Cholesterol is superior to 7-ketocholesterol or 7alpha-hydroxycholesterol as an allosteric activator for acyl-coenzyme A:cholesterol acyltransferase 1

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SUMMARY

We compared the abilities of cholesterol versus various oxysterols as substrate and/or as activator for the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), by monitoring the activity of purified human ACAT1 in response to sterols solubilized in mixed micelles or in reconstituted vesicles. The results showed that 5alpha, 6alpha-epoxycholesterol, and 7alpha-hydroxycholesterol are comparable to cholesterol as the favored substrates, while 7-ketocholesterol, 7beta-hydroxcholesterol, 5beta, 6beta-epoxcholesterol, and 24(S), 25-epoxycholesterol are very poor substrates for the enzyme. We then tested the ability of 7-ketocholesterol as an activator when cholesterol was measured as the substrate, and vice versa. When cholesterol was measured as the substrate, the addition of 7-ketocholesterol could not activate the enzyme. In contrast, when 7-ketocholesterol was measured as the substrate, the addition of cholesterol significantly activated the enzyme and changed the shape of the substrate saturation curve from sigmoidal to essentially hyperbolic. Additional results show that, as an activator, cholesterol is much better than all the oxysterols tested. These results suggest that ACAT1 contains two types of sterol binding sites; the structural requirement for the ACAT activator site is more stringent than it is for the ACAT substrate site. Upon activation by cholesterol, ACAT1 becomes promiscuous towards various sterols as its substrate.
INTRODUCTION

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is a membrane-bound enzyme located in the endoplasmic reticulum (ER). It is present in a variety of cell types and tissues, and utilizes two lipophilic substrates, cholesterol and long-chain fatty acyl-coenzyme A, to catalyze the formation of neutral lipid cholesteryl esters (CEs). In mammals, two ACAT isoforms exist (ACAT1 and ACAT2) (reviewed in (1)). The tissue distribution of ACAT1 is essentially ubiquitous while that of ACAT2 is more restricted. The physiological roles of these isoforms in various tissues are under active investigation. At the single cell level, ACAT participates in controlling the cellular membrane cholesterol level. Unlike many other enzymes involved in cholesterol metabolism, regulation of ACAT by sterol occurs at the post-translational level. In mixed micelles or in reconstituted vesicles, both ACAT1 and ACAT2 display a sigmoidal response to cholesterol as their substrates (2). These results are consistent with the concept that the ACAT activity is allosterically regulated by membrane cholesterol content in the ER (reviewed in (3)).

Oxysterols are sterols containing a second oxygen atom, present as a carbonyl, hydroxyl, or epoxide group in rings A or B or in the side chain, in addition to the C3 hydroxyl group. A large number of oxysterols are found in various locations, including food products, plasma, or inside the cells (reviewed in (4), (5)). They are produced by various enzymes in vivo, and/or by chemical oxidation in vitro.
hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 7alpha-
hydroxycholesterol are the 4 main oxysterols enzymatically derived (6), (5), and
can be found in the plasma and inside certain cell types. 7-ketocholesterol, 7alpha-
hydroxycholesterol, 7beta-hydroxycholesterol, and 27-hydroxycholesterol are the 4
major oxysterols found in human atherosclerotic lesions (5), (7). In addition, 7-
ketocholesterol, 7alpha-hydroxycholesterol, 7beta-hydroxycholesterol, 5alpha,
6alpha-epoxycholesterol and 5beta, 6beta-epoxycholesterol are the major
oxysterols present in oxidized low-density lipoprotein (LDL) preparations in vitro
(8), (9). Oxysterols possess a wide range of biological properties, and may play
regulatory roles in cholesterol metabolism. For example, when added to the
culture medium of intact cells, most oxysterols, including 7alpha-
hydroxycholesterol, 7-ketocholesterol and 25-hydroxycholesterol, greatly
suppressed cholesterol biosynthesis rate (10), (11). Several oxysterols are high
affinity ligands for the nuclear receptor LXR alpha 1 (13), (14), and one of them,
24(S), 25-epoxycholesterol, has been proposed as a participant in cholesterol
regulation in the liver (12). On the other hand, the roles of oxysterols in
controlling cholesterol homeostasis in vivo are still under debate, partly because in
various mammalian systems examined, various oxysterols are present in very low
concentrations, with much shorter half-lives relative to cholesterol (15).
Oxysterols have also been shown to have effects on ACAT activity. For example,
when added to medium of tissue culture cells, oxysterols such as 7-ketocholesterol
or 25-hydroxycholesterol, in addition to their suppressive effect on cholesterol biosynthesis rate, stimulated cholesterol esterification rate and increased ACAT activity (16). When cholesterol was added in the same manner, it failed to provide the same response. Despite numerous studies, the mechanism of oxysterol mediated activation of cholesteryl ester biosynthesis had not been clarified. It could be due to the presence of a putative oxysterol binding site present in ACAT1, or could be due to the ability of oxysterol to mobilize cellular cholesterol to the ER; other mechanism(s) could not be ruled out. We and other investigators used various crude cell extract systems and had demonstrated the apparent activation of ACAT by 25-hydroxycholesterol \textit{in vitro} (17), (18), (19). However, in these studies, the enzyme ACAT and the sterols (cholesterol and 25-hydroxycholesterol) serving as substrate and/or activator were present in different membranes. Thus one could not rule out the possibility that the apparent activation by 25-hydroxycholesterol was due to its ability to translocate cholesterol from a cholesterol-rich membrane to a cholesterol-poor membrane where ACAT is located (discussed in (19)). Studies were also performed attempting to determine the sterol substrate specificity of ACAT. When individual sterols were delivered in vesicle form to the enzyme, Cases and colleagues have shown that ACAT seemed to utilize various oxysterols much more efficiently than cholesterol (20). Again, these studies involved the use of crude enzyme extracts, with ACAT and sterols present in different membranes. Among various sterols, the ability to move
from the donor membrane to the ACAT-containing membrane differs greatly (21). The transfer rates for oxysterols were much faster (more than 10 times) than those for cholesterol; these differences could greatly mask the true sterol specificity for the enzyme. Thus, the intrinsic substrate specificity of ACAT could not be determined from these studies. In oxidized LDL loaded macrophages, large amounts of esterified oxysterols and esterified cholesterol are present in the cytosolic fraction of the cells; these cytosolic steryl esters are the products of ACAT reaction (7). How ACAT can utilize oxysterols in the presence of large amounts of cellular cholesterol is not clear, partly because the relative specificity of ACAT towards oxysterols and cholesterol as substrate or as activator has not been clarified.

In the current work, we compared the abilities of several selected oxysterols versus cholesterol as ACAT substrates or as ACAT activators. The oxysterols evaluated included, as representatives of ring A or B oxysterols, 7alpha-, and 7beta-hydroxycholesterol, 7-ketocholesterol, and 5alpha, 6alpha- and 5beta, 6beta-epoxycholesterol; and as a representative of side-chain oxysterols, 24(S),25-epoxycholesterol. We first placed sterols and the enzyme in mixed micelles (22), using human ACAT1 purified to homogeneity as the enzyme source, and analyzed the enzyme activity in response to varying sterol concentrations. In mixed micelles, the sterol is in direct contact with the enzyme in solution form (23). This assay system also avoids the formation of sterol microdomain(s). On the other
hand, the environment provided by the mixed micelles is not close to that of ACAT1 under physiological conditions, because ACAT1 is an integral membrane protein residing in the ER. We therefore tested the validity of information learned from using the mixed micelles system by using the reconstituted vesicle system (24), (25). The latter system provides an environment close to that of ACAT1 under physiological conditions. In addition, the sterol and the enzyme ACAT1 reside in the same vesicles, thus eliminating the sterol transfer step between two different vesicles prior to enzyme catalysis. Our results show that ACAT1 can accommodate cholesterol, 5alpha, 6alpha-epoxycholesterol, and 7alpha-hydroxycholesterol as its three preferred substrates. In contrast, for activation of ACAT1, cholesterol is superior to all the oxysterols tested, including 7-ketocholesterol, 7alpha-hydroxycholesterol, 7beta-hydroxycholesterol, 5alpha, 6alpha-epoxycholesterol, 5beta, 6beta-epoxycholesterol, or 7beta-hydroxycholesterol. Thus, the structural requirement for sterol as an ACAT activator is more stringent than it is for sterol as an ACAT substrate.

EXPERIMENTAL PROCEDURES

Materials

Cholesterol, 7beta-hydroxycholesterol, 5alpha, 6alpha-epoxycholesterol, 5beta, 6beta-epoxycholesterol, 7-ketocholesterol, beta-sitosterol, CHAPS, taurocholate, oleoyl-coenzyme A, egg phosphatidylcholine (PC), cholesteryl oleate, fatty acid-free bovine serum albumin, 4-dimethylaminopyridine (DMAP), imidazole, oleic
anhydride, triethylamine, and primulin dye were all from Sigma. 7alpha-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol were from Steraloids. 24(S), 25-epoxycholesterol was synthesized as previously described (26), (27). All the sterols showed single spots in thin-layer chromatographic (TLC) analysis, and were used without further purification. BHT (2,6-Di-tert.-butyl-p-cresol) was from Eastman Kodak. Centrisart ultrafiltration device (cut-off MW 300kDa) was from Sartorius. Organic solvents used were reagent grade and were from Fisher. Grace’s insect cell medium was from Invitrogen. The software program Prism (GraphPad Software, Inc.) was from Sigma.

Methods

Enzyme purification

The source of enzyme was recombinant human ACAT1 expressed in insect Hi5 cells and purified to electrophoretic homogeneity. The purification procedure was as described previously (22), (28), using nickel column chromatography and ACAT1 monoclonal column chromatography. For some of the experiments reported in this work (Fig. 4), the enzyme source used was HisACAT1$_{\delta1-65}$ (29). The ACAT1 monoclonal antibody only recognizes the N-terminal of the enzyme. The HisACAT1$_{\delta1-65}$ enzyme lacked the N-terminal and thus could only be partially purified, using nickel column chromatography (29).
ACAT enzyme activity assay

The enzyme was assayed in sterol/PC/taurocholate mixed micelles or in reconstituted vesicles conducted as described previously (22), using the radioactive substrate $^3$H-oleoyl coenzyme A at $4 \times 10^4$ dpm/nanomol. The mixed micelles were prepared as described previously (22), containing varying sterol concentrations, reported as mol % sterol/sterol +PC as indicated in various figures, in 11.2mM PC and 18.6 mM taurocholate. For assays that contained two different sterols, each sterol/PC/taurocholate mixed micelles sample was made separately, then mixed together and used within 6h. The reconstituted vesicles were prepared essentially as described previously (30), (31). In the current work, we used taurocholate instead of cholate as the detergent to prepare the bile salt/PC/sterol micelles, with taurocholate at 18.6 mM and the PC at 11.2 mM. We then used cholestyramine to remove bile salt from mixed micelles, which rapidly led to vesicle formation. Control experiments using radioactive taurocholate showed that after two treatments of cholestyramine, more than 99% of the taurocholate was removed from the resultant vesicles.

Examining the sterol solubility in mixed micelles

The sterol solubility in micellar solutions was monitored by subjecting the solutions to ultracentrifugation (at 50,000g for 40 min) followed by ultrafiltration through a Sartorius Centrisart ultrafiltration device that contains a membrane with a molecular weight cutoff of 300kDa as described (32). After the
ultracentrifugation and the ultrafiltration steps, the sterols were quantitated according to method described (33). Briefly, the lipids present in the untreated samples or in the supernatants after treatment were extracted with chloroform/methanol (2:1), spotted on to the TLC plate and separated using the solvent system hexane : acetone : acetic acid = 80 : 20 : 1(v/v/v). The plate was sprayed with a 0.05% solution of primulin dye, then scanned by using the STORM 860 imaging system to detect the laser-excited fluorescent signals. Spots of a given sterol were quantitated by integration of variable pixel intensities using the Imagequant software. Standard curves were produced by quantitating increasing amounts (10 to 200 microgram) of a given sterol sample spotted in parallel lanes.

*Chemical synthesis of sterol oleate esters*

Various nonradioactive sterol oleate esters were used as internal markers, and visualized by iodine staining after TLC analysis. Other than cholesteryl oleate and 25-hydroxycholesterol (from Steraloids), the sterol esters described in the current work were not commercially available. They were chemically synthesized based on the general instruction provided by Molecular Probes. Typically, 5 micromoles of a given sterol was weighed in an amber vial, 0.166ml of methylene chloride was added and swirled briefly. 0.075ml of triethylamine, 3mg of oleic anhydride and 0.042 mg of 4-dimethylaminopyridine (DMAP) as catalyst, and 2micro-liter of 0.1%BHT in methylene chloride as antioxidant were then added. The vial was sealed under nitrogen and stirred with magnetic stir bar overnight in the dark. 12 h
later, the same amounts of oleic anhydride, DMAP and BHT described before were added, and the reaction was continued for an additional 10 h. The sterol oleates synthesized were used as the internal markers for TLC without purification. When TLC plates were run in petroleum ether : ether : acetic acid = 90 : 10 : 1 solvent system, the Rf values for oleates of the following sterols were: cholesterol: 0.87; 5alpha, 6alpha-epoxycholesterol: 0.51; 5beta, 6beta-epoxycholesterol: 0.58; 7-ketoocholesterol: 0.36; 7alpha-hydroxycholesterol: 0.21; 7beta-hydroxycholesterol: 0.28; beta-sitosterol: 0.89; 25-hydroxycholesterol: 0.30; 24(S), 25 epoxycholesterol: 0.46.
RESULTS

In order to quantitatively compare the abilities of various sterols that serve as ACAT substrate and/or activator, a single-phase system comprised of the enzyme, phospholipid, and sterol is needed. We had previously developed a mixed micelles system for measuring ACAT activity. This system consisted of mixing egg PC, taurocholate, and cholesterol at appropriate ratios, followed by bath-sonication at cold temperature until optical clearance. The enzyme ACAT solubilized in low concentration of the detergent CHAPs is then added to the mixed micelles before the reaction begins. It is possible that the micelles prepared by sonication may still contain a minor portion of sterol in the form of supersaturated microcrystals, and/or multilamellar or unilamellar vesicles. To remove microcrystals and/or vesicles from the micelles, Moschetta and colleagues developed a simple and effective procedure that involved ultracentrifugation and ultrafiltration (32). We used this procedure to monitor the quality of the mixed micelles that contained either cholesterol, or 7-ketocholesterol, or 5alpha, 6alpha-epoxycholestanol. The results showed that essentially 100% of these three sterols existed in the micellar phase at concentrations from 0.25 mM to 2 mM (which is equivalent to 0.022 to 0.15 mol % sterol/sterol+PC) (data not shown). Other sterols, including 5beta, 6beta-epoxycholesterol, beta-sitosterol, 7alpha-hydroxycholesterol, and 7beta-hydroxycholesterol behaved in the same manner (results not shown). In contrast, oxysterols that contain the hydroxy groups at the side chain, including 24-
hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol, could only produce clear micellar solutions at no more than 0.5 mM (0.043 mol% sterol/sterol+PC). Therefore, in our current study, we have focused our effort on comparing various sterols that readily form mixed micelles. We compared the ability of 7 sterols to serve as substrates of ACAT by varying their concentrations from 0.047 to 2.0 mM, which amount to 0.004 to 0.15 mole% sterol/sterol+PC. In some experiments, the highest concentrations were extended to 2.3 mM (0.17 mole% sterol/sterol+PC). The solubility of 24(S), 25-epoxycholesterol was higher than that of oxysterols with hydroxy groups at the side chain; this property allowed us to study 24(S), 25-epoxycholesterol at concentrations between 0.0047 to 0.1 mole% sterol/sterol+PC. The results (Fig. 1) show that 5alpha, 6alpha-epoxycholestanol, cholesterol, and 7alpha-hydroxycholesterol are the three best substrates. All three sterols exhibit sigmoidal substrate saturation curves; the $K_{0.5}$ values (the concentration at which half-maximal velocity is achieved) for these three sterols were similar, varying between 0.7 and 0.9 mole% sterol/sterol+PC. At sterol concentrations above $K_{0.5}$, 5alpha,6alpha-epoxycholestanol is a slightly better substrate than cholesterol, while 7alpha-hydroxycholesterol is approximately 70% as efficient as cholesterol as the substrate (results of three independent experiments). The other 3 sterols tested, 7-ketocholesterol, 7beta-hydroxycholesterol, and beta-sitosterol (a major plant sterol) were all vastly inferior to cholesterol as the substrate. In addition, 24(S), 25-epoxycholesterol
tested at concentrations up to 0.1 mole% sterol/sterol+PC is also a poor substrate. The inset showed the same data using a smaller scale to report the ACAT activity. It demonstrates that ACAT1 can definitely use any of the latter 4 sterols as substrates, though in a far less efficient manner. We next tested the effect of cholesterol when 7-ketocholesterol serves as the substrate, and vice versa. The results (Fig. 2A) show that cholesterol added at low concentrations (from 0.01 to 0.1 mole% sterol/sterol+PC) significantly activated the enzyme when 7-ketocholesterol was measured as the substrate. It also changed the substrate saturation curve from being sigmoidal to essentially hyperbolic. Calculations (using the software program Prism) showed that the Hill coefficient decreased from 3.0 without cholesterol to 1.1 with cholesterol added at 0.1 mole% sterol/sterol+PC. The $K_{0.5}$ value decreased from 0.13 mole% sterol/sterol+PC without cholesterol to 0.04 mole% sterol/sterol+PC with cholesterol. The Vmax value increased from 150 nmol/min without cholesterol to 200 nmol/min with cholesterol. The activation effect by cholesterol shown in Fig. 2A could not be explained because cholesterol is a better substrate and binds to the catalytic site more efficiently. If this were the case, cholesterol should have caused severe inhibition when 7-ketocholesterol was measured as the substrate. When cholesterol was measured as the substrate, 7-ketocholesterol added at low concentrations (from 0.005 to 0.025 mole% sterol/sterol+PC) caused slight inhibition of the enzyme (Fig. 2B). The inhibition was more prominent at higher cholesterol concentrations.
Thus, the inhibitory effect of 7-ketocholesterol is presumably through substrate competition. 7-Ketocholesterol did not alter the sigmoidicity of the cholesterol substrate saturation curve. Calculations showed that the $K_{0.5}$ value for cholesterol stayed at 0.07 mole% sterol/sterol+PC, and the Hill coefficient stayed around 2.2, with or without 7-ketocholesterol added.

Human ACAT1 is a homotetrameric enzyme (34). We had previously shown that by deleting the first 65 a.a. residues from the N-terminal, the enzyme could be converted to a dimeric form. This form was designated as hACAT1$_{\text{delta 1-65}}$. The dimeric enzyme is 5 to 10 times more active than the native ACAT1 in terms of catalytic efficiency (29). We compared the effect of cholesterol and 7-ketocholesterol using hACAT1$_{\text{delta 1-65}}$ as the enzyme source. The results show (Fig. 3A) that cholesterol significantly activated the enzyme activity when 7-ketocholesterol was measured as the substrate, while 7-ketocholesterol added had minimal effect on the enzyme activity when cholesterol was measured as the substrate (Fig. 3B). Thus, the ability of cholesterol to activate the enzyme does not require the enzyme to exist at the tetrameric form.

We next tested the effect of cholesterol when 5alpha, 6alpha-epoxycholestanol was measured as the substrate, and vice versa. The results showed that cholesterol added at low concentrations significantly activated the enzyme when 5alpha, 6alpha-epoxycholesterol was used as the substrate, while 5alpha, 6alpha-epoxycholesterol added inhibited the enzyme when cholesterol was measured as
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the substrate (data not shown). This result suggested that cholesterol may be a better activator than 5alpha, 6alpha-epoxycholesterol, although the latter sterol is a slightly better substrate than cholesterol (Fig. 1). To test this interpretation, we next used 7-ketocholesterol as the substrate at two different concentrations (at 0.08 or 0.12 mole% sterol/sterol+PC), and compared cholesterol versus various other sterols as indicated, including 5alpha, 6alpha-epoxycholesterol, for their abilities to activate the enzyme. The results (Fig. 4) showed that among all the sterols tested, cholesterol is the only sterol that caused significant activation of the enzyme.

In intact cells, ACAT resides mainly in the ER and uses sterol present in the ER as the enzymatic substrate (3). To measure ACAT activity in a system that is similar to the ER, we next tested the effects of various sterols present in vesicle form. To avoid the step of sterol transfer between donor vesicles to the vesicles where ACAT resides, we had previously developed a reconstituted vesicle system by diluting the ACAT solubilized in detergent into a large excess of preformed vesicles with defined sterol and PC composition (24). We now used the reconstituted vesicle system to compare the abilities of cholesterol, 5alpha, 6alpha-epoxycholesterol, and 7-ketocholesterol to serve as ACAT substrates. The results show (Fig. 5) that 5alpha, 6alpha-epoxycholesterol and cholesterol are much better substrates than 7-ketocholesterol. For each of the sterols tested, the enzyme responded to the sterol content present in the vesicles in a sigmoidal-like manner.

We next tested the effect of cholesterol on ACAT activity when 7-ketocholesterol
was measured as the substrate, and vice versa. The results show that cholesterol added at low concentrations significantly activated the enzyme when 7-ketocholesterol (Fig. 6A) was measured as the substrate, while 7-ketocholesterol (Fig. 6B) added inhibited the enzyme, presumably through substrate competition, when cholesterol was measured as the substrate. In data not shown, we also tested the effect of cholesterol on ACAT activity when 5alpha, 6alpha-epoxycholestanol was measured as the substrate, and vice versa, and have obtained essentially the same results as described in Fig. 6A,B. To further test this finding using the reconstituted vesicle system, we used 7-ketocholesterol as the variable substrate, and compared cholesterol and 5alpha, 6alpha-epoxycholesterol, added at 0.4 mole% sterol/sterol+PC, for their ability to activate the enzyme. The results (Fig. 7) show that cholesterol is superior to 5alpha, 6alpha-epoxycholesterol as the activator. Thus, the results using the reconstituted vesicles system fully corroborated the results using the mixed micelles system.
DISCUSSION

In the current work, we employed the mixed micelles assay system to compare the abilities of various sterols to serve as a substrate for ACAT1. The results show that cholesterol and 5alpha, 6alpha-epoxycholesterol are the two best substrates tested. 7alpha-hydroxycholesterol is the third best substrate, being 70% as efficient, while other sterols such as 7-ketocholesterol were 10% or less as efficient. Modification of cholesterol by including the 7alpha-hydroxy moiety in steroid ring B is moderately tolerated as the enzymatic substrate. We then developed a method to compare the abilities of various sterols serving as ACAT activators. The results show that cholesterol is far superior as an activator to all other sterols tested, including 5alpha, 6alpha-epoxycholesterol or 7alpha-hydroxycholesterol. Thus, the structural specificity of the activator site is much more stringent than its sterol substrate site. The results obtained by using the mixed micelles have essentially been confirmed by using the reconstituted vesicles system.

In bile salt-based mixed micelles, sterol-specific microdomain(s) has not been reported. In PC-based vesicles, the physical properties of cholesterol, 5alpha, 6alpha-epoxycholestanol, 7alpha-hydroxycholesterol, 7beta-hydroxycholesterol, and 7keto-cholesterol have previously been shown to be similar to those of cholesterol (19), (35), (36). Therefore, we believe that the sterol-specificity demonstrated in our current work is mainly due to the intrinsic specificity of the
enzyme, not due to subtle difference in biophysical properties of sterols in micelles or in vesicles. We also show that the ability of cholesterol to serve as an activator is preserved when the oligomeric structure of ACAT changes from the tetrameric form to a dimeric form. To explain these results, we propose the following model: ACAT1 contains a sterol substrate site and an allosteric sterol activator site. The activator site is restricted to cholesterol only, while the substrate site is more promiscuous. When 7-ketocholesterol is the substrate, activation by cholesterol at low concentration decreases the $K_{0.5}$ value as well as increasing the $V_{\text{max}}$ value towards 7-ketocholesterol. When 5alpha, 6alpha-epoxycholesterol is the substrate, activation by cholesterol at low concentration decreases the $K_{0.5}$ value without affecting the $V_{\text{max}}$ value of the enzyme towards 5alpha, 6alpha-epoxycholesterol. In the future, using various biochemical approaches that include photoaffinity labeling and site-specific mutagenesis studies can test the validity of this model. In oxidized LDL loaded macrophages, a large amount of esterified oxysterols including 7-ketocholesterol are found; those present in the cytosol were derived from ACAT reaction (7). These observations could be explained based on our model: ACAT1 present in macrophages is activated by cholesterol in the cholesterol-rich environment, and becomes more efficient in using 7-ketocholesterol as an alternative substrate, thus causing an ample amount of 7-ketocholesterol to be esterified. In addition, as shown in our current work, beta-sitosterol (a major plant sterol) is a poor ACAT1 substrate and as an activator. Our
model predicts that beta-sitosterol may become a much better substrate of ACAT1 when the enzyme is under high cholesterol conditions. In the future, this prediction could be tested under various physiological conditions.

Related to our current work, Brown and colleagues (37) have recently shown that when added as cyclodextrin complex, cholesterol, 7-ketocholesterol, 7alpha-hydroxycholesterol, or other structurally related sterols added to the ER membranes in vitro causes conformational change of the sterol-sensing protein SCAP. Oxysterols with hydroxy groups at the side chain, such as 25-hydroxycholesterol or 27-hydroxycholesterol fail to initiate this change. SCAP is a key factor involved in sterol-specific transcriptional regulation of cholesterol biosynthesis. The sterol specificity demonstrated in their system strongly suggests that it is cholesterol itself that acts on the sterol regulatory machinery. The authors did not test the abilities of the sterols to cause conformational change of SCAP in a dose-dependent manner. Our results reported here show that cholesterol is superior to either 7-ketocholesterol or 7alpha-cholesterol as an ACAT activator in vitro. Both ACAT1 and SCAP are integral membrane proteins that mainly reside in the ER. Our results thus reinforce the results of Brown and colleagues, supporting the concept that the content of cholesterol rather than the content of an oxysterol such as 7-ketocholesterol or 7alpha-hydroxycholesterol located in the ER plays a pivotal role in the regulation of intracellular cholesterol metabolism. The fact that 7-ketocholesterol added in intact cells caused significant stimulation in
esterification of cholesterol (16) could be explained by its ability to cause translocation of cholesterol from the plasma membrane to the ER where ACAT resides (38), (3).

Due to their limited solubility in mixed micelles, we could not perform extensive testing on various oxysterols with hydroxy-moiety at the side chain. We did however find that 25-hydroxycholesterol at concentrations up to 0.05mole% was also a poor substrate for ACAT1. When cholesterol was measured as a substrate, 25-hydroxycholesterol added at low concentration failed to increase ACAT activity; while cholesterol added at low concentration greatly increased the ACAT activity in utilizing 25-hydroxycholesterol as the substrate (results not shown). In lipid membranes, the oxysterols with hydroxy groups at the side chain are very different from cholesterol in terms of their biophysical properties (21), (35), (36). Kauffman and colleagues proposed that 25-hydroxycholesterol and 27-hydroxycholesterol might prefer to interact with PC in “reverse orientation”; i.e., with its hydrophilic side chain lining up with the polar moiety of the PC molecules in membranes (39). Based on earlier studies and the current results, if oxysterols serve as important regulators for ACAT activity in intact cells, they may act through certain novel mechanism(s), rather than by incorporating into the ER membranes and being recognized by ACAT as a preferred activating molecule.
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ABBREVIATIONS

ACAT, acyl-coenzyme A:cholesterol acyltransferase; BHT, (2,6-Di-tert.-butyl-p-cresol); BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphatidylcholine
FIGURE LEGENDS

Fig. 1. Sterol substrate saturation curves of HisACAT1 in mixed micelles. The mixed micelles were prepared as described in Material and Methods. The ACAT activity assays were performed in duplicate. Points shown were averages of duplicate assays; error bars indicated variations from the mean. The results shown are representative of two separate experiments. The inset shows replot of the same data with smaller scale in the ACAT activity. Abbreviations used: 5α, 6α-epoxy: 5alpha, 6alpha-epoxycholesterol; 5β, 6β-epoxy: 5beta, 6beta-epoxycholesterol; 7-keto: 7-ketocholesterol; 7αOH: 7alpha-hydroxycholesterol; 7βOH: 7 beta-hydroxycholesterol; 24(S), 25-epoxy: 24(s), 25-epoxycholesterol; β-sito: beta-sitosterol.

Fig. 2. Sterol substrate saturation curves of HisACAT1 with both cholesterol and 7-ketocholesterol present in mixed micelles. The final concentrations of cholesterol or 7-ketocholesterol were as indicated. A. 7-ketocholesterol substrate saturation curve in the presence of indicated concentrations of cholesterol. B. Cholesterol substrate saturation curve in the presence of indicated concentrations of 7-ketocholesterol. The assays were done in duplicate. The results shown are representative of two separate experiments.
Fig. 3. Sterol substrate saturation curves of HisACAT1<sub>delta 1-65</sub>, with both cholesterol and 7-ketocholesterol present in mixed micelles. HisACAT1<sub>delta 1-65</sub> was partially purified as described in Material and Methods. A. 7-ketocholesterol substrate saturation curve in the presence of indicated concentration of cholesterol (at 0 or 0.04 mol%). B. Cholesterol substrate saturation curve in the presence of indicated concentration of 7-ketocholesterol (at 0 or 0.04 mol%). The assays were done in duplicate.

Fig. 4. Effects of various sterols as indicated (at 0.04 mol%) on HisACAT1 when 7-ketocholesterol was measured as the substrate. The concentration of 7-ketocholesterol was fixed at 0.08 mol% or at 0.12 mol%. The results shown were averages of duplicate assays.

Fig. 5. Sterol substrate saturation curves of HisACAT1 in reconstituted vesicles. Reconstituted vesicles containing cholesterol, or 7-ketocholesterol or 5alpha, 6alpha-epoxycholesterol at indicated concentrations were prepared as described in Material and Methods. Results were averages of duplicate assays; error bars indicate variations from the mean.

Fig. 6. Sterol substrate saturation curves of HisACAT1 with both cholesterol and 7-ketocholesterol present in reconstituted vesicles. The cholesterol/PC/taurocholate
and 7-ketocholesterol/PC/taurocholate mixed micelles were made separately, then mixed and treated with cholestyramine to produce the reconstituted vesicles.  A. 7-ketocholesterol substrate saturation curves with cholesterol at indicated concentration (0 or 0.04 mol%).  B. Cholesterol substrate saturation curves with 7-ketocholesterol at indicated concentration (0 or 0.04 mol%).

Fig. 7. 7-ketocholestrol substrate saturation curves of HisACAT1 with cholesterol or 5alpha, 6alpha-epoxycholesterol present in reconstituted vesicles. The final concentration of cholesterol or 5alpha, 6alpha-epoxycholesterol added was at 0.04 mol%. The assays were done in duplicate.
Figure 2

A

7-ketocholesteryl Oleate Formed (dpm)

7-keto/(7-keto+PC)

B

Cholesteryl Oleate Formed (dpm)

Chol/(Chol+PC)
Figure 3

(A) 7-ketocholesterol oleate formed (dpm) as a function of 7-keto/(7-keto+PC).

(B) Cholesterol oleate formed (dpm) as a function of Chol/(Chol+PC).
Figure 4

7-keto Oleate (dpm)

7-keto only
5α,6α-epoxy
5β,6β-epoxy
7αOH
7βOH
β-sitosterol
Cholesterol

7-keto/(7-keto+PC)
Figure 6

A

7-ketocholesteroyl oleate formed (dpm) vs. 7-keto/(7-keto+PC)

Chol

0

0.04

B

Cholesteryl oleate formed (dpm) vs. Chol/(Chol+PC)

7-keto

0

0.04
Figure 7

[Graph showing the formation of 7-ketocholesteryl oleate as a function of the 7-keto/(7-keto+PC) ratio with different conditions: none, +Chol, and +5α,6α-epoxy.]
Cholesterol is superior to 7-ketocholesterol or 7alpha-hydroxycholesterol as an allosteric activator for acyl-coenzyme A: cholesterol acyltransferase 1
Yi Zhang, Chunjiang Yu, Jay Liu, Thomas A. Spencer, Catherine C.Y. Chang and Ta-Yuan Chang

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