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Microsomal long chain fatty acyl-CoA transacylation: differential effect of sterol carrier protein-2

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Abstract

The recent discovery that sterol carrier protein-2 (SCP-2) binds long chain fatty acyl-CoA (LCFA-CoA) with high affinity (A. Frolov et al., J. Biol. Chem. 271 (1997) 31878–31884) suggests new possible functions of this protein in LCFA-CoA metabolism. The purpose of the present investigation was to determine whether SCP-2 differentially modulated microsomal LCFA-CoA transacylation to cholesteryl esters, triacylglycerols, and phospholipids in vitro. Microsomal acyl-CoA:cholesterol acyltransferase (ACAT) activity measured with liposomal membrane cholesterol donors depended on substrate LCFA-CoA level, mol% cholesterol in the liposomal membrane, and total amount of liposomal cholesterol. As compared to basal activity without liposomes, microsomal ACAT was inhibited 30–50% in the presence of cholesterol poor (1.4 mol%) liposomes. In contrast, cholesterol rich (>25 mol%) liposomes stimulated ACAT up to 6.4-fold compared to basal activity without liposomes and nearly 10-fold as compared to cholesterol poor (1.4 mol%) liposomes. Increasing oleoyl-CoA reversed the inhibition of microsomal ACAT by cholesterol poor (1.4 mol%) liposomes, but did not further stimulate ACAT in the presence of cholesterol rich (35 mol%) liposomes. In contrast, high (100 μM) oleoyl-CoA inhibited ACAT nearly 3-fold. This inhibition was reversed by LCFA-CoA binding proteins, bovine serum albumin (BSA) and SCP-2. SCP-2 was 10-fold more effective (mole for mole) than BSA in reversing LCFA-CoA inhibited microsomal ACAT. Concomitantly, under conditions in which SCP-2 stimulated ACAT it equally enhanced transacylation of oleoyl-CoA into phospholipids, and 5.2-fold enhanced oleoyl-CoA transacylation to triacylglycerols. In summary, SCP-2 appeared to exert its greatest effects on microsomal transacylation in vitro by reversing LCFA-CoA inhibition of ACAT and by differentially targeting LCFA-CoA to triacylglycerols. These data suggest that the high affinity interaction of SCP-2s with LCFA-CoA may be physiologically important in microsomal transacylation reactions.

Keywords: Cholesterol ester; Triacylglycerol; Phospholipid; Microsome; Sterol carrier protein-2

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; BSA, bovine serum albumin; LCFA, long chain fatty acid; LCFA-CoA, long chain fatty acyl-CoA; NMR, nuclear magnetic resonance; SCP-2, sterol carrier protein-2

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1. Introduction

As implied by its name, sterol carrier protein (SCP-2) is most often associated with functions involving the transfer of cholesterol between membranes (reviews in [1–9]), biliary cholesterol transport [10,11], steroidogenesis [7,11,12], lung surfactant formation [13], intestinal cholesterol absorption [14,15], diabetes [16], macrophage foam cell formation [17], and hypercholesterolemia [18]. The mechanism whereby SCP-2 elicits such effects has only recently begun to be resolved. With rare exception [19], most studies using radiolabel [20–22], fluorescence [20,23–25], and ^{13}C-NMR techniques ([1]; N.J. Stolowich, A. Frolov, A.I. Scott, J.T. Billheimer, F. Schroeder, Holo-sterol carrier protein-2: ^{13}C-NMR investigation of cholesterol and fatty acid binding sites, submitted) demonstrate that SCP-2 has a single sterol binding site with K_d reported as low as 6–10 nM ([1,25]; Stolowich et al., submitted). The binding of cholesterol appears to be required for SCP-2 to function as a sterol transfer protein [26].

The ability of SCP-2 to transfer sterols is also thought to be the mechanism whereby this protein interacts in vitro with microsomal acyl-CoA:cholesterol acyltransferase (ACAT) to stimulate cholesterol esterification over the range 2- to more than 10-fold, depending on the assay conditions used [23,27–31]. Since ACAT activity is limited by microsomal membrane cholesterol supply and the microsomal cholesterol pool is small (reviews in [32–34]), movement of cholesterol from donor membranes to microsomes is required for maximal ACAT activity. However, in many of the assays suggesting microsomal ACAT stimulation by SCP-2, the substrate cholesterol was solubilized with organic solvents, albumin, or detergents, as opposed to membrane cholesterol donors. Furthermore, recent demonstration that solvents such as ethanol inhibit the binding of ligands to SCP-2 [25,35] as well as albumin binding of lipophilic ligands such as cholesterol and LCFA-CoA complicates interpretation of SCP-2 mediated effects.

A less understood aspect of microsomal ACAT activity is the relationship between SCP-2 and the other microsomal ACAT substrate, long chain fatty acyl-CoA (LCFA-CoA). Early work based on regulation of microsomal ACAT in vitro suggested the importance of LCFA-CoA substrate level. High levels of LCFA-CoA inhibited ACAT while the LCFA-CoA binding protein bovine serum albumin (BSA) prevented LCFA-CoA's inhibition of microsomal ACAT (reviews in [32,36]). Recently, increasing data have begun to suggest hitherto unsuspected roles for SCP-2 in LCFA-CoA metabolism. First, SCP-2 may influence LCFA-CoA pool formation. SCP-2 binds the precursors of LCFA-CoA with high affinity: straight chain LCFA with $K_d = 180$–560 nM ([35,37]; Stolowich et al., submitted); branched chain LCFA with $K_d = 714$ nM [24,38]. Whether SCP-2 stimulates the conversion of LCFA to LCFA-CoA via LCFA-CoA synthetases (as do other LCFA or LCFA-CoA binding proteins) remains to be determined (review in [39]). Second, SCP-2 binds LCFA-CoAs with even higher affinity than LCFA: straight chain LCFA-CoA with $K_d = 2$–5 nM ([40]; Stolowich et al., submitted); branched long chain LCFA-CoAs with $K_d = 250$ nM [24]. The finding that the affinity of SCP-2 for long chain LCFA-CoAs (LCFA-CoA), $K_d = 2$–5 nM [40], is essentially the same as that of the highest affinity reported for cholesterol, 6–10 nM ([1,25]; Stolowich et al., submitted) suggests that SCP-2 stimulates microsomal transacylase reactions such as ACAT not only by enhancing cholesterol transfer to the enzyme, but equally important by interacting with the other ACAT substrate, LCFA-CoA. The major purpose of the present investigation was to examine this role and to also determine whether SCP-2 might differentially affect microsomal transacylase reactions forming cholesteryl esters, triacylglycerols, and phospholipids.

2. Materials and methods

2.1. Materials

Cholesterol (3β-hydroxyl-5-cholesten), triacylglycerol, cholesteryl oleate, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) were purchased from Avanti Polar-Lipids (Alabaster, AL, USA). [1-^{14}C]-Oleoyl-CoA (53.34 mCi/mmol) in 10 mM sodium acetate, pH 6.0, ethanol 1:1, v/v and [1-^{14}C]oleoyl-CoA (59.5 mCi/mmol) in 10 mM sodium acetate, pH 6.0 were obtained from DuPont New England Nuclear (Boston, MA, USA). Fatty acid free bovine

serum albumin and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). Sephacryl S-300 beads were purchased from Pharmacia (Uppsala, Sweden). Silica gel G thin-layer chromatography plates were purchased from Anal Tech (Newark, DE, USA). All other chemicals were reagent grade or better.

2.2. Methods

2.2.1. Recombinant human sterol carrier protein-2

Recombinant SCP-2 was isolated and purified as described earlier [41]. The protein concentration was determined by Bradford assay [42]. The SCP-2 stock solution was diluted to a 90 μM working solution.

2.2.2. Isolation of rat liver microsomes

Microsomes were prepared from male Sprague-Dawley rat (200–210 g) livers [36,41] and washed by gel filtration using 100 ml column volume of Sephacryl S-300 beads as described earlier [43]. Western immunoblotting showed that this preparation had no detectable SCP-2.

2.2.3. Liposome preparation

Designated amounts of POPC (in chloroform) and cholesterol (in ethanol) were mixed and evaporated to dryness under a stream of nitrogen. The mixed contents were dried further in vacuo for at least 2 h to remove any residual solvents. To the dried lipid mixture was added 1 ml of 100 mM potassium phosphate buffer, pH 7.4, 2 mM dithiothreitol. The mixture was vortexed followed by sonication for 3–5 min in a bath sonicator. The multilayer liposomes were further processed to yield single bilayer liposomes by sonication for 30 min with the microprobe tip and Fisher Sonic Dismembrator, Model 300 (Fisher, Pittsburgh, PA, USA). The liposome preparation was centrifuged for 30 min at 24°C, 36 000 rpm in an L7-55 Ultracentrifuge and Type 40 Ti rotor (Beckman Instr., Fullerton, CA, USA) to remove multilamellar vesicles and titanium particles released by the microtip.

2.2.4. ACAT assay

Unless indicated in the text, each reaction mixture contained 100 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, 15 μg of microsome protein, and 5 μl of liposome preparation. The endogenous acyl-CoA content in the microsomes was very low as evidenced by basal microsomal ACAT activity near 0 at time zero (see Section 3, Fig. 2B). Each ACAT reaction was initiated by addition of [1-^{14}C]oleoyl-CoA from a solution supplied directly by the manufacturer (59.5 mCi/mmol, in 10 mM sodium acetate, pH 6.0) to yield a final oleoyl-CoA concentration of 15 μM. No unlabeled oleoyl-CoA substrate was added to the reaction mixture. The final reaction volume was 50 μl. Incubation was performed at 37°C in a shaking water bath for 30 min, the reaction was stopped by adding 500 μl of chloroform:methanol 2:1 (v/v), vortexed, and allowed to sit at 4°C for at least 4 h or at −20°C for 2 h to extract lipids. The extracted lipids were then resolved by thin-layer chromatography (see Section 2.2.5). Blanks were assayed routinely in the absence of microsome. Each reaction was performed in triplicate.

2.2.5. Thin-layer chromatography

The extracted lipids were dried under a stream of N_2 and redissolved in 50 μl chloroform. The sample was spotted on an activated silica gel G plate and lipids were separated using the solvent system containing petroleum ether:diethyl ether:acetic acid (85:20:1 v/v/v). Lipids were visualized with 6-*p*-toluidine-2-naphthalenesulfonic acid (TNS, 1 mM) in 50 mM Tris (pH 7.4) [41]. Cholesteryl oleate, triacylglycerol, oleic acid, cholesterol, phosphatidic acid and phosphatidylcholine standards were run parallel with samples. The bands corresponding to the desired bands were removed by scraping and the radioactivity was determined by liquid scintillation counting using a Packard 1600 TR scintillation counter (Meridan, CT, USA).

3. Results

3.1. Cholesterol poor liposomes inhibit microsomal acyl-CoA cholesterol acyltransferase

The major purpose of this investigation was to determine the effect of sterol carrier protein-2 (in the absence of solvents, detergents, or albumin) on microsomal utilization of fatty acyl-CoA and cholesterol toward cholesteryl esters. Since endogenous mi-

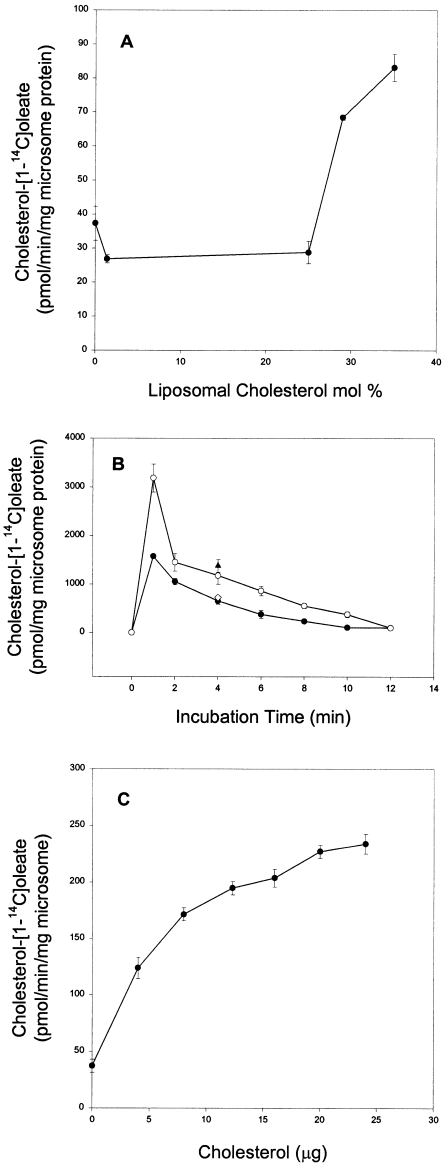

Fig. 1. Effect of liposomal cholesterol on microsomal acyl-CoA cholesterol acyltransferase activity. (A) Effect of liposomal mole% cholesterol on microsomal ACAT. ACAT activity was determined as described in Section 2.2 except that liposomal cholesterol donor cholesterol content was varied as follows: 0, 1.4, 25, 30, or 35 mole% cholesterol. (B) Effect of time of preincubating microsomes with liposomal cholesterol donors on ACAT activity. ACAT activity was determined with cholesterol poor liposomes (1.4 mol% cholesterol). Liposomes were preincubated with microsomes for 30 min (●) or 0 min (○) at 37°C, followed by initiation of ACAT by addition of 11.2 μM [1-^{14}C]oleoyl-CoA. The effect of preincubating liposomes with [1-^{14}C]oleoyl-CoA for 30 min at 37°C followed by microsome initiation vs. preincubating microsomes with liposomes followed by initiation with [1-^{14}C]oleoyl-CoA for 30 min at 37°C is shown by ▲ and ◇, respectively. (C) Effect of total liposomal cholesterol at constant mol% on microsomal ACAT. ACAT was determined as described in Section 2.2 using 35 mol% liposome as cholesterol donor and increasing the total liposomal cholesterol from 0 to 25 μg in the assay.
←

crosomal cholesterol is insufficient to saturate the microsomal ACAT, exogenous cholesterol must be provided in the assay [32]. In the present investigation, liposomal membranes were chosen as the cholesterol substrate vehicle because, unlike organic solvents, detergents, or lipoproteins, they more closely resemble a physiological cholesterol substrate, i.e. intracellular membranes [32].

Cholesterol poor (1.4 mole%) donor liposomes inhibited microsomal ACAT activity by 30% (Fig. 1A) as compared to basal activity without liposomes. Preincubation of the microsomes with cholesterol poor liposomes for 30 min at 37°C further increased this inhibition to 50% (Fig. 1B). This increased inhibition caused by preincubation was prevented when the microsomes were absent from the preincubation mixture. This suggested that preincubation of microsomes with cholesterol poor liposomes may have actually resulted in cholesterol transfer from the microsome to the cholesterol poor liposomes, thereby accounting for the greater inhibition of microsomal ACAT.

3.2. *Oleoyl-CoA prevents inhibition of microsomal acyl-CoA cholesterol acyltransferase by cholesterol poor liposomes*

The effect of oleoyl-CoA substrate level on microsomal ACAT activity in the presence of cholesterol poor liposomes was examined in order to determine whether increasing oleoyl-CoA substrate could overcome the inhibition of microsomal ACAT observed above with cholesterol poor (1.4 mol%) liposomes. As shown in Fig. 2A, measurement of microsomal cholesterol oleate production as a function of time in the presence of cholesterol poor liposomes showed maximal cholesterol oleate production near 6 min. Thereafter, the amount of cholesterol oleate formed decreased to level off at values near half-maximal, most likely due to endogenous cholesterol ester hydrolase present in the microsomes. Increasing the oleoyl-CoA substrate concentration dramatically in-

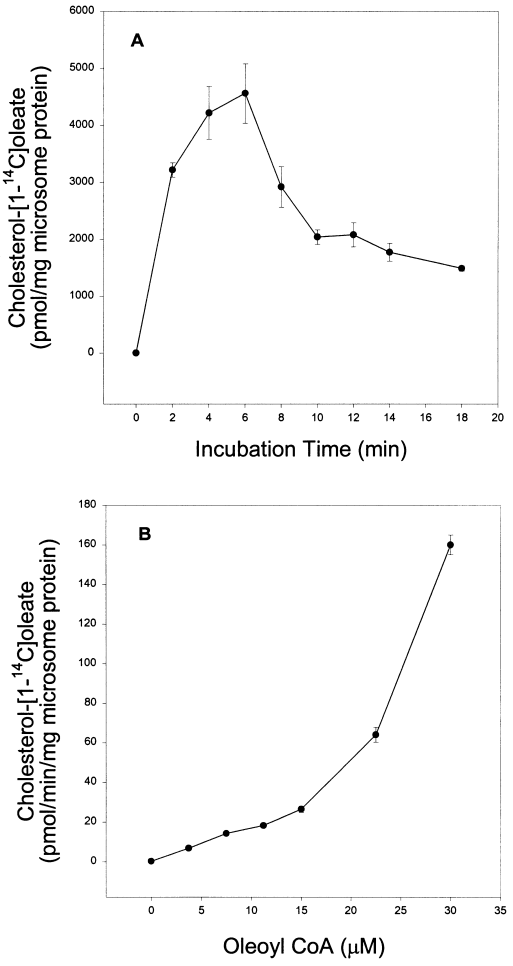

Fig. 2. Time course and effect of oleoyl-CoA on microsomal acyl-CoA cholesterol acyltransferase activity with cholesterol poor liposomes. Microsomal ACAT activity was measured as described in Section 2.2 except that cholesterol poor liposomes (1.4 mol%) were used. The ACAT reaction was initiated by addition of 11.2 μM [1-^{14}C]oleoyl-CoA in A and 0–30 μM [1-^{14}C]oleoyl-CoA in B.

creased the microsomal ACAT utilization of cholesterol from 1.4 mol% cholesterol poor liposomes (Fig. 2B). Even at 30 μM oleoyl-CoA, the microsomal ACAT activity near 160 pmol [1-^{14}C]cholesterol oleate/min/mg was not maximal. Thus, increasing oleoyl-CoA substrate overcame the inhibition of microsomal ACAT by cholesterol poor liposomes.

3.3. *Cholesterol rich liposomes stimulate microsomal acyl-CoA cholesterol acyltransferase*

In contrast to cholesterol poor liposomes, cholesterol rich liposomes stimulated microsomal ACAT. A critical threshold of mol% cholesterol in the donor liposomes appeared necessary for activation of microsomal ACAT. Increasing the liposomal cholesterol from 1.4 mol% to 25 mol% did not enhance microsomal ACAT (Fig. 1A). In contrast, further increasing the liposomal mol% cholesterol to 29% and 35% in the assay increased microsomal ACAT over basal activity by 1.8- and 2.2-fold, respectively (Fig. 1A). This suggested that, above 25 mol% cholesterol in the liposomal membrane, a critical threshold was reached such that the donor membrane cholesterol became available for utilization by microsomal ACAT.

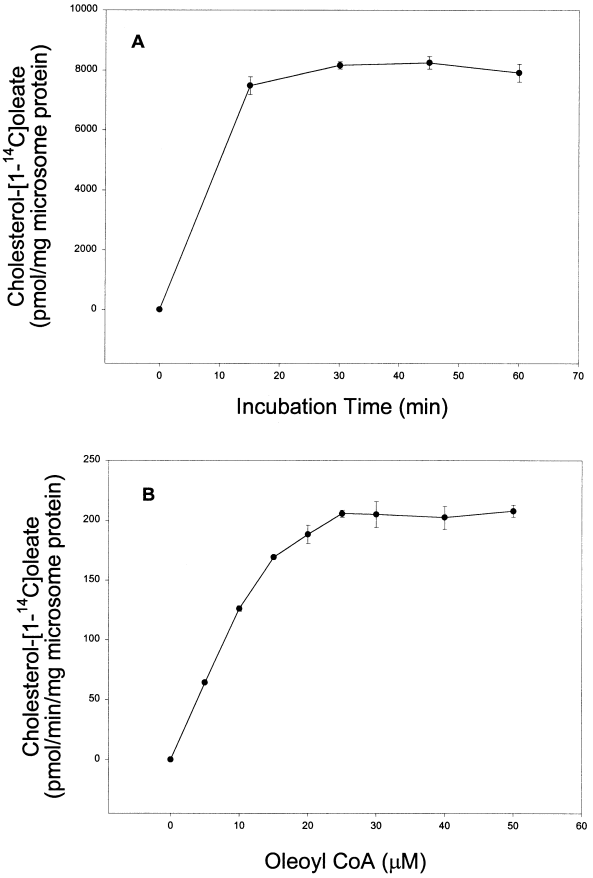

Fig. 3. Time course and effect of oleoyl-CoA on microsomal acyl-CoA cholesterol acyltransferase activity with cholesterol rich liposomes. All conditions were as described in Fig. 2 except that cholesterol rich liposomes (35 mole%) were used. (A) Time course of microsomal ACAT activity when 25 μl liposomes were added. (B) Effect of increasing [1-^{14}C]oleoyl-CoA from 0 to 50 μM on microsomal ACAT activity in the presence of 10 μl liposomes. Values represent the mean ± S.E.M. (n = 5–6).

The time dependence of microsomal ACAT activity measured in the presence of cholesterol rich (35 mol%) liposomes indicated that these liposomes increased microsomal ACAT rapidly and achieved maximal stimulation by 10 min incubation (Fig. 3A). This maximal activity was nearly 2-fold higher than that exhibited with cholesterol poor liposomes (Fig. 2B). Furthermore, the cholesterol rich liposomes increased microsomal ACAT to a plateau (Fig. 3A) while cholesterol poor liposomes exhibited a peak, but no plateau, in microsomal ACAT activity.

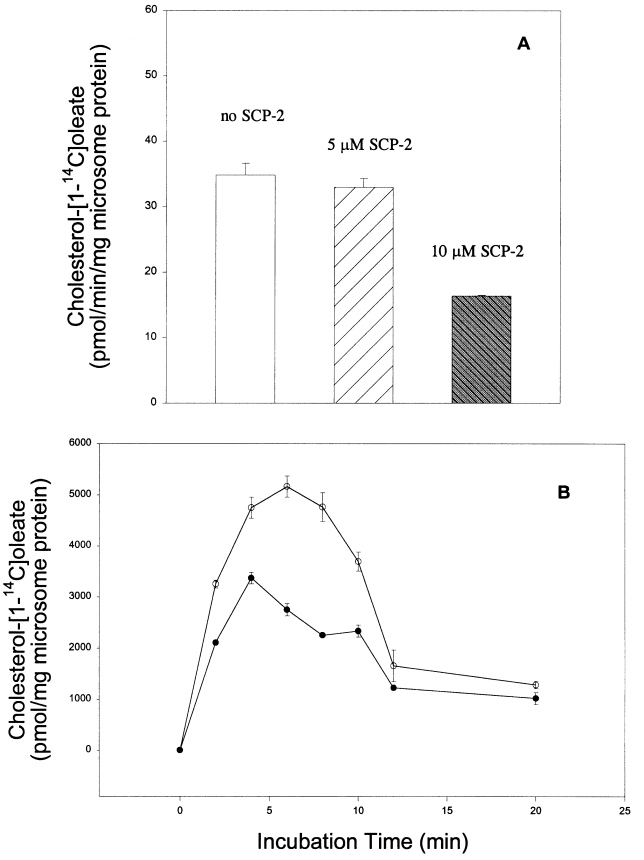

Fig. 4. The effect of sterol carrier protein-2 on microsomal acyl-CoA cholesterol acyltransferase activity with cholesterol poor liposomes. Microsomal ACAT activity was measured as described in Section 2.2 with cholesterol poor liposomes (1.4 mole%). In panel A the ACAT reaction was initiated by addition of 1.9 μM [1-^{14}C]oleoyl-CoA followed by incubation at 37°C for 30 min. The open bar indicates microsomal ACAT activity in the absence of sterol carrier protein-2 (SCP-2); the cross-hatched bar was in the presence of 5 μM SCP-2; the solid bar was in the presence of 10 μM SCP-2. In panel B, the ACAT reaction was initiated by addition of 11.2 μM [1-^{14}C]oleoyl-CoA followed by incubation at 37°C for the designated times. Open circles (○) refer to the absence of SCP-2 while closed circles (●) refer to the presence of 10 μM SCP-2. Values represent the mean ± S.E.M. (n = 5–6).

A liposomal dose-response curve was constructed to determine whether the 2.2-fold stimulation of microsomal ACAT was maximal. An increasing amount of cholesterol rich (35 mol% cholesterol) liposomes was added to the microsomal ACAT assay. Under these conditions, microsomal ACAT dramatically increased nearly 6.4-fold as compared to basal (no liposomes) (Fig. 1C) and nearly 10-fold as compared to cholesterol poor (1.4 mol%) liposomes (Fig. 1C vs. 1A). This [total donor membrane cholesterol] saturation curve was hyperbolic, with maximal cholesteryl ester formation near 250 pmol/min/mg protein (Fig. 1C).

3.4. *Oleoyl-CoA stimulates microsomal acyl-CoA cholesterol acyltransferase from cholesterol rich liposomes*

The effect of oleoyl-CoA substrate level on microsomal ACAT activity in the presence of cholesterol rich liposomes was examined in order to determine whether increasing oleoyl-CoA substrate could further enhance microsomal ACAT. In the presence of increasing the oleoyl-CoA the microsomal ACAT activity exhibited a hyperbolic saturation curve (Fig. 3B). Maximal stimulation of microsomal ACAT was obtained by 25 μM oleoyl-CoA. The maximal microsomal ACAT activity (near 210 pmol cholesteryl oleate/min/mg microsomal protein; Fig. 3B) obtained with cholesterol rich (35 mol%) liposomes required at least twice as much oleoyl-CoA as was necessary to obtain the maximal activity with cholesterol poor (1.4 mol%) liposomes (Fig. 2B).

3.5. *Differential effect of SCP-2 on microsomal ACAT utilization of liposomal cholesterol: cholesterol poor vs. cholesterol rich liposomes*

SCP-2 did not stimulate microsomal ACAT in the presence of cholesterol poor (1.4 mole%) liposomes at low oleoyl-CoA levels. In fact, SCP-2 inhibited microsomal ACAT by nearly 50% both at low (1.9 μM, Fig. 4A) and at higher (11.2 μM, Fig. 4B) oleoyl-CoA. Thus under conditions wherein the substrate

Table 1
Effect of sterol carrier protein-2 on microsomal acyl-CoA cholesterol acyltransferase activity in the presence of cholesterol rich liposomes

SCP-2	ACAT (pmol/min/mg protein)		
	Liposome (μl)		
	6	10	25
None	87 ± 5	229 ± 19	272 ± 7
5 μM	139 ± 8**	309 ± 16*	325 ± 5**
10 μM		382 ± 23**	311 ± 9**
Increase (%)			
5 μM	60	35	20
10 μM		67	14

Microsomal ACAT was determined with cholesterol rich liposomes (35 mol%) as described in Section 2.2. All reactions were initiated by addition of 15 μM [1-^{14}C]oleoyl-CoA. ACAT activity was expressed as pmol/min/mg microsomal protein. An asterisk and double asterisk refer to $P < 0.05$ and $P < 0.01$, respectively ($n = 3$), as compared to no SCP-2 added.

oleoyl-CoA was not inhibitory, SCP-2 inhibited microsomal ACAT utilization of cholesterol poor liposomal cholesterol.

In contrast, SCP-2 had the opposite effect on microsomal ACAT in the presence of cholesterol rich liposomes. When cholesterol rich (35 mol%) liposomes were used, SCP-2 stimulated microsomal ACAT modestly, up to 1.6-fold (6 μl liposomes, Table 1), at 15 μM oleoyl-CoA. Increasing the total cholesterol by 4-fold (25 μl liposomes, Table 1) further increased microsomal ACAT (see Fig. 1C). Thus in the presence of non-inhibitory levels of oleoyl-CoA, SCP-2 stimulated microsomal ACAT utilization of cholesterol from cholesterol rich liposomes.

3.6. Specificity of SCP-2 on oleoyl-CoA utilization by microsomal ACAT

Since acyl-CoAs can be utilized by several transacylases present in the endoplasmic reticulum, it was important to determine whether SCP-2 specifically stimulated microsomal ACAT transacylation of oleoyl-CoA to cholesteryl ester or whether SCP-2 simultaneously enhanced transacylation of oleoyl-CoA to other lipids, i.e. phospholipids or triacylglycerols. At short incubation time (5 min), SCP-2 stimulated microsomal incorporation of oleoyl-CoA into triacylglycerols nearly twice as well as into cholesterol esters, while stimulating oleoyl-CoA incorporation into phospholipid about half as well (Table 2). After 30 min incubation, SCP-2 stimulated oleoyl-CoA incorporation into cholesterol oleate and phospholipid nearly equally, about 35% (Table 2). Concomitantly, SCP-2 stimulated oleoyl-CoA incorporation into triacylglycerol 5.2-fold, from 63 to 330 pmol/mg microsome protein (Table 2). In summary, SCP-2 simultaneously stimulated microsomal oleoyl-CoA transacylation to form cholesterol ester and phospholipid equally well. However, SCP-2 stimulated oleoyl-CoA transacylation to form triacylglycerol nearly 15-fold more effectively than incorporation into cholesterol esters or phospholipids under conditions optimized for microsomal ACAT.

Table 2
Differential effect of sterol carrier protein-2 on microsomal oleoyl-CoA utilization

SCP-2	Time (min)	Cholesterol oleate	Triacylglycerol	Phospholipid
None	5	147 ± 13	24.0 ± 1.6	2125 ± 67
2.5 μM	5	191 ± 10*	38.4 ± 3.5**	2500 ± 49**
Increase (%)		30	60	18
None	30	29.4 ± 1.3	2.3 ± 0.2	373 ± 16
2.5 μM	30	39.8 ± 1.8**	12.0 ± 1.0**	504 ± 8**
Increase (%)		35	521	35

Microsomal utilization of [1-^{14}C]oleoyl-CoA was determined exactly as described for assay of microsomal ACAT activity. Reactions containing 20 μl of cholesterol rich liposomes (25 mol%), 15 μg microsomal protein and 15 μM [1-^{14}C]oleoyl-CoA were incubated for 5 or 30 min as indicated, respectively. The lipids were then extracted and resolved as described in Section 2.2. ^{14}C-Labeled cholesterol oleate, triacylglycerol, and phospholipids are in pmol/min/mg microsome protein. An asterisk and double asterisk refer to $P < 0.05$ and $P < 0.025$, respectively ($n = 3$–4), as compared to no SCP-2 added.

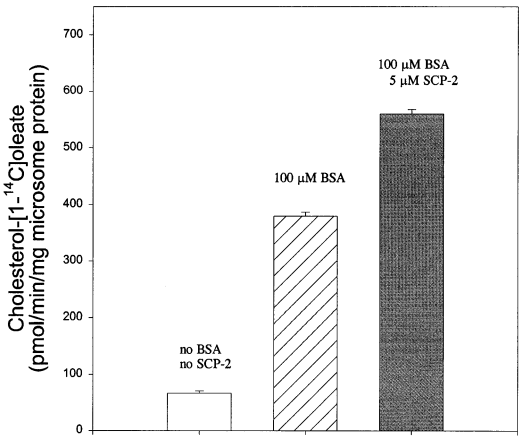

Fig. 5. Effect of long chain fatty acyl-CoA binding proteins on microsomal acyl-CoA cholesterol acyltransferase. Microsomal ACAT was measured as described in Section 2.2 except that cholesterol rich liposomes (35 mole%) were used. Each reaction mixture contained 6 μl of 35 mol% liposomal cholesterol donor, 15 μg microsome protein, and the ACAT reaction was initiated by addition of 100 μM [1-^{14}C] oleoyl-CoA. The open bar indicates microsomal ACAT activity in the absence of albumin (BSA) or sterol carrier protein (SCP-2); the cross-hatched bar was in the presence of 100 μM BSA; the solid bar was in the presence of 100 μM BSA/5μM SCP-2. Values represent the mean ± S.E.M. (n = 5–6).

3.7. *Inhibition of microsomal acyl-CoA cholesterol acyltransferase by high levels of oleoyl-CoA: effect of sterol carrier protein-2 and serum albumin*

Although long chain fatty acyl-CoA levels up to 50 μM did not inhibit microsomal ACAT (Fig. 3B), higher levels of long chain fatty acyl-CoAs inhibited microsomal ACAT. At a concentration of 100 μM oleoyl-CoA, microsomal ACAT activity with cholesterol rich (35 mol%) liposomes was dramatically inhibited more than 3-fold. The specific activity of microsomal ACAT at 50 (Fig. 3B) and 100 (Fig. 5) μM oleoyl-CoA was 207 ± 9 and 67 ± 5 pmol, respectively, cholesteryl ester/min/mg microsomal protein.

The effect of long chain fatty acyl-CoA binding proteins on microsomal ACAT inhibition by high oleoyl-CoA concentration was determined. BSA binds long chain fatty acyl-CoA [36,44,45]. BSA (100 μM) overcame the inhibition of microsomal ACAT by 100 μM oleoyl-CoA (Fig. 5). BSA increased microsomal ACAT 5.5-fold, from 67 (absence of BSA) to 380 (presence of BSA) pmol cholesteryl ester/min/mg microsomal protein (Fig. 5). However, the latter level was only 1.8-fold higher than the maximal obtained under non-inhibitory concentrations of oleoyl-CoA (Fig. 3B) above. Clearly, the inclusion of BSA in microsomal ACAT assays removed the inhibitory effect of high levels of long chain fatty acyl-CoA, confirming earlier findings with BSA [46,47].

SCP-2 also prevented the inhibition of microsomal ACAT by high oleoyl-CoA concentration. It was not possible to test the effect of 100 μM SCP-2 since SCP-2 aggregated under these conditions in the assay. However, addition of 5 μM of SCP-2 to the sample containing 100 μM BSA further reversed the inhibition of microsomal ACAT by 100 μM oleoyl-CoA. Microsomal ACAT was further stimulated by the 5 μM SCP-2 to 560 pmol cholesteryl ester/min/mg microsomal protein, i.e. an additional 180 pmol cholesteryl ester/min/mg microsomal protein (Fig. 5). Assuming that the effects of the two proteins were additive, BSA and SCP-2 stimulated the oleoyl-CoA inhibited microsomal ACAT by 0.055-fold/μmol BSA and 0.54-fold/μmol SCP-2. This suggested that SCP-2 may be nearly 10-fold more effective (mole for mole) than albumin in reversing the inhibition of microsomal ACAT by long chain fatty acyl-CoA. Equally important, BSA and SCP-2 stimulated microsomal ACAT 2–3-fold more than maximal microsomal ACAT activity observed under non-inhibitory levels of 20–50 μM oleoyl-CoA. Consequently, in the presence of cholesterol rich (35 mol%) liposomes and high oleoyl-CoA (100 μM) concentration, SCP-2 stimulated microsomal ACAT activity 8.4-fold (560 vs. 67 pmol cholesteryl ester/min/mg microsomal protein).

In summary, the apparent degree of SCP-2 mediated stimulation of microsomal ACAT in the presence of cholesterol rich liposomes was dependent on the level of long chain fatty acyl-CoA present in the assay.

4. Discussion

Although a large body of in vitro evidence has accumulated on putative functions of lipid binding proteins such as SCP-2 in microsomal cholesterol esterification, these data were most often obtained

under non-physiological conditions (presence of detergents, solvents, etc.) and almost exclusively with the SCP-2 from a single species, the rat [23,27–31]. Furthermore, the relevance of the recent discovery that SCP-2 binds long chain fatty acyl-CoA, rather than just cholesterol, to microsomal cholesterol esterification has not yet been established. The data presented herein contribute to our understanding of these issues.

First, the results showed for the first time that human recombinant SCP-2 stimulated microsomal esterification of liposomal-derived cholesterol. The data are significant in that they were obtained with membrane cholesterol donors and in the absence of cholesterol vehicles (solvents, detergents, BSA) that are not normally present in the cell cytoplasm. In addition, the SCP-2 concentrations at which stimulation was observed (i.e. 2.5–10 μM) were in the same range as those found in liver and a variety of peripheral tissues; i.e. 0.01–0.1% of cytosolic protein (i.e. 1–15 μM) (review in [48]). To our knowledge, all previous work on SCP-2 mediated microsomal esterification of liposomal-derived cholesterol [23,28,49] addressed the rat SCP-2. While the structure of SCP-2 is highly conserved among mammalian species, the human and rat SCP-2 differ in 12% of their amino acid sequence (review in [50,51]). Although little is known regarding the relative functions of SCP-2s from different species, some data suggest that the functions of SCP-2s from different species or sources may not necessarily be identical [50]: hepatoma SCP-2, but not liver SCP-2, exhibits pronounced sphingomyelin transfer activity; avian SCP-2 does not exhibit homology in the N-terminus with the mammalian SCP-2s and also appears to exhibit a preference for sphingomyelin transfer. Similarly, species differences in function have also been noted for other lipid transfer proteins, such as the acyl-CoA binding protein [52] and isoforms of fatty acid binding proteins [53–55]. In contrast, a comparison of the activities of the human and rat SCP-2s showed that these proteins maximally stimulated microsomal esterification from liposomal-derived cholesterol as much as 6.4-fold (present work) vs. 3.5–5-fold [23,28,49], respectively. Thus, the present findings established that the effects of SCP-2 on microsomal esterification of liposomal-derived cholesterol observed with the rat SCP-2 could be extended to those with the human SCP-2.

Second, the data showed for the first time that SCP-2 may inhibit, rather than stimulate microsomal esterification of liposomal-derived cholesterol. In the absence of inhibition by LCFA-CoA, the influence of SCP-2 on microsomal ACAT was determined by both the liposomal cholesterol/phospholipid ratio (i.e. mol% cholesterol) and the total cholesterol available. Cholesterol poor liposomes (1.4 mol%), SCP-2 inhibited (rather than stimulated) microsomal ACAT. An inhibitory effect of SCP-2, regardless of species of origin, has heretofore not been reported. This SCP-2 mediated inhibition of microsomal ACAT was overcome by: (i) increasing the mol% of cholesterol in the liposomes from 1.4 mol% to values greater than 25 mol% (e.g. 29 or 35 mol%); (ii) increasing the total amount of cholesterol rich liposomes (35 mol%). The effects of higher mol% cholesterol or higher total cholesterol may be explained in part by the cholesterol content of the membranes. For example, it is known from the literature (see above) that SCP-2 may either enhance cholesterol exchange and/or mass transfer down a cholesterol gradient. Thus, the SCP-2 mediated increase in microsomal ACAT activity may coincide with the mole% cholesterol in the donor liposomal membrane approaching and exceeding that of the acceptor microsome. This would facilitate SCP-2 mediated mass transfer of cholesterol from cholesterol rich liposomal membranes to microsomal ACAT. This would suggest that, when the exogenous cholesterol donor is cholesterol poor, SCP-2 binds cholesterol and enhances its transfer away from the microsomal membrane toward the liposomal membrane, thereby inhibiting ACAT. In contrast, when the exogenous donor membrane is cholesterol rich then SCP-2 binds the cholesterol therein and elicits its transfer to the microsomal ACAT.

Third, the data presented herein addressed for the first time the role of SCP-2 in modulating microsomal esterification of liposomal derived cholesterol via SCP-2 interaction with long chain fatty acyl-CoA. At low levels of liposomal cholesterol (cholesterol poor liposomes) increasing the oleoyl-CoA concentration reversed the SCP-2 mediated inhibition of microsomal ACAT. In contrast, the inhibitory effects of high levels of oleoyl-CoA were reversed by albumin and

SCP-2. While the mechanism accounting for these effects is not yet completely clear, it may be speculated to be due at least in part to the high affinity of SCP-2 for LCFA-CoA with K_d as low as 2–5 nM ([24,40]; Stolowich et al., submitted). Although LCFA-CoA is much more water soluble than cholesterol (mM vs. nM critical micellar concentration), the majority of LCFA-CoA is still membrane bound (review in [48]). However, as shown herein and by others [46,47] LCFA-CoA are potent detergents and can inhibit ACAT at physiological LCFA-CoA concentrations (review in [48]). Thus, SCP-2 could enhance microsomal ACAT either by solubilizing LCFA-CoA from membranes and transfer to ACAT and/or by removal of inhibitory influence of LCFA-CoA. As shown herein and by others (review in [32]), BSA prevents the inhibition of microsomal ACAT by high levels of LCFA-CoA, apparently by BSA interacting directly with LCFA-CoA [36,44,45,56]. Consequently, BSA is included in many microsomal ACAT assays (review in [32]). However, since BSA is not the normal cytoplasmic LCFA-CoA binding protein, the physiological relevance of this observation is unclear. Instead, increasing data suggest the presence of several intracellular LCFA-CoA binding proteins in most tissues. One of these intracellular proteins, SCP-2, was only recently discovered to be a LCFA-CoA binding protein ([24,40]; Stolowich et al., submitted). The data presented herein show for the first time that SCP-2 was as much as 10-fold more effective (mole for mole) than BSA in reversing inhibition of microsomal ACAT by high levels (100 μM) of LCFA-CoA. These observations of LCFA inhibitory effects on microsomal ACAT in vitro are in the range of LCFA-CoA concentration normally found in tissues such as liver or heart where levels up to 110–152 μM LCFA-CoA have been reported under normal conditions (review in [48]). (LCFA-CoA levels in other tissues appear 2–10-fold lower than in liver or heart.) Furthermore, starvation, diabetes, LCFA, and peroxisome proliferator agents increase liver LCFA-CoA levels from 1.2- to 3.7-fold, while ischemia and hypoxia increase heart LCFA-CoA levels 1.6–3.1-fold (review in [48]).

The physiological significance of SCP-2 mediated regulation of microsomal ACAT is supported by the localization of SCP-2 within the cell. While it is recognized that SCP-2 is highly concentrated in peroxisomes, significant amounts of SCP-2 have also been colocalized to endoplasmic reticulum of tissues and cells. Data from this ([1,57]; O. Starodub, B.P. Atshaves, J.B. Roths, J. Schoer, C.J. Jolly, A.B. Kier, F. Schroeder, Sterol carrier protein-2 expression and intracellular localization in transfected L-cell fibroblasts, submitted) as well as other [58–65] laboratories agree that the organelle in which SCP-2 is most highly concentrated is the peroxisome. However, peroxisomes account for less than 1% of cellular organelles in most tissues and substantial SCP-2 is extra-peroxisomal. Both immunogold electron microscopy [58–65] and double immunolabeling laser scanning confocal microscopy ([57]; Starodub et al., submitted) of tissues and cells show much extra-peroxisomal SCP-2 is present colocalized with the endoplasmic reticulum and with mitochondria. Double immunolabeling and laser scanning confocal microscopy of transfected L-cell fibroblasts overexpressing the 15 kDa pro-SCP-2 gene construct, showed that about 20% of SCP-2 colocalized with rough endoplasmic reticulum (Starodub et al., submitted). This issue has been discussed in several recent reviews [1,50]. In summary, significant SCP-2 is localized in the endoplasmic reticulum, the organelle where cholesterol is esterified by ACAT.

While the above data obtained in vitro support a role for SCP-2 in stimulating microsomal cholesterol esterification by microsomal ACAT, we recognize that there are competing microsomal enzymes that also use long chain fatty acyl-CoAs. Although it would be desirable to examine the effect of SCP-2 on the cholesterol and long chain fatty acyl-CoA utilization by purified ACAT, the ACAT enzyme is an integral membrane protein which cannot be solubilized without the use of detergent [66,67]. Furthermore, the purified ACAT is inactive unless reconstituted into model phospholipid membranes. However, the activity of the reconstituted ACAT varies with the lipid composition of the model membrane and it is not known how such activities might relate to the physiological situation in an intact microsomal membrane. Consequently, the present investigation utilized a microsomal preparation maintaining a more physiological membrane environment for ACAT.

The varied effects of SCP-2 in vitro depend upon

the experimental conditions, suggesting that extrapolation to the in vivo situation might also be complex. Indeed, recent studies with transfected cells and gene ablated animals are consistent with this expected complexity. For example, several studies showed that SCP-2 overexpression increased cholesterol ester formation via ACAT. (i) Transfected L-cells overexpressing the 15 kDa pro-SCP-2 (precursor of SCP-2) displayed 1.4-fold increased conversion of exogenous ^{3}H-cholesterol to ^{3}H-cholesterol ester; 1.6-fold increased esterification of plasma membrane derived ^{3}H-cholesterol; 1.6-fold enhanced microsomal esterification of ^{3}H-oleic acid to plasma membrane derived cholesterol; 2-fold greater equilibrium mass of intracellular cholesteryl esters [68]. (ii) L-cells overexpressing the 58 kDa SCP-x (partially processed intracellularly to SCP-2) had not only the expected increase in 58 kDa SCP-x, but also 2-fold increased 13 kDa SCP-2. These cells displayed a 2.3- and 2.5-fold increase in cholesterol ester formation from exogenous ^{3}H-cholesterol and ^{3}H-oleic acid, respectively [57]. Conversely, SCP-2 gene ablation in mice showed 2-fold decreased liver cholesteryl ester mass [24]. In contrast, overexpression of 15 kDa pro-SCP-2 in transfected hepatoma cells resulted in decreased cholesterol esterification in the absence of changes in the expression of ACAT or cholesterol ester hydrolase [63]. The differences in SCP-2 effects observed in the transfected L-cell fibroblasts vs. transfected hepatoma cells may be due to the free cholesterol available for esterification. It has been shown in macrophages that esterification of cholesterol occurs only after the free cholesterol pool expands 25% above basal levels [69–71]. When the various cell lines were compared based on the effects of SCP-2 on increasing free cholesterol content, they were consistent with the observations made in macrophages. For example, overexpression of 15 kDa pro-SCP-2 (the complete SCP-2 gene product) in L-cells resulted in 28% increase in free cholesterol content of the cell and, concomitantly, a 38% increase in cholesterol ester mass [68,72]. In contrast, overexpression of the 13 kDa SCP-2 (incomplete SCP-2 gene product) did not increase either free cholesterol or cholesterol ester [68,72], most likely due to improper intracellular targeting [73]. This relationship of increased free cholesterol correlating with increased ACAT can also be extended to the transfected hepatoma cells overexpressing 15 kDa pro-SCP-2, wherein neither free cholesterol nor cholesterol ester content were significantly increased [63].

Finally, the data showed for the first time that SCP-2 stimulated differential utilization of LCFA-CoA by microsomal acyltransferases under conditions wherein LCFA-CoA was not inhibitory. SCP-2 differentially targeted LCFA-CoA 15-fold more toward triacylglycerols than toward cholesteryl esters or phospholipids in vitro. These data obtained in vitro suggesting a new potential role for SCP-2 in other microsomal transacylation reactions, are supported by functional data obtained with transfected cells and gene ablated animals: (i) transfected L-cells overexpressing the 15 kDa pro-SCP-2 (precursor of SCP-2) showed a 2-fold increased equilibrium mass of intracellular triglycerides [68]; (ii) SCP-2 gene ablation in mice decreased liver triacylglycerol mass 2-fold [24].

In summary, the in vitro assay data presented herein suggest that SCP-2 is a potential intracellular regulator of microsomal LCFA-CoA transacylation reactions, especially those giving rise to cholesteryl esters and triacylglycerols. These observations are consistent with and are supported by data obtained with intact transfected cells overexpressing SCP-2 and by studies in vivo with SCP-2 gene ablated mice. Furthermore, the studies presented herein suggest that SCP-2's high affinity for LCFA-CoA may also play a part in the physiological regulation of these transacylation reactions, not only by binding the substrate cholesterol and/or LCFA-CoA, but also by removing the inhibitory influence of high levels of LCFA-CoA.

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