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Expert Review

Potential Role of Acyl-Coenzyme A:Cholesterol Transferase (ACAT) Inhibitors as Hypolipidemic and Antiatherosclerosis Drugs

Carlos Leon,[1] John S. Hill,[2] and Kishor M. Wasan[1,3]

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Abstract. Acyl-coenzyme A:cholesterol transferase (ACAT) is an integral membrane protein localized in the endoplasmic reticulum. ACAT catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl coenzyme A. The cholesteryl esters are stored as cytoplasmic lipid droplets inside the cell. This process is very important to the organism as high cholesterol levels have been associated with cardiovascular disease. In mammals, two ACAT genes have been identified, ACAT1 and ACAT2. ACAT1 is ubiquitous and is responsible for cholesteryl ester formation in brain, adrenal glands, macrophages, and kidneys. ACAT2 is expressed in the liver and intestine. The inhibition of ACAT activity has been associated with decreased plasma cholesterol levels by suppressing cholesterol absorption and by diminishing the assembly and secretion of apolipoprotein B-containing lipoproteins such as very low density lipoprotein (VLDL). ACAT inhibition also prevents the conversion of macrophages into foam cells in the arterial walls, a critical event in the development of atherosclerosis. This review paper will focus on the role of ACAT in cholesterol metabolism, in particular as a target to develop novel therapeutic agents to control hypercholesterolemia, atherosclerosis, and Alzheimer's disease.

KEY WORDS: acyl-coenzyme A:cholesterol transferase; atherosclerosis; cholesterol; hypercholesterolemia; lipoproteins.

INTRODUCTION

High serum cholesterol levels have been associated with cardiovascular disease (CD), a leading cause of death and disability in the Western world (1). Cholesterol metabolism has also been implicated in the development of Alzheimer's disease (AD) (2), a neurodegenerative condition that affects 5 million individuals and is the fourth leading cause of death in the United States. Cholesterol levels are affected by the rate of endogenous cholesterol synthesis, biliary cholesterol excretion, and dietary cholesterol absorption (3).

Several lipid lowering strategies, especially with cholesterol synthesis inhibitors ("statins"), have been developed (4) and are currently in use. Nevertheless, a substantial number of patients who receive a statin monotherapy, do not achieve the treatment goals (5). Moreover, augmenting the dose of statins may also increase adverse side effects (6). Given the limitations of the statins and other lipid agents such as fibrates and bile acid sequestrants, the research on novel lipid lowering agents is ongoing and is currently targeting the inhibition of intestinal cholesterol absorption. Compounds that lower cholesterol absorption include plant sterols and stanols (7), ACAT inhibitors (8), microsomal triglyceride transfer protein (MTP) inhibitors (9), and ezetimibe (10).

Several studies have demonstrated that ACAT inhibitors limit cholesterol absorption in animal models (11,12). The development of ACAT knockout mouse models (13,14) has confirmed the important role of ACAT in cholesterol absorption, in particular, in a high fat diet scenario (15). A better understanding of ACAT expression and activity is the cornerstone for the development of novel inhibitors to treat conditions such as hypercholesterolemia, atherosclerosis, and Alzheimer's disease.

IDENTIFICATION OF ACAT

Acyl-coenzyme A:cholesterol transferase (ACAT), also known as sterol *o*-acyltransferase (SOAT; EC 2.3.1.26) is responsible of the esterification of cholesterol with fatty acids (16). This reaction takes place in the endoplasmic reticulum of a variety of cells and tissues (17). By esterifying cholesterol into cholesteryl ester, ACAT allows the storage of the otherwise toxic free cholesterol (a polar lipid) into the highly

[1] Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.
[2] Department of Pathology and Laboratory Medicine, Faculty of Medicine, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.
[3] To whom correspondence should be addressed. (e-mail: Kwasan@interchange.ubc.ca)

ABBREVIATIONS: ACAT, acyl-coenzyme A:cholesterol transferase; AD, Alzheimer's disease; Aβ, amyloid beta peptide; CD, cardiovascular disease; CE, cholesteryl esters; DGAT, diacylglycerol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; PBR, peripheral-type benzodiazepine receptor; SOAT, sterol *o*-acyltransferase; TG, triglyceride; TMD, transmembrane domain; VLDL, very low density lipoprotein.

nonpolar cholesteryl esters. Initial studies suggested a transmembrane localization of ACAT because it required detergents for its solubilization (18). However, reduced ACAT expression levels and enzyme inactivation by detergents during purification limited the rate of progress in elucidating its mechanism of action (18).

The understanding of cholesterol esterification was triggered by the identification of the human ACAT gene cDNA by Chang *et al.* (19) using an expression cloning strategy. For these studies, an ovary hamster cell line deficient in ACAT activity was generated by mutagenesis. Stable transfection with human cDNA fragments complemented the ACAT deficiency in these cells, leading to the isolation of an ACAT cDNA. This cDNA predicted a 550-amino acid integral membrane protein. Subsequently, the expression of this cDNA in insect cells, devoid of cholesterol esterification activity, coupled to ACAT enzymatic assays determined that this gene encoded for a catalytic subunit (20). Based on this initial finding, other mammalian ACAT genes were cloned (21,22), showing that mammalian ACAT proteins share more than 87% homology. At least three independent lines of evidence have led to the realization of the existence of more than one functional ACAT isoform. Kinnunen *et al.* (23) suggested the presence of at least two different types of ACAT in rabbits based on biochemical studies using ACAT isolated from aorta and liver that showed a 40-fold difference in sensitivity to the same inhibitor. The identification of two sterol esterification genes in yeast (ARE1 and ARE2), which were functional in the absence of the other one (24), also supported the possibility of more than one human gene. Another evidence was the isolation of a mouse ACAT homolog (25) and expression studies in different tissues, as ACAT was only marginally expressed in the liver and small intestine, which also presented significant ACAT activity (13,22).

The unequivocal evidence of a second ACAT gene came with the disruption of the mouse ACAT gene by Meiner *et al.* (13). The inactivation of this gene resulted in decreased cholesterol esterification in ACAT-deficient fibroblasts, macrophages, and adrenal tissues, whereas livers of ACAT-deficient mice contained significant amounts of cholesteryl esters and no apparent reduction in ACAT activity. Phenotypic changes were also absent in the intestines of ACAT-deficient mice. The answer to this puzzle came through the work of Cases *et al.* (26) who reported the cloning, expression, and characterization of a second mouse ACAT gene. This new gene, designed ACAT2, was 44% identical to the first cloned mouse ACAT (renamed ACAT1). ACAT2 was primarily expressed in mouse liver and intestine, as shown by Northern blot and RT-PCR analysis, thus explaining the normal cholesteryl ester levels in these tissues in the ACAT1 knockout mice.

ACAT STRUCTURE

Human ACAT1 encodes a 550-amino acid protein and its calculated molecular mass is 69 kDa. However, its relative molecular weight in denaturing gel electrophoresis is 46 kDa; this difference might be explained by its high isoelectric point and/or its high hydrophobicity (20). Human ACAT2 is a 552-amino-acid-long polypeptide, with a predicted molecular mass of 60.7 kDa, although its relative molecular weight is smaller than predicted, as in the case of ACAT1 (27). ACAT1 and ACAT2 share high homology near the carboxyl terminus but not in the amino terminus. The homology between the nucleotide sequences of the human isoforms is 55%. Figure 1A depicts some of their characteristics, including predicted transmembrane domains as well as putative phosphorylation sites.

Both ACAT1 and ACAT2 are integral membrane proteins. This conclusion arises from the original biochemical studies that required the use of the detergent deoxycholate for their isolation (18) and is supported by the hydropathy studies of the predicted amino acid sequence that suggested that both enzymes may contain multiple transmembrane domains (28,29). Lin *et al.* (28) used a human ACAT1 protein individually tagged at various hydrophilic residues to transfect a CHO cell line deficient in the endogenous gene. The transfected cells were permeabilized with detergent to allow the access of antibodies to the cytosol, and immunofluorescence microscopy was used to investigate the topography of the tagged ACAT1 proteins. This approach suggested that ACAT1 presents seven transmembrane domains (TMDs). Joyce *et al.* (29) used a different model of monkey ACAT1 proteins, truncated at the end of the TMDs, tagged with a glycosylation, and expressed *in vitro* in microsomal membranes. This study concluded that ACAT1 presents only five TMDs. These five TMDs were detected by both approaches; however, only Lin *et al.* reported the presence of TMDs comprising the regions of amino acids 325–342 and 471–486. One important difference between the two studies was that the epitope tagged approach confirmed the expression and enzymatic activity, whereas the truncated approach did not report these parameters. In the case of ACAT2, Joyce *et al.* (29) reported five TMDs in monkey ACAT2, whereas Lin *et al.* (28) found only two TMDs. The role of the amino terminal region of ACAT1 was analyzed by Yu *et al.* (30). They found that the N-terminal region, comprising 131 amino acids, resided in the cytoplasm and contained the dimer-forming motif. When this region was deleted, the enzyme converted from a homotetramer to a homodimer. Nevertheless, it did not lose its ability to bind cholesterol, suggesting that the substrate recognition site is not located in this region. Based on studies where ACAT1 was expressed as a histidine-tagged protein in insect cells and the use of coimmunoprecipitation and chemical cross-linking, it was concluded that the human ACAT1 is a homotetrameric protein (31).

There are three studies that have looked to determine the localization of potential substrate binding sites in ACAT. Cao *et al.* (32) used a hamster ACAT1 construct with a S269L mutation for transfection studies in CHO cells. This residue was conserved in ACAT2 and in diacylglycerol acyltranferase (DGAT). The mutant ACAT1 was associated with decreased enzymatic activity, but also decreased expression levels when compared to the wild-type enzyme. Joyce *et al.* (29) showed that the same mutation, but in this case in ACAT2, also led to a loss of activity. Guo *et al.* (33) analyzed a motif, FYxDWWN, present in the carboxyl terminal region of ACAT and DGAT of yeast, mammals, and plants. A mutation of the two tryptophan residues (WW527,528AS) in yeast ACAT gene resulted in a total loss

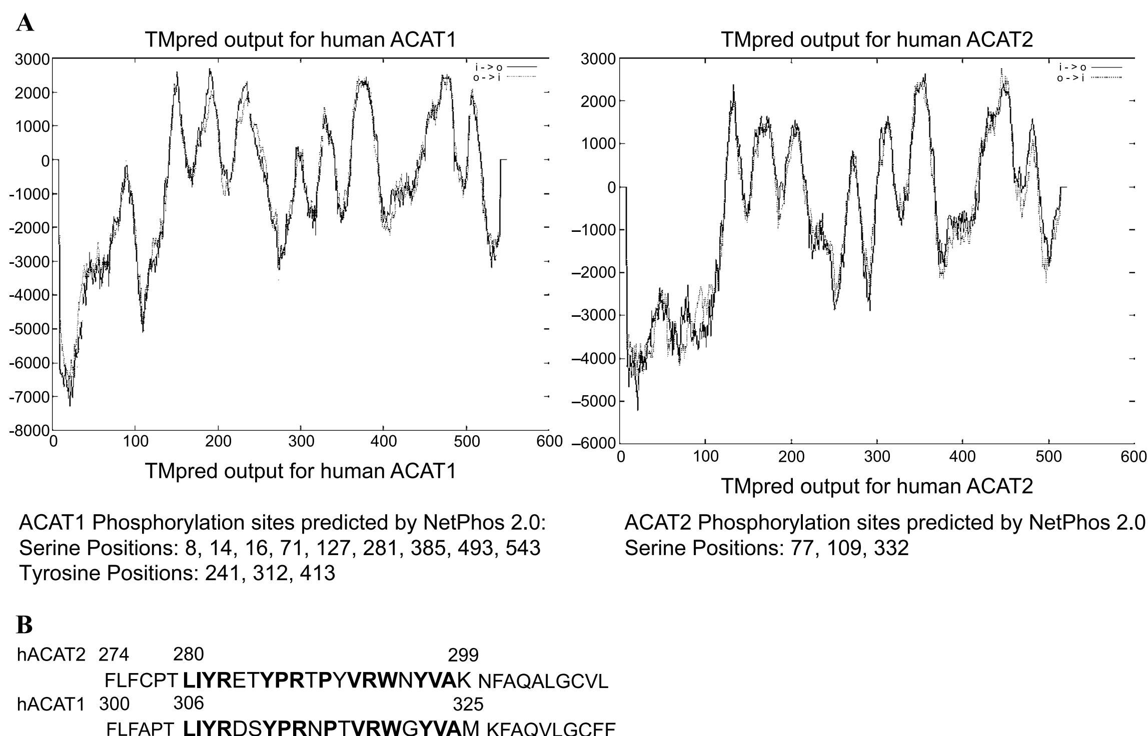

Fig. 1. (A) Transmembrane domains of human acyl-coenzyme A:cholesterol transferase 1 (ACAT1) and ACAT2 as predicted by TMpred. The sequences of human ACAT1 and ACAT2 were analyzed for the presence of transmembrane domains by using the program TMpred. The number of transmembrane domains predicted for ACAT1 coincides with the results by Lin *et al.* (28). In the case of Joyce *et al.* (29), they predicted five transmembrane domains for ACAT2, whereas this analysis renders six transmembrane regions. Both sequences were analyzed for the presence of serine, threonine, and tyrosine phosphorylation sites using the NetPhos program. (B) Localization of the putative cholesterol binding sites in human ACAT1 and ACAT2. The location of the cholesterol binding sites in the ACAT isforms is currently unknown. Several studies have tried to address this issue and are discussed in the text. Using an algorithm derived from the work of Li and Papadopoulos (34), we scanned the sequence of ACAT1 and ACAT2 and found a putative cholesterol binding site.

of enzymatic activity without compromising the microsomal localization of expression levels. Similarly, the change of tyrosine to alanine (Y524A) in both the human and yeast genes led to loss of activity, with no effect on protein levels or microsomal targeting. When an *in vitro* substrate saturation assay was carried out using increasing concentrations of oleoyl-CoA, the results indicated that this mutation significantly reduced the apparent oleoyl-CoA affinity. Another motif was studied, HSF, which is conserved in the human and yeast ACAT genes and is involved in the sterol esterification in simian ACAT1 and ACAT2 (29). An S339L mutation of the ARE2 yeast isoform did not catalyze esterification of sterols. In this case, the expression levels were drastically reduced in comparison with the wild-type enzyme, although the extent of the reduction in expression may not completely account for the total loss in activity. Interestingly, this residue is absent from DGAT, whose role is to esterify diacylglycerol and does not bind cholesterol. Li and Papadopoulos (34), while studying the peripheral-type benzodiazepine receptor (PBR) by mutagenesis, proposed the existence of a cholesterol binding motif [lv]-X(1,5)-Y-X(1,5) -[rk], where [lv] represents leucine or valine, X(1,5) corresponds to any sequence of one to five amino acids, Y represents tyrosine, and [rk] represents arginine or lysine.

This algorithm was deducted after analyzing the sequence of 20 different cholesterol binding proteins and determining the ability of a mutagenized PBR to transport cholesterol. We used this algorithm to scan the sequence of ACAT1 and ACAT2 and surprisingly we found two of these motifs in a "tandem" arrangement separated by four amino acids (Fig. 1B) in both ACAT1 and ACAT2 (homology between human ACAT tandem repeats 84%). When this 20-amino acid sequence was compared to the sequences present in the database, we found a high homology with other ACAT family members (95%) from species such as mouse and rat. The putative cholesterol binding sites in ACAT2 were located in the cytoplasmic region, according to the membrane distribution in the tridimensional model created by Lin *et al.* (28).

Further site-directed mutagenesis and crystallographical studies will contribute to determine the exact location of the active binding sites and will undoubtedly provide new ideas about the design of novel ACAT inhibitors.

ACAT EXPRESSION AND REGULATION

The human ACAT1 and ACAT2 genes present several differences in their localization and expression. ACAT1 is located in two different chromosomes, 1 and 7, which is an unusual feature for human genes (35). The ACAT1 gene, which spans over 200 kb, presents four transcripts of different sizes: 2.8, 3.6, 4.3, and 7 kb. A P1 promoter controls the 2.8-

and 3.6-kb transcripts, while a P7 promoter controls the expression of the 4.3-kb transcript.

Yang *et al.* (36) demonstrated that sequences located in these two chromosomes were required to produce a novel 56-kDa ACAT isoenzyme that was located in the endoplasmic reticulum and was enzymatically active.

The human ACAT2 gene located in chromosome 12, spans over 18 kb and presents 15 exons (37). Several potential motifs recognized by transcription factors were found in the 5′ flanking sequence upstream of the ACAT2 start codon, including sites for GAT-1, Cdx-2, and HNF-3b (37). Cdx-2 is an interesting candidate as regulator of ACAT2 expression, as suggested by serial deletion of the promoter and lost of transcription when the Cdx-2 binding region was absent. Song *et al.* (37) also demonstrated the binding of a Cdx-2 antibody to the promoter region of ACAT2. Interestingly, ACAT2 expression is correlated with the differentiation of the enterocytes, and Cdx-2 expression is enhanced by the enterocyte differentiation process (38). Sterol can regulate the expression of genes containing sterol responsive elements (SREs) in their promoter regions (39). However, no SRE could be found in the promoter regions of ACAT1 or ACAT2, consistent with the experiments where sterol did not modulate ACAT gene transcription (35).

Addition of cholesterol *in vitro* has been shown to potentiate ACAT (17). When particular oxygenated sterols were added to cells, an enhancement of cholesterol esterification was noted; there was no similar response when cholesterol was added under similar conditions (40). This suggests that maybe inside the cells, the ACAT activator is an oxysterol derived from cholesterol, rather than cholesterol itself. This was further supported by a study involving the expression of human ACAT1 in insect cells (20), which showed that recombinant ACAT activity was activated by oxysterol in intact cells and *in vitro*.

As pointed out above, the tissue distribution of ACAT1 and ACAT2 is quite different. ACAT1 is more ubiquitous and has been found in macrophages, adrenal glands, hepatocytes, enterocytes, renal tubule cells, and neurons (41). ACAT1 expression is upregulated during monocytic differentiation into macrophages (42), which is consistent with studies on undifferentiated THP-1 cells, a monocytic human cell line, demonstrating that different agonists that can trigger the differentiation process can upregulate ACAT1 expression. These agonists included 1,25-dihydroxyvitamin D3 (43), phorbol 12-myristate, and interferon gamma (44). Treatment of THP-1 cells with all-*trans*-retinoic acid and interferon gamma, a proatherogenic cytokine, induced the expression of the 2.8- and 3.6-kb transcripts (44). This is consistent with the presence of a site on the promoter P1 recognized by STAT1, the transcriptional activator that mediates many of the interferon gamma responses (45).

ACAT2 has been found in the apical region of the intestinal villi (46) and in the hepatocyte (47). Chang *et al.* (46) showed by immunodepletion, immunoblot, and activity assays that ACAT1 was the main ACAT isoform expressed in the human hepatocyte; however, immunohistochemistry studies were not able to confirm these findings. Using immunoblotting, immunofluorescence, and activity assays, Parini *et al.* (47) demonstrated that ACAT2 is the main ACAT isoform expressed in human liver. ACAT2 expression in the liver and the intestine is consistent with its roles in lipoprotein assembly and secretion of cholesteryl esters. Based on these studies, the tissue distribution of the ACAT isoforms is virtually the same between humans, mice, and primates.

ACAT expression can also be affected in disease states. ACAT1 is expressed in macrophage-derived foam cells present in human atherosclerotic lesions (48). Interestingly, adiponectin, an adipocytokine that has been shown to inhibit foam cell formation (49), can downregulate ACAT1 expression in macrophages derived from human peripheral mononuclear cells (50). Interestingly, all four ACAT1 transcripts were decreased by the adiponectin treatment and this paralleled with a drastic reduction in cholesteryl ester accumulation.

ACAT2 expression was shown to be upregulated in a puromycin-induced rat nephrotic syndrome model (51). This study reported a correlation between the ACAT2 protein expression and the plasma total cholesterol. The same group also described a diet-induced syndrome X rat model (52). In this study, the group of animals that developed the syndrome X (also known as metabolic syndrome), presented obesity and hyperlipidemia, accompanied with increased expression of ACAT2 and the scavenger receptor SR-B1. Cholesterol 7α-hydroxylase and LDL receptor levels were decreased.

Wilcox *et al.* 2001 (53) reported the ability of the flavonoids, naringenin, and hesperetin to decrease the expression and activity of ACAT2 and MTP. The decrease in ACAT2 messenger was specific, as ACAT1 expression was unaffected by these compounds.

The characterization of compounds that modulate the expression of ACAT1 and ACAT2 will be very helpful in understanding their gene regulation, both in basal and disease states.

UNDERSTANDING THE ROLE OF ACAT IN CHOLESTEROL METABOLISM USING ACAT INHIBITORS AND KNOCKOUT MICE MODELS

Cellular cholesterol content is the net result of local *de novo* synthesis, influx, and efflux. These components consist of separate metabolic pathways, depicted in Fig. 2, which depend on the abundance of specific lipoproteins that deliver or accept cholesterol.

The liver plays an important role in the regulation of plasma lipoprotein concentration. The liver and the brain are the major organs responsible for synthesizing and processing cholesterol. After lipolysis of their triglyceride core by lipoprotein lipase, the VLDL remnants are either removed by the liver or transformed to LDL particles, which may be taken up by peripheral tissues via the LDL receptor. In contrast to the pathway describing the delivery of cholesterol to peripheral tissues, there is a process termed reverse cholesterol transport that describes the transfer of cholesterol from peripheral tissues back to the liver. High density lipoproteins (HDL) are thought to have key role in the transport of cholesterol from the periphery back to the liver, where cholesterol may be secreted into the bile and ultimately excreted in the feces. The presence of these two components, a forward and a reverse transport, leads to a fined-tuned regulation of cholesterol metabolism.

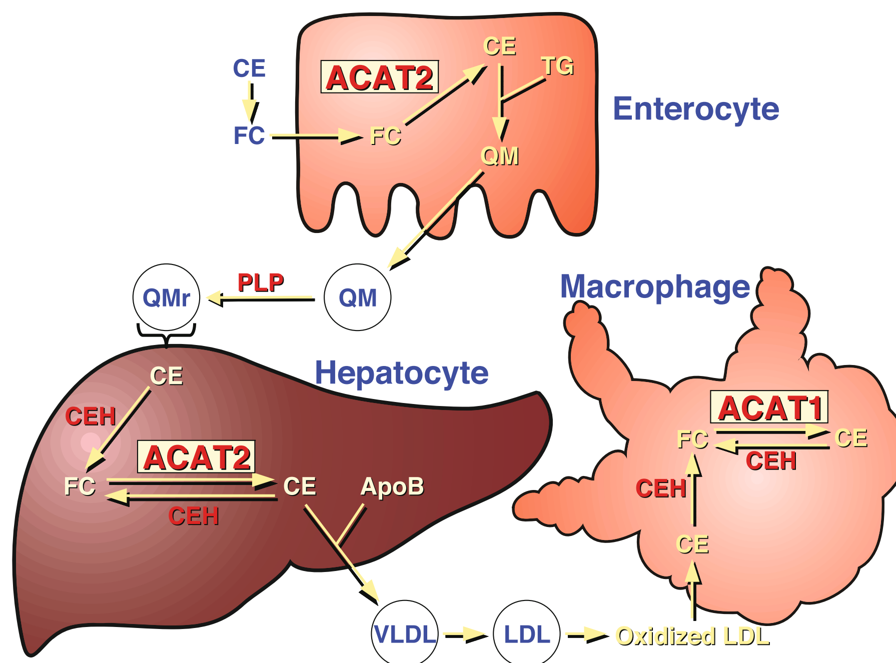

Fig. 2. Cholesterol metabolism and the role of ACAT1 and ACAT2. Cellular cholesterol content is the net result of local *de novo* synthesis, influx, and efflux. This figure illustrates some of cholesterol metabolic pathways involved in the liver, macrophage, and small intestine. Cholesterol metabolism in the brain is briefly discussed in the text.

In the liver, ACAT produces cholesteryl esters that constitute the core lipid in the very low density lipoprotein (VLDL). Craig *et al.* (54) demonstrated that β-VLDL or chylomicron remnants caused an increase in cellular cholesteryl esters and stimulated secretion of newly produced apo-B. The use of ACAT inhibitors has supported this idea as avasimibe, a potent ACAT inhibitor, decreased the synthesis of cholesteryl esters, inducing the accumulation of apoB in a HepG2 cell model (55). *In vivo* studies in rabbits (11), hamsters (12), and monkeys (56) support these *in vitro* results.

The major part of cholesterol entering the intestine, biliary, and dietary cholesterol, is reabsorbed (57). Intestinal cholesterol absorption was thought to be a passive process. However, there is increasing evidence of an active transport of cholesterol; for example, it has been shown to be saturable (58), susceptible to inhibition (59), and to be associated with individual variability (60). Several proteins have been implicated as potential cholesterol influx transporters, such as the scavenger receptors SR-BI and CD36, but the data on knockout mouse models do not support their involvement in cholesterol flux (61,62). Another protein, NPC1L1, was recently implicated in cholesterol absorption (10). Once cholesterol is absorbed in the small intestine, ACAT is involved in converting dietary and biliary cholesterol into cholesteryl ester that constitute the core lipid in chylomicrons. VLDL and chylomicrons are the most important lipoprotein carriers for triacylglycerol transport in the blood.

In the macrophage, ACAT also modulates the cholesterol/cholesteryl ester ratio, and its expression is increased in the early stages of atherosclerotic plaque formation, characterized by the accumulation of cytoplasmic lipid droplets in both macrophages and smooth muscle cells. Macrophages entering the early atherosclerotic plaque take up large amounts of the free cholesterol that is required to be stored as cholesteryl esters. Thus under conditions where free cholesterol cannot be esterified, i.e., by using ACAT inhibitors, there is cell death (63).

The brain can also synthesize cholesterol. In average, the human brain contains 20 mg of cholesterol per gram of tissue, which is six times the amount present in the liver (64). Because cholesterol is synthesized *de novo* in the brain and its metabolism is very slow (a half-life of 1 year in human brains compared to a few hours in the blood), serum cholesterol fluctuations do not affect central nervous system cholesterol (65). This dichotomy between serum cholesterol and brain cholesterol metabolism explains how there is data that suggest that cholesterol metabolism affects the development of Alzheimer's disease (AD), while at the same time serum cholesterol does not correlate well with AD (2,66). The hypothesis that lowering peripheral cholesterol could affect the onset or progression of AD was supported by a direct correlation between cellular cholesterol and Aβ secretion (67). Aβ is a peptide that aggregates in the brains of AD patients. It is toxic to neurons, and mutations on enzymes involved in its metabolism have been associated with AD (68).

Polymorphisms in genes involved in cholesterol metabolism, such as ABCA1 (69), apoE (70), and LRP-associated protein (71), have been associated with AD. The association between cholesterol metabolism and AD was tested in retrospective studies using statins. Patients taking statins showed a 70% lower prevalence of AD (72). However, a large prospective study involving patients at risk for cardiovascular disease did not show any effect of statins in the

prevention of AD onset (73). The causes of the onset of AD are not clear, but the peptide Aβ is clearly implicated. For example, studies in guinea pigs showed that lowering cholesterol levels using statins (74) reduced Aβ production.

The role of ACAT1, the main cerebral ACAT isoform, in the processing of the amyloid precursor protein was recently reviewed by Puglielli *et al.* (75). As discussed below, the inhibition of ACAT, which lowers cholesterol absorption, reduces Aβ production and is considered as a target to treat AD.

ACAT INHIBITORS AS POTENTIAL THERAPEUTIC AGENTS

During the last 30 years, there has been intensive work on the development of ACAT inhibitors designed to lower lipid absorption (8,76). The ACAT inhibitors can be broadly divided into three groups: (1) the fatty acid anilide derivatives, (2) the urea-derived compounds, and (3) compounds that show enhanced solubility. We will provide some representative examples of each class of ACAT inhibitor and discuss their development.

CI-976 is a fatty acid anilide derivative that was developed in 1992 (77). In a rat model, it was shown to lower plasma cholesterol by 60%, and simultaneously to increase HDL levels (94%). In a rabbit model of atherosclerosis, this ACAT inhibitor both blocked the progression of the lesion and reduced plasma cholesterol levels. Recently, Vaziri and Lang (51) showed that ACAT inhibition using CI-976 drastically reduced proteinuria, hyperlipidemia and LDL receptor deficiencies in a rat model of nephrotic syndrome.

The novel ACAT inhibitor F-12511 was tested for antiatherosclerotic properties in New Zealand white rabbits consuming a high fat diet. Treatment with this inhibitor reduced total plasma cholesterol by 50% and led to a 50% reduction in the number of aortic lesions (78). Eflucimibe (F-12511) is currently undergoing clinical trials (79).

CP 113,818 is another example of an anilide derivative that showed promising results in animal models (12,80). In a recent report, pellets containing CP-113,818 were inserted surgically under the dorsal skin of control mice and an AD transgenic mouse model to allow for the continuous and controlled release of the active compound over an established period of time (81). This compound markedly reduced the formation of amyloid plaques. The development of ACAT inhibitors for the treatment of Alzheimer's disease is an interesting approach that is being fuelled by novel delivery systems set up for unrelated drugs already approved by the FDA for the treatment of this disease (82). Current drug delivery methods targeting the blood–brain barrier and the *in vitro* blood–brain barrier models were recently reviewed by Cucullo *et al.* (83). However, research to date in this area is very preliminary and quite speculative, requiring further investigation.

The results with ACAT inhibitors presenting a urea motif have been very encouraging *in vitro*, but have not been very successful in clinical trials. For example, Peck *et al.* (84) developed compound 447C88, which—after preliminary animal studies—was tested in a clinical trial. The results did not show a significant change in plasma triglycerides, or total HDL or LDL after treatment with the drug.

Sulfonylureas, drugs used to treat noninsulin-dependent diabetes mellitus, have also been tried for ACAT inhibition. Ohgami *et al.* (89) tested the effects of glibenclamide (GB) on cholesterol esterification (CE) in macrophage-derived cells. GB inhibited intracellular accumulation of CE induced by acetylated LDL or oxidized LDL in J774 cells. The direct effect of GB on ACAT was corroborated in the cell-free reconstitution ACAT assay. Furthermore, GB effectively inhibited the ACAT activity of PMA-stimulated THP-1, suggesting that GB inhibited the activity of both isozymes.

Avasimibe (CI-1011) is a member of the group of ACAT inhibitors that present enhanced solubility and was reviewed recently (90). Developed by Pfizer, it is currently undergoing clinical trials (phase III). Its safety has been shown in rat, dogs, and humans (90). In clinical trials involving a cohort of men and women with hyperlipidemia and hypoalphalipoproteinemia, avasimibe significantly reduced total triglyceride and VLDL cholesterol (91), but not total plasma cholesterol. The effect of avasimibe in combination with statins was analyzed in a rabbit atherosclerosis model (92). These investigators found no added effect on the combination therapy in terms of decrease in total plasma cholesterol, but there was an enhanced effect in reduction of aortic lesion, suggesting that a combined therapy targeting both ACAT and HMG-CoA reductase may prove beneficial in inducing atherosclerotic lesion regression. Tardiff *et al.* (93) analyzed the effect of avasimibe on human coronary atherosclerosis. They found that the drug did not favorably alter coronary atherosclerosis, as assessed by intravascular ultrasound.

The development of ACAT inhibitors has undoubtedly helped researchers to understand the role of these enzymes in cholesterol metabolism. In Fig. 3, we summarize some of the biological effects of ACAT inhibition.

Novel developments in the design of ACAT inhibitors using bioinformatics tools (94), large-scale preparations of these enzymes (95), as well as methods to determine isoform specificity of novel ACAT inhibitors (96), will certainly contribute to the discovery of new drugs and a better understanding of the role of ACAT in cholesterol metabolism.

ACAT KNOCKOUT MODELS

Essentially, all the ACAT inhibitors that have been developed show a similar potency to both isoforms. In this respect, the use of knockout mouse models has allowed the characterization of specific roles for ACAT1 and ACAT2. It is also an indication of the potential safety of an agent that will specifically block ACAT activity, as the viability and fertility, as well as other physiological parameters were not significantly affected. In ACAT1 null mice (13), ACAT activity was absent in adrenal glands, testes, ovaries, and macrophages, although there was significant ACAT activity in the liver and intestine. Studies in mouse models lacking Apolipoprotein E (97) or LDL receptor (98), which were backcrossed with the ACAT1 knockout mice, were not able to demonstrate conclusively whether ACAT1 deficiency contributed to decrease atherosclerosis. These studies on the double knockouts rather showed several abnormalities such as cutaneous xanthomatosis, cholesterol accumulation in the skin, skin loss, and dry eyes. These findings and another

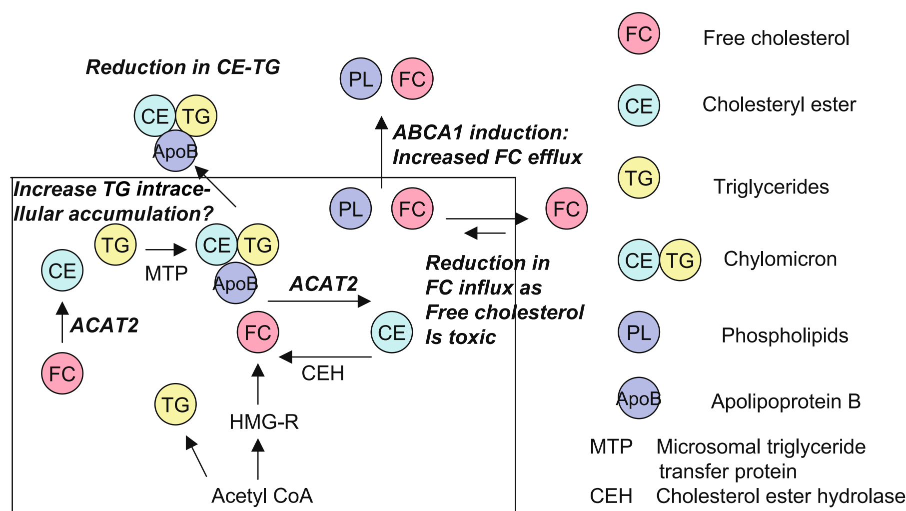

Fig. 3. Consequences of ACAT inhibition in intestinal cells. Free cholesterol (FC) is esterified by ACAT2 to cholesteryl ester (CE). In the presence of ACAT inhibition, the FC is not esterified and FC accumulates. Because free cholesterol is toxic to the cell, ABCA1 expression and activity are increased to efflux FC. ACAT inhibition could lead to a reduced influx of cholesterol but the mechanisms involved are presently unknown. The reduction in cholesteryl esters leads to a reduction in the assembly of CE–TG–ApoB complexes, thus limiting their plasma concentration. There are some reports on increased triglyceride accumulation in the cell.

study that demonstrated the role of macrophages on xanthomatosis in the ACAT1 knockout mouse (98) clearly indicate that ACAT1 inhibitors used at extreme doses may be associated with adverse side effects. In this report, we also found a deposition of free cholesterol in the brain supporting the role of ACAT1 in brain cholesterol homeostasis. The role in atherogenesis of disrupting ACAT1 in macrophages was assessed by transplanting bone marrow cells from a double ACAT1-LDL receptor knockout into irradiated LDL receptor knockout mice (99). Interestingly, ACAT1 disruption in the macrophage led to enhanced diet induced atherosclerosis. The possible explanation could be that the accumulation of free cholesterol, unable to esterify in the absence of ACAT1, led to a proatherogenic inflammatory response.

An ACAT2−/− mouse model was developed by Buhman *et al.* (14). This study provided the genetic evidence that ACAT2 plays a pivotal role in dietary cholesterol absorption. In mice fed a high cholesterol diet, ACAT2 deficiency led to resistance to diet-induced hypercholesterolemia and cholesterol gallstone formation.

Studies in mouse models of atherosclerosis showed reduced levels of atherosclerosis in the ACAT2-deficient mice (100), whereas there was no significant reduction in ACAT1−/− mice (97). A monkey model has shown a correlation between hepatic cholesteryl ester secretion and coronary artery atherosclerosis (101). The results on suppression of plasma cholesterol levels and development of atherosclerosis in rabbits using diverse ACAT inhibitors (E-5324 and HL-004) support the findings on knockout mice models (86,102). Recently, Repa *et al.* (15) confirmed that ACAT2-deficient mice absorbed less cholesterol compared to wild-type mice; however, there was no change in bile acid synthesis or hepatic cholesterol absorption. Interestingly, they found an induction in ABCA1 expression, an efflux transporter, in the knockout mice, irrespective of the diet. This increased ABCA1 expression could mediate the efflux of toxic unesterified cholesterol, and has also been reported in another model of ACAT inhibition (103), although there are recent data that challenge this possibility (94).

SOLUBILITY AND BIOAVAILABILITY ISSUES REGARDING ACAT INHIBITORS

There has been limited work on solubility and bioavailability issues regarding ACAT inhibitors published in the public domain. There has been a great interest in developing ACAT inhibitors with enhanced solubility. Based on urea derivatives, Trivedi *et al.* (85) substituted their phenyl moieties with groups that were amenable to form salts and polar groups, thereby maintaining the ACAT inhibitory properties but drastically enhancing the solubility and bioavailability.

An interesting example is compound FR182980, potent both *in vitro* and *in vivo*, which showed significant adrenal toxicity in dogs (86). This compound was redesigned by inserting a pyrazole ring, which increased its polarity without compromising its ACAT inhibitory properties (87). Moreover, this new compound showed a reduced adrenal toxicity. Because ACAT1 is the main isoform in the adrenal tissues, it would be interesting to compare the isoform specificity of the parent compound and the novel entity.

A modified urea inhibitor that presents increased water solubility, PD 132301-2, decreased cholesterol absorption in animal models, but was also found to be adrenotoxic (88).

CONCLUSIONS AND FUTURE PERSPECTIVES

ACAT is a key enzyme in controlling cholesterol metabolism. ACAT inhibition is a promising target to reduce cholesterol absorption, a key element in the treatment of hypercholesterolemia, atherosclerosis, and possibly Alzheimer's

disease. The current cholesterol-lowering drugs of choice, the statins, have proved safe and efficacious in treating cardiovascular diseases. Because they act through a mechanism different than that of ACAT control (HMG-CoA reductase inhibition), and based on experimental data (104), it was proposed that a combination therapy could reduce cholesterol levels and atherosclerotic lesions more efficiently and at safer lower dosages.

Poor bioavailability and adrenotoxicity (8,105,106) have limited the use of ACAT inhibitors as lipid-lowering drugs. The lack of information in the public domain about the solubility and bioavailability of ACAT inhibitors is of concern, and further research in this area is warranted. In addition, to date, ACAT inhibitors have not been proven to be as clinically effective as had been expected and many have failed in early clinical stages because of unexpected side effects. However, development of bioinformatics strategies and tissue-specific drug delivery systems may help overcome these limitations and contribute to the use of these potentially promising drugs.

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