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Macrophage Cholesteryl Ester Mobilization and Atherosclerosis

Shobha Ghosh^{*}, Bin Zhao, Jinghua Bie, and Jingmei Song

Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA 23298-0050

Abstract

Accumulation of cholesteryl esters (CE) stored as cytoplasmic lipid droplets is the main characteristic of macrophage foam cells that are central to the development of atherosclerotic plaques. Since only unesterified or free cholesterol (FC) can be effluxed from the cells to extracellular cholesterol acceptors, hydrolysis of CE is the obligatory first step in CE mobilization from macrophages. This reaction, catalyzed by neutral cholesteryl ester hydrolase (CEH), is increasingly being recognized as the rate limiting step in FC efflux. CEH, therefore, regulates the process of reverse cholesterol transport and ultimate elimination of cholesterol from the body. In this review, we summarize the earlier controversies surrounding the identity of CEH in macrophages, discuss the characteristics of the various candidates recognized to date and examine their role in mobilizing cellular CE and thus regulating atherogenesis. In addition, physiological requirements to hydrolyze lipid droplet-associated substrate and complexities of interfacial catalysis are also discussed to emphasize the importance of evaluating the biochemical characteristics of candidate enzymes that may be targeted in future to attenuate atherosclerosis.

Keywords

Macrophage; Cholesteryl Ester Hydrolase; Atherosclerosis; Reverse Cholesterol Transport

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in United States and according to the Heart and Stroke Statistics released by American Heart Association, one in three American Adults (~80,000,000) has one or the other form of cardiovascular disease, including CAD, and it accounts for one in 2.8 deaths, that is equal to all deaths due to cancer, chronic lower respiratory disease, accidents and diabetes combined. The underlying cause of CAD is atherosclerosis that is characterized by deposition of lipids in the artery wall.

Atherosclerosis is a chronic disease that often starts during early teens and progresses silently without any overt clinical symptoms till about age 40 when it manifests as heart attack or even stroke. Accumulation of lipid-laden macrophage foam cells in the artery wall is the hall mark of atherosclerosis. Two different, though not mutually exclusive, theories are proposed to describe the events involved in atherogenesis. According to “response to injury” hypothesis, the initiating event is injury to the endothelial lining of the artery wall leading to the subsequent migration of monocytes and circulating lipoproteins mainly the low density lipoprotein (LDL) into the intimal space followed by the unregulated uptake of modified LDL by monocyte derived macrophages leading to the formation of foam cells (Ross et al, 1977, Ross, 1993).

^{*}Corresponding Author: Shobha Ghosh, Ph.D., FAHA, Professor of Medicine and Physiology, Department of Internal Medicine, Division of Pulmonary and Critical Care, Virginia Commonwealth University, Richmond, VA 23298-0050, Ph: 804-827-1012, Fax: 804-628-1204, shobha@vcu.edu.

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“Response to retention” hypothesis, on the other hand, proposes that LDL migrates into the intimal space and is retained by association with the proteoglycans and is modified. Subsequent uptake by infiltrating macrophages results in development of foam cells (Williams and Tabas, 1995). Regardless of the sequence of events, the end result is accumulation of foam cells which initiates the formation of fatty streaks. With continued that accretion of foam cells these fatty streaks develops into an atherosclerotic plaque. Lipid burden of an atherosclerotic plaque not only contributes to the volume but also enhances plaque associated inflammation and thus determines its vulnerability to rupture (Davies and Thomas, 1985). Therefore, reduction in the lipid core of the plaque is a favored strategy to decrease plaque volume as well as increase plaque stability (Libby and Aikawa, 1998). Currently used therapies for CAD include cholesterol lowering drugs such as statins that reduce plasma cholesterol by inhibiting the endogenous cholesterol synthesis. Although one of the pleiotropic effects of statins is to reduce inflammation and improve plaque stability, no direct strategy is presently available to reduce the lipid burden of atherosclerotic plaque. Cleveland Clinic study by Nissen et al (Nissen et al, 2003) provided the first direct evidence of the clinical benefit of enhancing removal of cholesterol from macrophage foam cells. Accumulation of cholesterol as cholesteryl esters (CE) and mobilization of CE to generate free cholesterol (FC) for efflux is regulated by several interactive pathways, the understanding of which is critical to develop therapeutic strategies directed towards enhancing FC efflux and decreasing lipid burden of the plaques.

CE accumulation and mobilization in macrophages

While like any other peripheral tissue, macrophages take up LDL via LDL-receptor, a pathway regulated at the levels of LDL receptor (LDLR) expression by cellular cholesterol content, macrophages also express scavenger receptors such as CD-36 and SR-A that permit unregulated uptake of modified LDL. This scavenging function is initially important in clearance of modified lipoproteins but if persistent, leads to massive accumulation of CE in macrophages resulting in the formation of foam cells. CEs are stored in the cytoplasm as lipid droplets and are in dynamic equilibrium with FC via a process first described as cholesteryl ester cycle by Brown et al (Brown et al, 1980). Lipoprotein derived CEs are hydrolyzed in late endosomes/lysosomes by acid cholesteryl ester hydrolase and the FC released is re-esterified on the endoplasmic reticulum by acyl CoA:cholesterol acyltransferase-1 (ACAT1). The resulting CE are stored in the cytoplasm as lipid droplets and hydrolyzed by neutral cholesteryl ester hydrolase (CEH) to release FC for efflux. Excess FC is, once again, re-esterified by ACAT1 to prevent FC associated cell toxicity. Thus, cellular FC can be considered to have two fates namely re-esterification by ACAT1 or efflux to extracellular acceptors (Figure 1). Inhibiting ACAT was one of the first strategies to reduce cellular CE accumulation and pharmacological inhibition of ACAT as a means to prevent or attenuate foam cell formation has been extensively pursued (Matsuda, 1994, Matsuo et al, 1995, Nicolosi et al, 1998, Sliskovic and White, 1991). An alternate approach would be to enhance CE hydrolysis by increasing CEH activity. While intuitively similar with respect to reducing cellular CE content, these two approaches are fundamentally different. Under conditions of ACAT inhibition, decrease in cellular FC can only be achieved by extracellular acceptor mediated FC efflux. Thus, ACAT inhibition results in increase in cellular FC accumulation, both in plasma membrane and in the endoplasmic reticulum resulting in toxicity, ER stress and apoptosis (Figure 1, Bottom right). These effects of ACAT inhibition and associated FC-induced toxicity can be partially relieved by increasing the concentration of extracellular cholesterol acceptors (Warner et al 1995). On the other hand, with increased CEH-mediated CE hydrolysis, the resulting FC can either be re-esterified by ACAT (Fate 1) or effluxed from the cell (Fate 2). Thus, even under conditions of limiting acceptor concentration, FC released by CEH mediated hydrolysis is re-esterified by functional ACAT1 preventing any increase in cellular FC.

Data from direct comparison of these two strategies on cellular FC and CE content establishing the fundamental differences are shown in Figure 2. Using agmACAT1 cells with stable over-expression of ACAT1, we have compared the effects of ACAT inhibition and transient CEH over-expression under conditions of limited (0.5% BSA) or sufficient (10% FBS) extracellular FC acceptors. Under conditions of ACAT inhibition FC readily accumulates in the cells (Panel A, Compare data from ACAT-sufficient and ACAT-inhibited cells). Yet, CEH over-expression under these conditions still reduced CE content without further increasing cellular FC content. More importantly, in the presence of sufficient extra-cellular acceptors, CEH over-expression resulted in significantly higher CE mobilization which was further increased in ACAT-inhibited cells and there was no increase (rather a small decrease) in cellular FC levels. These data provide direct experimental evidence that, unlike ACAT inhibition, CEH over-expression does not lead to increase in cellular FC content and that these two intuitively similar strategies for reducing cellular CE content are fundamentally different (Figure 1). As will be discussed below, these differences manifest as different outcomes in vivo; ACAT inhibition or deficiency leading to FC accumulation associated toxicity and xanthoma formation and transgenic over expression of CEH resulting in attenuation of CE accumulation and atherosclerosis.

Even prior to the identification of two genes for ACAT (ACAT1 in macrophages as well as other tissues and ACAT-2 restricted to liver and intestine), pharmacological inhibition of ACAT was pursued to reduce intracellular accumulation and several inhibitors were developed and tested for the potential to reduce foam cell formation (Matsuda, 1994, Matsuo et al, 1995, Nicolosi et al, 1998, Sliskovic and White, 1991). However, in vivo preferential pharmacological inhibition of macrophage ACAT or ACAT-1 led to an increase plaque formation in mouse and rabbit models of atherosclerosis (Perrey et al, 2001). Mice deficient in ACAT1 displayed marked systemic abnormalities in lipid homeostasis in hypercholesterolemic Apo-E deficient and LDL-receptor deficient mice, leading to extensive deposition of free cholesterol in skin and brain (Accad et al, 2000, Yagu et al, 2000) consistent with the cellular effects of ACAT inhibition shown in Figure 2B. Fazio *et al* reported increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophage (Fazio et al, 2001). The reduced content of macrophage and neutral lipids in lesions lacking ACAT1 may have beneficial effects on lesion stability (Boan et al, 2000). However, the increase in lesion area and the systemic lipid abnormalities observed in ACAT1-deficient mice suggest that deficiency of ACAT1 could have detrimental effects (Glass and Witztum, 2001) consistent with increase in cellular FC associated toxicity.

Several lines of evidence are available to suggest that intracellular CE hydrolysis is an important step in regulating CE mobilization. Macrophages with high neutral CEH activity accumulate less cholesterol esters in the presence of atherogenic β -migrating very low-density lipoproteins (β -VLDL) in comparison to macrophages with low CEH activity (Ishii et al, 1992). Animal models of atherosclerosis, such as the hypercholesterolemic rabbit and the White Carneau pigeon, appear to possess macrophages in which stored cholesterol esters are resistant to hydrolysis and subsequent mobilization (Mathur et al, 1985, Yancey and St. Clair, 1994). Hakamata et al attributed the species-specific differences in macrophage foam cell formation to differences in CE mobilization (Hakamata et al, 1994). Despite compelling evidence for the importance of CEH-mediated CE mobilization as a critical step in regulating foam cell formation and atherosclerosis, efforts to evaluate the role of CEH to attenuate cellular CE levels were hindered by the controversies surrounding the identity of the enzyme responsible for macrophage CE hydrolysis. Some of these controversies and the potential candidate enzymes catalyzing macrophage CE hydrolysis are discussed below.

Macrophage CE Hydrolysis

Brown et al demonstrated for the first time that in macrophage foam cells, CE hydrolysis is extra lysosomal and defined the “need” for a neutral cholesteryl ester hydrolase (CEH) that can release FC from the lipid droplet associated CE (Brown et al, 1980). Since then, several candidate enzymes have been proposed as the likely candidates responsible for this activity and Table 1 summarizes the characterization of these possible candidates. Based on the observations that cAMP enhances FC efflux from macrophages and hormone sensitive lipase (HSL) is activated by cAMP, HSL was thought to be the likely candidate for macrophage CE hydrolysis (Goldberg and Khoo, 1990, Small et al, 1989). HSL is expressed in murine macrophages (Khoo et al, 1993) but its expression in human macrophages remains controversial (Johnson et al, 2000, Li and Hui, 1997, Reue et al 1997). We were unable to detect HSL expression in human macrophage cell line THP1 and in human blood derived monocyte macrophages (unpublished observations). Harte et al reported very low expression of HSL in rabbit macrophages suggesting that HSL expression is limiting in atherosclerosis-susceptible animals. Over-expression of HSL by transient transfection in RAW 264.7 murine macrophages led to increased mobilization of CE in the presence of an ACAT inhibitor (Escary et al 1998) but macrophage-specific transgenic expression of HSL led to a paradoxical increase in atherosclerosis and macrophages isolated from these mice stored 2–3 fold higher CE when incubated with AcLDL in vitro (Escary et al 1999). In addition, macrophages from HSL deficient mice did not have reduced CE mobilization suggestive of a limited, if any, role of HSL in macrophage CE mobilization (Osuga et al 2000). Since the effects of HSL deficiency on atherosclerosis have not been studied, it remains to be seen whether HSL plays a role in atherogenesis.

The second candidate enzyme speculated to play a role in macrophage CE mobilization was carboxyl ester lipase or CEL. CEL was first characterized from pancreas as a bile salt dependent cholesteryl esterase (Gallo, 1981, Kissel, 1989). It was also present in other tissues such as mammary glands and was secreted in milk to facilitate digestion of CE in infants and newborns (Hui and Kissel, 1990). Li and Hui reported the absence of HSL in human monocyte/macrophage cell line THP1 as well as primary blood derived monocyte macrophages and demonstrated the presence of CEL in these cells (Li and Hui, 1997). However, this is a secretory enzyme and was thought to play a limited role in intracellular CE metabolism.

We purified and cloned rat liver neutral cytosolic CEH that belonged to the carboxylesterase family and distinctly different from HSL and CEL (Ghosh and Grogan, 1991, Ghosh et al, 1995). Using the strategy of homology cloning, we identified human macrophage CEH (Official gene symbol *CES1*, Accession number *NG_012057*) and demonstrated its expression in the THP1 human monocyte/macrophage cell line, as well as in human peripheral blood monocyte/macrophages (Ghosh 2000). This enzyme associated with the surface of lipid droplets in lipid-laden cells (its physiological substrate) and hydrolyzed CE present in lipid droplets (Zhao et al, 2005). Over-expression of this enzyme resulted in mobilization of cellular CE (Ghosh et al, 2003) demonstrating its role in regulating cellular CE accumulation. Stable over-expression of this CEH in human monocyte/macrophage cell line, THP1, resulted in significantly higher FC efflux to ApoAI, HDL and serum demonstrating that FC released by CEH-mediated hydrolysis of intracellular CE is available for efflux by all known pathways (Zhao et al, 2007). Taken together, these data support the role of this enzyme in regulating macrophage CE content and FC efflux.

Carboxylesterases as Cellular Neutral CEHs

Carboxylesterases (gene symbol *CES*) have long been considered as non-specific esterases involved in xenobiotic metabolism (see Satoh and Hosokawa, 1998 for detailed review) known

to hydrolyze aromatic and aliphatic esters. Further, these enzymes also play a role in detoxification in the lung and/or protection of the central nervous system from ester or amide compounds. Accordingly, purified enzymes were not tested for their ability to hydrolyze acyl esters of glycerol or cholesterol and the commonly used substrate to test for enzyme activity is p-nitro phenyl ester. Identification of rat hepatic CEH as a member of carboxylesterase family, for the first time, demonstrated the role of these enzymes in lipid metabolism. In humans, there are at least four CES genes present on chromosome 16 (*CES1*, *CES2*, *CES4* and *CES7*) although complete characterization and knowledge of the physiological role of the respective gene products is lacking except for *CES1*. In addition to catalyzing the hydrolysis of ester and amide bonds in cocaine and heroin (Pindel et. al. 1997), we demonstrated that human *CES1* also hydrolyzes the acyl ester bonds in CE and triacylglycerols and subsequent work from our laboratory established the role of this enzyme in cellular CE mobilization from macrophage foam cells as described above. In addition, we have also cloned and characterized *CES1* from human liver (Zhao et al, 2005) and demonstrated that this enzyme can enhance the hydrolysis of HDL-delivered CE, increase reverse cholesterol transport and boost bile acid excretion in the feces (Zhao et al 2008).

In mice, Dolinsky et al were the first to characterize a carboxylesterase from liver that hydrolyzed triacylglycerol and based on its activity it was designated as triglyceride hydrolase or TGH (Dolinsky, 2001). Analysis of mouse genome suggests that at least 9 different genes with homology to TGH are present on chromosome 8 and these belong to the carboxylesterase family of genes. The official gene symbol for TGH is *Ces3*. Similar to human carboxylesterase family, these enzymes were also characterized for their ability to detoxify xenobiotics (Sato and Hosokawa, 1998). It is, therefore, tempting to speculate that in mice the higher number of these genes is in accordance with the scavenging feeding habits of mice that increase the exposure to xenobiotics. Once again, the enzymes characterized to date with the exception of TGH, have only been tested for their ability to hydrolyze ester and amide bonds in xenobiotics and their role in metabolism of endogenous lipids remains to be established. In addition to CEH and TGH, another enzyme with CE hydrolytic activity was recently described (Okazaki et al, 2008). This was originally identified as arylacetamide deacetylase-like 1 (AADACL1), a gene present on human chromosome 3 and with partial homology to a gene on mouse chromosome 3. Although there is no sequence homology between AADACL1 and CEH or TGH, it appears to share the same catalytic triad that is required for the hydrolysis of ester bonds. Since these enzymes catalyze the hydrolysis of hydrophobic substrates, the activities are often measured using simple water soluble esters such as p-nitro phenyl esters or substrates presented in the form of emulsions containing phospholipids and detergents such as bile salts. It needs to be emphasized that CE in macrophage foam cells are present as droplets and for any of these enzymes to be physiologically relevant, it has to catalyze hydrolysis of the substrate presented in the same physiological form. Thus, despite nucleotide or protein structure differences, any enzyme that can hydrolyze CE presented as lipid droplets would function as an intracellular CE hydrolase and enhance CE mobilization. In addition, keeping in view that several candidate enzymes can potentially hydrolyze intracellular CE, it is more critical to recognize the importance of CE hydrolysis than to associate this activity with one candidate protein.

Complexity of Intracellular lipid droplet associated CE hydrolysis

In foam cells, CE-rich lipid droplets accumulate in the cytoplasm. CEs are synthesized by ACAT1 on the endoplasmic reticulum (ER) and it is currently that these newly synthesized CE as well as triglycerides initially accumulate in between the two leaflets of ER membrane. Lipid droplets (LD) are subsequently released by budding into the cytoplasm together with part of the cytoplasmic leaflet or monolayer of the ER membrane (Figure 3). PAT (Perilipin, Adipophilin and Tip47) family of LD associated proteins such as perilipin, ADRP (adipose

differentiation-related protein) or Tip47 are then required for the stabilization of these LDs. Enzymes involved in the hydrolysis of TG or CE contained within the LDs must gain access to these substrates by associating with LD. CEL is secreted and thus, cannot associate with intracellular LDs. HSL that is present in the cytoplasm, once phosphorylated by Protein kinase A, associates with LD in a perilipin-dependent manner and facilitates TG hydrolysis in adipocytes and may be even CE hydrolysis in macrophages (Clifford et al 2000). We have demonstrated translocation of cytoplasmic CEH to lipid droplets upon lipid loading (Zhao et al 2005). Newly identified enzyme KIAA1363 with CE hydrolytic activity resides in the ER and it remains to be seen as to how this enzyme gains access to its LD associated substrate (Igarashi et. al. 2009).

Enzymes acting on a lipid/water interface have stringent requirements for the surface characteristics for efficient binding and catalysis and therefore, activities are critically dependent on the substrate presentation (Figure 4). Although detailed description of interfacial catalysis is beyond the scope of this review, a few relevant and important considerations are discussed here (For detailed review, see Reis et al, 2009). Lipolytic enzymes undergo a conformational change upon adsorption at the lipid/water interface and recent structural studies have shown that the active site is covered with a α -helical loop, i.e., a lid that renders it inaccessible for the substrate until the enzymes bind to the hydrophobic substrate (Belle et al 2007). Presence of surfactants and detergents on the interface as well as accumulation of some catalytic products can expel the enzyme from the surface and reduce enzyme activity (Gargouri et al, 1983). The reaction rates are therefore, controlled by the overall composition of the interfacial microenvironment. Thus, in vitro lipolysis of triglycerides is decreased when substrate is presented as lecithin containing emulsions and non ionic detergents such as Triton X-100 displace the enzyme from the surface of the substrate and reduce activity (Reis et al, 2008). Deykin and Goodman demonstrated very early in CE hydrolytic enzyme(s) characterization that highest enzymatic activity is obtained when substrate is presented as droplets (dissolved in acetone). Presentation of the substrate as an emulsion containing detergents such as Triton x-100 or bile salts significantly reduced enzyme activity (Deykin and Goodman, 1962). Thus, in addition to appropriate sub-cellular localization, ability to hydrolyze CE in its physiological droplet form becomes an important criterion for any CE hydrolase to be physiologically relevant and play a role in intracellular CE hydrolysis and subsequent mobilization. We have characterized CEH using substrate presented as droplets and shown that this enzyme can hydrolyze pure CE droplets as well as mixed droplets containing CE and TG. Inability to detect activity of CEH and TGH, as rationalized by Okazaki et al to identify a new candidate CE hydrolase (KIAA1363) using proteomics approach (Okazaki et al, 2008) (42), may as well be a manifestation of the assay used where the substrate was presented as emulsions containing lecithin as well as detergents (Hajjar et al, 1983). The enzymatic activity of HSL or KIAA1363 towards physiologically relevant physical state of the CE substrate remains to be evaluated.

Role of CE hydrolysis in atherosclerosis

Despite the apparent uncertainty of the major CE hydrolase, its sub-cellular localization and its ability to associate with its physiological substrate (CE associated with cytoplasmic LDs), the importance of this step in mobilization of CE and thereby attenuating atherosclerosis cannot be over-emphasized. This hypothesis was initially tested by development of HSL transgenic mice (Escary et al, 1999). Paradoxically, these mice had increased atherosclerosis and it was thought to be due to limiting extra cellular acceptors. Subsequently, Choy et al developed ApoA IV and HSL double transgenics in C57BL/6 background and demonstrated a decrease in diet-induced atherosclerosis compared to HSL transgenics (Choy et al, 2003). HSL deficiency, however, did not have any effect on macrophage CE hydrolytic activity (Osuga et al, 2000) precluding its role in macrophage cholesterol homeostasis (Contreras, 2002) and no studies

have been performed to date to directly assess the development of atherosclerosis in HSL deficient mice. Contrary to their earlier data and conclusions, Sekiya et al recently reported a decrease in macrophage CE hydrolytic activity in macrophages from HSL^{-/-} mice and a modest but non-significant increase in diet-induced atherosclerosis in LDLR^{-/-} mice reconstituted with HSL^{-/-} bone marrow. The exact role of HSL in atherogenesis, thus, awaits a more systematic study (Sekiya et. al. 2009).

Since CEL expression was demonstrated in human macrophages, Kodvawala et al developed macrophage-specific CEL transgenic mice to evaluate the role of this CE hydrolase in the development of atherosclerosis (Kodvawala et al, 2005). In athero-susceptible ApoE^{-/-} background, CEL transgenic mice displayed an approximate 4-fold higher atherosclerotic lesion area than ApoE^{-/-} mice without the CEL transgene. It was speculated that perhaps its extracellular location or its hydrolysis of ceramide and lysophosphatidylcholine leads to increased cholesterol esterification and decreased cholesterol efflux resulting in increased atherosclerosis. Regardless of the underlying mechanisms, these studies demonstrate a minimal role for CEL in macrophage CE mobilization and thereby in atherogenesis.

To evaluate the role of CEH in foam cell formation and atherogenesis, we developed macrophage-specific CEH transgenic mice and crossed them into an atherosusceptible LDLR^{-/-} background. High-fat high-cholesterol diet induced atherosclerosis was evaluated and we reported almost a 50% reduction in lesion area in LDLR^{-/-}-CEH transgenic mice compared to LDLR^{-/-} mice (Zhao et al, 2007). In addition, CEH-mediated increase in CE mobilization also reduced lesion necrosis. It should be pointed out that the decrease in atherosclerosis and lesion necrosis was observed in the presence of physiological concentration of extracellular cholesterol acceptors. This is in contrast to HSL transgenic mice where, in the absence of ApoA IV over-expression, there was a paradoxical increase in atherosclerosis (Escary et al 1999) and only in ApoA IV HSL double transgenics a reduction in lesion development was observed (Choy et al, 2003). Consistent with our *in vitro* studies, macrophages from CEH transgenic mice showed higher FC efflux and decreased cellular CE levels upon loading with modified LDL. Over-expression of CEH in macrophages alone increased the process of reverse cholesterol transport and there was increased elimination of cholesterol in the feces of CEH transgenic mice (Zhao et al, 2007). Transgenic mice over-expressing the newly identified CE hydrolase (KIAA1363 or NCEH) have not been developed yet and the role of this enzyme in affecting atherogenesis remains to be evaluated. Sekiya et al have, however, reported an increase in Western-diet induced atherosclerosis in NCEH^{-/-} mice in an ApoE^{-/-} background (Sekiya et al 2009). These studies, however, did not evaluate the effects of NCEH deficiency in C57BL/6 background and direct consequence of NCEH ablation on increasing the risk for the development of atherosclerosis is not apparent. It also needs to be emphasized that despite total lack of NCEH expression, the macrophage CE hydrolytic activity as well as FC efflux was only reduced by less than 40% providing support for the concept of macrophage CE hydrolysis being a multi-enzyme process.

The success in attenuating atherosclerosis and lesion necrosis by transgenic expression of CEH underscores the importance of the role of CE hydrolysis in atherogenesis. It is noteworthy that advanced lesions from CEH transgenic mice still contained significant number of viable macrophages indicating that CEH is likely to play a role in modulating the characteristics of advanced lesions. Future studies will define the role of CEH in improving plaque stability. However, for clinical relevance and benefit, it is essential to understand the mechanisms involved in the endogenous regulation of CE hydrolysis such that these pathways can subsequently be targeted for pharmacological intervention.

Regulation of macrophage CE hydrolysis

CE hydrolysis is the rate-limiting step in FC efflux from macrophages (Yancey and St. Clair, 1994) and as noted above, in athero-susceptible species, macrophage CEs are resistant to mobilization (Mathur et al, 1985, Yancey and St. Clair, 1994) and intracellular CE hydrolytic activity negatively correlates with cellular CE content (Zhao et al, 2003). Therefore, it is often recommended to measure cellular FC efflux in the presence of ACAT inhibitor (Rothblat et al, 2002) and majority of FC efflux studies are conducted in the presence of ACAT inhibitors. These measurements, however, only report on the processes involved in FC transport to and through the plasma membrane to the extracellular acceptors and do not truly reflect the ability of a given cell to efflux FC under physiological conditions where ACAT will be active and stored cholesterol will be in the form of CE and not predominantly FC. To gain better understanding of FC efflux from macrophages, it is critical to not only measure it in the absence of ACAT inhibitors to assess the rate of endogenous CE hydrolysis, but also to elucidate mechanisms or pathways involved in regulating intracellular CE hydrolysis. One of the first interventions reported to increase intracellular CE hydrolysis was by exposure of macrophages to cyclic AMP (Colbran et al, 1986, Bernard et al, 1991) and it was concluded that the enzyme responsible for this hydrolysis is activated by reversible phosphorylation providing the rationale for considering HSL as the candidate CE hydrolase (Khoo et. al. 1981, Small et al, 1989). Since then, increase in cellular CE hydrolysis has been achieved by exposure to prostaglandin E1 (Goldberg and Khoo, 1990), 17-beta estradiol (Napolitano et al, 2001, Tomita et al, 1995) and 15- lipooxygenase-1 (Weibel et al, 2009). However, being a multi-enzyme process, only very limited knowledge is available on the regulation of individual CE hydrolases either at transcriptional or at the enzyme activity level. HSL is known to be activated by protein kinase A dependent reversible phosphorylation (Belfrage et al, 1982) as is CEH which is also phosphorylated by protein kinase C (Ghosh and Grogan, 1989). Although CEL is also phosphorylated but this modification is thought to be required for its secretion and its effects on enzyme activity *per se* has not been evaluated (Pasqualini et al, 1997, 2000). Effects of post-translational modifications such as phosphorylation on newly identified CE hydrolase, KIAA1363, have yet to be determined. Limited knowledge is currently available on the transcriptional regulation of the various CE hydrolases. Grober et al reported the cloning of the HSL proximal promoter (Grober et al, 1997) and recently Yajima et al demonstrated that HSL gene is transcriptionally regulated by PPAR gamma/RXR alpha heterodimers (Yajima et al, 2007). Further, Deng et al reported that up-regulation of HSL by PPARgamma requires the involvement of Sp1 (Deng et al 2006). We cloned the proximal promoter of human CEH and identified functional PPAR response elements (Ghosh and Natarajan 2001). In contrast to HSL, PPAR gamma as well as PPAR alpha agonists down-regulated CEH promoter activity. Recent studies from our laboratory have also identified a functional LXR response element on the CEH promoter (Figure 5). Luciferase activity was significantly increased when cells transfected with luciferase reporter constructs driven by 1686 bp long CEH proximal promoter were exposed to LXR ligand TO901317. This increase was amplified when cells were co-transfected with LXR α expression vector suggesting that LXR α activation increases transcriptional activity of CEH promoter. LXR α agonists have been shown to enhance FC efflux and this effect is thought to be due to an increase in the expression of cholesterol transporters ABCA1 and ABCG1 (Tall 2008). It can be hypothesized that LXR α activation enhances CEH expression as well as FC transporters (ABCA1 and ABCG1) thereby increasing CE hydrolysis and stimulating the efflux of released FC. Continuing studies in our laboratory will not only provide further experimental evidence for this mechanism of regulation of CEH and FC efflux but also explore other potential mechanisms that can be targeted to enhance or activate CEH and thereby increase FC efflux.

Conclusions and Future directions

Significant attenuation of atherosclerosis and lesion necrosis in LDLR^{-/-}CEHTg mice confirms the hypothesis that increasing intracellular CE mobilization is anti-atherogenic and identifies CEH as a potential target for pharmacological intervention. We have also demonstrated that over-expression of CEH in liver enhances *in vivo* reverse cholesterol transport and increases cholesterol elimination from the body (Zhao et al, 2008) suggesting that interventions targeted at increasing CEH will at least have beneficial effects in two major tissues involved in whole body cholesterol homeostasis. Unpublished results from our lab have demonstrated that macrophage-specific transgenic expression of CEH also leads to attenuation of systemic and plaque associated inflammation further establishing CEH as an important anti-atherogenic target. Identification of endogenous mechanisms that regulate the expression of CEH are, therefore, absolutely critical. As described above, currently we have very limited knowledge of how any of these candidate CE hydrolases are regulated *in vivo*. Continued characterization of proximal promoters and covalent modifications will provide the required information on the transcriptional and post-translational regulation of these enzymes.

Throughout this review, we have alluded to the presence of multiple candidate enzymes responsible for cellular CE hydrolysis and discussed data related to the physiological role of these enzymes obtained using different *in vitro* and *in vivo* models. In addition, we have also described the specific issues related to the ability of these enzymes to hydrolyze CE present in the cytoplasm as lipid droplets and stringent requirements for the physico-chemical properties of the interface for maximal activity of these enzymes that bring about catalysis at a water-lipid interface. It would be extremely important that further characterization of known or yet to be identified CE hydrolases includes close attention to these biochemical characteristics especially in the studies where conclusions are made regarding the major CE hydrolase in a given cell type. To date, the individual contributions of the various CE hydrolases have not been defined. Studies with targeted deletion of individual genes may be inconclusive if there is a compensatory increase in the expression or activity of other enzymes. Therefore, it needs to be emphasized that, while it may be important to define the individual contributions of HSL, CEL, CEH, KIAA1363 and yet unidentified candidates, enhancing the expression/activity of any one of these will be sufficient in attenuating atherosclerosis as illustrated by our data with LDLR^{-/-}CEHTg mice. Thus, from a biochemical stand point, it is important to identify *all* CE hydrolases that regulate intracellular CE accumulation. However, for a clinical benefit, it is more critical to determine ways to enhance *total* CE hydrolysis and not focus on which, if any, enzyme is the major CE hydrolase in macrophages or liver. Establishing a strategy to increase the transcription or activity of any one CE hydrolase and demonstrating that this increase leads to enhanced mobilization of intracellular CE, whether or not this is the major CE hydrolase, would be of profound clinical relevance.

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References

1. Accad M, Smith S, Newland D, Sanan D, King JL, Linton M, Fazio S, Farese JR. Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase-1. *J Clin Invest* 2000;105:711–719. [PubMed: 10727439]
2. Belfrange P, Fredrikson G, Olsson H, Strålfors P. Hormonal regulation of adipose tissue lipolysis by reversible phosphorylation of hormone-sensitive lipase. *Prog Clin Biol Res* 1982;102(Pt C):213–223. [PubMed: 6300929]

3. Belle V, Fournel A, Woudstra M, Ranaldi S, Prieri F, Thomé V, Currault J, Verger R, Guigliarelli B, Carrière F. Probing the opening of the pancreatic lipase lid using site-directed spin labeling and EPR spectroscopy. *Biochemistry* 2007;46:2205–2214. [PubMed: 17269661]
4. Bernard DW, Rodriguez A, Rothblat GH, Glick JM. cAMP stimulates cholesteryl ester clearance to high density lipoproteins in J7774 macrophages. *J Biol Chem* 1991;266:710–716. [PubMed: 1845991]
5. Bocan TM, Krause BR, Rosebury WS, Mueller SB, Lu X, Dagle C, Major T, Lathia C, Lee H. The ACAT inhibitor avasimibe reduces macrophages and matrix metalloproteinase expression in atherosclerotic lesion of hypercholesterolemic rabbits. *Arterioscle Thromb Vasc Biol* 2000;20:70–79.
6. Brown MS, Ho YK, Goldstein JL. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J Biol Chem* 1980;255:9344–9352. [PubMed: 7410428]
7. Choy HA, Wang XP, Schotz MC. Reduced atherosclerosis in hormone-sensitive lipase transgenic mice overexpressing cholesterol acceptors. *Biochim Biophys Acta* 2003;1634:76–85. [PubMed: 14643795]
8. Clifford GM, Londos C, Kraemer FB, Vernon RG, Yeaman SJ. Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. *J Biol Chem* 2000;275:5011–5015. [PubMed: 10671541]
9. Colbran RJ, Garton AJ, Cordle SR, Yeaman SJ. Regulation of cholesterol ester hydrolase by cyclic AMP-dependent protein kinase. *FEBS Lett* 1986;201:257–261. [PubMed: 3011511]
10. Contreras JA. Hormone sensitive lipase is not required for cholesteryl ester hydrolysis in macrophages. *Biochem Biophys Res Comm* 2002;292:900–903. [PubMed: 11944899]
11. Davies MJ, Thomas AC. Plaque fissuring the cause of acute myocardial infarction, sudden ischaemic death and crescendo angina. *Br Heart J* 1985;53:363–373. [PubMed: 3885978]
12. Deng T, Shan S, Li PP, Shen ZF, Lu XP, Cheng J, Ning ZQ. Peroxisome proliferator-activated receptor-gamma transcriptionally up-regulates hormone-sensitive lipase via the involvement of specificity protein-1. *Endocrinology* 2006;147:875–884. [PubMed: 16269451]
13. Deykin D, Goodman DS. The hydrolysis of long-chain fatty acid esters of cholesterol with rat liver enzymes. *J Biol Chem* 1962;237:3649–3656. [PubMed: 14027461]
14. Dolinsky VW, Sipione S, Lehner R, Vance DE. The cloning and expression of a murine triacylglycerol hydrolase cDNA and the structure of its corresponding gene. *Biochim Biophys Acta* 2001;1532:162–72. [PubMed: 11470237]
15. Escary J, Choy HA, Reue K, Wang X, Castellani LW, Glass CK, Lusis AJ, Schotz MC. Paradoxical effect on atherosclerosis of hormone-sensitive lipase overexpression in macrophages. *J Lipid Res* 1999;40:397–404. [PubMed: 10064727]
16. Escary JL, Choy HA, Reue K, Schotz MC. Hormone-sensitive lipase overexpression increases cholesteryl ester hydrolysis in macrophage foam cells. *Arterioscler Thromb Vasc Biol* 1998;18:991–998. [PubMed: 9633942]
17. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RV. Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest* 2001;107:163–171. [PubMed: 11160132]
18. Gallo LL. Sterol ester hydrolase from rat pancreas. *Methods Enzymol* 1981;71:664–74. [PubMed: 7278675]
19. Gargouri Y, Julien R, Bois AG, Verger R, Sarda L. Studies on the detergent inhibition of pancreatic lipase activity. *J Lipid Res* 1983;24:1336–1342. [PubMed: 6644184]
20. Ghosh S. Cholesteryl ester hydrolase in human monocyte/macrophage: cloning, sequencing and expression of full-length cDNA. *Physiol Genomics* 2000;2:1–8. [PubMed: 11015575]
21. Ghosh S, Grogan WM. Activation of rat liver cholesterol ester hydrolase by cAMP-dependent protein kinase and protein kinase. *C Lipids* 1989;24:733–736.
22. Ghosh S, Grogan WM. Rapid three-step purification of a hepatic neutral cholesteryl ester hydrolase which is not the pancreatic enzyme. *Lipids* 1991;10:793–798. [PubMed: 1795600]
23. Ghosh S, Mallonee DH, Hylemon PB, Grogan WM. Molecular cloning and expression of rat hepatic neutral cholesteryl ester hydrolase. *Biochim Biophys Acta* 1995;1259:305–312. [PubMed: 8541339]
24. Ghosh S, Natarajan R. Cloning of the human cholesteryl ester hydrolase promoter: identification of functional peroxisomal proliferator-activated receptor responsive elements. *Biochem Biophys Res Commun* 2001;284:1065–1070. [PubMed: 11409902]

25. Ghosh S, St Clair RW, Rudel LL. Mobilization of cytoplasmic CE droplets by over-expression of human macrophage cholesteryl ester hydrolase. *J Lipid Res* 2003;44:1833–1840. [PubMed: 12837853]
26. Glass CK, Witztum JL. Atherosclerosis: The road ahead. *Cell* 2001;104:503–516. [PubMed: 11239408]
27. Goldberg DI, Khoo JC. Stimulation of neutral cholesteryl ester hydrolase by cAMP in P388D1 macrophages. *Biochim Biophys Acta* 1990;1042:132–137. [PubMed: 2153410]
28. Grober J, Laurell H, Blaise R, Fabry B, Schaak S, Holm C, Langin D. Characterization of the promoter of human adipocyte hormone-sensitive lipase. *Biochem J* 1997;328:453–461. [PubMed: 9371701]
29. Hajjar DP, Minick CR, Fowler S. Arterial neutral holesteryl esterase. A hormone-sensitive enzyme distinct from lysosomal cholesteryl esterase. *J Biol Chem* 1983;258:192–198. [PubMed: 6848493]
30. Hakamata H, Miyazaki A, Sakai M, Suginoara Y, Sakamoto Y, Horiuchi S. Species difference in cholesteryl ester cycle and HDL-induced cholesterol efflux from macrophage foam cells. *Arterioscler Thromb* 1994;14:1860–1865. [PubMed: 7947613]
31. Harte RA, Hultén LM, Lindmark H, et al. Low level expression of hormone-sensitive lipase in arterial macrophage-derived foam cells: potential explanation for low rates of cholesteryl ester hydrolysis. *Atherosclerosis* 2000;149:343–350. [PubMed: 10729384]
32. Hui DY, Kissel JA. Sequence identity between human pancreatic cholesterol esterase and bile salt-stimulated milk lipase. *FEBS Lett* 1990;276:131–134. [PubMed: 2265692]
33. Igarashi M, Osuga JI, Isshiki M, Sekiya M, Okazaki H, Takase S, Takanashi M, Ohta K, Kumagai M, Nishi M, Fujita T, Nagai R, Kadowaki T, Ishibashi S. Targeting of neutral cholesterol ester hydrolase to the endoplasmic reticulum via its N-terminal sequence. *J Lipid Res*. 2009 Jul 10; [Epub ahead of print].
34. Ishii I, Oka M, Katto N, Shirai K, Saito Y, Hirose S. Beta-VLDL-induced cholesterol ester deposition in macrophages may be regulated by neutral cholesterol esterase activity. *Arterioscler Thromb* 1992;12:1139–1145. [PubMed: 1390586]
35. Johnson WJ, Jang SY, Bernard DW. Hormone sensitive lipase mRNA in both monocyte and macrophage forms of the human THP-1 cell line. *Comp Biochem Physiol B Biochem Mol Biol* 2000;126:543–52. [PubMed: 11026666]
36. Khoo JC, Mahoney EM, Steinberg D. Neutral cholesterol esterase activity in macrophages and its enhancement by cAMP-dependent protein kinase. *J Biol Chem* 1981;256:12659–12661. [PubMed: 6273401]
37. Khoo JC, Reue K, Steinberg D, Schotz MC. Expression of hormone-sensitive lipase mRNA in macrophages. *J Lipid Res* 1993;34:1969–1974. [PubMed: 8263420]
38. Kissel JA, Fontaine RN, Turck CW, Brockman HL, Hui DY. Molecular cloning and expression of cDNA for rat pancreatic cholesterol esterase. *Biochim Biophys Acta* 1989;1006:227–236. [PubMed: 2688744]
39. Kodvawala A, Ghering AB, Davidson WS, Hui DY. Carboxyl ester lipase expression in macrophages increases cholesteryl ester accumulation and promotes atherosclerosis. *J Biol Chem* 2005;280:38592–38598. [PubMed: 16166077]
40. Li F, Hui DY. Modified low density lipoprotein enhances the secretion of bile salt-stimulated cholesterol esterase by human monocyte-macrophages. Species-specific difference in macrophage cholesteryl ester hydrolase. *J Biol Chem* 1997;272:28666–28671. [PubMed: 9353334]
41. Libby P, Aikawa M. New insights into plaque stabilization by lipid lowering. *Drugs* 1998;56(Suppl 1):9–13. [PubMed: 9740536]
42. Mathur SN, Field FJ, Megan MB, Armstrong HL. A defect in mobilization of cholesteryl esters in rabbit macrophages. *Biochim Biophys Acta* 1985;834:48–57. [PubMed: 3978114]
43. Matsuda K. ACAT inhibitors as antiatherosclerotic agents. *Med Res Rev* 1994;14:271–305. [PubMed: 8007738]
44. Matsuo M, Ito F, Konto A, Aketa M, Tomoi M, Shimomura K. Effect of FR145237, a novel ACAT inhibitor on atherogenesis in cholesterol fed and WHHL rabbits. Evidence for a direct effect on the arterial wall. *Biochem Biophys Acta* 1995;1259:254–260. [PubMed: 8541332]

45. Napolitano M, Blotta I, Montali A, Bravo E. 17 β -estradiol enhances the flux of cholesterol through the cholesteryl ester cycle in human macrophages. *Biosci Rep* 2001;21:637–652. [PubMed: 12168771]
46. Nicolosi RJ, Wilson TA, Krause BR. The ACAT inhibitor, CI 1011 is effective in the prevention and regression of aortic fatty streak in hamsters. *Atherosclerosis* 1998;137:77–85. [PubMed: 9568739]
47. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC, Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* 2003;290:2292–2300. [PubMed: 14600188]
48. Okazaki H, Igarashi M, Nishi M, Sekiya M, Tajima M, Takase S, Takanashi M, Ohta K, Tamura Y, Okazaki S, Yahagi N, Ohashi K, Amemiya-Kudo M, Nakagawa Y, Nagai R, Kadowaki T, Osuga J, Ishibashi S. Identification of neutral cholesterol ester hydrolase, a key enzyme removing cholesterol from macrophages. *J Biol Chem* 2008;283:33357–33364. [PubMed: 18782767]
49. Osuga J, Ishibashi S, Oka T, Yagyu H, Tozawa R, Fujimoto A, Shionoiri F, Yahagi N, Kraemer FB, Tsutsumi O, Yamada N. Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc Natl Acad Sci USA* 2000;97:787–792. [PubMed: 10639158]
50. Pasqualini E, Caillol N, Mas E, Bruneau N, Lexa D, Lombardo D. Association of bile-salt-dependent lipase with membranes of human pancreatic microsomes is under the control of ATP and phosphorylation. *Biochem J* 1997;327:527–535. [PubMed: 9359426]
51. Pasqualini E, Caillol N, Valette A, Lloubes R, Verine A, Lombardo D. Phosphorylation of the rat pancreatic bile-salt-dependent lipase by casein kinase II is essential for secretion. *Biochem J* 2000;345:121–128. [PubMed: 10600647]
52. Perrey S, Legendre C, Matsuura A, Guffroy C, Binet J, Ohbayashi S, Tanaka T, Ortuno JC, Matsukura T, Laugel T, Padovani P, Bellamy F, Edgar AD. Preferential pharmacological inhibition of macrophage ACAT increases plaque formation in mouse and rabbit models of atherogenesis. *Atheroscler* 2001;155:359–370.
53. Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF. Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* 1997;272:14769–14775. [PubMed: 9169443]
54. Reis P, Holmberg K, Miller R, Krägel J, Grigoriev DO, Leser ME, Watzke HJ. Competition between lipases and monoglycerides at interfaces. *Langmuir* 2008;24:7400–7407. [PubMed: 18547084]
55. Reis P, Holmberg K, Watzke H, Leser ME, Miller R. Lipases at interfaces: a review. *Adv Colloid Interface Sci* 2009;147–148:237–250.
56. Reue K, Cohen RD, Schotz MC. Evidence for hormone-sensitive lipase mRNA expression in human monocyte/macrophages. *Arterioscler Thromb Vasc Biol* 1997;17:3428–32. [PubMed: 9437189]
57. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993;362:801–809. [PubMed: 8479518]
58. Ross R, Glomset J, Harker L. Response to injury and atherogenesis. *Am J Pathol* 1977;86:675–684. [PubMed: 842616]
59. Rothblat GH, de la Llera-Moya M, Favari E, Yancey PG, Kellner-Weibel G. Cellular cholesterol flux studies: methodological considerations. *Atherosclerosis* 2002;163:1–8. [PubMed: 12048116]
60. Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Ann. Rev Pharmacol Toxicol* 1998;38:257–288.
61. Sekiya M, Osuga J, Nagashima S, Ohshiro T, Igarashi M, Okazaki H, Takahashi M, Tazoe F, Wada T, Ohta K, Takanashi M, Kumagai M, Nishi M, Takase S, Yahagi N, Yagyu H, Ohashi K, Nagai R, Kadowaki T, Furukawa Y, Ishibashi S. Ablation of neutral cholesterol ester hydrolase 1 accelerates atherosclerosis. *Cell Metab* 2009;10:219–228. [PubMed: 19723498]
62. Sliskovic DR, White AD. Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends Pharmacol Sci* 1991;12:194–199. [PubMed: 1862535]
63. Small CA, Goodacre JA, Yeaman SJ. Hormone sensitive lipase is responsible for the neutral cholesteryl ester hydrolase activity in macrophages. *FEBS Lett* 1989;247:205–208. [PubMed: 2541013]

64. Tall AR. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *J Intern Med* 2008;263:256–273. [PubMed: 18271871]
65. Tomita T, Sawamura F, Uetsuka R, Ikeda M, Tomita I. The regulation of cholesteryl ester metabolism by 17 beta-estradiol in macrophages. Activation of neutral cholesterol esterase. *Ann N Y Acad Sci* 1995;748:637–639. [PubMed: 7695221]
66. Warner GJ, Stoudt G, Bamberger M, Johnson WJ, Rothblat GH. Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol. *J Biol Chem* 1995;270:5772–5778. [PubMed: 7890706]
67. Wei E, Alam M, Sun F, Agellon LB, Vance DE, Lehner R. Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice. *J Lipid Res* 2007;48:2597–2606. [PubMed: 17878493]
68. Weibel GL, Joshi MR, Alexander ET, Zhu P, Blair IA, Rothblat GH. Overexpression of human 15 (S)-lipoxygenase-1 in RAW macrophages leads to increased cholesterol mobilization and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 2009;29:837–842. [PubMed: 19325142]
69. Williams KJ, Tabas I. The Response-to-Retention Hypothesis of Early Atherogenesis. *Arterioscler Thromb Vasc Biol* 1995;15:551–561.
70. Yagu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perry S, Tamura Y, Ohyashi K. Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem* 2000;275:21324–21330. [PubMed: 10777503]
71. Yajima H, Kobayashi Y, Kanaya T, Horino Y. Identification of peroxisome-proliferator responsive element in the mouse HSL gene. *Biochem Biophys Res Commun* 2007;352:526–531. [PubMed: 17134676]
72. Yancey PG, St Clair RW. Mechanism of the defect in cholesteryl ester clearance from macrophages of atherosclerosis-susceptible White Carneau pigeons. *J Lipid Res* 1994;35:2114–2129. [PubMed: 7897310]
73. Zhao B, Fisher BJ, St Clair RW, Rudel LL, Ghosh S. Redistribution of macrophage cholesteryl ester hydrolase from cytoplasm to lipid droplets upon lipid loading. *J Lipid Res* 2005;46:2114–2121. [PubMed: 16024911]
74. Zhao B, Natarajan R, Ghosh S. Human liver cholesteryl ester hydrolase: cloning, molecular characterization, and role in cellular cholesterol homeostasis. *Physiol Genomics* 2005;23:304–310. [PubMed: 16131527]
75. Zhao B, Song J, Chow WN, St Clair RW, Rudel LL, Ghosh S. Macrophage-specific transgenic expression of cholesteryl ester hydrolase significantly reduces atherosclerosis and lesion necrosis in Ldlr mice. *J Clin Invest* 2007;117:2983–2992. [PubMed: 17885686]
76. Zhao B, Song J, Ghosh S. Hepatic overexpression of cholesteryl ester hydrolase enhances cholesterol elimination and in vivo reverse cholesterol transport. *J Lipid Res* 2008;49:2212–2217. [PubMed: 18599737]
77. Zhao B, Song J, St Clair RW, Ghosh S. Stable Over-Expression of Human Macrophage Cholesteryl Ester Hydrolase (CEH) Results in Enhanced Free Cholesterol Efflux from Human THP1-macrophages. *Am J Physiol Cell Physiol* 2007;292:C405–412. [PubMed: 16971496]

Figure 1. Fundamental Difference between ACAT inhibition and CEH over-expression as strategies for reducing macrophage CE content

Intracellular CE are in a dynamic equilibrium with FC and this equilibrium is maintained by the activities of ACAT1 and CEH. FC released by the action of CEH has two fates - indicated as 1 (re-esterification by ACAT1 and 2 (efflux to extracellular cholesterol acceptors). Under conditions of ACAT1 inhibition (bottom right), there is reduction in cellular CE content. However, since FC can no longer be re-esterified, FC has only one fate (2) – efflux to extracellular acceptors. This leads to an increase in intracellular FC. Accumulation of FC in plasma membrane or in endoplasmic reticulum leads to cellular toxicity. On the other hand, if CEH is over-expressed, the released FC still has the two fates (1 and 2). The cellular FC remaining after efflux can be re-esterified by functional ACAT1 preventing any increase in cellular FC although reducing the net intracellular CE content.

A. In the Presence of Limiting Acceptor (0.5% BSA)

B. In the Presence of Sufficient Acceptor (10% FBS)

Figure 2. Over-expression of CEH in cells with stable over-expression of ACAT1 (agmACAT1 cells) reduces cellular CE content without increasing the cellular FC levels

agmACAT1 cells were transiently transfected either with an empty vector pCMV or with CEH expression vector in the presence or absence of ACAT1 inhibitor. Twenty four hours post-transfection, medium was replaced with either serum free medium supplemented with 0.5% BSA (limiting acceptor) or with complete growth medium containing 10% FBS (sufficient acceptor). Total lipids were extracted after additional 48 hours and cellular free (FC) and esterified cholesterol (EC) content determined by GLC. It should be noted that content of FC and EC is higher when 10% FBS is included in the medium due to lipoprotein uptake. An increase in FC is only seen when ACAT1 was inhibited. CEH over-expression led to a slight

decrease and not an increase in cellular FC. Cellular EC content was reduced with CEH over-expression and the magnitude of this decrease was greater in ACAT1 inhibited cells. * $p < 0.05$

Figure 3.
Intracellular localization of candidate CE hydrolases and their potential association with lipid droplets – the physiological substrate.

Hydrolysis at the lipid-water interface is regulated by:

1. Binding and retention of the enzyme at the interface
 - Surfactants or detergents can expel the enzyme from the interface
 - Accumulation of some catalytic products results in the desorption of enzyme
2. Change in enzyme conformation to expose the otherwise inaccessible active site
 - Opening of the α -helical loop or the "lid" domain that covers the active site
 - Activation of enzymes by binding to insoluble substrates
3. Characteristics of the interfacial microenvironment
 - Alteration of the "quality of the interface" by accumulated hydrophobic products
 - Changes in the interface by exogenous molecules such as phospholipids and detergents

Figure 4.
Regulation of interfacial catalysis by a lipolytic enzyme.

Figure 5. Activation of CEH promoter activity by LXR α agonist (TO-901317)

COS-7 cells were co-transfected with either Luciferase reporter construct driven tandem LXR response elements (LXRE-Luc) or by 1686 bp long CEH proximal promoter (CEH-1686-Luc) along with an empty vector or LXR α expression vector. Twenty four post-transfection, cells were exposed to LXR α ligand TO-901317. Luciferase activity was measured in cell lysates after an additional 24 hours and normalized to β -galactosidase. Fold increase in promoter activity by ligand dependent activation of LXR α are indicated.

Table 1

Summary of all candidate CE hydrolase studied to date

#	Enzyme Name	Originally Characterized From	Sub- Cellular Localization	Substrate Used for Assay	Atherosclerosis In Transgenic	Atherosclerosis in Knockout mice	Citation Number	Key Findings/Conclusions
1	HSL	Mouse Adipose Tissue	Cytosol	TG, PC and Detergent Emulsion	Increased	Not Studied	27, 63, 37, 40, 15, 10, 49, 61	May be the neutral CEH in macrophages Not detected in human macrophages Increased atherosclerosis due to limiting acceptor concentration No difference in macrophage CE hydrolytic activity in HSL ^{-/-} mice Neutral CEH in mouse macrophages due to enzyme other than HSL Contrary to earlier data from the same group (Ref. #48), HSL ^{-/-} macrophages show reduced CE hydrolytic activity and FC efflux
2	CEH	Rat Pancreas	Secreted	CE and Bile acid Micelle	Increased	Not Studied	40, 39	Expressed in human macrophages; may have a limited role in intracellular CE hydrolysis since it is secreted Increased atherosclerosis in transgenic mice due to decreased availability of FC for efflux
3	TGH(CES3)	Pig Liver	ER Lipid Droplets	TG, Bile salts and albumin	Not Studied	Not Studied	14, 67	Involved in TG hydrolysis in liver Liver specific Transgenic expression of human TGH minigene slightly increases VLDL Secretion; Expected to be pro-atherogenic
4	CEH(CES1)	Human Macrophages	Cytosol Lipid Droplets	Droplets	Significantly reduced	Under Investigation	20, 73, 77, 75	Neutral CEH in human macrophages Associated with cytoplasmic lipid droplets - the physiological substrate Over-expression mobilizes cellular CE and increase FC efflux by all pathways in vitro Reduced atherosclerosis, Increase reverse cholesterol

#	Enzyme Name	Originally Characterized From	Sub- Cellular Localization	Substrate Used for Assay	Atherosclerosis In Transgenic	Atherosclerosis in Knockout mice	Citation Number	Key Findings/Conclusions
5	KIAA1363 (AADACL1)	Proteomics	ER	CE, PC and Detergent Emulsion	Not Studied	Increased but only in ApoE ^{-/-} or LDLR ^{-/-} background and after Western Diet feeding	48 33 61	transport and cholesterol excretion in vivo Over expression reduces cellular CE, present in foam cell in plaques Anchored in the ER membrane; mechanism by which the enzyme access the cytosolic lipid droplet substrate not established Reduction in atherosclerosis in ApoE ^{-/-} background, Effect of deficiency per se in C57BL/6 background not established>60% FC efflux still maintained in macrophages from knockout mice indicating presence of other enzymes