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Macrophage Cholesterol Homeostasis and Atherogenesis: Critical Role of Intracellular Cholesteryl Ester Hydrolysis

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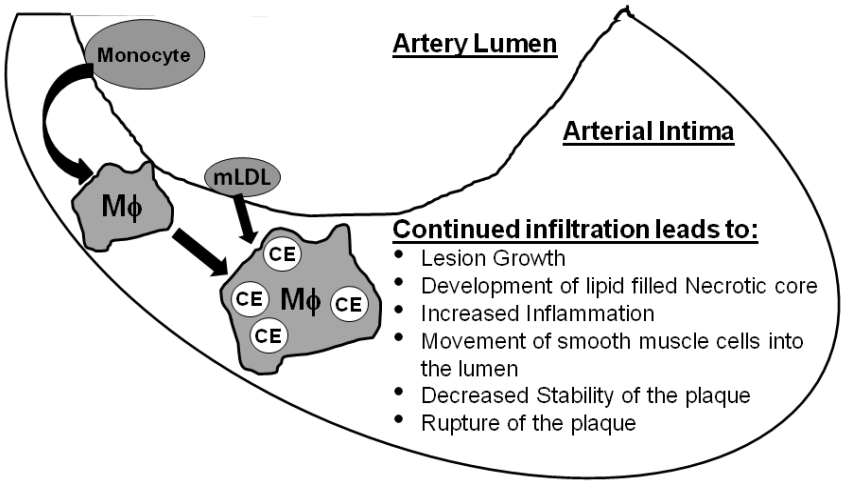
1. Introduction

Coronary artery disease (CAD) is the leading cause of death in United States and contributes to significant mortality around the world. According to the Heart Disease and Stroke 2011 update, released by the American Heart Association, 82,600,000 American adults (>1 in 3) have 1 or more type of cardiovascular disease and >2200 Americans die of CAD every day which is an average of 1 in every 39 seconds. These are alarming statistics and underscore the importance of continued understanding of the processes involved in the development of CAD. Atherosclerosis, characterized by increased lipid accumulation in the artery wall, is the major underlying cause of CAD.

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. While it is well established that accumulation of lipid-laden macrophage foam cells in the artery wall is the hall mark of atherosclerosis, two different theories are proposed to describe the events leading to infiltration of macrophages and subsequent development of foam cells within the artery wall. 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. This is followed by the unregulated uptake of modified LDL (mLDL) by monocyte derived macrophages leading to the formation of foam cells (Ross et al, 1977, Ross, 1993). "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 in the intimal space of the artery wall. This initiates the formation of fatty streaks which develops into an atherosclerotic plaque with continued accretion of foam cells. Figure 1 below summarizes these major events that lead to the formation of atherosclerotic plaque.

Continuous accumulation of lipid laden foam cells in an atherosclerotic plaque not only contributes to its 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 an obvious strategy to target reduction in plaque volume as

well as enhance plaque stability. However, no therapies are currently available to reduce the lipid burden of atherosclerotic plaque. The presently used therapies for CAD include cholesterol lowering drugs such as statins that reduce plasma cholesterol by inhibiting the endogenous cholesterol synthesis. As a consequence, there is reduction in plasma LDL cholesterol levels which limits further foam cell formation and progression of atherosclerotic plaque. Nonetheless, these cholesterol lowering drugs do not increase removal of lipid from existing plaques and, therefore, cannot achieve plaque regression. 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. A detailed understanding of the cellular mechanisms that regulate lipid accumulation within macrophage foam cells and processes that are critical for mobilization of stored lipid is central to future development of targeted strategies to reduce atherosclerotic plaque burden with the aim to reduce CAD.



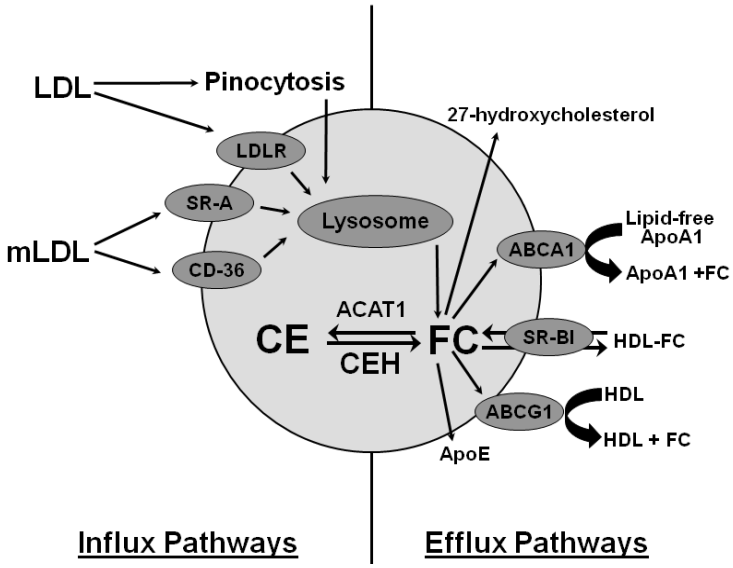
CE: Cholesteryl esters; Mϕ: macrophage; mLDL: modified LDL

Fig. 1. Schematic showing the events in the artery wall that lead to the formation of atherosclerotic plaque

2. Macrophage cholesterol homeostasis

Cholesterol homeostasis in macrophages and other peripheral cells is maintained by a balance between the influx and efflux processes. Cholesterol influx occurs by receptor and non-receptor mediated uptake of both normal and modified lipoproteins. The uptake of native LDL via the LDL receptor is regulated by the feedback inhibition of LDL receptor expression by cellular cholesterol levels and most peripheral cells limit their cholesterol uptake by this mechanism. Excess LDL returns to the liver where it is, once again, taken up via LDL receptor. Under conditions of increased dietary intake or increased endogenous synthesis of cholesterol, circulating LDL that cannot be taken by peripheral tissues or liver due to the inhibition of the expression of LDL receptor, becomes modified and is no longer a ligand for LDL receptor. Macrophages play a critical role in the removal of this modified

LDL. Macrophages express scavenger receptors, namely, scavenger receptor A (SR-A) and CD-36, that facilitate the uptake of modified LDL. Scavenger-receptor mediated uptake of modified LDL is largely unregulated and should be regarded as a mechanism that evolved to effectively clear modified lipoproteins from circulation and thus preventing the associated toxicity. Non-receptor mediated uptake pathways have recently been identified such as phagocytosis of aggregated LDL and macropinocytosis of native LDL that can also potentially contribute to lipid accumulation in macrophage foam cells. Cholesteryl esters (CE) associated with LDL are hydrolyzed in late endosomes/ lysosomes to free cholesterol (FC) which then traffics to and integrates into the plasma membrane. Excess membrane cholesterol and also a fraction of LDL-derived FC is transported to endoplasmic reticulum where it is re-esterified by acyl CoA:cholesterol acyltransferase-1 (ACAT1) and stored in cytoplasmic lipid droplets. While this re-esterification of cholesterol is initially beneficial to the cells in preventing the FC-associated cell toxicity, under conditions of unregulated or increased uptake of modified LDL, it leads to excessive accumulation of CE present as cytoplasmic lipid droplets giving the cells their characteristic "foamy" appearance. Cellular CE undergo constant cycle of hydrolysis and re-esterification (cholesteryl ester cycle) with a half life of 24h. Brown et al demonstrated for the first time that hydrolysis of stored CE is extra lysosomal and defined the "need" for a neutral cholesteryl ester hydrolase (CEH) (Brown et al, 1980) that can release FC from the lipid droplet associated CE which can either be re-esterified again by ACAT-1 or removed from the cells by extracellular acceptor-mediated cholesterol efflux. This acceptor mediated FC efflux is the major mechanism for the removal of cellular cholesterol. Efflux of FC occurs either by aqueous diffusion or via several transporters namely ATP binding cassette transporter A1 (ABCA1) and ABCG1. Bidirectional flux of FC also occurs through scavenger receptor BI (SR-BI). Since FC is extremely hydrophobic, its efflux from the cells is coupled to its association with specific extracellular acceptors. Macrophages secrete apolipoprotein E (ApoE) and it is believed that ApoE serves as the extracellular acceptor for FC that is effluxed via aqueous diffusion pathway. Serum albumin, present in the interstitial fluid, also serves as an important acceptor for FC effluxed via non-transporter mediated pathways. Apolipoprotein A1 that is synthesized in the liver and secreted as minimally lipidated particle containing small amount of phospholipids is the acceptor for FC effluxed via ABCA1. After acquiring some amount of FC, ApoA1 can no longer accept FC from ABCA1. Bulk of FC efflux occurs via ABCG1 and ApoA1 with some FC or high density lipoprotein (HDL) serve as the extracellular acceptor for this process. A small amount of FC is also converted into 27-hydroxycholesterol which can readily diffuse out of the cell and contribution of this pathway towards total FC removal from macrophages remains undefined. Figure 2 summarizes the various influx and efflux pathways involved in macrophage cholesterol homeostasis. Under normal physiological conditions a balance between these influx and efflux pathways maintains the levels of cellular cholesterol in macrophages and prevents foam cell formation. However, an imbalance between influx and efflux, occurring by either increased influx (under conditions with high levels of circulating LDL such as hypercholesterolemia) or decreased efflux (under conditions of aberrant cellular processes reducing FC availability for efflux or decreased levels of extracellular FC acceptors), cellular accumulation of CE containing lipid droplets increases resulting in the formation of foam cells and initiating atherogenesis. Improved understanding of these processes is, therefore, critical to developing new strategies to not only prevent foam cell formation but also to reduce CE burden of existing foam cells that may be associated with atherosclerotic lesions.



ABC: ATP binding cassette transporter; ACAT: Acyl-CoA cholesterol acyltransferase; ACEH: Acid cholesteryl ester hydrolase; ApoE: Apolipoprtoein E; CE: Cholesteryl ester; CEH: Cholesteryl ester hydrolase; FC: Free cholesterol; LDLR: LDL receptor; mLDL: Modified LDL; PL: Phospholipids; SR-A: Scavenger receptor type A; SR-BI: Scavenger receptor class B type I.

Fig. 2. Macrophage Cholesterol Homeostasis: Influx and Efflux Pathways

2.1 Cholesteryl ester accumulation

Cellular CEs exist in dynamic equilibrium with FC in a futile cholesteryl ester cycle with a half life of 24h. The two enzymes involved in this cycle are Acyl-CoA cholesterol acyltransferase (ACAT) and cholesteryl ester hydrolase (CEH). Two distinct genes have been identified for ACAT; ACAT1 is ubiquitously expressed in all cell types including macrophages and ACAT2 expression is restricted to liver and intestine. Since foam cells are characterized by accumulation of CE and ACAT1 is responsible for formation of CE, pharmacological inhibition of ACAT was initially pursued as a means to prevent or attenuate foam cell formation (Matsuda, 1994, Matsuo et al, 1995, Nicolosi et al, 1998, Sliskovic and White, 1991). However, increased plaque formation by preferential pharmacological inhibition of ACAT-1 was noted in mouse and rabbit models of atherosclerosis (Perrey et al, 2001). Ablation of ACAT1 gene resulted in marked systemic abnormalities in lipid homeostasis in hyper-cholesterolemic 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). Further, ACAT1-deficient mice also displayed an increase in lesion area and the systemic lipid abnormalities. Inhibition or deficiency of ACAT1 also results in increased intracellular FC since it cannot be esterified to CE which is its inert and storage form. A rise in FC above its physiological concentration is associated with cellular toxicity (Glass and Witztum, 2001). Enrichment of the endoplasmic reticulum (ER) with FC induces ER stress leading to apoptosis. Therefore, although intuitively a logical strategy to limit cellular CE accumulation, inhibition or deficiency of ACAT1 has detrimental effects.

Alternatively, reduction in CEs stored in macrophage foam cells can also be achieved by enhancing mobilization. The first and the rate limiting step in cellular CE mobilization is CEH-mediated hydrolysis. Consistently, 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). Further, 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). While increased hydrolysis of CE will also lead to increase in FC but there is a fundamental difference between CEH activation and ACAT1 inhibition. Under conditions of ACAT1 inhibition, cellular FC has only one fate, namely, efflux to extracellular acceptors. However, FC generated as a result of CEH activation can either be re-esterified by ACAT1 or be effluxed to extracellular acceptor (two fates). Thus, CEH activation or over-expression does not result in an increase in cellular FC and is not associated with toxicity seen with ACAT1 inhibition indicating that CEH-mediated increase in cellular CE mobilization is a valid approach to target attenuation of foam cell formation (Ghosh et al, 2009).

2.2 Cholesteryl ester hydrolysis and cellular CE mobilization

Unregulated uptake of modified lipoproteins by macrophages can be regarded as a defence mechanism by which removal of these potentially toxic particles is facilitated. As discussed above, efflux of FC from macrophage foam cells is the major mechanism by which this CE accumulation can be reversed underscoring the importance of intracellular CE hydrolysis. Since the first description for the "need" of an extra lysosomal neutral CEH, several candidate enzymes have been identified as potential CE hydrolases in macrophages. Based on the observations that cAMP enhances FC efflux from macrophages and hormone sensitive lipase (HSL), an enzyme initially characterized from adipose tissue, requires protein kinase A and cAMP dependent activation, 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). Over-expression of HSL by transient transfection in 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. 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 et al, 1989) that was also present in other tissues and was secreted in milk to facilitate digestion of CE in infants and newborns (Hui and Kissel, 1990). Li and Hui demonstrated the presence of CEL in human monocyte/macrophage cell line THP1 as well as primary blood derived monocyte macrophages and reported the absence of HSL in these cells (Li and Hui, 1997). However, since CEL is a secretory enzyme and it was thought to play a limited role in intracellular CE metabolism.

Pursuing the characterization of neutral CEH, we purified and cloned rat liver neutral cytosolic CEH which belonged to the carboxylesterase family and was distinctly different from HSL and CEL although it shared the same catalytic triad (Ghosh and Grogan, 1991, Ghosh et al, 1995). Using the strategy of homology cloning, we subsequently 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.

Stating the inability to measure CE hydrolytic activity associated with human CEH and its murine orthologue triglyceride lipase (TGL), Okazaki et al used a proteomics approach to identify another enzyme containing lipase consensus motifs and α/β-hydrolase folds and reported the characterization of murine orthologue of AADACL1 (a deacetylase) also known as KIAA1363 (Okazaki et al, 2008). While its deficiency significantly reduced the deacetylase activity it did not affect CE hydrolase activity (Buchebner et al, 2010). These authors also found no difference in lipid droplet formation and cellular CE and FC content of HSL-deficient macrophages. Based on the data obtained with HSL and AADACL1 or KIAA1363 deficient macrophages, it can be concluded that macrophage CE hydrolysis is likely a multi-enzyme process. Considering the importance of CE hydrolysis in cellular CE mobilization, natural redundancy in enzymes capable of catalyzing this reaction is probably a protective mechanism evolved to prevent pathological consequences of lipid accumulation.

2.3 CEH and atherogenesis

Despite the apparent uncertainty surrounding the identity of macrophage CE hydrolase, the importance of this step in mobilization of CE and thereby attenuating atherosclerosis cannot be over-emphasized. The hypothesis that enhancing CE hydrolysis will reduce foam cell formation and consequently attenuate atherogenesis, 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 levels of 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.

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 atherosusceptible 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 total cholesterol and CE content of the lesions resulting in significantly reduced lesion necrosis. 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). The success in attenuating atherosclerosis and lesion necrosis by transgenic expression of CEH underscores the importance of the role of CE hydrolysis in atherogenesis. Transgenic mice over-expressing the newly identified CE hydrolase (AADACL1 or KIAA1363) have not yet been developed and the role of this enzyme in affecting atherogenesis remains to be evaluated.

2.4 CEH and elimination of cholesterol from the body

While hydrolysis of CE stored in macrophage represents the first step towards removal of FC from lesion associated foam cells, final removal of FC occurs from the liver either via direct secretion into bile or by conversion into bile acids, both of which are excreted in the feces. Figure 3 below summarizes the different steps involved in the movement of FC from macrophages to liver for final elimination, a process called Reverse Cholesterol Transport. FC effluxed from macrophages becomes associated with extracellular cholesterol acceptors and apolipoprotein A1 (ApoA1) is the major acceptor. Lipid free ApoA1 accepts FC transported via ABCA1 and this partially lipidated ApoA1 or nascent HDL (High density lipoprotein) then becomes the acceptor of FC transported via ABCG1. Bulk of FC efflux to HDL occurs via ABCG1 although bidirectional flux of FC also occurs through SR-BI. In the plasma compartment, FC in HDL particle is esterified by plasma Lecithin cholesterol acyltransferase (LCAT) and CE constitutes greater than 80% of total cholesterol carried by the HDL particle. In the liver, HDL binds to its receptor SR-BI and delivers its lipid "cargo", and lipid poor HDL is presumed to return into circulation to further serve as the extracellular cholesterol acceptor and facilitate removal of cholesterol from peripheral tissues including artery wall associated macrophage foam cells. This function of HDL is responsible for its alias "Good cholesterol" in contrast to LDL or "Bad cholesterol" that delivers cholesterol to the peripheral tissues and is responsible for CE accumulation in macrophages.

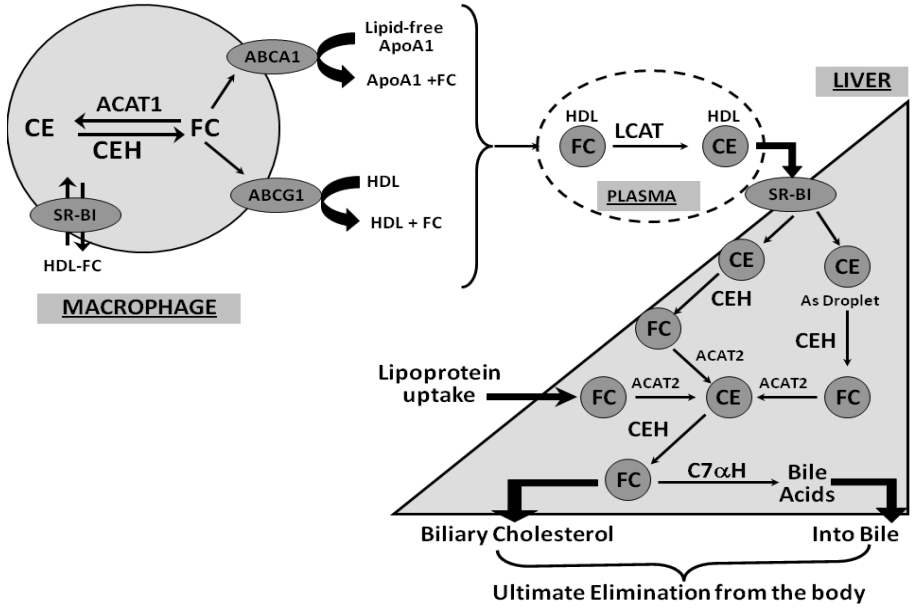


Fig. 3. Reverse Cholesterol Transport

FC delivered to the liver via SR-BI is believed to be directly secreted into bile (Kozarsky et al, 1997). On the other hand, for generation of FC, the HDL-delivered CE, once again, needs to be hydrolyzed underscoring the importance of CEH in hepatic CE metabolism. FC enters the hepatic metabolic pool where it is either re-esterified by ACAT2 (distinct from ACAT1 present in macrophages) or secreted into bile or converted into bile acids where the rate-limiting step is catalyzed by cholesterol 7α hydroxylase (C7αH). It should be emphasized that although humans and other mammals can synthesize cholesterol starting from acetate, they lack the enzymes required to degrade the steroid nucleus. Extremely hydrophobic cholesterol requires several modifications to increase its water solubility and to facilitate its excretion. Conversion of cholesterol to water soluble bile acids is one such mechanism which along with direct secretion of cholesterol in bile represents the major route of cholesterol elimination from the body.

2.4.1 Hepatic CE hydrolysis

Based on immunological cross reactivity and observed variations in CE hydrolytic activities in rat liver, Harrison speculated that hepatic CE hydrolytic activity is due to the uptake of pancreatic cholesterol esterase and that liver does not synthesize a similar enzyme (Harrison, 1988). This concept gained further support by the observed similarity between pancreatic and liver enzymes (Camulli, 1989, Chen 1997). However, we purified and characterized a rat hepatic CEH that was immunologically distinct from pancreatic CEH (Ghosh 1991). Molecular cloning of this rat liver CEH identified it as a member of the carboxylesterase family of enzymes (Ghosh et al., 1995). Biochemical, physiological and developmental characterization of this enzyme confirmed its role in regulating hepatic cholesterol homeostasis (Natarajan et al, 1996, 1998, Ghosh et al 1998). Homology cloning,

based on this rat liver CEH, was used to identify the human macrophage CEH described above (Ghosh 2000). We also cloned CEH from human liver and demonstrated that, unlike ACAT, hepatic and macrophage CEH are products of the same gene *Ces1* (Zhao et al., 2005). To evaluate the role of hepatic CEH in regulating hepatic cholesterol metabolism, we over expressed CEH in mouse liver using adenovirus. Our data demonstrated a significant increase in bile acid secretion following adenovirus-mediated over-expression suggesting that CEH plays an important role in regulating this last step of RCT and enhances the elimination of cholesterol from the body (Zhao et al, 2008). To address the question whether enhancing CEH mediated CE hydrolysis in liver can increase in vivo RCT, we utilized "macrophage-to-feces" in vivo RCT model described by Zhang et al (Zhang et al, 2003). Adenovirus mediated over-expression of CEH led to a significant increase in the flux of cholesterol from macrophages to feces, predominantly in fecal bile acids (Zhao et al, 2008). These data demonstrate that increase in hepatic CEH alone can increase the flux of cholesterol from peripheral tissues such as macrophages to liver and ultimate elimination from the body into feces. Such enhanced elimination of cholesterol from the body should be potentially anti-atherogenic and to test the hypothesis that increase in hepatic CEH alone can reduce atherosclerosis, we have developed liver-specific CEH transgenic mice and studies are in progress to evaluate the effects on diet-induced atherosclerosis in these transgenic mice in an LDLR-/- background.

Initially HSL was thought be not expressed in liver (Holm et al, 1987), but re-evaluation of HSL-/- mice has provided some evidence that suggests that HSL might be expressed in liver and may contribute to CE hydrolysis in hepatocytes (Sekiya et al 2008, Fernandez et al 2008). However, to date, there are no reports of direct evaluation of HSL in regulating bile acid synthesis precluding the determination of its role in RCT and cholesterol elimination from the body. Expression of AADLAC1 or KIAA1363 is negligible in human and mouse liver and it is unlikely to play a significant role in either hepatic cholesterol homeostasis or in regulating the terminal step of RCT, namely hydrolysis of HDL-delivered CE.

2.5 Metabolic consequence of CE accumulation in macrophages

Intracellular accumulation of CE or cholesterol is considered to enhance the expression of pro-inflammatory mediators. Fazio and Linton proposed a feedback loop linking macrophage cholesterol balance and inflammatory mediators and suggested that a primary defect in cellular cholesterol balance may induce changes in the inflammatory status of the macrophage (Fazio and Linton 2001). Close relationship between macrophage cholesterol efflux and inflammation is also exemplified by attenuation of in vitro efflux and in vivo RCT by endotoxins (McGillicuddy et al 2009). In addition, pro-inflammatory cytokines such as IL-1β and TNFα enhance lipid accumulation and foam cell formation by reducing lipid catabolism (Persson et al 2008). Consistently, under conditions of enhanced cholesterol accumulation, for example by deficiency of cholesterol transporter ABCA1, there was a marked increase in TNF-α secretion from macrophages (Koseki et al 2007) and increased inflammation with resulting loss in pancreatic beta cell function (Tang and Oram, 2009). It is also noteworthy that macrophages from ldlr-/- Abca1-/- mice with 80-fold increase in cellular CE content display an exaggerated inflammatory response (Francone et al, 2005). Thus, accumulation of CE in macrophages is likely to be central to the development of multiple metabolic disorders including atherogenesis, diabetes and hepatic steatosis (Figure 4).

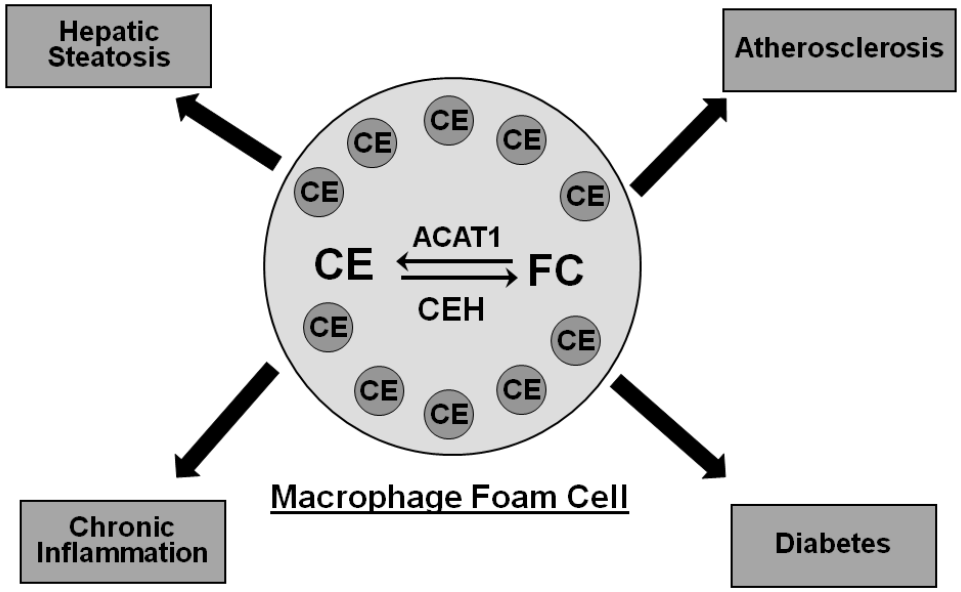


Fig. 4. Role of macrophage foam cell in regulating multiple metabolic disorders

2.5.1 CEH-mediated CE mobilization and inflammation

In contrast to CE accumulation, over-expression of ApoA1 that enhances CE removal and decreases cellular cholesterol levels results in attenuated response to pro-inflammatory insult by LPS (Levine et al 1993). Further, a recent clinical study demonstrated beneficial effects of re-constituted HDL infusions that enhance cholesterol efflux from cells on suppression of inflammation (Patel et al 2009) underscoring the importance of cholesterol removal in modulating inflammatory processes.

The observed decrease in lesion necrosis in CEH transgenic mice (Zhao et al 2007) was the first indication that CEH-mediated increase in macrophage CE mobilization is likely to be anti-inflammatory. We, therefore, directly examined the effects of CEH mediated hydrolysis of intracellular CE on reducing inflammation and inflammation-linked pathologies. Macrophage-specific transgenic expression of CEH significantly improved glucose tolerance and insulin sensitivity in LDLR-/- mice (Bie et al, 2010) demonstrating a critical role for CE mobilization in the development of insulin resistance and Type-2 diabetes mellitus (T2DM). Chronic low grade inflammation is increasingly being recognized as a key step in the pathogenesis of obesity-induced insulin resistance and T2DM and expanding adipose tissue was initially recognized as the site of production of pro-inflammatory mediators (Trayhurn and Wood 2004, Weiss 2004) responsible for this low grade inflammation. However, recent studies have demonstrated that majority of adipose tissue derived cytokines (TNFα, IL-6 and IL-1β) actually originate in non-fat cells and among them, infiltrated macrophages play the most prominent role. This low-grade inflammation is mediated by the activation and recruitment of macrophages into the expanding adipose tissue (Bouloumié et al 2005). The level of macrophages within a tissue represents a balance between recruitment, survival/expansion and emigration which is facilitated by several chemokines and growth

factors produced by adipocytes as well as infiltrated resident macrophages. These include monocyte chemoattracttant protein 1 (MCP-1) (Kanda et al, 2006), macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Kim et al, 2008) that recruit and assist in expansion or colonization of macrophages, respectively. Whether accumulation of cholesterol in macrophages can affect macrophage recruitment into the adipose tissue due to increased production of these pro-inflammatory mediators has not been explored. Subramanian et al have recently reported that addition of relatively small amount (0.15%) of dietary cholesterol resulted in marked increase in accumulation of macrophages in adipose tissue (Subramanian et al, 2008). Although this study provides the first evidence that cholesterol plays an important role in macrophage infiltration into the adipose tissue, the role of macrophage cholesterol balance in regulating this process remains undefined. We demonstrated that CEH-mediated increase in CE mobilization in macrophages decreased activation of pro-inflammatory transcription factor NF-κB resulting in decreased expression of pro-inflammatory chemokines (e.g., MCP-1) and cytokines (e.g., IL-1β and IL-6) resulting in significant reduction in circulating cytokines. Macrophage infiltration into expanding adipose tissue was also significantly reduced in CEH transgenic mice. Further, insulin signaling in adipocytes was not dramatically perturbed by CEH over-expressing macrophages (Bie et al, 2010). Collectively, these data led us to propose the following model (Figure 5) whereby CEH-mediated reduction in macrophage CE levels regulate infiltration of macrophages into the expanding adipose tissue. Reduction in the number of macrophages limits the deleterious effects of macrophage-adipocyte interactions that are central to the development of insulin resistance.

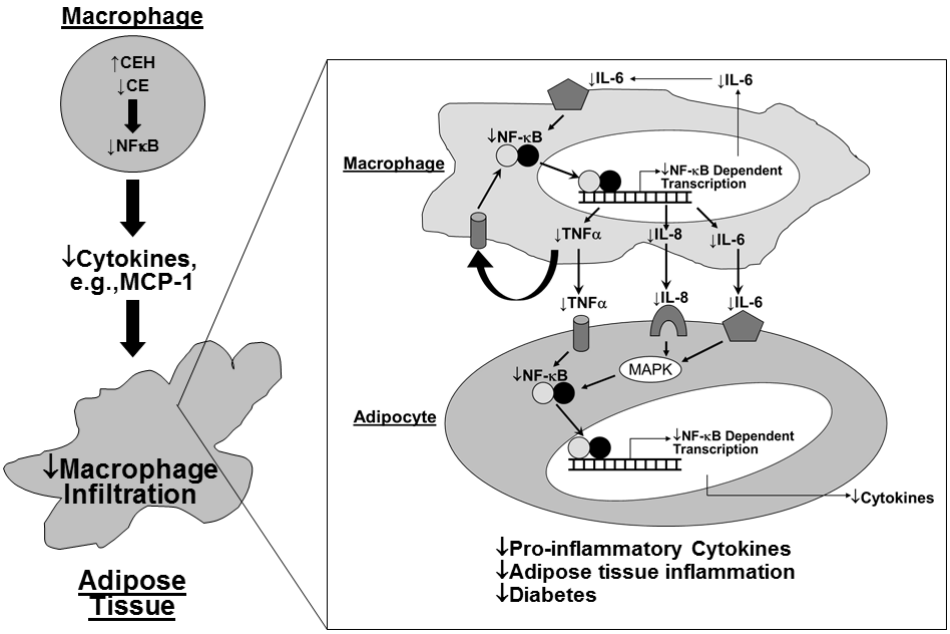


Fig. 5. Proposed model for Improved Insulin Sensitivity by CEH-mediated macrophage CE mobilization

Besides hypercholesterolemia, chronic low grade inflammation, obesity and T2DM are additional risk factors for development of atherosclerosis and CAD. Our data showing CEH-mediated attenuation of insulin resistance and systemic as well as adipose tissue inflammation establishes macrophage CE mobilization as an important therapeutic target that can simultaneously affect multiple and linked metabolic disease processes.

2.5.2 CEH-mediated CE mobilization and hepatic steatosis

In addition to the development of obesity and insulin resistance, both of which are risk factors for atherosclerosis, excessive intake of high calorie or high fat food also leads to the ectopic deposition of excess fat in tissues other than the physiological fat depots such as adipose tissue. Increased accumulation of lipids in liver results in hepatic steatosis that is central to the development of non-alcoholic fatty liver disease and which progresses into non-alcoholic steatohepatitis. The etiology of this disease is distinct from a similar liver pathology seen with alcoholism and hence the name.

Upon excessive lipid storage liver overproduces multiple cardiovascular risk factors such as glucose, very low density lipoproteins, plasminogen activator inhibitor-1 (PAI-1) coagulation factors and C-reactive protein (CRP). In addition, liver fat content is closely related to fasting insulin concentrations and direct measures of hepatic insulin sensitivity and increase in hepatic lipid contents predicts T2DM (Yki-Jarvinen, 2005). The mechanism(s) responsible for increased intra-hepatic triglyceride accumulation are not completely understood. It has been suggested that dysfunctional adipose tissue, characterized by adipocyte hypertrophy, macrophage infiltration, impaired insulin signaling, and insulin resistance, releases a host of inflammatory adipokines and excessive amounts of free fatty acids (FFA) that promote ectopic fat deposition in liver (Cusi, 2010). Alterations in FFA uptake regulated at the level of fatty acid transporter, CD36 expression is thought to be involved in increased triglyceride accumulation and hepatic CD36 expression is directly correlated with liver fat in human subjects (Greco et al, 2008).

Kupffer cells are resident macrophages present in the liver and, therefore, changes in macrophage metabolism and/or phenotype will also affect kupffer cells. However, the role of hepatocyte microenvironment namely the effect of Kupffer cells in regulating triglyceride accumulation in hepatocytes is not completely defined. Pro-inflammatory cytokines (namely, IL-1β, IL-6 or TNFα) secreted by Kupffer cells activate hepatocytes (Scott et al, 2005), affect gluconeogenesis (Yerkovich et al, 2004), increase the expression of acute phase proteins (Knolle et al, 1995) and enzymes involved in xenobiotic metabolism (Milosevic et al, 1999). On the other hand, anti-inflammatory cytokine IL-10 secreted by Kupffer cells is essential for hepatocyte homeostasis and its loss under conditions of Kupffer cell depletion is associated with increased STAT3-dependent signalling and steatosis leading to decreased insulin signalling (Clementi et al, 2009). These studies underscore the importance of understanding the mechanisms involved in regulating the inflammatory status of Kupffer cells. To directly evaluate the role of kupffer cells, we examined hepatocyte triglyceride (TG) accumulation in livers of leptin-deficient obese (ob/ob) mice that were crossed with macrophage-specific CEH transgenic mice. Leptin deficiency in these ob/ob mice leads to increased obesity resulting in significant ectopic lipid accumulation in the liver and, thus, providing an ideal model to test the effects of decreased CE accumulation in kupffer cells as a result of transgenic expression of CEH on hepatocyte TG metabolism. Accumulated lipids are stored as cytoplsamic lipid droplets that continue to occupy greater cellular area and

total area occupied by lipid was used as a measure of total lipid accumulation and percent area occupied by lipid was significantly reduced by transgenic expression of CEH (Table 1). These data were confirmed by direct quantification of TG in total hepatic lipids where a significant decrease in hepatic TG content was observed in ob/ob-CEH transgenic mice. It is important to note that expression of CEH transgene in this model is macrophages-specific and, therefore, the observed effects are due to the changes in kupffer cell CE metabolism.

Gentotype	Percent area occupied by lipid	Total TG (μg/mg tissue)
ob/ob	45.4 ± 5.3	32.3 ± 4.3
ob/ob-CEH Transgenic	39.5 ± 3.1**	23.1 ± 4.2**

Table 1. Macrophage-specific transgenic expression of significantly decreases hepatic lipid accumulation **P<0.05

Consistent with a reduction in hepatic lipid content, ob/ob-CEH transgenic mice showed improved glucose tolerance and insulin sensitivity.

Macrophage polarization plays a pivotal role in the development of insulin resistance and it is promoted by a transition from an alternative M2 activation state maintained by STAT6 and PPARs to a classical M1 activation state driven by NF-κB, AP1, and other signal-dependent transcription factors that play crucial roles in innate immunity (Olefsky and Glass, 2010). While IL-1β, IL-6 and TNFα are the predominant cytokines secreted by M1 macrophages, M2 macrophages secrete anti-inflammatory cytokines IL-10 and IL-13. M1 activation by LPS via Toll-like receptor 4 (TLR4) or by free fatty acids as identified recently, triggers inflammatory responses (Bilan et al, 2009). Intracellular lipid content, specifically the cholesterol content, is increasingly being recognized as another major factor that contributes to macrophage activation. While cholesterol loading of macrophages activated TLR4 (Sun et al, 2009), HDL or ApoA1-mimetic stimulated removal of cellular cholesterol abolished LPS-induced mRNA expression of pro-inflammatory mediators such as MCP-1, MIP-1, RANTES, IL-6, and TNF-alpha but significantly up-regulated LPS-induced anti-inflammatory IL-10 expression (Smythies et al, 2010). Interaction of ApoA1 with cholesterol transporter ABCA1, suppressed the ability of LPS to induce pro-inflammatory cytokine expression suggesting that cholesterol removal from macrophages is an anti-inflammatory event (Tang et al, 2009). CEH-mediated increase in CE mobilization and subsequent decrease in cellular cholesterol content is, therefore, likely to polarize the macrophages to an M2 or anti-inflammatory phenotype. This is consistent with our earlier data demonstrating a decrease in expression of pro-inflammatory cytokines via reduced activation of NF-κB and AP-1 in CEH transgenic macrophages (Bie et al, 2010) that leads to a decrease in the levels of pro-inflammatory cytokines (e.g., IL-6) in circulation and reduction in systemic inflammation. Based on these data, we propose the following model (Figure 6) to integrate the cellular events and interactions that lead to the increased accumulation of TG in the liver and how CEH-mediated reduction in cellular CE content resulting in M2 polarization of Kupffer cells as well as decrease in circulating cytokines such as IL-6 can attenuate this process.

The studies described above and the recent developments linking cellular CE content to macrophage phenotype and inflammation underscore the importance of macrophage cholesterol homeostasis in regulating multiple metabolic disorders.

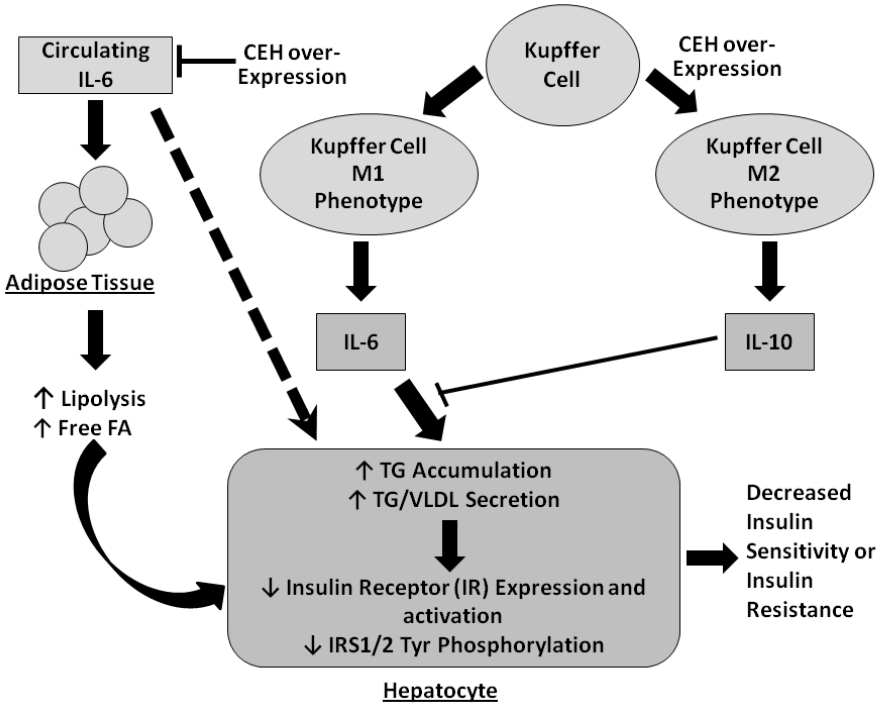


Fig. 6. Proposed model by which CEH-mediated macrophage CE mobilization attenuates hepatic TG accumulation and improves insulin sensitivity.

2.5.3 CEH-mediated CE mobilization and atherosclerotic plaque stability

In recent years, there has been a shift in the paradigm of arterial re-modelling during progression of atherosclerosis and improved understanding of the mechanism underlying acute coronary syndromes or sudden heart attacks. The earlier notion of gradual narrowing of the arterial lumen that eventually occludes the coronary artery resulting in heart attacks is being replaced by the concept of a developing plaque in the arterial wall (that may or may not affect the lumen) that becomes unstable and prone to rupture. Thrombotic events resulting from the sudden release of lipid contents of the plaque into circulation is now considered as the primary cause of acute coronary events. Large necrotic cores as well as increased inflammation associated with plaques are the two main factors that determine plaque stability.

Macrophage content of plaque is variable during the development of plaques (from fatty streaks to complex plaques) and is determined by processes involved in macrophage recruitment, apoptosis and egress. A balance between these processes limits plaque progression and is also likely to maintain favourable plaque characteristics. With increased lipid accumulation, plaque-associated foam cells undergo apoptosis and functional macrophages are required for efficient clearance of apoptotic cells (or efferocytosis). Inability to effectively clear the apoptotic cells results in accumulation of released lipids and other inflammatory mediators that lead to secondary necrosis. Growth of acellular necrotic core

not only destabilizes the plaque by altering its physical characteristics, but it also limits smooth muscle cell proliferation required to maintain the integrity of the fibrous cap of the plaque. Our earlier studies have demonstrated a decrease in lesion necrosis in macrophage-specific CEH transgenic mice. These lesions also contained increased number of macrophages and significantly less number of apoptotic cells (Zhao et al, 2007). Collectively, these characteristics are suggestive of a more stable plaque.

Khallou-Laschet et al evaluated the phenotype of macrophages associated with progression of atherosclerosis in mice and demonstrated that early lesions were infiltrated with alternatively activated and anti-inflammatory M2 macrophages which favoured smooth cell proliferation and deposition of extra-cellular matrix (tissue repair phenotype). However, a phenotypic switch of existing macrophages within the plaques from M2 to M1 (classically activated macrophages with pro-inflammatory properties) occurred during plaque progression resulting in increased inflammation (Khallou-Laschet et al, 2010). M1 macrophages, identified by high expression of Ly6C surface antigen ($Ly6C^{Hi}$), increase with hypercholesterolemia, actively adhere to endothelium, become lesional macrophages and represent a newly recognized component of the inflammatory response in atherosclerosis (Swirski et al, 2007). We examined the phenotype of diet-induced lesion associated macrophages isolated from LDLR-/- and LDLR-/-CEH transgenic mice. Consistent with our earlier results described above, increased polarization towards M2 phenotype was noted in lesional macrophages from CEH transgenic mice. Studies are in progress to determine the effects of CEH-mediated shift in macrophage polarization on lesion-associated inflammation.

Increase in inflammation with increasing cellular cholesterol content has been known for a long time. Recent studies have also shown that deficiency of cholesterol transporter ABCA1 in LDLR-/- mice that results in massive CE accumulation, leads to an exacerbated response to lipopolysaccharide and increased inflammation (Francone et al, 2005). Increased inflammatory gene expression was also noted in macrophages from ABCG1 deficient macrophages (Balden et al, 2008). However, cellular mechanisms that directly link changes in cellular cholesterol homeostasis to inflammatory pathways remain undefined. In cells with increased CE accumulation, we demonstrated that CEH-mediated CE mobilization directly attenuated NF-κB and AP1-driven gene expression suggesting that pro-inflammatory transcription factor-driven gene expression is directly affected by cellular CE content (Bie et al 2010). However, the intracellular processes that are involved in "sensing" and "linking" of cellular CE content to inflammatory gene expression still remain to be elucidated.

3. Conclusions

Maintaining macrophage cholesterol homeostasis is central to atherogenesis. While unregulated uptake of modified LDL results in increased cellular CE content and foam cell formation, CEH-mediated mobilization of CE represent an important mechanism by which macrophages reduce the lipid burden. Given the importance of this step, intracellular CE hydrolysis is a multi-enzyme process and several enzymes catalyze this reaction. It is extremely important that the future focus is maintained on intracellular CE hydrolysis and not on establishing the "major" CE hydrolase in either human or mouse macrophages. Current strategies of single gene ablation are likely to be inconclusive not only due to the presence of multiple enzymes but also due to as yet unrecognized compensatory

mechanisms that may become operative under conditions of single gene deficiency leading to erroneous dismissal of the candidate CE hydrolase as unimportant .

Removal of cholesterol from arterial wall associated macrophage foam cells represents the first step in prevention of atherosclerotic plaque formation or regression of existing plaques. Our results with CEH over-expression conclusively demonstrate the anti-atherogenic role of this enzyme in mobilizing stored CE from macrophages and attenuating atherogenesis. Cholesterol removed from the periphery including artery wall associated macrophages is returned to the liver via HDL and liver is the only organ that facilitates final elimination of cholesterol from the body. Since 80% of the total cholesterol delivered by HDL is CE, hepatic hydrolysis of CE is crucial to further metabolism of cholesterol. Consistently, increase in hepatic CE hydrolysis enhances elimination of cholesterol as bile acids in faeces. More importantly, increasing CE hydrolysis in liver alone stimulates movement of cholesterol from macrophages to faeces. Thus, hydrolysis of CE is an extremely important step in removal of cholesterol from the body and intracellular CE hydrolysis represents an important anti-atherogenic step that should be targeted for therapeutic benefit.

Although macrophage cholesterol homeostasis is intuitively linked to atherogenesis as the primary disease process, continuing characterization of macrophage-specific CEH transgenic mice has revealed novel roles of CEH in regulating several disease processes and has established the central role of macrophage cholesterol homeostasis. By modulating infiltration of macrophages into adipose tissue and thus affecting adipose tissue as well as systemic inflammation, mobilization of CE from macrophages improves glucose tolerance and insulin sensitivity. Similarly, by altering the polarization towards a more anti-inflammatory phenotype, increased CE mobilization from kupffer cells attenuates hepatic steatosis. Altered polarization towards M2 phenotype in atherosclerotic lesions also leads to decreased lesion necrosis and increased macrophage survival. Collectively, these studies clearly establish CEH as an important therapeutic target with a potential to simultaneously affect multiple disease processes. Ongoing studies will establish the direct link(s) between cellular CE content and inflammatory pathways and identify the intracellular pathways involved.

From a clinical and translational perspective, future studies are necessary to delineate endogenous mechanisms that regulate these CE hydrolases such that these can be specifically targeted (e.g., by pharmacological means) to increase the activity in vivo.

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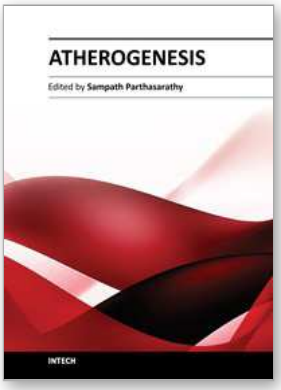

Atherogenesis

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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