophages were preincubated for 14 h with 50 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE*) or presence (*FC*) of compound 58035. The cells were then incubated for 5 h with 100 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence or presence of compound 58035, respectively, with no further additions (*Control*) or with 10 μg/ml cycloheximide, 50 μM ALLN, or 50 μM lactacystin as indicated. Aliquots of cell lysates were then assayed for ABCA1 and β-actin protein by immunoblot analysis. *C*, macrophages were preincubated in medium containing 0.5 mM 8-bromo-cAMP for 18 h to induce expression of ABCA1. The 8-bromo-cAMP was then removed, and the cells were incubated for an additional 5 h in medium containing 10 μg/ml cycloheximide alone (*CHX*) or in the same cycloheximide medium containing 100 μg/ml acetyl-LDL (*CHX*/*CE*) or 100 μg/ml acetyl-LDL + 10 μg/ml compound 58035 (*CHX*/*FC*). Cell lysates were then probed for ABCA1 and β-actin by immunoblotting.

was treated with 2% methyl-β-cyclodextrin for 15 min at 37 °C immediately following the 5-h incubation with [^{3}H]cholesterol-labeled acetyl-LDL plus the ACAT inhibitor. This treatment removed ~30% of the [^{3}H]cholesterol. As shown in Fig. 1*E*, the methyl-β-cyclodextrin treatment completely reversed the defect in apoA-I-mediated phospholipid efflux in FC-loaded macrophages. Taken together, the data in Fig. 1 demonstrate that FC loading leads to severe impairment of ABCA1 function in macrophages.

FC Loading of Macrophages Leads to a Decrease in ABCA1 Protein, but Not in AbcA1 mRNA

To determine whether the amount of ABCA1 protein was decreased by FC loading, macrophages were incubated with acetyl-LDL in the absence (CE loading) or presence (FC loading) of an ACAT inhibitor for 5 or 7 h and then probed for ABCA1 protein by immunoblotting. As shown in Fig. 2*A*, the total amount of ABCA1 protein was clearly decreased in FC-loaded macrophages at both 5 and 7 h. By densitometry, using β-actin as a protein loading control, there was a 72 ± 5% (n = 11) decrease in ABCA1 in the FC-loaded macrophages at 5 h. At 7 h, CE-loaded macrophages had a 2.4-fold increase in ABCA1 protein compared with the 5-h CE-loaded macrophages, which was expected given that sterol loading increases ABCA1 expression (33). However, the amount of ABCA1 protein in the 7-h FC-loaded macrophages was further diminished, resulting in a >90% decrease in ABCA1 protein at this time point. Using a 4 °C biotinylation protocol, we assayed the amount of ABCA1 on the surface of macrophages that had been incubated with acetyl-LDL ± ACAT inhibitor for 5 h. In the experiment displayed in Fig. 2*B*, cell-surface ABCA1 was barely detectable in FC-loaded macrophages; in repeat experiments, cell-surface ABCA1 was detectable in FC-loaded macrophages, but was substantially diminished compared with that in CE-loaded macrophages.

Although the decrease in ABCA1 protein occurred prior to the onset of cellular toxicity, we tested whether this event could possibly be a consequence of caspase activation by assessing the effects of a broad caspase inhibitor, BAF, and an inhibitor of effector caspases, Z-DEVD-fmk. We have previously shown that DEVD blocks FC-induced apoptosis in macrophages (21). As shown in Fig. 2*C*, neither BAF nor Z-DEVD-fmk blocked the decrease in ABCA1 protein in FC-loaded macrophages. Thus, this cellular event is not downstream of caspase activation.

Quantitative PCR was conducted to determine whether the decrease in ABCA1 protein was associated with a decrease in *AbcA1* mRNA. As shown in Fig. 3*A*, the mRNA levels were virtually identical in CE- and FC-loaded macrophages. To distinguish between decreased translation of *AbcA1* mRNA *versus* increased degradation of ABCA1 protein, we examined ABCA1 protein levels in cycloheximide-treated cells. As shown in Fig. 3*B* (*upper two panels*), ABCA1 protein was decreased to a similar level in the absence or presence of cycloheximide (73% *versus* 74% decrease, respectively), indicating an increase in turnover of ABCA1 protein.

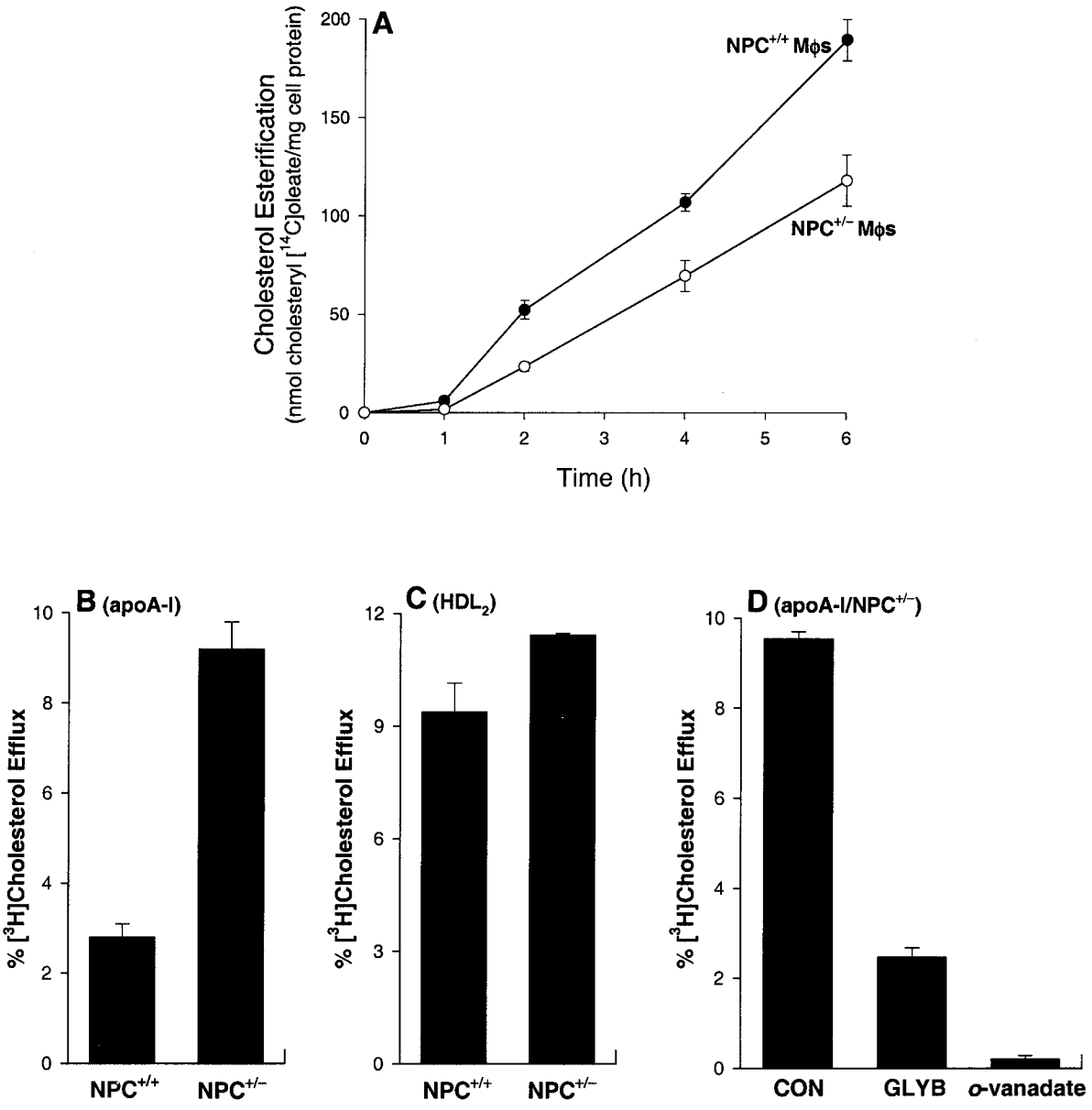

FIG. 4. **Partial *npc1* deficiency restores ABCA1-mediated cholesterol efflux in FC-loaded macrophages.** *A*, macrophages (*Mϕs*) from wild-type and heterozygous NPC mice, all on the apoE knockout/C57 background (see Footnote 2), were incubated in medium containing 100 μg/ml ^{125}I-acetyl-LDL for 1, 2, 4, or 6 h, after which cholesterol esterification was assayed. In this experiment, the uptake and degradation of ^{125}I-acetyl-LDL and *in vitro* ACAT activity in the presence of excess cholesterol were similar in the two cell types. *B–D*, macrophages from wild-type and heterozygous NPC mice, all on the apoE knockout/C57 background, were incubated for 5 h with 100 μg/ml [^{3}H]cholesterol-labeled acetyl-LDL in DMEM and 0.2% BSA in the presence of 10 μg/ml compound 58035. The macrophages were then incubated for 18 h in the same medium containing 15 μg/ml apoA-I (*B* and *D*) or 20 μg/ml HDL_2 (*C*), and efflux of [^{3}H]cholesterol was measured. In *D*, the 18-h apoA-I incubation was done in the presence of 200 μM glyburide (*GLYB*) or 200 μM orthovanadate as indicated. [^{3}H]Cholesterol efflux was assayed as described in the legend to Fig. 1. *CON*, control.

To begin to explore which cellular proteases may be involved in FC-induced ABCA1 degradation, we tested the effects of ALLN, which blocks cysteine proteases and inhibits proteasomal degradation, and lactacystin, which is a specific inhibitor of proteasomal degradation. Both inhibitors partially prevented the decrease in ABCA1 levels in FC-loaded macrophages (Fig. 3*B*, *lower two panels*). Compared with the 72 ± 5% decrease in ABCA1 in FC-loaded *versus* CE-loaded macrophages (see above), ABCA1 was decreased by only 21 ± 9% (n = 4) in lactacystin-treated FC-loaded cells and by only 22 ± 3% (n = 4) in ALLN-treated FC-loaded cells. Calpeptin (40 μM) and PD150606 (25 μM), specific inhibitors of the cysteine protease calpain, did not prevent the decrease in ABCA1 in FC-loaded macrophages (data not shown). In summary, FC loading of macrophages leads to a substantial decrease in ABCA1 protein levels. The mechanism appears to be an increase in ABCA1 degradation, at least partially by the proteasomal pathway.

An alternative explanation for these data is that CE loading stabilizes ABCA1 and that the normal turnover of ABCA1 occurs in FC-loaded macrophages. To directly test this idea, we needed a control in which macrophages were incubated without acetyl-LDL. However, because macrophages incubated without lipoproteins have very little expression of ABCA1 due to lack of transcriptional induction (24–26, 33), the design of the experiment needed to be altered. For this purpose, the macrophages were preincubated with 8-bromo-cAMP to transcriptionally induce ABCA1 in the absence of lipoproteins (46). The cells were then incubated with cycloheximide, to prevent further translation of ABCA1, in the absence of acetyl-LDL, with acetyl-LDL alone (CE), or with acetyl-LDL plus compound 58035 (FC).

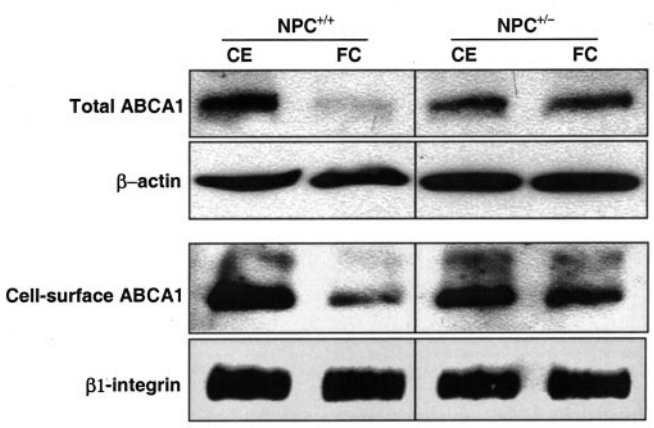

FIG. 5. **Partial *npc1* deficiency restores the level of ABCA1 protein in FC-loaded macrophages.** Macrophages from wild-type and heterozygous NPC mice, all on the apoE knockout/C57 background (see Footnote 2), were incubated for 5 h with 100 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE*) or presence (*FC*) of 10 μg/ml compound 58035. Aliquots of total cell protein (*upper panels*) or cell-surface protein (*lower panels*) were then subjected to immunoblot analysis for ABCA1 and the standards β-actin and β_1-integrin.

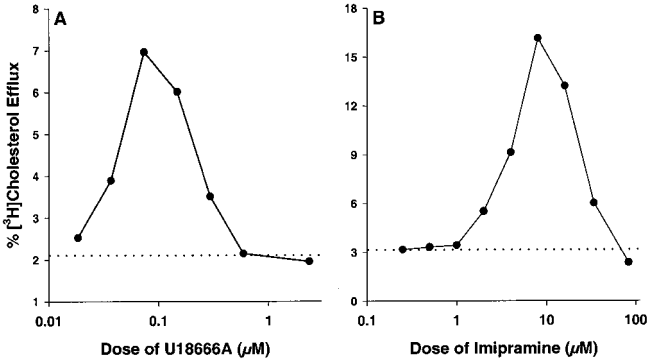

FIG. 6. **Low-dose amphipathic amines restore ABCA1-mediated cholesterol efflux in FC-loaded macrophages.** Peritoneal macrophages from C57 mice were incubated for 5 h with 100 μg/ml [^{3}H]cholesterol-labeled acetyl-LDL in DMEM and 0.2% BSA in the presence of 10 μg/ml compound 58035. The macrophages were then incubated for 6 h in the same medium containing 15 μg/ml apoA-I in the absence or presence of the indicated concentrations of U18666A (*A*) or imipramine (*B*), and efflux of [^{3}H]cholesterol was measured. The *dotted line* in each graph indicates the percentage of [^{3}H]cholesterol efflux in the absence of U18666A or imipramine.

Compared with macrophages incubated without acetyl-LDL, ABCA1 was modestly increased in CE-loaded macrophages, but was markedly decreased in FC-loaded macrophages (Fig. 3*C*). Thus, whereas CE loading may cause a modest increase in the stability of ABCA1, FC loading causes a marked increase in ABCA1 turnover independently of this effect.

Decreases in ABCA1-mediated Cholesterol Efflux and ABCA1 Protein Levels Are Prevented by Subtle Defects in Cholesterol Trafficking

Studies with Heterozygous Niemann-Pick C (NPC) Macrophages—In FC-loaded macrophages, cholesterol accumulates in perinuclear organelles, presumably late endosomes or lysosomes, and also traffics to peripheral sites such as the plasma membrane and endoplasmic reticulum (47). To begin to understand how FC loading leads to defective ABCA1-mediated cholesterol efflux and increased turnover of ABCA1, we investigated the relationship between intracellular cholesterol trafficking and these FC-induced events. The hypothesis was that the induction of ABCA1 degradation in FC-loaded macrophages requires the trafficking of cholesterol from late endosomes/lysosomes to a peripheral cellular site.

In this context, we first conducted a series of experiments with macrophages from heterozygous NPC (NPC$^{+/-}$) mice.[2] NPC1, the protein defective in type I Niemann-Pick C disease, is required for the normal trafficking of cholesterol out of late endosomal and/or lysosomal compartments. We have previously shown that cholesterol efflux via both ABCA1 and non-ABCA1 pathways is severely disrupted in macrophages from *homozygous* NPC mice, presumably because cholesterol transport from late endosomes/lysosomes to the ABCA1 efflux pathway in the plasma membrane is defective (48). However, several initial cholesterol trafficking experiments suggested that macrophages from *heterozygous* NPC mice might be useful to test our hypothesis. In particular, using methyl-β-cyclodextrin to monitor plasma membrane cholesterol (49), we showed that trafficking of acetyl-LDL-derived cholesterol to the plasma membrane was blocked by only ~10–15% in NPC$^{+/-}$ *versus* NPC$^{+/+}$ macrophages (data not shown). However, trafficking to the endoplasmic reticulum, using ACAT-mediated cholesterol esterification as an assay, was decreased by as much as 50% in the NPC$^{+/-}$ macrophages (Fig. 4*A*); note that this difference is not due to defective ACAT enzyme in NPC$^{+/-}$ macrophages as assessed by *in vitro* assays. Thus, these macrophages should maintain sufficient cholesterol trafficking to the ABCA1 efflux pathway, yet might prevent the FC-induced decrease in ABCA1 protein and function if this process requires uninterrupted cholesterol trafficking to other peripheral sites.

Accordingly, FC-loaded NPC$^{+/+}$ and NPC$^{+/-}$ macrophages were incubated for 5 h with [^{3}H]cholesterol-labeled acetyl-LDL plus compound 58035 and then assayed for efflux to apoA-I and HDL_2. Importantly, [^{3}H]cholesterol loading was similar in both cell genotypes. As shown in Fig. 4*B*, cholesterol efflux to apoA-I was markedly increased in NPC$^{+/-}$ macrophages compared with NPC$^{+/+}$ macrophages. As expected from our previous data, efflux to HDL_2 was already relatively high in NPC$^{+/+}$ FC-loaded macrophages, and it was increased only slightly by the heterozygous NPC mutation (Fig. 4*C*). To verify that the increased cholesterol efflux to apoA-I observed in NPC$^{+/-}$ macrophages was mediated by ABCA1, we showed that this efflux was blocked by two inhibitors of the ABCA1 efflux pathway, glyburide and orthovanadate (Fig. 4*D*).

To relate the cholesterol efflux data to ABCA1 protein levels, we assayed total and cell-surface ABCA1 in CE-loaded (*i.e.* control) and FC-loaded NPC$^{+/+}$ and NPC$^{+/-}$ macrophages. Consistent with our previous data, we observed substantial decreases in both total and cell-surface ABCA1 in FC-loaded NPC$^{+/+}$ macrophages (Fig. 5, *left panels*). In NPC$^{+/-}$ macrophages, however, there was much less of a decrease in both total and cell-surface ABCA1. By densitometry, total ABCA1 was decreased by only 30 ± 7% (n = 4) in FC-loaded *versus* CE-loaded NPC$^{+/-}$ macrophages. In conclusion, the data in Figs. 4 and 5 are consistent with a model in which the induction of ABCA1 degradation by FC loading, with the resulting defective cholesterol efflux to apoA-I, requires intact trafficking of this FC to a peripheral cellular site.

Studies with Low-dose Amphipathic Amines—To further test the idea that partial disruption of cholesterol trafficking can protect ABCA1-mediated cholesterol efflux and ABCA1 protein in FC-loaded macrophages, we took advantage of the ability of

[2] For these studies, the laboratory took advantage of macrophages from available mice that had been bred into the apoE knockout/C57BL6 genetic background for a separate *in vivo* study (16). However, similar results were found with macrophages from apoE wild-type mice on the Balb/c background, and the same principles were demonstrated with apoE wild-type mice on the C57 background using low-dose U18666A, as shown below.

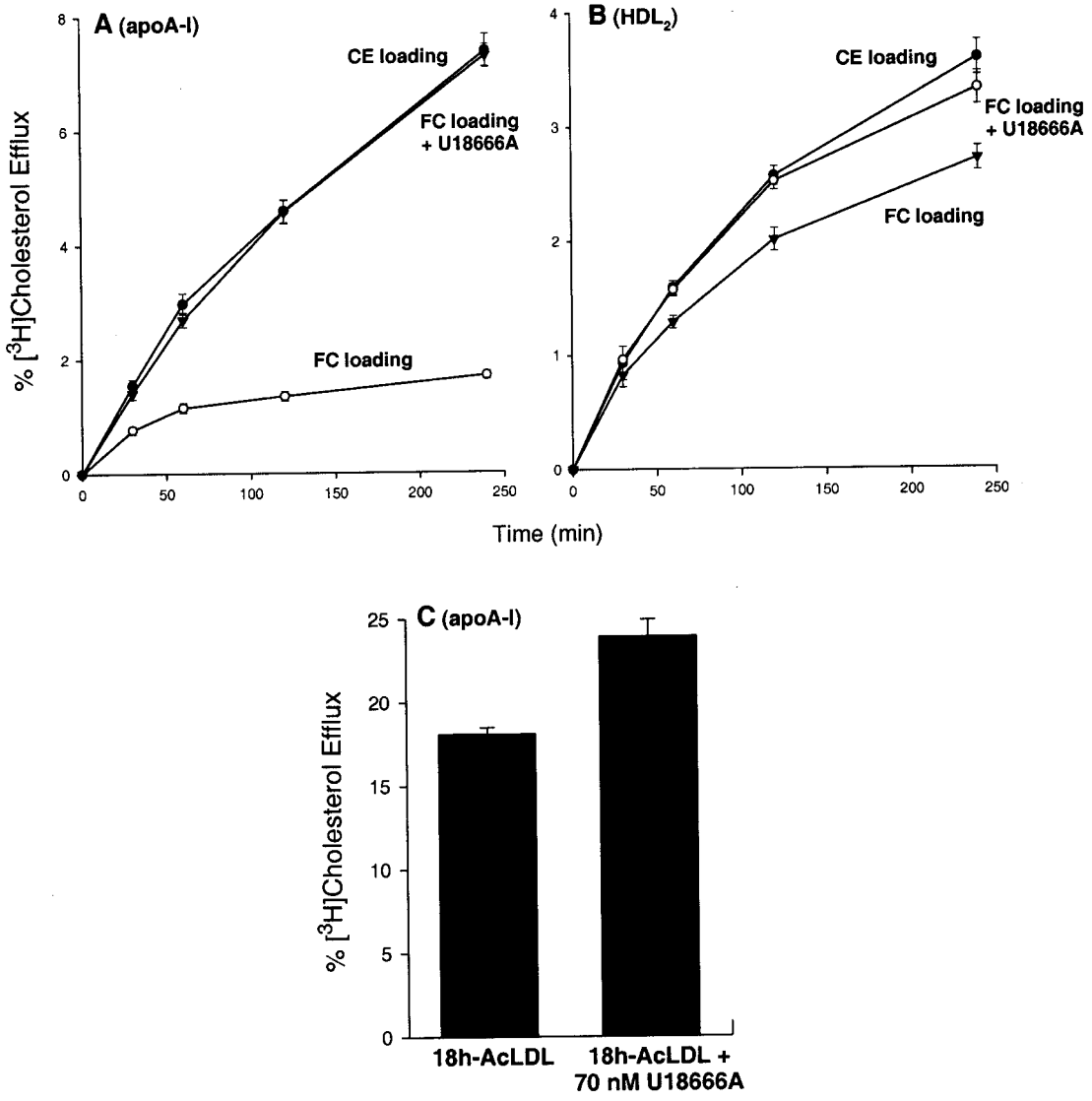

FIG. 7. **70 nM U18666A restores ABCA1-mediated cholesterol efflux in FC-loaded macrophages and enhances efflux in macrophages incubated long-term with acetyl-LDL.** The experiment in *A* was conducted as described for Fig. 6*A*, except that 70 nM U18666A was used, and the apoA-I incubation time was varied as indicated. In *B*, 20 μg/ml HDL_2 was the cholesterol acceptor. In *C*, the macrophages were incubated with 100 μg/ml acetyl-LDL (*AcLDL*) without compound 58035 for 5 h and then incubated for a further 18 h with acetyl-LDL in the absence or presence of 70 nM U18666A. [^{3}H]Cholesterol efflux was assayed as described in the legend to Fig. 1.

certain types of amphipathic amines to mimic the NPC phenotype (49, 50). Examples of such compounds include U18666A and imipramine (49, 50). As expected, a high level of inhibition of cholesterol trafficking by high-dose amines would be expected to block cholesterol efflux due to a marked defect in cholesterol trafficking to the plasma membrane (51). Based on the data from the heterozygous NPC macrophages, however, we predicted that a low concentration of these compounds would increase cholesterol efflux in FC-loaded cells by protecting ABCA1 from increased degradation.

To test this idea, we examined the effect of various concentrations of U18666A and imipramine on ABCA1-mediated cholesterol efflux in FC-loaded macrophages. As shown in Fig. 6*A*, U18666A concentrations in the low-dose range dramatically increased the level of lipoprotein·cholesterol efflux to apoA-I in FC-loaded cells; peak efflux was observed at 70 nM U18666A. At high doses, U18666A gradually inhibited ABCA1-mediated efflux, presumably due to a severe blockage of cholesterol trafficking to the plasma membrane. Similar results were found with imipramine (Fig. 6*B*). Consistent with our overall hypothesis, with the heterozygous NPC data in Fig. 5, and with the

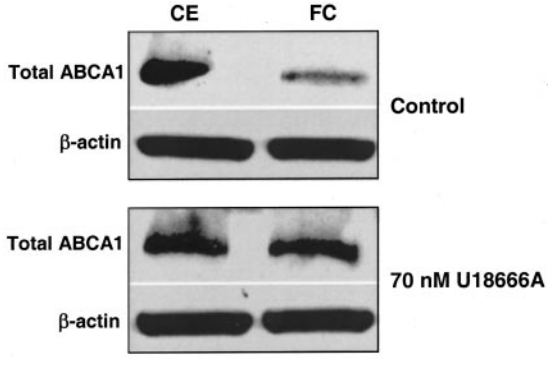

FIG. 8. **70 nM U18666A restores the level of ABCA1 protein in FC-loaded macrophages.** Macrophages were preincubated for 14 h with 50 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence (*CE*) or presence (*FC*) of compound 58035. The cells were then incubated for 5 h with 100 μg/ml acetyl-LDL in DMEM and 0.2% BSA in the absence or presence of compound 58035, respectively, with no further additions (*Control*) or with 70 nM U18666A. Aliquots of total cell protein were subjected to immunoblot analysis for ABCA1 and β-actin.

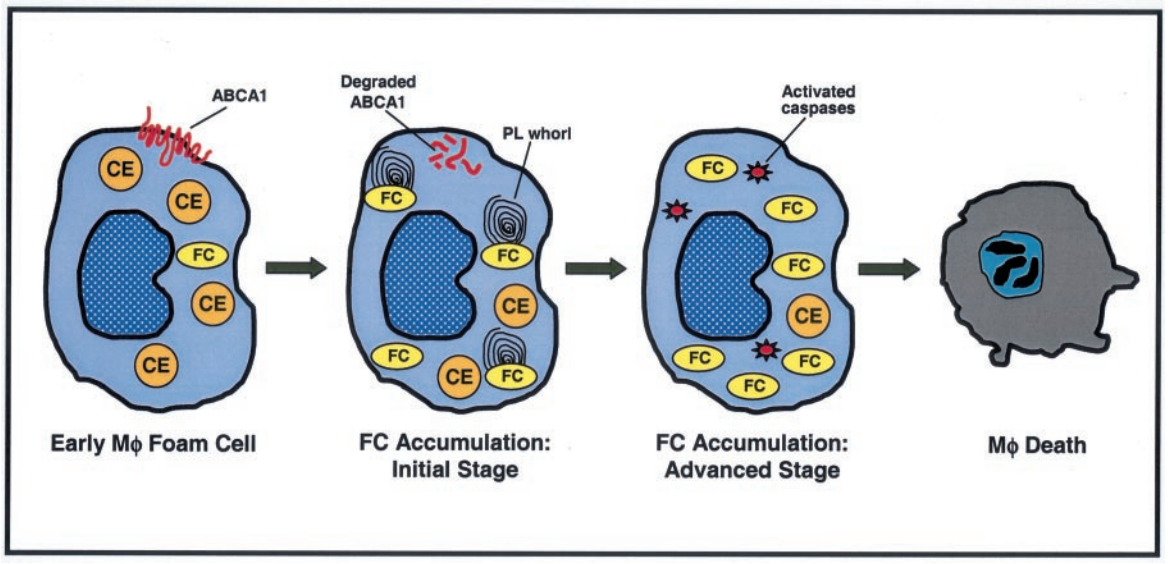

FIG. 9. **Stages of adaptation and death in FC-loaded macrophages.** In early foam cells, uptake of atherogenic lipoproteins results in mostly CE accumulation. There is transcriptional induction of ABCA1 (24–27, 33), which may function to limit cellular FC levels. As FC begins to accumulate, there is an adaptive phospholipid (*PL*) stage consisting of increased phospholipid biosynthesis, leading to increased phospholipid mass in the form of intracellular membrane whorls (18). There may also be increased unsaturation of phospholipid fatty acyl chains (19). Both responses would help neutralize the stiffening effects of excess FC on cellular membranes. As described herein, however, FC loading also leads to an increase in the degradative turnover of ABCA1, which may further exacerbate FC accumulation. With more massive FC accumulation, phospholipid biosynthesis decreases, and an FC-induced death program is induced (8, 9). This program consists of activation of Fas ligand, mitochondrial dysfunction, mitochondrial cytochrome *c* release, and caspase activation (21, 22). *Mϕ*, macrophage.

data of Liscum and co-workers (51), we demonstrated that 70 nM U18666A resulted in an ~90% decrease in cholesterol trafficking to the endoplasmic reticulum,[3] yet blocked trafficking to the plasma membrane by only ~10%.

Time courses of lipoprotein·cholesterol efflux to apoA-I or HDL_2 in CE-loaded macrophages, FC-loaded macrophages, and FC-loaded macrophages treated with 70 nM U18666A are shown in Fig. 7 (*A*, apoA-I; and *B*, HDL_2). With apoA-I, the relatively low level of efflux in FC-loaded macrophages was restored to a level almost identical to that in CE-loaded macrophages by treatment of the FC-loaded cells with low-dose U18666A. Consistent with the data in Figs. 1*B* and 4*C*, FC-loaded macrophages had only a small defect in cholesterol efflux to HDL_2, which, nonetheless, was increased by a small amount to the level seen in CE-loaded cells. Interestingly, 70 nM U18666A also increased cholesterol efflux to apoA-I by ~30% in macrophages incubated for a prolonged period with acetyl-LDL without an ACAT inhibitor (Fig. 7*C*). These data raise the possibility that the amount of FC that naturally accumulates under these conditions may be enough to cause modest dysfunction of the ABCA1 cholesterol efflux pathway by the mechanisms described above.

The effect of 70 nM U18666A on ABCA1 protein levels was investigated in CE- and FC-loaded macrophages. As shown in Fig. 8, the decrease in ABCA1 in FC-loaded macrophages was substantially abrogated by treatment with 70 nM U18666A. By densitometry, total ABCA1 was decreased by only 17 ± 12% ($n = 4$) in FC-loaded *versus* CE-loaded macrophages in the presence of 70 nM U18666A. Similar data were obtained with cell-surface ABCA1 (data not shown). These data, together with those obtained with heterozygous NPC macrophages, support a model in which the cholesterol that accumulates in FC-loaded macrophages must traffic to a peripheral cellular site other than the plasma membrane, perhaps the endoplasmic reticulum, to trigger the degradation of ABCA1 and the decrease in ABCA1 function.

[3] P. M. Yao and I. Tabas, manuscript in preparation.

DISCUSSION

The findings reported herein provide new insight into several areas of macrophage cell biology related to atherosclerosis. On one level, the results of this study add an important step to the progression of events that constitute the pathobiology of the FC-loaded macrophage (Fig. 9). In this context, there are interesting parallels to events surrounding changes in phospholipid metabolism. As described previously, phosphatidylcholine is induced in FC-loaded macrophages as an adaptive response, *viz.* to equalize the FC/phospholipid ratio in cellular membranes (52). This adaptive response eventually fails, however, leading to acceleration of FC-induced macrophage death (52). Similarly, cholesterol loading of macrophages leads initially to the induction of ABCA1 (24–27, 33), which would appear to be an adaptive response that protects the cells from FC accumulation. As shown in this report, however, this adaptive response also eventually fails. In the case of phosphatidylcholine biosynthesis, the initial FC-induced induction is a post-translational signaling event involving dephosphorylation and activation of CTP:phosphocholine cytidylyltransferase and perhaps other regulatory proteins (53). The mechanism of the eventual decrease in CTP:phosphocholine cytidylyltransferase activity has not yet been investigated. In the case of ABCA1, the initial induction occurs at the transcriptional level via activation of LXR (24–27), and the eventual decline in ABCA1 activity is, as shown here, related to FC-induced accelerated degradation of the protein. It is also possible that other events contribute to the decline in ABCA1 activity with more advanced FC loading, such as a decline in ABCA1 activity due to the "stiffening" effects of excess FC on membranes surrounding ABCA1. Furthermore, because ABCA1 requires ATP binding for activity (54), a decrease in cellular ATP levels that results from FC-induced mitochondrial dysfunction may also contribute to the decline in ABCA1 function. Importantly, both the decrease in CTP:phosphocholine cytidylyltransferase activity and the degradation of ABCA1 are triggered before the macrophages show overt biochemical and morphological signs of cytotoxicity, such as the drop in mitochondrial membrane potential, caspase ac-

tivation, cell shape changes, and membrane permeability disruption. Indeed, as shown in Fig. 2*C*, the decrease in ABCA1 protein is not prevented by caspase inhibitors. Thus, we propose that the combined decreases in the activities of CTP: phosphocholine cytidylyltransferase and ABCA1 are critical factors in the acceleration of FC-induced death in macrophages (Fig. 9).

The data in this study also add additional insight into the post-translational regulation of ABCA1. Although much emphasis has been placed on the transcriptional regulation of ABCA1, new studies are beginning to reveal the role of alterations of ABCA1 turnover as an important regulatory mechanism. ABCA1 has a relatively rapid turnover rate, and Wang and Oram (28) reported that unsaturated fatty acids accelerate the degradation of ABCA1 and thus decrease apoA-I-mediated efflux. The proteases that mediate this accelerated degradation were not identified. Moreover, Arakawa and Yokoyama (29) have very recently shown that apoA-I itself stabilizes ABCA1 by retarding its degradation. In their study, ALLN, but not lactacystin, blocked the turnover of ABCA1 in the absence of apoA-I, and these authors speculated that a non-proteasomal thiol protease was involved. In our study, the decrease in ABCA1 protein in FC-loaded macrophages was blocked to similar degrees by ALLN and lactacystin, suggesting an important role for proteasomal degradation in this process. A important area of future investigation will be to determine the molecular mechanisms linking FC loading to proteasomal degradation of ABCA1, including the possibility that FC loading triggers ubiquitination of ABCA1.

Along these lines, our current work strongly suggests that the triggering of ABCA1 degradation requires trafficking of cholesterol from late endosomes/lysosomes to a peripheral cellular site. We base this conclusion on the results of two complementary experiments, one using a molecular genetic mutation and the other using a pharmacological agent. It is formally possible, however, that 70 nM U18666A may work by partially blocking *npc1* and that the mechanism whereby partial inhibition of *npc1* (by the low-dose drug or by the heterozygous mutation) blocks FC-induced ABCA1 degradation involves an *npc1*-related event independent of cholesterol trafficking. If cholesterol trafficking is involved, the destination site mediating ABCA1 degradation is not yet known. Although our studies with the heterozygous *npc1* mutation and 70 nM U18666A suggest that a peripheral site like the endoplasmic reticulum may be more important than the plasma membrane, we cannot rule out a role for a low-abundance subdomain of the plasma membrane. Defining this site and its FC-induced signaling molecules will be a major emphasis of our future studies in this area.

Last, the findings in this report may provide new insight into the molecular events that occur during the progression of atherosclerosis. Although there are other cholesterol efflux pathways that are independent of ABCA1, such as those involving HDL, the apoA-I/ABCA1 pathway appears to be particularly important. For example, patients with Tangier disease have a selective defect in ABCA1-mediated cholesterol efflux and a higher risk than normal for atherosclerotic vascular disease (26). Moreover, humans with heterozygous ABCA1 deficiency were found to have decreased cholesterol efflux from fibroblasts, which correlated with increased carotid artery intima-media thickness (55). Likewise, transgenic mice that overexpress ABCA1 have decreased atherosclerosis (56), and bone marrow transplantation studies have revealed that decreased expression of macrophage ABCA1 enhances atherosclerosis without affecting plasma lipid levels (31, 32). These findings, together with our data, raise the possibility that the accumulation of FC that is known to occur in lesional macrophages may, prior to causing cell death, accelerate atherosclerosis by decreasing ABCA1-mediated cholesterol efflux from these cells. Indeed, this scenario may explain why mice transplanted with ACAT-negative macrophages have not only increased lesional macrophage death, but also increased lesion size *per se* (17). Once the pathway of FC-induced ABCA1 degradation is further elucidated, this model can be further tested by genetically manipulating molecules in this pathway. These studies may also lead to novel strategies to promote cholesterol efflux from atherosclerotic lesions and thus limit lesion progression.

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