Physiological Difference in Autophagic Flux in Macrophages From 2 Mouse Strains Regulates Cholesterol Ester Metabolism

Peggy Robinet, Brian Ritchey, Jonathan D. Smith

Objective—DBA/2 apoE\(^{-/-}\) mice have \(=10\)-fold larger lesions than AKR apoE\(^{-/-}\) mice. The objective of this study was to determine whether macrophages from these 2 strains had altered cholesterol metabolism that might play a role in their divergent atherosclerosis susceptibility.

Approach and Results—AKR and DBA/2 macrophages incubated with acetylated low-density lipoprotein resulted in higher cholesterol ester (CE) and lower free cholesterol accumulation in the DBA/2 cells. However, these strains had equivalent acetylated low-density lipoprotein uptake and cholesterol esterification activity. Cholesterol efflux from unloaded cells to apolipoprotein A-I or high-density lipoprotein was similar in the 2 strains. However, on acetylated low-density lipoprotein loading, cholesterol efflux was impaired in the DBA/2 cells, but this impairment was corrected by loading in the presence of an inhibitor of cholesterol esterification. Thus, the cholesterol efflux capabilities are similar in these strains, but there seemed to be a defect in lipid droplet–stored CE mobilization in DBA/2 cells. Lalstat 1, a specific inhibitor of lysosomal acid lipase, completely blocked the hydrolysis of lipid droplet–stored CE, implying that lipid droplet autophagy is responsible for CE turnover in these cells. CE turnover was 2-fold slower in DBA/2 versus AKR cells. Autophagic flux, estimated by a fluorescent light chain 3-II reporter and the increase in p62 levels after chloroquine treatment, was higher in AKR versus DBA/2 macrophages, which had an apparent decrease in autophagosome fusion with lysosomes. When autophagy was activated by amino acid starvation, CE levels decreased in DBA/2 cells.


Key Words: atherosclerosis ■ autophagy ■ cholesterol ester ■ foam cell

Atherosclerosis, the primary cause of cardiovascular disease and the leading cause of death worldwide,\(^1\) is characterized by the progressive buildup of cholesterol-rich plaques in the arteries. Apolipoprotein E (apoE)-deficient mice develop aortic lesions that progress from fatty streaks to fibrous plaques with a predilection to form at regions with disturbed flow\(^2\) and are a commonly used model for this disease. Atherosclerosis in various mouse models is sensitive to the genetic background, and we have previously shown that apoE-deficient DBA/2 mice have 10-fold larger lesion areas than apoE-deficient AKR mice when fed with a chow diet for 16 weeks.\(^3\) As an early step in the formation of atherosclerotic lesions is the transformation of arterial wall macrophages into lipid-loaded foam cells,\(^4\) we studied cholesterol metabolism after cholesterol loading with acetylated low-density lipoprotein (AcLDL) in bone marrow–derived macrophages from apoE-deficient AKR and DBA/2 mice in vitro.

We found that DBA/2 versus AKR macrophages accumulated more cholesterol esters (CE) in lipid droplets (LD) attributable to a decreased rate of LD-stored CE hydrolysis. Recently, Ouimet et al\(^5\) characterized that macrophage foam cell LD are delivered to lysosomes via autophagy, where LD-stored CE is hydrolyzed into free cholesterol (FC) by lysosomal acid lipase. We found that the same pathway is responsible for the majority of LD-stored CE hydrolysis in apoE-deficient AKR and DBA/2 macrophages, but that autophagic flux was slower in DBA/2 cells resulting in more CE accumulation in LD. These studies imply that the regulation of autophagy may play an important physiological role in foam cell formation and atherogenesis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.
Results

Macrophage Cholesterol Accumulation and Uptake
To examine cholesterol metabolism in apoE-deficient bone marrow–derived macrophages (hereafter described as macrophages) from the atherosclerosis-resistant AKR and atherosclerosis-sensitive DBA/2 backgrounds, we incubated these cells for 24 hours in presence of increasing concentrations of AcLDL and measured total cholesterol, FC, and CE levels. Total cholesterol levels increased on AcLDL loading in both strains, and this loading seemed to be saturable, as expected because it is receptor mediated (Figure 1A). The amount of loading during 24 hours was variable using different batches of AcLDL, but in general, unloaded cells contained ≈30 to 50 μg/mL cholesterol/mg cell protein, whereas loaded cells contained from 100 to 300 μg/mL cholesterol/mg cell protein. However, using different batches of AcLDL, we consistently observed that the distribution of FC and CE was significantly different between the 2 strains at all doses of AcLDL. DBA/2 macrophages had lower levels of FC (Figure 1B) and higher CE/FC ratios of ≈2 to 3 in DBA/2-loaded macrophages and <1 in AKR-loaded macrophages (Figure 1D).

We hypothesized that this strain effect on cholesterol loading and CE storage might be attributable to differences in the following: (1) lipoprotein uptake; (2) AcLDL-bound CE hydrolysis to FC, transport of FC to the endoplasmic reticulum or subsequent cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT); (3) FC efflux to extracellular acceptors; or (4) CE hydrolysis from LD.

Figure 1. Acetylated low-density lipoprotein (AcLDL) loading of macrophages from apoE-deficient AKR and DBA/2 mice. Macrophages from AKR (open square and dash line) or DBA/2 (black square and solid line) mice were loaded for 24 hours at 37°C with 0, 50, 100, or 150 μg/mL AcLDL. Total cholesterol (TC; A), free cholesterol (FC; B), cholesterol ester (CE; C), and CE/FC ratio (D) are expressed as the means±SD of triplicates. *P<0.05; **P<0.01; ***P<0.001 by 2-tailed t test comparing the 2 strains at each dose.

To test our first hypothesis, we measured the uptake of DiI-labeled AcLDL by macrophages from both strains using flow cytometry after a 30-minute incubation. We found similar levels of AcLDL uptake by AKR and DBA/2 macrophages (Figure IA in the online-only Data Supplement). We confirmed this result with an alternate method measuring [3H]-AcLDL uptake (Figure IB in the online-only Data Supplement). Thus, we ruled out AcLDL uptake as the mechanism responsible for the differences observed in cholesterol loading at 50 μg/mL AcLDL and, in general, for the differences in CE/FC distribution in AKR and DBA/2 macrophages.

AcLDL-Bound CE Metabolism and Re-Esterification
We next addressed the second hypothesis that the strain difference in cholesterol loading might be attributable to altered levels of AcLDL-bound CE catabolism, delivery of FC to the endoplasmic reticulum, or activity of ACAT, the endoplasmic reticulum enzyme in macrophages that esterifies FC to CE for storage in LDs. To determine whether there was a strain effect on lysosomal hydrolysis of endocytosed CE in AcLDL, we measured the percentage of CE remaining in cells after AcLDL loading (50 μg/mL) for 16 hours in the presence of ACAT inhibitor (ACATi). Both AKR and DBA/2 macrophages contained <6% of their total cholesterol in CE (not significant versus each other), demonstrating that lysosomal hydrolysis of AcLDL-derived CE was similar in these strains and ruling out a lysosomal storage disorder in DBA/2 cells. We then assessed ACAT activity in 2 assays using either broken cell lysates or living cells. This was important because there is a well-characterized ACAT protein polymorphism in the AKR strain, although this N-terminal 33 residue truncation is not associated with altered ACAT activity.6,7 In broken cell lysates made from cholesterol-loaded cells, we found no difference in ACAT activity between the 2 strains (6.7±0.3 and 6.5±0.9 dpm CE/mg cell protein in AKR and DBA/2 macrophages, respectively; P=0.70; Figure 2A). To confirm these results and to verify that the CE/FC ratio difference we observed in loaded cells was not attributable to altered delivery of FC to the endoplasmic reticulum, we measured the initial ACAT activity in live unloaded or preloaded AKR and DBA/2 macrophages by incubation with [3H]cholesterol-AcLDL for 1 hour. In both AKR and DBA macrophages, CE formation was induced in preloaded cells versus unloaded cells by >10-fold (P<0.0001; Figure 2B). However, there were no significant differences in ACAT activity between the 2 strains in either unloaded or preloaded cells. These results confirm the data in the broken cell lysates and imply that the delivery of FC to the site of ACAT in the endoplasmic reticulum is comparable in both strains. Thus, AcLDL CE metabolism and re-esterification does not seem to be responsible for the observed strain effects on CE/FC ratio.

Macrophage Cholesterol Efflux
We evaluated efflux of cholesterol to apoAI and high-density lipoprotein (HDL) in unloaded cells, where most cholesterol is in FC, and in preloaded cells, where CE stores accumulate. In the unloaded cells, there was no significant difference
between AKR and DBA/2 macrophages in terms of efflux to 10 μg/mL apoAI (1.9±0.8% versus 1.6±0.4%, respectively) or 100 μg/mL HDL (8.1±0.6% versus 7.6±0.5%, respectively, Figure 3A). However, when loaded cells were incubated with these acceptors, AKR macrophages, versus DBA/2 macrophages, had increased efflux to both apoAI (4.8±0.8% versus 3.0±0.3%; P<0.05) and HDL (8.1±1.5% versus 5.7±0.6%; P<0.01; Figure 3B). To determine whether this strain effect on the difference in efflux between the unloaded and loaded cells was attributable to CE stores, we repeated the efflux study in cells in which AcLDL loading was performed in the presence of an ACATi to prevent CE formation. As in the unloaded cells, the macrophages loaded in the presence of ACATi had no significant strain differences in their efflux to either apoAI or HDL (Figure 3C). Thus, there is no inherent difference in efflux capacity between AKR and DBA/2 macrophages when the cells contain mostly FC; however, in conditions where CE stores are present, the DBA/2 macrophages have impaired efflux activity. Taken together, the results suggest that the strain effect on CE/FC distribution may be attributable to different efficiencies in CE hydrolysis.

Macrophage CE Hydrolysis

Esterified cholesterol in cholesterol-loaded macrophages is stored in LD and for this CE to be mobilized for efflux, it must first undergo hydrolysis to FC. A recent report by Ouimet et al.\(^5\) highlights the involvement of autophagy in regulating LD-stored CE hydrolysis and cholesterol efflux from cholesterol-loaded macrophages. This study demonstrated the engulfment of LD by autophagosomes delivering LD-stored CE to lysosomal acid lipase via the formation of autolysosomes. To test the involvement of lysosomal acid lipase in the hydrolysis of LD-stored CE in AKR and DBA/2 macrophages, we followed a protocol similar to Ouimet et al.,\(^5\) in which AcLDL-loaded macrophages, with concomitant CE stores, were chased for 24 hours with apoAI in absence or presence of ACATi, or in the presence of ACATi plus lalistat 1, a specific inhibitor of lysosomal acid lipase.\(^8,9\) For the chase in the absence of ACATi, CE levels were 2-fold higher in DBA/2 versus AKR cells (133.0±12.2 versus 64.8±5.6 μg/mg cell protein; P<0.001, respectively, Figure 4A), representative of the higher initial CE storage of DBA/2 macrophages (compare with Figure 1C). When ACATi was added to the chase media, to prevent the re-esterification of hydrolyzed LD-stored CE, the CE levels dropped in both strains but still resulted in significantly more CE in DBA/2 versus AKR macrophages.
ACATi led to a 63% decrease in CE in the AKR cells versus a 51% decrease in DBA/2 cells (P < 0.001 for both strains by ANOVA posttest). In the presence of both ACATi and lalistat 1 during the chase, LD-stored CE hydrolysis was inhibited, and the CE levels in both strains were similar to those observed in the absence of ACATi. These results suggest that lysosomal acid lipase is responsible for the hydrolysis of LD-stored CE in these foam cells and that LD-stored CE hydrolysis via lysosomal acid lipase may be slower in DBA/2 macrophages.

To more precisely measure LD-stored CE hydrolysis rates, we measured cellular CE levels after AcLDL loading (0-hour chase) or 24 hours after chasing with apoAI in the presence of ACATi to block FC re-esterification. We varied the AcLDL loading dose to load the AKR and DBA/2 macrophages with similar levels of CE; nevertheless, we normalized our data to the CE content of the cells at 0 hour. Combining the data from 3 independent experiments, we observed 41±8% and 66±14% reductions in CE content after the 24-hour chase in the AKR and DBA/2 cells, respectively (P=0.01 by t test; Figure 4B). This corresponds to a CE half life of 13.9 hours in AKR cells, which was doubled in DBA/2 macrophages to 30.4 hours, thus confirming that LD-stored CE hydrolysis is slower in DBA/2 cells.

We further addressed the role of lysosomal acid lipase in LD-stored CE turnover qualitatively using fluorescent microscopy. Unloaded AKR and DBA/2 macrophages or cells loaded with 50 µg/mL AcLDL in the absence or presence of ACATi were stained with Nile red to visualize LD and costained with DAPI (nuclear stain). The results, presented in Figure 4C, show that loaded cells accumulate LD in both AKR and DBA/2 cells, but to a greater extent in DBA/2, consistent with the CE biochemical measurement in Figure 1C. After a 24-hour chase in presence of ACATi, the number of LD is dramatically reduced in AKR cells and their number seems moderately decreased in DBA/2 macrophages, but still higher than in the AKR cells, again consistent with the biochemical CE measures in Figure 4A. When lalistat 1 and ACATi were added to the chase media, there was no discernable decrease in the number of LD versus the loaded cells in both strains. These results confirm the role of lysosomal acid lipase in CE LD turnover in both strains and suggest that either decreased...
activity of lysosomal acid lipase or decreased autophagy of LD may be responsible for decreased LD-stored CE turnover in DBA/2 macrophages. We also examined small early foam cell lesions in the aortic root of chow diet–fed AKR and DBA/2 mice using Bodipy to stain CE-laden LD. In a preliminary quantitative study, we observed that the LD% of the foam cell area was >2-fold larger in DBA/2 versus AKR lesions (P<0.01; Figure II in the online-only Data Supplement). This finding is consistent with our cell culture observation of more CE content in DBA/2 versus AKR AcLDL-loaded macrophages (Figure 4).

Cholesterol esterase activity was measured in cell lysates from AKR and DBA/2 macrophages. In both strains, there was >2-fold more acidic than neutral cholesterol esterase activity (P<0.01 by ANOVA posttest), but there was no significant strain difference between the acidic or neutral cholesterol esterase activities (Figure 4D). Thus, the activity of lysosomal acid lipase does not seem to be limiting in the DBA/2 macrophages. These results are also consistent with the absence of defect in AcLDL-bound CE hydrolysis in DBA/2 cells.

**Autophagy Role in Strain Difference in CE Ester Hydrolysis**

To investigate whether autophagy may play a role in the observed impairment of LD-stored CE hydrolysis in DBA/2 cells, the key autophagy proteins microtubule-associated protein 1 light chain 3 (LC3) and p62 were assessed using Western blot, normalized to GAPDH used as a loading control. LC3 is synthesized as a precursor and is cleaved to form the cytosolic form LC3-I, which is then covalently modified with phosphatidylethanolamine to generate LC3-II, which specifically binds to the elongating autophagosome and remains bound through to fusion with the lysosome where it is degraded. Low levels of LC3-II can result from low conversion from LC3-I, which occurs when autophagy is blocked at an early stage leading to LC3-I accumulation. We compared the LC3-II/LC3-I ratio in AcLDL-loaded macrophages and found in both cell types that there was ≈5-fold higher levels of LC3-II than LC3-I (Figure 5A), indicating that both cell types have sufficient LC3-II to initiate autophagy. The levels of p62 can be used as an indication of autophagic flux because p62 targets protein aggregates to the autophagosome and is degraded along with the aggregate; thus, low levels of p62 that are increased by chloroquine treatment, which blocks its lysosomal degradation, are associated with high levels of autophagic flux. We assessed p62 levels in AcLDL-loaded macrophages in the absence or presence of a 4-hour treatment with 30 μM chloroquine. Chloroquine treatment induced p62 levels 2.1-fold in AKR macrophages (P<0.01), whereas this treatment led a nonsignificant 13% increase in p62 levels in DBA/2 macrophages (Figure 5B). This result indicated a lower level of autophagic flux in the DBA/2 cells, agreeing with the observed slower rate of LD-stored CE hydrolysis (Figure 4B). In the absence of chloroquine, we observed that p62 levels were 2-fold higher in DBA/2 versus AKR macrophages (Figure 5B; P<0.01). This finding is also consistent with lower rates of degradation via autophagy in DBA/2 versus AKR cells.

**Figure 5.** DBA/2 vs AKR macrophages have impaired autophagic flux. A, Light chain (LC)3-II to LC3-I protein ratio in AKR (open bar) and DBA/2 (solid bar) cells loaded for 24 hours with 50 μg/mL acetylated low-density lipoprotein (AcLDL). Results represent the mean±SD of triplicates. B, p62 protein expression in AcLDL-loaded AKR (white bars) and DBA/2 (black bars) cells incubated for 4 hours with (solid bars) or without (cross-hatched bars) chloroquine (Cq). Results represent the means±SD of triplicate cell samples. 1 vs 2, P<0.01 by ANOVA posttest. C, Bifluorescent LC3 assay for autophagic flux as describe in Methods in the online Data Supplement. Fluorescent puncta in the red channel indicate LC3-II in autophagosomes and autolysosomes, whereas green puncta indicate autophagosome bound LC3-II only. D, Mean puncta red fluorescent protein (RFP)/green fluorescent protein (GFP) fluorescence ratio per cell in AcLDL-loaded AKR and DBA/2 macrophages in the absence or presence of a 1-hour amino acid starvation. Higher ratios indicate autophagosome fusion with lysosomes. Different numbers above the data points represent P<0.05 by ANOVA posttest. EGFP indicates enhanced GFP, and mRFP, monomeric RFP.
pH-sensitive enhanced green fluorescent protein fluorescence diminishes, leaving puncta with red/green fluorescence ratio >1. On AcLDL loading of the transfected AKR macrophages, epifluorescent microscopy revealed that the median number of fluorescent puncta was similar in AKR (7 puncta) and DBA/2 (8 puncta) macrophages (P=0.61 by nonparametric t test after counting puncta in 19 AKR and 21 DBA/2 transfected cells; Figure III in the online-only Data Supplement). Thus, autophagosome formation seemed similar in the 2 strains. Image analysis revealed a broad distribution of cells with mean fluorescent puncta red/green ratios ranging from ≈1 to ≈2.7. This is indicative of cells with nonlysosomal LC3 localization (red/green ratio ≈1) and lysosomal LC3 localization (red/green ratio >1), the latter consistent with relatively rapid autophagic flux, and enhanced green fluorescent protein emission decreased in the low pH lysosome environment. However, DBA/2 foam cells had puncta with red/green ratios of ≈1 (P<0.05 versus AKR cells by ANOVA posttest), indicative of relatively slow autophagic flux (Figures 5C and 5D). Thus, this assay suggests that the impaired step in autophagic clearance of LD-stored CE in DBA/2 cells is the fusion of autophagosomes with lysosomes.

Our finding of higher basal levels of p62 in DBA/2 versus AKR macrophages (Figure 5B) is consistent with the proposed impairment in the DBA/2 strain at the level of autolysosome formation. To validate this bifluorescent reporter assay, we induced autophagic flux in both strains by amino starvation. Image analysis revealed increased cellular red/green puncta ratios in both strains (P<0.05 versus nonstarved cells of the same strain by ANOVA posttest; Figure 5D). Thus, both p62 Western blot and LC3 bifluorescent reporter revealed slower autophagic flux in DBA/2 versus AKR macrophages.

We then subjected DBA/2 preloaded macrophages to amino acid starvation, a commonly used activator of autophagy. A 2-hour chase in the presence of apoAI and ACATi was performed in complete DMEM or in amino acid-free EBSS, and CE levels were reduced by 29.8% in the EBSS compared with the control cells (80.4±4.3 and 114.5±11.3 μg/mg cell protein, respectively; P<0.05 by ANOVA posttest; Figure 6A). When we added chloroquine during the EBSS chase to block autophagy at the level of autolysosome function, CE levels went back up to 131.6±14.1 μg/mg cell protein (non-significant versus nonstarved controls), indicating that the EBSS-induced LD-stored CE hydrolysis was dependent on autolysosome function. LC3-II levels also decreased by 26% on amino acid starvation (P<0.05 by ANOVA posttest), which was reversed to basal levels by the addition of chloroquine (Figure 6B). Thus it seems that DBA/2 cells have decreased basal autophagic flux leading to slower LD-stored CE turnover but that on induction of autophagy by amino acid starvation, DBA/2 macrophages are capable of clearing LD-stored CE more efficiently.

**Discussion**

We characterized altered cholesterol metabolism in AKR versus DBA/2 apoE-deficient bone marrow macrophages. On cholesterol loading with AcLDL, DBA/2 macrophages, compared with AKR macrophages, accumulated more CE attributable to decreased LD-stored CE hydrolysis leading to less cholesterol efflux to apoAI and HDL. There were no apparent strain differences in AcLDL uptake or ACAT activity, the latter measured both in live cells and cell lysates. The AKR strain is known to harbor a mutation in the ACAT gene leading to exon skipping that results in a 33 amino acids deletion at the N terminus of the protein. However, Meiner et al reported that this protein change is not associated with a change in adrenal ACAT activity, and our results confirm equivalent activity in macrophages carrying this truncated ACAT isoform.

Recently, Ouimet et al demonstrated that lysosomal acid lipase is the predominant activity responsible for the hydrolysis of CE in foam cell LD, and we confirmed this finding by the use of lalistat 1, a specific lysosomal acid lipase inhibitor. We found that AKR and DBA/2 cells had equal amounts of lysosomal acid lipase activity; thus, the activity or expression of this enzyme does not seem to be responsible for our observed strain effect on LD-stored CE hydrolysis. However, alterations in human lysosomal acid lipase, encoded by the LIPA gene, may play a role in coronary artery disease (CAD) susceptibility. Three genome-wide association studies have identified LIPA intronic single nucleotide polymorphisms, rs141244 or rs2246949 (which are in complete linkage disequilibrium with each other and thus coinherited on the same allele), as associated with CAD. Although the effect of the risk allele was not very strong with CAD odds ratio of ≈1.1, these findings were all...
highly significant, exceeding the conservative genome-wide threshold of significance of $P<10^{-8}$. LIPA gene expression was ascertained in whole blood or monocytes by microarrays, and the CAD risk allele of the LIPA gene was found to be associated with higher expression of LIPA mRNA.16,17 The direction of this effect on LIPA gene expression does not match with our findings, in which increased lysosomal lipase activity would be expected to promote CE turnover, cholesterol efflux, and foam cell regression. However, LIPA mRNA levels in blood cells are far removed from lysosomal acid lipase activity in cholesterol engorged arterial foam cells; thus, it is unknown whether these single nucleotide polymorphisms play a role in human atheroma CE metabolism, and the mechanism responsible for their CAD association has not been determined. Nevertheless, we ruled out lysosomal acid lipase activity differences as being responsible for our observed strain effects on LD-stored CE turnover.

The role of autophagy in LD metabolism was first demonstrated by Singh et al in olate-treated hepatocytes and fibroblasts, in which autophagy was shown to decrease cellular triglyceride levels, LD size and number, and promote fatty acid oxidation.18 Oumit et al9 extended this observation to foam cells, in which mobilization of macrophage foam cell LD-stored CE was shown to be via autophagy. Three recent studies have highlighted the role of autophagy in atherosclerosis. Atg5 is one of the genes required for formation of the autophagic isolation membrane and engulfment. Macrophage specific Atg5-deficient mice were created via Cre-lox method-

Autophagy regulation in either direction seems competent to play a physiological role in macrophage CE metabolism. Thus, it is attractive to speculate that some of rapamycin’s antiatherogenic activity stems from induction of autophagy in arterial foam cells, promoting hydrolysis of LD-stored CE, cholesterol efflux, and foam cell regression.

In conclusion, we demonstrated that LD-stored CE hydrolysis is mediated by lysosomal acid lipase, and our data support the notion that the delivery of LD-stored CE to the lysosome is mediated by autophagy. We found that the higher CE levels in DBA/2 macrophages were caused by a decrease in CE hydrolysis from LD attributable to slower basal autophagic flux in these cells, leading to lower rates of cholesterol efflux. Thus, the physiological regulation of autophagy plays an important role in foam cell CE metabolism, which may play a role in the increased atherosclerosis susceptibility of the DBA/2 versus the AKR mouse strain. The identification of the genes and mechanisms responsible for this strain effect on macrophage autophagy is of great interest and may aid in identifying new therapeutic targets.

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Disclosures

None.

References


Atherosclerosis is a complex disease with genetic and environmental risk factors, and much of our knowledge about the pathogenesis of this disease has come from the study of animal models. Previously, we observed 10-fold larger aortic root lesions in apoE-deficient mice on the DBA/2 versus the AKR genetic background. Here, we identified an intermediate phenotype, the excess storage of cholesterol esters in cultured macrophages derived from DBA/2 mice. We determined that the mechanism for this phenotype is the slower hydrolysis of cholesterol esters DBA/2 versus the AKR genetic background. Here, we identified an intermediate phenotype, the excess storage of cholesterol esters in cultured

Significance

Atherosclerosis is a complex disease with genetic and environmental risk factors, and much of our knowledge about the pathogenesis of this disease has come from the study of animal models. Previously, we observed 10-fold larger aortic root lesions in apoE-deficient mice on the DBA/2 versus the AKR genetic background. Here, we identified an intermediate phenotype, the excess storage of cholesterol esters in cultured macrophages derived from DBA/2 mice. We determined that the mechanism for this phenotype is the slower hydrolysis of cholesterol esters stored in lipid droplets, which is in turn mediated by a lower rate of lipid droplet autophagy, specifically at the autophagosome to lysosome fusion step. This novel finding that physiological factors regulate autophagy to impact foam cell cholesterol ester metabolism demonstrates that this pathway may play an important role in atherosclerosis susceptibility. This knowledge may be leveraged to find novel diagnostics and therapeutics for atherosclerosis.
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Supplemental Figures for "Physiological difference in autophagic flux in macrophages from two mouse strains regulates cholesterol ester metabolism"

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Supplemental Figure I– AcLDL uptake by macrophages from apoE-deficient AKR and DBA/2 mice. (A) Macrophages from AKR (open bars) or DBA/2 (solid bars) were incubated with DiI-AcLDL and uptake was assessed using flow cytometry. The mean fluorescence intensity±SD of duplicates is shown on the graph. Cells incubated in absence of DiI were used as a control. (B) Macrophages from AKR (open bar) or DBA/2 (solid bar) were incubated with $[^3H]$-AcLDL and uptake was assessed by liquid scintillation counting. Results show the mean±SD of duplicates.
Supplemental Figure II – Bodipy staining of lipid droplets in foam cells from AKR and DBA/2 apoE-deficient aortic root lesion sections. (A) Lipid droplets in foam cells from aortic root lesions were stained using Bodipy (green) and slides were mounted with Vectashield (Vector Labs) containing DAPI, staining nuclei blue. Images obtained with 63x water immersion lens. (B) The area of the lipid droplets was assessed using Image-Pro plus 7.0 and expressed as percentage of foam cell area (N=4 lesions for AKR and N=3 lesions for DBA/2).
Supplemental Figure III – Mean puncta number per cells in AKR and DBA/2 apo-E deficient macrophages. Cells with ≥20 puncta were difficult to count accurately and assigned a value of 20 puncta. Data not normally distributed, lines show median values, not significantly different by non-parametric Mann-Whitney t-test.
Materials and methods

Bone marrow macrophages. Bone marrow derived macrophages were obtained from apoE-deficient mice on the AKR and DBA/2 background. Bone marrow cells were flushed from the femur and humerus bones and re-suspended in macrophage growth medium (DMEM, 10% FBS, 20% L-cells conditioned media as a source of MSCF) as previously described\(^1\) and plated. The media was renewed twice per week. Cells were used for experiments 10 to 14 days after plating when the bone marrow cells were confluent and fully differentiated into macrophages. During some experiments, serum free DMEM media was supplemented with 10ng/mL MCSF to aid cell survival.\(^3\)

Lipoprotein preparations and labeling. Human LDL (1.019 < d < 1.063 g/mL) and human HDL (1.063 < d < 1.21g/mL) were prepared by ultracentrifugation. LDL was acetylated as described previously.\(^4\),\(^5\) HDL, LDL and AcLDL were dialyzed against PBS with 100µM EDTA and 20µM BHT. Protein concentrations of lipoproteins were determined using an alkaline Lowry assay.\(^6\) For DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate) labeling, 10 mg/mL DiI stock in DMSO was diluted in PBS and added to AcLDL at a final concentration of approximately 10 µg/mg of protein. The mixture was incubated at 37°C for 25min and centrifuged for 2min to pellet any insoluble debris. The supernatant was collected and filtered sterilized before use. For [\(^3\)H] labeling, [\(^3\)H]-cholesterol in ethanol was pre-incubated with undiluted AcLDL or FBS at a final concentration of 0.5 µCi/mL for 15min at 37°C before adding to DMEM at a final concentration of 50 µg/mL AcLDL or 1% FBS, respectively.

AcLDL uptake. Cells were plated in 24-well plates and differentiated as described above. Cells were washed twice with DMEM and incubated in DMEM containing 10 ng/mL MCSF and 50 µg/mL DiI-AcLDL. Lipoprotein uptake proceeded for 30min at 37°C. The cells were washed twice with cold DMEM and detached from the plate using cellstripper\(^\text{TM}\) (Cellgro). The cell suspension was then place in a 5 mL tube and wash twice using flow cytometry media (PBS, 0.5 mM EDTA, 0.5% BSA). The cells were resuspended in 200 µL of flow cytometry media and were analyzed by flow cytometry (LSRII, Becton-Dickinson). Fluorescence was gated for individual live cells and the fluorescence associated with 10,000 cells was determined for each sample. The data collected from the cells were analyzed with Flowjo software. Alternatively, cells were incubated with [\(^3\)H]-AcLDL for 30min, cholesterol was extracted using hexane:isopropanol (3:2; v:v) and protein was solubilized using 0.2N NaOH. The radioactivity in the cells was determined by liquid scintillation counting and results expressed as dpm/µg cell protein.

Acyl-CoA cholesterol acyl transferase (ACAT) activity assay. To investigate ACAT activity, cells were plated in 6-well (cell lysate assay) or 12-well plates (live cell assay) and differentiated as described above.

Cell lysate assay – Cells were loaded for 48h using 50 µg/mL AcLDL, harvested and lysed by sonication. The ACAT activity was determined as described before with small modification.\(^7\) Briefly, the lysates were incubated for 15min at 37°C with cholesterol-rich phosphatidylserine liposomes and an additional 15min at 37°C after the addition of [\(^14\)C]-oleyl-CoA in presence of fatty acid free BSA. Cholesterol was extracted using
methanol:chloroform (2:1), and the organic phase was dried down and resuspended in hexane. CE was separated using thin layer chromatography and the radioactivity of this fraction assessed by liquid scintillation counting. Proteins were dissolved with 0.2 N NaOH and the results are normalized to the protein content, evaluated by BCA assay.

**Live cell assay** – Cells were incubated overnight with DMEM, 10 ng/mL MCSF and 1% FBS or 50 µg/mL AcLDL. Then, cells were incubated for 1h with [³H]cholesterol-AcLDL and the lipids were extracted in hexane:isopropanol (3:2). CE and protein were analyzed as described above to determine ACAT activity.

**Cholesterol efflux.** Cells were cholesterol labeled with 1% [³H]-FBS or 50 µg/mL [³H]-AcLDL in DMEM containing 10 ng/mL MCSF for 16h at 37°C. After labeling, cells were chased for 4h at 37°C in DMEM with or without acceptors (10 µg/mL apolipoprotein A1 or 100 µg/mL HDL). At the end of this chase period, the radioactivity in the medium and cells was determined by liquid scintillation counting, and the percent efflux was calculated as 100 x (medium dpm)/(medium dpm + cell dpm). Finally, percent efflux to acceptors was calculated as (percent efflux to acceptors)-(percent efflux in absence of acceptors). All treatments were performed in triplicate. When indicated, the loading and the chase were done in presence of 2 µg/mL ACAT inhibitor 58035 (ACATi).

**Cholesterol mass quantification.** Cells were plated in 12-well plates and differentiated as described above. The cells were incubated 24h at 37°C with macrophage culture media without or with varying doses of AcLDL. When indicated, the loading period was followed by a chase period in DMEM media containing 10 ng/mL MCSF, 10 µg/mL apoA1 and 2 µg/mL ACATi (chase media) with or without the addition of 10 µM lalistat 1, a lysosomal acid lipase inhibitor (kind gift from Fred Maxfield and Paul Helquist). Lipids were extracted from cells, and esterified cholesterol and protein amounts were evaluated as described previously.

**Fluorescence microscopy studies.** For lipid droplet visualization, cells were plated on 4-chamber slides and differentiated as described above. Three wells of cells were loaded for 24h with 50 µg/mL AcLDL and unloaded cells were used as a control. Two of the loaded wells were then chased for 24h in chase media with or without the addition of 10 µM lalistat 1. Finally, cells were fixed at room temperature for 20min in 10% buffered formalin phosphate (Fisher scientific), lipid droplets were stained with Nile red (100 ng/mL), and nuclei were stained with DAPI. For EGFP and mRFP tagged LC3-II reporter transfection studies, differentiated cells were scraped from a plate and transfected with ptfLC3 (Addgene plasmid #21074) using a nucleofection kit from Lonza specifically designed for macrophages (kit #VPA-1009). Cells were re-plated, and 24h later the cells were AcLDL loaded for 16h before epifluorescent imaging on live cells. Some wells were amino acid starved by incubation in EBSS for 1h prior to imaging. To determine the relative RFP/GFP puncta fluorescence for each cell, we used Adobe Photoshop to measure the red and green intensity at the center of each punctum and calculated the average red/green ratio of all puncta per cell. Aortic root sections of formalin fixed hearts were stained with Bodipy (10 µg/ml) for 1h, washed in PBS with 0.05% Tween-20 and PBS alone, and mounted with Vectashield (Vector Labs)
containing DAPI. The area of lipid droplets was assessed using Image-Pro plus 7.0 (Media Cybernetics) and expressed as percentage of foam cell area.

**Lysosomal acid lipase activity assay.** Lysosomal acid lipase activity was measured as described previously with a few modifications. Briefly, cell lysates were incubated with liposomes containing phosphatidylcholine, cholesterol oleate and cholesterol-[1-14C]-oleate at pH 5 or 7.4. Free fatty acids were separated from CE by addition of 0.1 M oleic acid and extraction in hexane:chloroform:methanol (370:185:444, vol:vol:vol) and 0.3 M NaOH. After separation, the upper fatty acid phase was collected and [14C]-counts assessed using a scintillation counter. Activity was normalized to protein levels in the cell lysates.

**Western blot.** Cells were plated in 12-well plates and differentiated as described above. After indicated treatment, cells were lysed directly in the plate using Laemmli buffer. The lysates were heated at 95°C for 5-10 min and loaded on 14% tris-glycine gels without prior normalizing for total protein content (this protocol improves LC3-II stability). After transfer, membranes were probed with antibodies against LC3 (NB100-2220, Novus Biologicals) and p62 (NBP1-48320, Novus Biologicals). GAPDH levels (antibody FL-335, Santa Cruz) were used to normalize for protein loading. When needed, membranes were stripped with Restore™ western blot stripping buffer (Thermo Scientific) as described by the manufacturer. Western blot results were analyzed using the ImageQuant TL software (GE healthcare).

**Statistics.** Comparison of two conditions was performed by student t-test, and comparison of multiple conditions was performed by ANOVA with Newman-Keuls posttest. Statistics were performed using GraphPad Prism software.

**References**


