

Inhibition of acyl-coenzyme A:cholesterol acyltransferase stimulates cholesterol efflux from macrophages and stimulates farnesoid X receptor in hepatocytes

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Abbreviations: ACAT, acyl-coenzyme A: cholesterol acyltransferase; BC, biliary cholesterol; FXR, farnesoid X receptor; GS, guggulsterone; OAA, oleic acid anilide; TMCM, THP-1 macrophage conditioned medium

Abstract

We investigated the mechanism of spontaneous cholesterol efflux induced by acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibition, and how an alteration of cholesterol metabolism in macrophages impacts on that in HepG2 cells. Oleic acid anilide (OAA), a known ACAT inhibitor reduced lipid storage substantially by promotion of cholesterol catabolism and repression of cholesteryl ester accumulation without further increase of cytotoxicity in acetylated low-density lipoprotein-loaded THP-1 macrophages. Analysis of expressed mRNA and protein revealed that cholesterol 7 α -hydroxylase (CYP7A1), oxysterol 7 α -hydroxylase (CYP7B1), and cholesterol 27-hydroxylase (CYP27) were highly induced by ACAT inhibition. The presence of a functional cytochrome P450 pathway was confirmed by quantification of the biliary cholesterol mass in cell monolayers and extracellular medium. Notably, massively secreted biliary cholesterol from macrophages suppressed the expression of CYP7 proteins in a farnesoid X receptor (FXR)-depend-

ent manner in HepG2 cells. The findings reported here provide new insight into mechanisms of spontaneous cholesterol efflux, and suggest that ACAT inhibition may stimulate cholesterol-catabolic (cytochrome P450) pathway in lesion-macrophages, in contrast, suppress it in hepatocyte via FXR induced by biliary cholesterol (BC).

Keywords: bile; cholesterol; cytochrome P-450 enzyme system; farnesoid X-activated receptor; oleoyl-anilide; sterol O-acyltransferase

Introduction

Macrophage foam cells, the hallmark of an early atherosclerotic lesion, results from unregulated uptake of modified low-density lipoprotein (LDL), such as acetylated LDL (acLDL), via the macrophage scavenger receptor A (MSRA) (Goldstein *et al.*, 1979). This increased cholesterol influx activates ACAT-1, which is responsible for cholesterol esterification in macrophages (Buhman *et al.*, 2000), and leads to formation of large amounts of intracellular cholesteryl ester (CE) (Cignarella *et al.*, 2005). The only way for macrophages to maintain cholesterol homeostasis and to prevent cytotoxicity due to accumulation of cholesterol is for them somehow to efflux the excess cholesterol into the extracellular space, which is the first step of reverse cholesterol transport (von Eckardstein *et al.*, 2001). Especially, spontaneous cholesterol efflux from macrophages may be significant within atherosclerotic lesions where the availability of specific subclasses of high-density lipoproteins (HDL) as lipid acceptors is limited (Cignarella *et al.*, 2005), but the process of efflux is not well understood.

Unlike the negative effects of ACAT inhibitors on the formation of foam cells in rodent macrophages via accumulation of free cholesterol (FC) (Warner *et al.*, 1995; Feng and Tabas, 2002), ACAT inhibition has been shown in many studies to repress the accumulation of total cholesterol (TC) in human macrophages by lowering the uptake of acLDL and facilitating FC efflux (Rodriguez *et al.*, 1999; Rodriguez and Usher, 2002; Cignarella *et al.*, 2005). Moreover, Cignarella *et al.* (2005) demonstrated that cholesterol efflux is not a simple consequence

of the availability of FC.

The current study was designed to: (i) find novel factors involved in spontaneous cholesterol efflux stimulated by ACAT inhibition in acLDL-loaded macrophages; (ii) investigate the mechanism by which these factors are regulated; (iii) examine how an alteration of cholesterol metabolism in macrophages impacts on that in HepG2 cells.

Materials and Methods

Materials

Oleic acid anilide (OAA), a known ACAT inhibitor, was synthesized by one of the authors as described (Roth *et al.*, 1992). [$1\text{-}^{14}\text{C}$]Oleoyl-CoA (56 Ci/mol) and [$4\text{-}^{14}\text{C}$]cholesterol (50 Ci/mol) were purchased from Amersham Biosciences (UK). The radioactivity of [$1\text{-}^{14}\text{C}$]oleoyl-CoA, [$4\text{-}^{14}\text{C}$]cholesterol, and related products was measured using a liquid scintillation counter (1450 Microbeta Trilux Wallac Oy, Turku, Finland). Blood was collected from normolipidemic volunteers with permission according to the Guidelines of Blood Donation Program for a Research of the Korea Red Cross Blood Center. LDL was isolated from the plasma by sequential ultracentrifugation (Hatch and Lees, 1968). acLDL was prepared by repeated addition of acetic anhydride to LDL (Basu *et al.*, 1976), and sterilized by filtration through a membrane with a pore size of 0.45 μm . The completeness of acetylation was assessed from electrophoretic mobility on agarose gels. acLDL was stored at 4°C and used within 1 month.

Cell cultures

Human acute monocytic leukemia THP-1 cells and HepG2 cells (American Type Tissue Culture Collection, Rockville, MO) were grown in RPMI-1640 medium (Gibco BRL, Carlsbad, CA) containing 10% FBS (Hyclone, Logan, UT) and DMEM (Gibco BRL) containing 10% FBS, respectively. THP-1 cells in suspension were plated in RPMI-1640 medium with 10% FBS and 50 ng/ml of PMA (Sigma, St. Louis, MO) for 3 days to become fully differentiated macrophages before use in experiments (Cheon *et al.*, 2007). In the majority of experiments, macrophages were enriched with cholesterol by addition of acLDL (100 $\mu\text{g/ml}$) in RPMI-1640 medium containing 10% lipoprotein-deficient serum (LPDS) for 48 h.

Whole-cell cholesterol esterification assay

THP-1 macrophages were pretreated overnight with

or without OAA in complete RPMI-1640 medium, followed by incubation in serum-free RPMI-1640 medium containing 0.2 $\mu\text{Ci/ml}$ of [$1\text{-}^{14}\text{C}$]oleoyl CoA-BSA (0.2%) complex and 100 $\mu\text{g/ml}$ of acLDL with or without OAA for 18 h. The [$1\text{-}^{14}\text{C}$]oleoyl CoA-BSA complex was prepared as described (Batetta *et al.*, 2003). The cells were washed with PBS and extracted with hexane/ isopropyl alcohol (3:2, v/v). The extracts containing esterified products were isolated by thin-layer chromatography (Tabas *et al.*, 1987). Whole-cell cholesterol esterification activity was assessed by determining the radioactivity of the cholesteryl [$1\text{-}^{14}\text{C}$]oleate produced.

Parallel artificial membrane-permeation assay (PAMPA)

The permeability of OAA was measured by the parallel artificial membrane-permeation assay (PAMPA) (Kansy *et al.*, 1998; Avdeef, 2005), which is based on the use of 96-well membrane filter-based plate (Millipore), in 5% DMSO/PBS, pH 7.4.

[$4\text{-}^{14}\text{C}$]cholesterol efflux assay

The cholesterol efflux assay was performed essentially as described (Feng and Tabas, 2002) with slight modification. Briefly, 1 mg of acLDL was incubated with 10 μCi of [$4\text{-}^{14}\text{C}$]cholesterol for 30 min at 37°C, and then 10 ml of RPMI-1640 medium was added. The THP-1 macrophages were incubated in this medium for 48 h with or without OAA, washed three times with PBS, and then incubated in RPMI-1640 medium containing 0.2% fatty acid-free BSA (Sigma, St. Louis, MO) overnight. After equilibration of the cholesterol pool, the cells were washed with PBS and incubated in RPMI-1640 medium containing 0.2% BSA with or without OAA. Efflux incubations were performed for up to 24 h in 6-well plates.

Quantification of intracellular and secreted cholesterol and biliary cholesterol

To quantify intracellular storage of cholesterol and non-cholesterol 3- α -hydroxysteroid (biliary cholesterol), macrophages were harvested after incubation for 48 h in RPMI-1640 medium with or without 100 $\mu\text{g/ml}$ of acLDL or oleic acid anilide (OAA). For quantification of secreted sterols, the cells were washed extensively with PBS and incubated for an additional 24 h in RPMI-1640 medium with or without OAA. The medium was collected and centrifuged at 13,000 g for 30 min at 4°C to remove detached cells and cell debris. A portion of

the cells was assayed for protein using the Bio-Rad DC protein assay kit, and the volume of cell suspension containing 1 mg of protein and the corresponding medium were analyzed for mass of steroids. TC and FC were quantified by an enzymatic-spectrophotometric method (Wako) after extraction with hexane/isopropyl alcohol (3:2, v/v) (Bligh and Dyer, 1959), and CE mass was calculated from the difference between the measurements. The mass of 3- α -hydroxysteroid (3 α HS) was quantified also by an enzymatic-spectrophotometric method (DCLchem) after extraction with hexane/isopropyl alcohol (3:2, v/v) (Bligh and Dyer, 1959), and the mass of biliary cholesterol (BC) was calculated by subtraction of the mass of FC from the mass of 3 α HS. Neutral lipids deposited in the cells were visualized by staining with oil red O as described (Rong *et al.*, 2005).

Real-time quantitative reverse transcription-polymerase chain reaction

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed to determine the expression of genes involved in cholesterol metabolism and mobilization in THP-1 macrophages, encoding for apoE (BC-003557), ABCA1 (AF165281), ABCG1 (BC029158), CYP7A1 (X56088), CYP7B1 (AF127090), CYP27 (M62401) with a rotor-gene 3000 (Corbett Research). The cells were incubated for 48 h with or without OAA as indicated, in the presence of 100 μ g/ml of acLDL. The following sets of primers and probes were used (forward and reverse, respectively) in qRT-PCR: β -actin, 5'-agc-gcg-gct-aca-gct-tca-3' and 5'-cat-ttg-cgg-tgg-acg-atg-3'; apoE, 5'-cgc-ctg-gtg-cag-tac-cg-3' and 5'-tga-ttg-tcg-ctg-ggc-aca-g-3'; ABCA1, 5'-cta-gga-tgg-caa-tca-tgg-tc-3' and

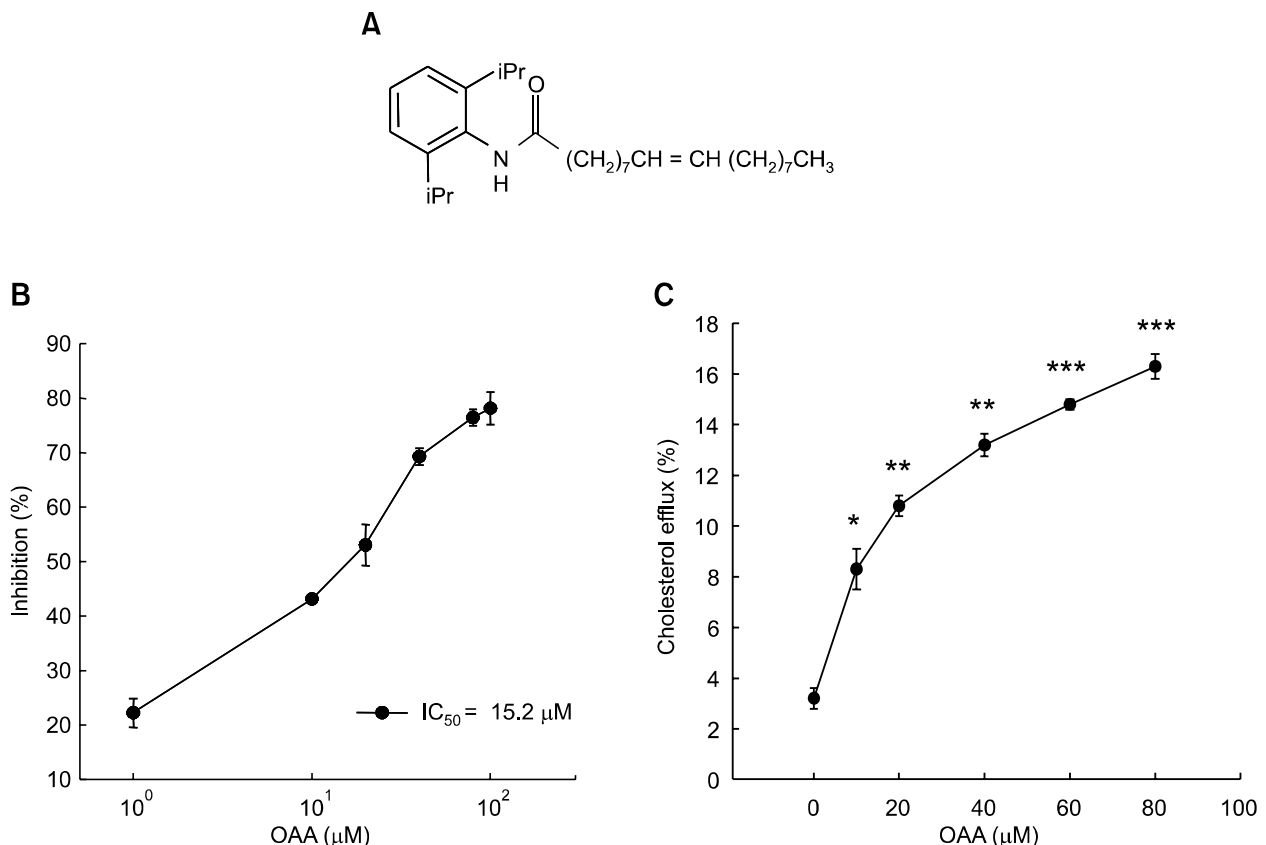


Figure 1. The ACAT inhibitor OAA promotes spontaneous cholesterol efflux from THP-1, cultured macrophages. (A) The structure of OAA. Whole-cell ACAT activity (B) and cholesterol efflux (C) in THP-1, cultured macrophages were determined. ACAT activity was measured by determining the incorporation of [14 C]oleoyl-CoA into labeled cholesteryl oleate. The values shown are expressed as the percentage inhibition of ACAT activity in control incubations. The cholesterol efflux are expressed as the percentage of total cellular [14 C]cholesterol. * P < 0.05, ** P < 0.01, *** P < 0.001 versus acLDL-loaded cells.

5'-aac-tgc-aac-gtc-cac-tac-tg-3'; ABCG1, 5'-gga-aga-tgt-agg-cag-att-gg-3' and 5'-aat-gtc-tgc-atg-gct-cag-tg-3'; CYP7A1, 5'-cag-aag-caa-tga-aag-cag-cta-ctg-3' and 5'-tgt-att-cac-aaa-tgc-ttg-aat-tta-tat-tta-3'; CYP7B1, 5'-gct-tcc-tta-tct-tgg-agt-gg-3' and 5'-gag-ctg-cag-aat-gga-tac-ag-3'; CYP27, 5'-cct-gtt-cga-gaa-acg-cat-tg-3' and 5'-tcc-ttt-gag-agg-tgg-tac-ag-3.

Statistical analysis

Results are given as mean \pm S.D. Statistical analysis was done using Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant. The experiments were repeated three times (separate cell preparations) unless noted otherwise.

Results

OAA (Figure 1A) inhibited ACAT activity in THP-1 macrophages with an IC_{50} value of 15.2 μ M (Figure 1B), which is a much higher value than that from an *in vitro* assay (hACAT1, $IC_{50} = 140$ nM) (Cho *et al.*, 2003). OAA showed a medium permeability in the parallel artificial membrane-permeation assay (PAMPA) with a $\text{Log } P_e$ value of -5.18 ± 0.02 . As the result, only 3 μ mol of OAA was shown to be able to cross the biological membrane from 100 μ mol of OAA in the donor compartment. Therefore, the reason why OAA exhibits a relatively lower ACAT inhibition activity in the cell system could be explained by the poor membrane permeability. But there is no doubt that OAA inhibits CE formation in acLDL-loaded macrophages.

The extent of cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) into the extracellular medium with an LDH assay kit (Takara) or the formation of MTT formazan (Liu and Hong, 2005). As the result, acLDL loading decreased cell viability by about 20%, while the addition of OAA to the medium containing acLDL did not cause decrease in cell viability (data not shown).

Decrement of CE mass dominates the negative effect of the accumulation of FC

The results from the staining of the cells with oil red O showed that acLDL-loading led to massive cell formation in THP-1 macrophages while the addition of OAA appeared to deplete storage lipid from the cells in a dose-dependent manner (Figure 2A). Next, we measured the cholesterol mass to investigate the effect of ACAT inhibition on intracellular CE and FC accumulation, and FC

secretion to the medium. As shown in Figure 2B, acLDL-loading increased cellular CE mass by 2.7-fold (354 ± 15 μ g/mg versus 128 ± 5 μ g/mg of cell protein, $P < 0.001$) and free cholesterol secretion about 1.9-fold (33 ± 2 μ g/mg versus 17 ± 2 μ g/mg of cell protein, $P < 0.01$), but did not cause change in the cellular content of FC significantly (135 ± 16 μ g/mg versus 122 ± 6 μ g/mg of cell protein). OAA reduced CE mass significantly in acLDL-loaded cells in a dose-dependent manner. The 80% decrease of ACAT activity by addition of 80 μ M OAA in the cells (Figure 1B) resulted in a substantial decrease of CE formation to a level lower than that for the control cells (99 ± 16 μ g/mg of cell protein, $P < 0.001$) (Figure 2B), but increased slightly the accumulation of FC in the cell monolayer, by 1.5-fold (200 ± 12 μ g/mg versus 135 ± 16 μ g/mg of cell protein, $P < 0.05$), and secretion of FC into the extracellular space, by 1.2-fold (56 ± 5 μ g/mg versus 33 ± 2 μ g/mg of cell protein, $P < 0.05$). A moderate increase of FC efflux is not sufficient to explain a marked reduction of CE accumulation.

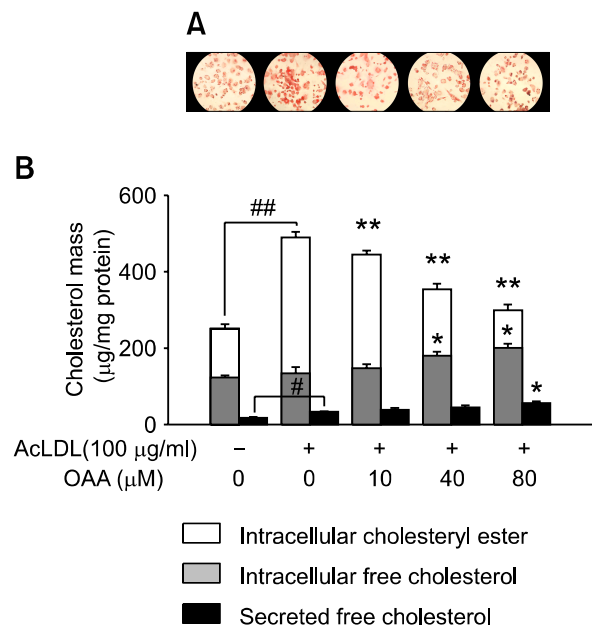


Figure 2. CE accumulation in acLDL-loaded macrophages is repressed during ACAT inhibition, which dominates FC accumulation. To quantify intracellular storage of cholesterol, THP-1 macrophages were incubated for 48 h in RPMI medium containing 10% LPDS with or without 100 μ g/ml of acLDL and the indicated concentrations of OAA. For quantification of secreted cholesterol, the cells were washed extensively and incubated for a further 24 h without acLDL. (A) Cells were fixed with 4% paraformaldehyde and stained with oil red O. Magnification $100\times$. (B) Cholesterol mass of hexane/isopropyl alcohol (3:2, v/v) extracts from the cells and media. # $P < 0.01$, ## $P < 0.001$ versus control cells; * $P < 0.05$, ** $P < 0.001$ versus acLDL-loaded cells.

Thus, it is speculated that FC might have been secreted after conversion into other molecules. Interestingly, the calculated ratio of efflux of cholesterol to total cholesterol derived from exogenous acLDL was increased from 7% to 80% during ACAT inhibition; i.e. it resulted mainly from reduction of the TC mass and not from an increase of FC efflux. Nevertheless, this observation was quite different from that of the [¹⁴C]cholesterol efflux experiment where cholesterol efflux was increased from 3.2% to 16.3% by ACAT inhibition ($P < 0.01$) (Figure 1C). It was concluded that the cholesterol mass data and the [¹⁴C]cholesterol efflux data might not be correlated simply, because [¹⁴C]cholesterol might have been secreted simultaneously, unlike acLDL, which was processed in various

cellular organelles.

ACAT inhibition triggers changes in expression of genes involved in cholesterol catabolism and mobilization

Quantitative real-time PCR analysis was conducted to investigate the levels of the relevant mRNA of various genes after incubation for 48 h with 100 µg/ml of acLDL plus the concentration of OAA indicated. As shown in Figure 3A, the levels of *ApoE* and *Abcg1* were unaffected by acLDL plus OAA compared with acLDL alone, which is not consistent with the results of previous studies (Cignarella *et al.*, 2005). In contrast, the mRNA level of cytochrome P450 family genes *Cyp7a1*,

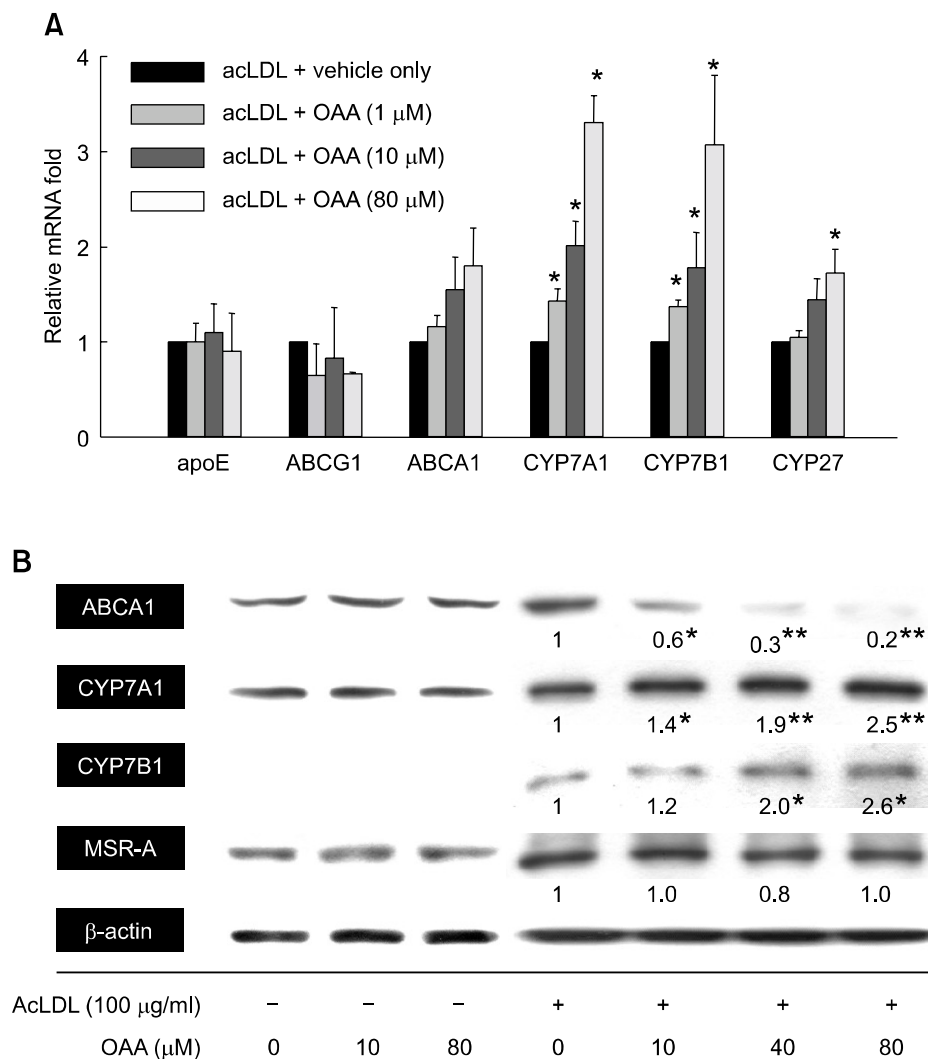


Figure 3. ACAT inhibition regulates the expression of genes involved in cholesterol catabolism and mobilization in cultured macrophages. Cells were incubated for 48 h with acLDL and the indicated concentration of OAA. (A) The levels of mRNA were measured by qRT-PCR and normalized by β-actin. * $P < 0.05$ versus acLDL-loaded cells. (B) Each protein expression level was analyzed by Western blot. The intensity of the detected bands was compared using a Calibrated Densitometer GS800 (Bio-Rad) with Quantity One software (version 4.4.0) and demonstrated as an average of three independent experiment. The intensity of the β-actin band was used as an internal control. * $P < 0.05$, ** $P < 0.01$ versus acLDL-loaded cells.

Cyp7b1, and *Cyp27* were increased in a dose-dependent manner. Especially, *Cyp7a1* and *Cyp7b1* were highly induced by 3.3-fold and 3.1-fold, respectively ($P < 0.05$), in the presence of 80 μM OAA, while *Cyp27* was induced by 1.7-fold ($P < 0.05$). The mRNA levels of *Abca1* were not

significantly induced.

Western blot analysis was performed to determine whether ACAT inhibition caused a change in the post-transcriptional process, and whether quantitative mRNA levels were correlated with protein levels. OAA itself did not influence the expression of any genes tested in THP-1 macrophages. The protein level of ABCA1, the mRNA expression of which tends to increase, was decreased substantially by ACAT inhibition in acLDL-loaded macrophages (Figure 3B). This result is in agreement with that of an earlier study, which demonstrated that ACAT inhibition induced the degradation of ABCA1 protein due to membrane stiffening effect (Feng and Tabas, 2002). The protein expressions of CYP7A1 and CYP7B1 were induced by 80 μM OAA treatment (2.5-fold, $P < 0.01$ and 2.6-fold, $P < 0.05$, respectively) in a very similar way to transcriptional regulation (Figure 3B). Translation of MSRA remains unchanged suggesting that ACAT inhibition does not affect the acLDL uptake into the cells.

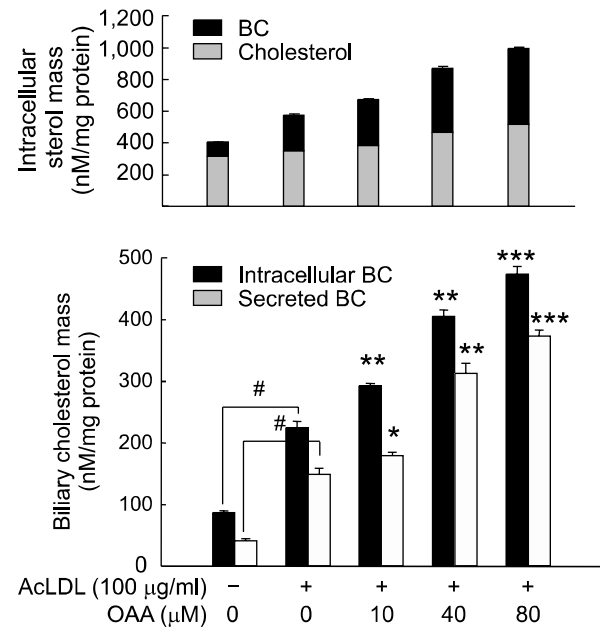


Figure 4. ACAT inhibition increases the mass of intracellular and secreted BC in cultured macrophages. The effect of ACAT inhibition on the production of BC was measured in the macrophages treated as described for Figure 2. (A) Intracellular cholesterol mass and biliary cholesterol mass are compared in the same graph. (B) The intracellular and secreted BC mass were analyzed and calculated as described in Materials and Methods. # $P < 0.01$ versus control cells; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus acLDL-loaded cells.

ACAT inhibition promotes cholesterol catabolism into BC

To investigate whether ACAT inhibition increased functional CYP7A1 and CYP7B1, and stimulated cholesterol catabolism into BC, the products of the cytochrome P450 pathway, we quantified the mass of intracellular and secreted BC using an enzymatic-spectrophotometric method (Figure 4). We observed that acLDL-loading induced formation of BC (225 ± 10 nmol/mg versus 87 ± 3 nmol/mg of cell protein, $P < 0.01$) that was more intensified during ACAT inhibition. The intracellular mass of

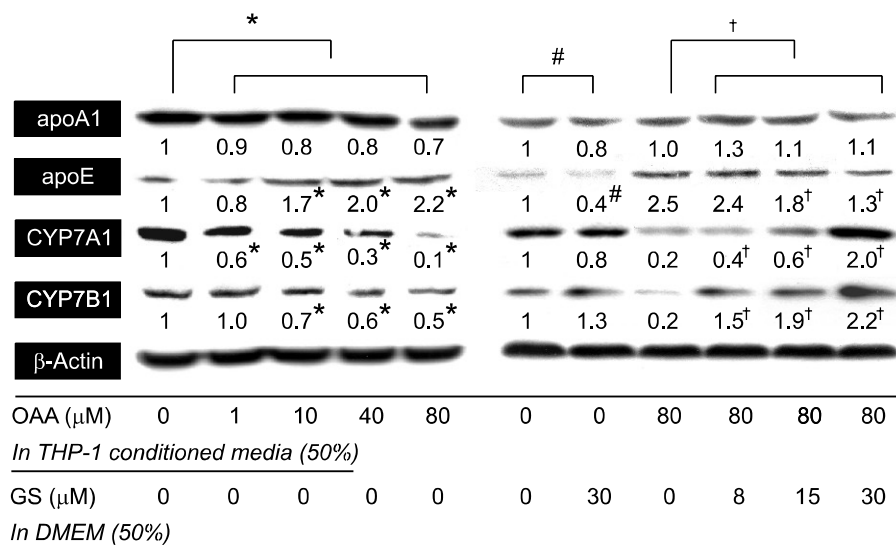


Figure 5. BC excreted from macrophages regulates the expressional level of apoE, CYP7A1 and CYP7B1 in an FXR-dependent manner in HepG2 cells. The HepG2 cells (5×10^6) were incubated with 50% TCM, derived from Figure 4, and 50% DMEM containing 10% LPDS with or without the indicated concentration of GS for 48 h. Each level of protein expression was analyzed by Western blot. The intensity of the detected bands is demonstrated as an average of three independent experiments. #, † $P < 0.01$ versus the indicated control.

BC was increased in proportion to ACAT inhibition. While FC was secreted by 20~30% of intracellular FC (Figure 2B), BC was secreted easily from cells to the medium, 70~80% of intracellular BC. These novel effects of ACAT inhibition may explain the reduction of lipid accumulation in THP-1 macrophages loaded with acLDL.

BC secreted from macrophages controls the gene expression in an FXR-dependent manner in HepG2 cells

In liver cells, BC could be a ligand of FXR (Nishimaki-Mogami *et al.*, 2004), which promotes apoE expression (Mak *et al.*, 2002) and represses the expression of apoA1 and the enzymes that catalyze bile acid synthesis, including CYP7A1 (Goodwin *et al.*, 2000) and CYP7B1 (Stedman *et al.*, 2006). Guggulsterone (GS) is a plant sterol from the *Commiphora mukul* tree and has been widely used to treat hyperlipidemia in humans (Urizar and Moore, 2003). It is well established that GS can act as an FXR antagonist and decrease expression of FXR target genes (Urizar *et al.*, 2002; Wu *et al.*, 2002). It has also been demonstrated that the hepatic lipid-lowering effect of GS was mediated through FXR using FXR knockout mice (Urizar *et al.*, 2002). To address the question as to whether BC secreted from macrophages could modulate the FXR pathway in HepG2 cells, the cells were incubated with 50% THP-1 macrophage conditioned medium (TMCM), which confirmed the presence of BC (Figure 4). The concentration of BC in TMCM was increased by 2.5 fold with 80% inhibition of ACAT activity (149 ± 10 nM/mg versus 373 ± 10 nM/mg of cell protein, $P < 0.001$). OAA itself did not influence the expression of any gene tested in HepG2 cells (data not shown), like the THP-1 macrophages. As shown in Figure 5, among the tested FXR-mediated genes, CYP7A1, CYP7B1, and apoE were regulated in proportion to the amount of BC included in TMCM. The expression of CYP7A1 and CYP7B1

was decreased by 75% and 50% with maximal concentration of BC in TMCM (Figure 4). In contrast, apoE expression was increased 3-fold. On the same concentration of BC, the FXR pathway seems to be inactivated by GS in a dose-dependent manner, and the expression of CYP7A1, CYP7B1, and ApoE were restored.

ACAT inhibition exhibits different regulation of cytochrome P450 gene expression between macrophages and HepG2 cells

Next, we investigated the direct effects of ACAT inhibition and the combinational effect of ACAT inhibition and TMCM treatment on HepG2 cells. Interestingly, we observed that the expression of CYP7A1 and CYP7B1 was mildly repressed by acLDL treatment, which is sustained by same expression level during ACAT inhibition and that TMCM treatment repressed those gene expressions (Figure 6). This result was distinct with that in macrophages, suggesting quite different regulation of CYP pathway between lesion-macrophages and HepG2 cells (Figure 6).

Discussion

The first part of this study showed that OAA effectively reduced cholesterol accumulation in THP-1 macrophages by inhibiting CE formation without increased cytotoxicity compared with acLDL alone. Also, the fluctuation of intracellular CE decrease is much bigger than that of secreted FC increase. To better understand about cholesterol flux as a consequence of ACAT inhibition and to investigate, if any, novel factors involved in spontaneous cholesterol efflux in human THP-1 macrophages, we performed a microarray experiment using GenePloer TwinChip Human-8K (Digital genomicsTM, Seoul, Korea). Analyzed levels of the expressed mRNA of genes related to lipid catabolism and mobilization, such as CYP7B1 and

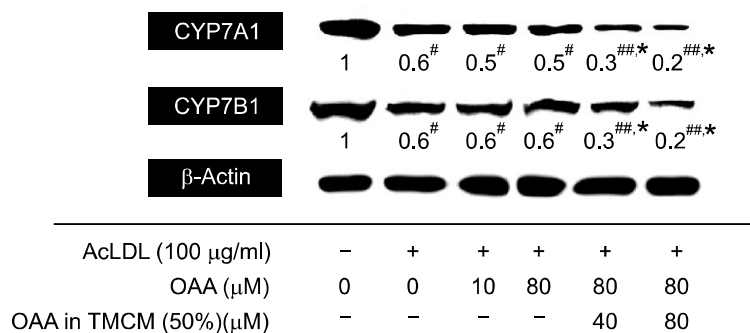


Figure 6. ACAT inhibition does not affect the expression level of CYP7A1 and CYP7B1 in HepG2 cells. Cells were incubated for 48 h with or without acLDL, the indicated concentration of OAA and/or 50% TMCM. Each protein expression level was analyzed by Western blot. The intensity of the detected bands is demonstrated as an average of three independent experiments. [#] $P < 0.05$, ^{##} $P < 0.01$ versus control cells; ^{*} $P < 0.05$ versus acLDL-loaded cells.

apoC1, were induced by 2-fold during even mild ACAT inhibition (40%) (data not shown). This result led us to focus on the catabolic pathway to BC in acLDL-loaded macrophages during ACAT inhibition. Similarly, we discovered that CYP7A1, CYP7B1, and CYP27 were highly expressed during ACAT inhibition. Our data showed for the first time that ACAT inhibition activated the cytochrome P450 pathway in acLDL-loaded macrophages, and thus the cells were rendered resistant to accumulation of cholesterol by increased catabolism to BC, which is immediately secreted out of the extracellular space.

Cytochrome P450 pathway is accomplished via two pathways, the classic pathway and the alternative pathway, where CYP7A1 and CYP7B1 function as rate-limiting enzymes, respectively (Nishimaki-Mogami *et al.*, 2004). In mammals, the CYP7A1 pathway accounts for the majority of cholesterol that is metabolized and removed from the body (Siperstein *et al.*, 1952), and predominantly causes the formation of cholate and chenodeoxycholate (CDCA) (Li *et al.*, 1990). Also, the CYP7B1 pathway contributes considerably to the total bile acid mass in humans and leads predominantly to the formation of CDCA (Axelson and Sjövall, 1990). These CYP7 proteins have been shown to be liver-specific enzymes (Russell, 1999), and have been believed not to function in non-hepatic cells under normal conditions (Zhang *et al.*, 1995). Notably, avasimibe, a known ACAT inhibitor, increased the expression of CYP7A1 and bile acid synthesis in rat hepatocytes (Post *et al.*, 1999). Transgenic expression of CYP7A1 in McArdle rat hepatoma cells (Labonte *et al.*, 2000) and in the livers of mice (Miyake *et al.*, 2002) could prevent massive accumulation of cholesterol. Most importantly, RAW264.7 macrophages, which express rat CYP7A1 stably, displayed a complete resistance to accumulation of cholesterol via both increased metabolism and efflux of cholesterol with no adverse effect on cell growth or viability (Moore and Davis, 2002). These studies support the concept that the cytochrome P450 pathway might be critical in the maintenance of cholesterol homeostasis in lesion-macrophages as well as in hepatocytes. In this research, we found that the intracellular mass of BC was increased by 3-fold with only acLDL-loading (Figure 4). The result demonstrated that macrophages have a functional cytochrome P450 pathway as a defense mechanism against the cholesterol accumulation. It is generally accepted that *Cyp7a1* is regulated by LXR α in the hepatocytes, although the action of LXR α in the macrophages has not been fully elucidated. LXR α signaling might be activated by oxysterol transformed from

cholesterol during ACAT inhibition. It is not clear whether oxysterol is generated simply by an intracellular oxidative mechanism concurrent with a general increase of the cellular cholesterol level or by a more specific manner when ACAT is inhibited in macrophages. It is certain, however, that inhibition of ACAT enhances the pool of free cholesterol available for conversion into oxysterol (Post *et al.*, 1999). Notably, 27-hydroxycholesterol has been identified as a ligand of LXR in cholesterol-loaded, monocyte-derived macrophages (Fu *et al.*, 2001). In this study, we observed that ACAT inhibition up-regulated CYP27 expression mildly but significantly. Thus, it is acceptable that at least 27-hydroxycholesterol among the various oxysterols might have a role as a ligand for LXR α .

Interestingly, CDCA, a major end-product of the cytochrome P450 pathway, is the most potent physiological ligand of FXR, a negative regulator of bile acid synthesis (Chiang *et al.*, 2000) and excretion (Stedman *et al.*, 2006). Activation of FXR result in decreased expression of CYP7A1 (Goodwin *et al.*, 2000), CYP7B1 (Stedman *et al.*, 2006), and apoA-1 (Claudel *et al.*, 2002), and increased expression of apoE (Mak *et al.*, 2002). FXR deletion in cholestasis model mice improved cholestatic liver disorders by increased excretion of bile acid from the body (Stedman *et al.*, 2006). In this study, it has shown that FXR down-regulates the multidrug resistance-associated proteins 1 and 4, which are postulated to act as alternative basolateral bile acid efflux transporters, and ABCG5 and ABCG8, which is a major pathway for cholesterol elimination. The results implicated that FXR antagonism possibly has a significantly increased capacity to export bile acids out of the hepatocyte back into the circulation and ability to excrete cholesterol into bile. Also, FXR deficiency in *Ldlr*^{-/-} mice resulted in a reduction in size of atherosclerotic lesions in the aorta, mainly via a decreased level of plasma LDL cholesterol, and a decrease of the accumulation of neutral lipid in peritoneal macrophages (Zhang *et al.*, 2006). There have been many conflicting results, depending on the experimental animal model in research areas related to atherosclerosis, which might originate from different mechanisms of cholesterol metabolism between species. It has been reported that rodents have a very hydrophilic bile acid pool, which is less potent in activation of FXR; thus, the LXR α could function as an important regulator of CYP7A1 in mice (Chiang *et al.*, 2001). In contrast, CYP7A1 expression was down-regulated by a high-cholesterol diet in African green monkeys (Rudel *et al.*, 1994) and in rabbits (Xu *et al.*, 2003), since the inhibitory effect of FXR may override the stimulatory effect of LXR α . There are another

regulator of bile acid synthesis, named steroid and xenobiotic receptor pregnane X receptor (PXR), which induces human cytochrome P450A4 in drug metabolism and represses CYP7A1 in bile acid synthesis in the liver. PXR is activated by a large number of endogenous and exogenous chemicals including naturally occurring steroids, antibiotics, antimycotics, bile acids, and the herbal antidepressant St. John's wort (Kliwer *et al.*, 2002). It is possible that various steroids released from acLDL might stimulate PXR, which down-regulated CYP proteins in HepG2 cells. These results led us to propose that the disappointing results of ACAT inhibitors, avasimibe (Tardif *et al.*, 2004) and pactimibe (Nicholls *et al.*, 2006) shown

in several clinical studies might result from activation of FXR, owing to the elevated pool of ligand for FXR; as consequence, cholesterol could not be excreted from the body. In this study, we discovered that BC secreted from acLDL-loaded macrophages during ACAT inhibition behaved as an FXR activator and regulated the expression of apoE, CYP7A1, and CYP7B1, and that these effects were abolished by the FXR antagonist, GS. Therefore, it is potential that ACAT inhibition promotes secretion of BC from macrophages but represses bile acid synthesis in hepatocytes via the activation of FXR as presented in Figure 7. Nishimaki-Mogami *et al.* (2004) demonstrated that some BC, which is metabolized further than 27

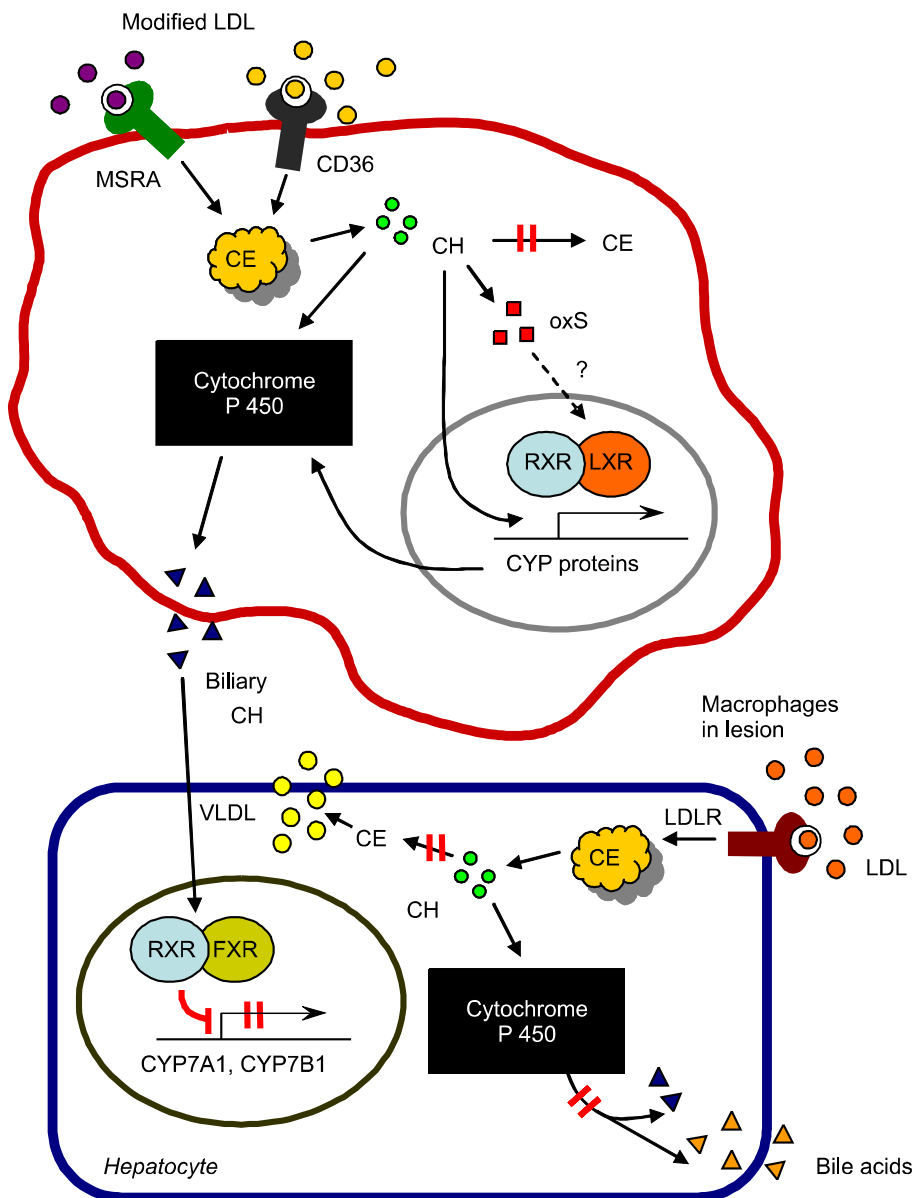


Figure 7. Schematic presentation of hypothesis proposed in this paper. In lesion-macrophages, unregulated cholesterol influx from modified LDL activates ACAT-1 and leads to formation of large amounts of intracellular CE. Inhibition of ACAT enhances the pool of free cholesterol available for conversion into oxysterol, thus LXR α signaling might be activated by oxysterol. ACAT inhibition induce cytochrome P450 proteins in acLDL-loaded macrophages, and thus the cells were rendered resistant to accumulation of cholesterol by increased catabolism to BC, which is immediately secreted out of the extracellular space. In liver cells, BC could be a ligand of FXR, which represses the expression of the enzymes involved in bile acid synthesis, including CYP7A1 and CYP7B1. Finally, ACAT inhibition could repress bile acid synthesis in hepatocytes and cholesterol excretion from the body via the activation of FXR. CH: cholesterol, CE: cholesteryl ester, oxS: oxysterol, BC: biliary cholesterol.

hydroxylation in the classic pathway of bile acid synthesis, exhibited activity for FXR comparable to that of CDCA; conversely, early intermediates in the bile acid synthesis pathway, such as 7 α -hydroxycholesterol and 27-hydroxycholesterol, showed no activity. Thus, we could consider that cholesterol was metabolized at least further than 27-hydroxylation in macrophages during ACAT inhibition, which is why the small change of absolute values of BC in the TMCM could activate the FXR pathway of HepG2 cells dramatically.

Our results also imply that the combination therapy of an ACAT inhibitor and an FXR antagonist *in vivo* may prove to have clinical benefit in the treatment of atherosclerosis by decreasing the accumulation of cholesterol in lesion-macrophages through enhancing the efflux of BC, and by facilitating the excretion of cholesterol out of the body.

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