Regulation of Lipid Droplet Cholesterol Efflux From Macrophage Foam Cells

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Abstract—Cholesterol efflux from macrophages is the first and potentially most important step in reverse cholesterol transport, a process especially relevant to atherosclerosis and to the regression of atherosclerotic plaques. Increasingly, lipid droplet (LD) cholesteryl ester (CE) hydrolysis is being recognized as a rate-limiting step in cholesterol efflux. The traditional view on macrophage CE hydrolysis is that this pathway is entirely dependent on the action of neutral hydrolases, and numerous candidate CE hydrolases have been proposed to play a role in lipid hydrolysis in macrophages and atherogenesis. Although the exact identity of macrophage-specific CE hydrolases remains to be clarified, a common point to all of these studies is that enhancing LD-associated CE hydrolysis increases cholesterol efflux and is antiatherogenic. Understanding how cholesterol is mobilized from LDs offers new steps for modulating cholesterol efflux, and recently a role for autophagy and lysosomal acid lipase in macrophage lipolysis has emerged. Autophagy and lysosomal acid lipase thus represent novel therapeutic targets to enhance macrophage reverse cholesterol transport. This review discusses our current understanding of the relationship between macrophage LDs and atherosclerosis and presents recent insights into the mechanisms for LD CE hydrolysis in macrophage foam cells. (Arterioscler Thromb Vasc Biol. 2012;32:575-581.)

Key Words: atherosclerosis ■ foam cells ■ lipoproteins ■ macrophages

Atherosclerosis is the major underlying factor leading to cardiovascular events such as heart attacks and strokes, and it is well established that macrophages play a crucial role in atherogenesis through the uptake of modified low-density lipoprotein (LDL) and secretion of inflammatory modulators, cytokines, and matrix-degrading enzymes.1 The primary cellular event of lesion initiation is the formation of macrophage foam cells, resulting from internalization of various forms of modified LDL that accumulate in the artery. Clearance of the arterial cholesterol deposits by macrophages is beneficial during the early stages of atherogenesis, but as the cholesterol homeostasis machinery becomes overwhelmed, the macrophages accumulating CE-LDS become foam cells that contribute to disease progression and the establishment of chronic inflammation.2

There is strong evidence to support the idea that enhancing macrophage reverse cholesterol transport (RCT), the removal of cholesterol from peripheral macrophages and its transport to the liver for clearance into the bile and ultimately the feces, is a good antiatherogenic strategy. Cholesterol efflux from macrophages is the first and potentially most important step in macrophage RCT, and accordingly the macrophage ATP binding cassette (ABC) transporter ABCA1 has been identified as being critical for this process.3

Foam Cell Lipid Droplets

Because excess free cholesterol can disrupt membranes and is cytotoxic, the cell has evolved complex mechanisms to tightly control its abundance and distribution.4 Under conditions of abundant cholesterol, cellular feedback mechanisms are in place to reduce cholesterol uptake and synthesis and increase cholesterol efflux.5 If these compensatory mechanisms fail to reduce free cholesterol levels appropriately, excess free cholesterol will be esterified and stored as noncytotoxic cholesteryl esters (CEs) in lipid droplets (LDs) to maintain the proper membrane free cholesterol concentration required for normal cellular functions to proceed.6 LDs are recognized as metabolically active but atypical intracellular organelles made up of a hydrophobic core of neutral lipids surrounded by a phospholipid monolayer coated with specific proteins.6 Several proteomic studies of LD proteins have identified signature coat proteins, including the PAT family of proteins (perilipin, adipophilin, TIP47). In keeping with the postulated endoplasmic reticulum (ER) origin of the LD, several enzymes of lipid synthetic pathways that reside in the ER have also been found in association with this organelle, including acyl-coenzyme A:cholesterol acyl transferase (ACAT).7,8

The cholesterol content and nature of LDs differ among cell types, reflecting the specialized nature of lipid storage.
Macrophages primarily accumulate CE in LDs on incubation with CE-enriched lipoproteins, such as acetylated or aggregated LDLs, whereas they predominantly accumulate triglyceride (TG)-enriched LDs on exposure to TG-rich lipoproteins, such as very-low-density lipoprotein and remnant lipoproteins.9 Neutral lipid storage in macrophages occurs in mixed CE and TG droplets; interestingly, cholesterol from CE in mixed droplets is effluxed more efficiently than from droplets resulting from LD TG depletion or CE enrichment.10 Irrespective of the nature of the neutral lipid accumulating in the macrophage LD, lipoprotein loading leads to increased adipophilin expression.9 The influence of adipophilin, the major macrophage LD coat protein, on atherogenesis was recently uncovered. Whole-body as well as macrophage-specific adipophilin ablation inhibits foam cell formation and protects against atherosclerosis development.11 Importantly, specific adipophilin ablation inhibits foam cell formation and recently uncovered. Whole-body as well as macrophage-specific adipophilin ablation inhibits foam cell formation and protects against atherosclerosis development.11 Importantly, specific adipophilin ablation inhibits foam cell formation and protects against atherosclerosis development.11

The CE Futile Cycle

Newly synthesized cholesterol, as well as lipoprotein-derived cholesterol, can be incorporated into LDs in the ER, where the ER-resident protein ACAT catalyzes the esterification of excess cholesterol for storage in LDs. Cholesterol in the LD undergoes constitutive cycles of esterification hydrolysis, which controls cholesterol availability for cell membranes and for efflux, whereby unesterified cholesterol released from the LD by CE hydrolysis can be effluxed to a cholesterol acceptor if one is present—which results in net CE hydrolysis—or otherwise reesterified by ACAT.12,13 The original studies that characterized this “futile cycle” concluded that cytoplasmic CE hydrolysis in macrophage foam cells was mediated by extralysosomal, cytoplasmic neutral CE hydrolases (CEHs).12,14

Neutral Lipolysis

Since the macrophage foam cell CE cycle was first characterized in the 1970 to 1980s, numerous candidate neutral hydrolases have been proposed. Despite general agreement that LD lipolysis is a critical step in the regulation of foam cell accumulation and atherosclerosis, significant advancements in our understanding of this process have been impeded by the controversies surrounding the exact identity of the macrophage neutral hydrolase.15 First, CEH, or carboxylesterase 1 (CES1) (the human homolog of the murine TG hydrolase), is expressed in human (but not in murine) macrophages and exerts CEH activity. Because hydrolytic enzymes must first gain access to the LD neutral lipid core to exert functional lipolytic activity, an important finding is that CEH translocates from the cytoplasm to LDs on lipid loading.16 CEH overexpression in macrophages reduces CE accumulation and enhances cholesterol efflux,17 whereas macrophage-specific CEH overexpression in vivo increases RCT and reduces atherosclerosis.18

Second, hormone-sensitive lipase (HSL), present in murine but not in human macrophages, is a longstanding candidate enzyme for macrophage CE hydrolysis, although its role in macrophage lipolysis remains controversial.19 HSL is a cytoplasmic enzyme that translocates to LDs on activation.20 Macrophage-specific HSL and apolipoprotein A-IV transgenic expression in mice fed an atherogenic diet reduces the size of aortic lesions,21 similar to what is observed on increased macrophage CEH activity.

A third neutral hydrolase reported to mediate CE hydrolysis in both murine and human macrophages is neutral cholesterol ester hydrolase 1 (Nceh1), with KIAA1363 being its human ortholog.22,23 Absence of this enzyme in macrophages challenged with acetylated LDL results in increased cellular CE content and decreased cholesterol efflux.23 Like CEH, Nceh1 is a member of the carboxylesterase family, and these are microsomal enzymes with active sites facing the ER lumen, raising interesting but unresolved questions as to how these enzymes gain access to cytosolic LDs.24 Although the mechanism of this association is unclear, this interaction possibly arises from a close association of LDs with the ER or, alternatively, occurs via presently uncharacterized associating molecules.20 Together, knock down of Nceh1 and HSL in macrophages nearly completely abolishes all CE hydrolysis in cell-free assays.23 In vivo, macrophage-specific ablation of Nceh1 increases atherosclerosis plaque development, as does macrophage-specific HSL ablation, and together a compound lack of macrophage Nceh1 and HSL has an additive effect on promoting atherogenesis.23 A contradictory report stating that whereas HSL plays a role in macrophage CE hydrolysis Nceh1 exhibits no CEH activity emphasizes that the identity or specificity of macrophage neutral CEHs is still a matter of debate.25,26 In summary, neutral LD CE hydrolysis in macrophages appears to be a multi-enzyme process, with HSL, Nceh1, and CEH each contributing to this process to varying degrees in murine and human macrophages. Additional studies are required to fully elucidate the exact role of each of these important candidates in macrophage lipolysis.

An important limitation in understanding the pathways that mediate foam cell lipolysis stems from the frequent use of cell-free enzymatic assays, which can be problematic. When neutral hydrolases come into contact with their substrate, LDs, they undergo a conformational change on adsorption at the water/lipid interface.15 The interfacial microenvironment is thus an important factor for enzymatic activity, and surfactants or detergents can expel interfacial enzymes from the surface of LDs and reduce their activity.15 In a number of cell-free assays, detergents are included, which can affect the overall lipolytic activity of these interfacial enzymes and impede accurate measurements of hydrolysis. The ideal way to characterize CE hydrolytic enzymes in cell-free assays is to thus present the substrate in its physiological form (as droplets), a methodology that has unfortunately not widely been used.15 Although the exact identity of macrophage-specific CEHs remains to be clarified, a common point to all of these studies is that enhancing LD-associated CE hydrolysis increases cholesterol efflux and is antiatherogenic. Understanding how cholesterol is mobilized from LDs offers new options for modulating cholesterol efflux.

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Acid Lipolysis

Following receptor-mediated endocytosis, lipoprotein-associated CE and TG are hydrolyzed by an active cholesterol esterase with optimal activity in the acid pH of the lysosomal lumen. Lysosomal acid lipase (LAL) is the critical enzyme for the hydrolysis of neutral lipids delivered to lysosomes, and mutations in the gene encoding this enzyme lead to cholesterol storage disorders. LAL deficiency in humans leads to 2 phenotypes: CE storage disease (CESD) and Wolman disease. An infantile onset disorder, Wolman disease has a more severe phenotype, with death usually occurring within the first year of life because of massive CE and TG accumulation in the liver and small intestine, resulting in cachexia due to malabsorption. CESD is a milder, later onset disorder, characterized by CE accumulation in visceral tissues; higher residual LAL activity in patients affected by CESD as compared with Wolman disease may be the basis for the milder phenotype of CESD.

Because efforts were concentrated on measuring candidate CEH enzymatic activity in cell-free assays, potential endocytic pathways that contribute to this process—requiring cytoskeletal integrity to transpire—were not discovered. An effective way to characterize the hydrolytic arm of the CE cycle is to incubate cells in the presence of an extracellular cholesterol acceptor and an ACAT inhibitor (to prevent cholesterol reesterification following hydrolysis), and to measure the amount of CE remaining as compared with the initial amount of cellular CE; in this way, dissipation of CE can be expressed as percentage of hydrolysis and the contribution of various enzymes/pathways to this process can be measured in a biologically relevant context, using LDs as substrate. Using this method, we observed that paraoxon—an inhibitor of all neutral lipases—did not abolish all lipolysis in macrophage foam cells. This led to the discovery that lysosomes and, more specifically, LAL hydrolyze LD CE.

Autophagy

Autophagy is an ancient, evolutionary conserved process that plays a major role in the degradation of cellular components. Essentially, autophagy is a trafficking pathway that delivers cytosolic constituents to the cell’s lytic compartments. Autophagy recycles stable cytosolic macromolecules to supply nutrients and maintain essential cellular functions under starvation conditions, and it has an important housekeeping function in the clearance of damaged proteins and organelles, long-lived proteins and protein aggregates, as well as serving to combat infection by a number of pathogens. There exist different forms of autophagy: (1) chaperone-mediated autophagy, where single proteins are recognized by cytosolic chaperones and delivered to lysosomes; (2) microautophagy, where cellular components are taken into lysosomes by invagination and pinching of the lysosomal membrane into the lumen; and (3) macroautophagy, where cargo is sequestered in de novo formed vesicles called autophagosomes, which subsequently fuse with lysosomes.

Even though autophagy was originally described in the 1960s, the first proteins contributing to this process—autophagy-related proteins, or Atgs—were identified only in 2008, making autophagy a relatively new and thriving field of study. There are now more than 30 Atg proteins identified; these organize into functional complexes that oversee the autophagic process, first controlling the formation of single lipid bilayer membranes (limiting membranes, or phagophores) that bud from preexisting organelles, such as the ER, and then modulating membrane elongation to form cup-shaped structures that engulf the cytoplasm and fuse at the mouth, giving rise to spherical autophagosomes. Subsequent autophagosomal fusion with lysosomes releases the autophagic body into the lysosome lumen, where it is degraded.

Macroutophagy (hereafter referred to as autophagy), can sequester cytosol in bulk or selectively. Autophagy’s contribution to lipid metabolism was recently elucidated through the discovery of macroautophagy, which constitutes the engulfment of neutral LDs by the autophagic machinery and their delivery to lysosomal lipases for breakdown in the lysosomal lumen. Impaired autophagy in the liver leads to abnormally high levels of hepatic cholesterol along with aberrant TG deposition because of the defective clearance of LDs. Whereas hepatic lipolysis has been largely viewed as a process regulated by cytosolic lipases, this seminal report forces us to reconsider this traditional view. That autophagy regulates cholesterol homeostasis is an emerging concept that has important implications in regards to atherosclerosis and cardiovascular disease. Indeed, sterol depletion has directly been shown to induce autophagy in human fibroblasts, although the exact mechanism by which cholesterol depletion initiates autophagy was not clear. Recently, sterol regulatory element binding protein 2 was linked to activation of the autophagy gene network in response to starvation, whereby several autophagy genes were identified as sterol regulatory element binding protein 2 targets. Thus, the LD can be viewed, in addition to de novo cholesterol synthesis and LDL receptor-mediated uptake, as a third source of cellular cholesterol regulated by sterol regulatory element binding protein 2. Whereas it is clear that cholesterol depletion initiates autophagy, the role of autophagy under conditions of intracellular lipid accumulation, of particular relevance to many diseased states, such as atherosclerosis, remains uncertain.

Autophagy, LAL, and Cholesterol Efflux

We have recently shown that under lipid-loading conditions, autophagy mediates the delivery of cytoplasmic LDs to lysosomes in macrophages and that LAL in the lysosomal lumen hydrolyzes LD CE to generate free cholesterol for efflux. It is surprising that the contribution of the lysosome to LD-cholesterol efflux was overlooked for so long, but in fact evidence that this pathway is active in macrophage foam cells exists throughout the literature. Using electron microscopy to characterize the CE cycle in murine macrophage foam cells, lamellar arrangements adjacent to LDs, projecting into the matrix of the droplets, often appearing in continuity with ER juxtaposed to LDs, were frequently observed (approximately 20% of LDs contained these specialized mem-
brane structures). Could these represent nascent autophagosomes at the LD periphery? Similarly, in another study describing CE accumulation in macrophages, although membrane-bound LDs were occasionally observed, most of them were not surrounded by a classical membrane. Could these occasional membrane-bound LDs represent LDs sequestered in autophagosomes? In 1999, Avart et al reported that the hydrolysis of cytoplasmic CE in CE-enriched macrophages occurs both in the cytoplasm and in lysosomes. In this report, the authors had no explanation for these “puzzling” observations and stated that “these data raise the question of why cytoplasmically synthesized LDs should find their way to lysosomes at all.”

In perfect accordance with the report from Avart et al, when we tested the functional importance of this observation, we found that a fraction of the cytoplasmic CE hydrolysis in CE-enriched macrophages could be attributed to neutral lipases whereas the other was mediated by a chloroquine-sensitive lysosomal function (Figure). Similar to what Avart et al described in their study, we also observed a portion of the cytoplasmic LD pool in lysosomes by electron microscopy. We have found autophagy to be specifically induced in response to atherogenic lipoprotein loading, and thus the autophagy-LAL pathway for LD CE hydrolysis is one that is explicitly triggered in foam cells. Autophagy induction by oxidized LDL has previously been reported in human vascular endothelial cells, and autophagy was proposed to play a role in oxidized LDL processing on its uptake by these cells. Conversely, we did not uncover a requirement for autophagy in oxidized LDL or acetylated LDL degradation, because lipoprotein-CE was hydrolyzed and excess lipoprotein-derived cholesterol was reesterified to accumulate as cytoplasmic CE to a similar extent in WT and Atg5−/− macrophages, which lack the autophagy-essential Atg5 protein and in which autophagy consequently cannot ensue. This is in accordance with the classical pathway by which lipoprotein-derived neutral lipids are first lysosomally hydrolyzed. Nevertheless, a common conclusion to both studies is that atherogenic lipoproteins trigger autophagy.

At any given time in the macrophage foam cell, only a small proportion of LDs are transported to lysosomes, but cumulatively this results in a significant contribution to LD breakdown and cholesterol efflux. The movement of cholesterol out of lysosomes is a poorly understood process, but it involves the cholesterol trafficking proteins Niemann-Pick type C 1 and 2. Mutations in either of these key Niemann-Pick type C proteins are a cause of lysosomal storage disorders, characterized by late endosomal/lysosomal cholesterol retention. Thus, LAL in the lysosomal lumen is critical for generating both lipoprotein- and LD-derived free cholesterol, which is subsequently released from the late endosome/lysosomal compartment via Niemann-Pick type C proteins, ABCA1 (see below), or both for efflux or reesterification.

The export of cholesterol mobilized by autophagy reveals itself to be an ABCA1-dependent process. Whereas ABCA1 mediates the transfer of phospholipids and cholesterol to apolipoprotein A-I (the major high-density lipoprotein apolipoprotein), which is associated with very little or no lipid, at
the plasma membrane, it can also acquire lipid from intracellular sites, such as the endosomal/lysosomal compartment and from the Golgi. ABCA1 traffics between the cell surface and late endocytic vesicles and preferentially mobilizes cholesterol deposited in late endosomes/lysosomes to stimulate cholesterol efflux. Specifically, the internalization and shuttling of ABCA1 is functionally important for the efflux of cholesterol out of endosomal compartments. This is corroborated by the observation that in Niemann-Pick type C1-deficient cells, where cholesterol accumulates in late endosomes/lysosomes, enhancing ABCA1 expression is able to overcome the impaired flux of cholesterol out of these compartments. Shuttling of the ABCA1 transporter from the plasma membrane to late endosomal/lysosomal compartment is increased in lipid-loaded cells as compared with unloaded cells, highlighting the importance of this pathway in cells that have accumulated excess lipoprotein-derived cholesterol. Incidentally, autophagy is explicitly triggered in lipid-loaded macrophages, thereby redistributing cytoplasmic LD cholesterol to lysosomes for removal by ABCA1, which is particularly effective at mobilizing cholesterol from intracellular compartments for efflux under these conditions.

The recent discovery of a lysosomal pathway for LD hydrolysis contradicts earlier findings that CE hydrolysis in macrophage foam cells is extralysosomal and requires a neutral CEH. Indeed, when cholesterol efflux is carried out in LDL- or acetylated LDL-loaded macrophages for 4 hours, chloroquine does not inhibit cholesterol efflux; it is only during longer efflux periods that chloroquine and LAL inhibition reduces cholesterol efflux, and only in lipid-loaded macrophages where autophagy is specifically induced. The trafficking of ABCA1 from the plasma membrane to endolysosomal compartments to transfer free cholesterol released from LDs through the enzymatic action of LAL may be limiting and explains why this efflux proceeds relatively slowly as compared with cytoplasmic lipolysis, which presumably generates free cholesterol primarily accessible to the endosomal recycling compartment and plasma membrane for rapid efflux. Additionally, the sequence of events leading to LD delivery to LAL by autophagy is a lengthy process compared with cytoplasmic hydrolysis. It requires sequestration of LDs in autophagosomes, fusion of LD-containing autophagosomes with endosomes or lysosomes, acidification of the newly fused endocytic compartments, and degradation of the autophagosomal membrane to release the cargo in the lysosomal lumen for degradation. Alternatively, cholesterol efflux may begin with readily available free cholesterol at the plasma membrane and in the endosomal recycling compartment as a primary source, forming a cholesterol gradient in the membranes of these organelles; following this, efflux from LAL-derived LD cholesterol in the endolysosomal network ensues.

A new role for LAL in the hydrolysis of LD cholesterol for efflux suggests that LAL activity in macrophage foam cells of atherosclerotic lesions could promote macrophage RCT and exhibit antiatherogenic properties. Overexpression of LAL has been shown to prevent atherogenesis and to promote the regression of established atherosclerotic lesions. Consequently, enhancing LAL activity has been proposed as a promising antiatherogenic therapy, similar to overexpression of neutral cholesterol ester hydrolases. Interestingly, recent results show that LAL activity contributes to the regulation of ABCA1 expression and high-density lipoprotein formation, which could explain why low plasma high-density lipoprotein cholesterol and premature atherosclerosis often occur in CESD patients. On the other hand, 2 recent genome-wide association studies report links of a gain-of-function LAL mutation with enhanced susceptibility to coronary artery disease. Elevated LAL expression, presumably leading to increased LAL activity, was associated with lower high-density lipoprotein cholesterol levels. Follow-up functional studies are required to clarify how either low or high LAL activity is protective or causative of premature coronary artery disease and to further evaluate how macrophage-specific LAL activity influences atherosclerotic lesions, but it is clear that LAL plays an important role in the pathogenesis of atherosclerosis and coronary artery disease.

**The Future of LD Cholesterol Efflux and Heart Disease**

Macrophage LDs cannot be considered passive fat deposits that store cholesterol. They are dynamic, highly motile organelles that frequently come into contact with vesicles of the endolysosomal network. We have long understood that the macrophage LD CE pool undergoes a continual cycle of hydrolysis and reesterification. Whereas the presence of a cholesterol acceptor interrupts the CE cycle, promoting net CE hydrolysis and cholesterol excretion, this net hydrolysis is achieved primarily via a reduced rate of ACAT-mediated cholesterol reesterification rather than an increase in the rate of hydrolysis per se. Evidence for regulation of the hydrolysis arm of the CE cycle has thus far been lacking. That autophagy is triggered in macrophage foam cells provides evidence that macrophage LD CE hydrolysis is not exclusively a constitutive process—its inherent plasticity provides exciting new ways to manipulate it to promote cholesterol efflux. Unraveling the precise molecular mechanisms that underlie cholesterol mobilization from LDs in macrophage foam cells could have a tremendous impact on the development of new therapeutics for the treatment of atherosclerosis.

**Conclusion**

Understanding the pathways that regulate the mobilization of cholesterol from macrophage LDs will bring novel therapeutic strategies that promote arterial macrophage RCT and reduce the lipid burden in atherosclerotic plaques. Both cytoplasmic and lysosomal lipolysis are critical for the efficient removal of cholesterol from macrophage foam cells, and future development of clinical strategies to enhance macrophage RCT should target both pathways. Autophagy and LAL represent new targets for the regulation of macrophage RCT, and future studies to elucidate their role in foam cell biology will certainly provide new insights into atherogenesis, coronary artery disease, and potential therapeutic avenues to treat them.
Disclosures

None.

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