Sterol Carrier Protein-2 Expression Modulates Protein and Lipid Composition of Lipid Droplets*

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Despite the critical role lipid droplets play in maintaining energy reserves and lipid stores for the cell, little is known about the regulation of the lipid or protein components within the lipid droplet. Although immunofluorescence of intact cells as well as Western analysis of isolated lipid droplets revealed that sterol carrier protein-2 (SCP-2) was not associated with lipid droplets, SCP-2 expression significantly altered the structure of the lipid droplet. First, the targeting of fatty acid and cholesterol to the lipid droplets was significantly decreased. Second, the content of several proteins important for lipid droplet function was differentially increased (perilipin A), reduced severalfold (adipose differentiation-related protein (ADRP), vimentin), or almost completely eliminated (hormone-sensitive lipase and proteins >98 kDa) in the isolated lipid droplet. Third, the distribution of lipids within the lipid droplets was significantly altered. Double labeling of cells with 12-(N-methyl)-N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-octadecanoic acid (NBD-stearic acid) and antisera to ADRP showed that 70, 24, and 13% of lipid droplets contained ADRP, NBD-stearic acid, or both, respectively. SCP-2 expression decreased the level of ADRP in the lipid droplet but increased the proportion wherein ADRP and NBD-stearic acid colocalized by 3-fold. SCP-2 expression also decreased the lipid droplet fatty acid and cholesterol mass (nmol/mg protein) by 5.2- and 6.6-fold, respectively. Finally, SCP-2 expression selectively altered the pattern of esterified fatty acids in favor of polyunsaturated fatty acids within the lipid droplet. Displacement studies showed differential binding affinity of ADRP for cholesterol and fatty acids. These data suggested that SCP-2 and ADRP play a significant role in regulating fatty acid and cholesterol targeting to lipid droplets as well as in determining their lipid and protein components.

Because of the detergent and regulatory properties of unesterified fatty acid, a variety of organisms including animals, plants, and yeast have evolved mechanisms to regulate closely the intracellular level of unesterified fatty acids (1, 2). One such mechanism is the storage in lipid droplets of fatty acids in esterified form as triacylglycerides and sterol esters. Lipid droplets are especially prevalent in adipose tissue (including mammary gland) and steroidogenic tissues, whereas lower levels are found in cells of liver, kidney, intestine, and muscle (heart and skeletal muscle) (reviewed in Ref. 3). Normally lipid droplets represent a storage site for energy (fatty acids), membrane phospholipid (triacylglycerol), and steroidogenesis (cholesterol, cholesteryl-ester) (reviewed in Refs. 4–7). In contrast, abnormalities in intracellular lipid storage are associated with obesity (reviewed in Ref. 8), cardiovascular disease (reviewed in Refs. 9 and 10), diabetes (reviewed in Ref. 11), neutral lipid storage disease (4), and Niemann Pick C disease (reviewed in Refs. 12–14). Despite the importance of lipid droplets in normal lipid metabolism and disease, relatively little is known about the structure, lipid, and protein composition or factors that regulate these parameters in lipid droplets.

It is generally assumed that the lipid components of lipid droplets are assembled as a core of neutral lipid (triacylglycerol and/or cholesterol ester) surrounded by a surface monolayer composed of polar lipids (cholesterol, phospholipids, and fatty acids) (reviewed in Refs. 3 and 5). Two major types of lipid droplets are recognized in animal cells. Lipid droplets from steroidogenic cells, rich in cholesteryl esters, supply a ready source of cholesterol for steroid hormone synthesis (7, 15, 16). Steroidogenic cells are rich in sterol carrier protein-2 (17). SCP-2 stimulates cholesteryl ester formation in vitro, in intact cells, and animals (reviewed in Ref. 17). SCP-2 may thereby also contribute to the composition of lipid droplets in steroidogenic cells (15) and to cholesterol transfer between lipid droplets and mitochondria in steroidogenic cells (7, 16, 18). In contrast, lipid droplets from adipocytes, rich in triacylglycerides, are either secreted into milk or retained within the cell to provide a source of fatty acids for signaling/gene regulation (1), fatty acids and glycerides for membrane phospholipid synthesis (4), as well as fatty acids for mitochondrial and peroxisomal oxidation to produce energy (reviewed in Refs. 3 and 19). Adipose tissue is poor in sterol carrier protein-2, a factor that correlates with the paucity of cholesterol and cholesteryl esters in adipocyte lipid droplets (17). In contrast, neither the properties nor regulation of lipid droplets in other cell types have been reported.

Increasingly it is recognized that a unique group of lipid

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¶ The abbreviations used are: SCP-2, sterol carrier protein-2; ADRP, adipose differentiation-related protein; NBD-stearic acid, 12-(N-methyl)-N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-octadecanoic acid; HDL, high density lipoprotein; PAGE, polyacrylamide gel electrophoresis; GLC, gas-liquid chromatography; BSA, bovine serum albumin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FAME, fatty acid methyl esters.
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droplet-specific proteins are localized as a protein “capsule” localized at the surface monolayer of the lipid droplet (reviewed in Ref. 5). These proteins include the perilipins (5), adipose differentiation-related protein (ADRP) (5, 20–22), P200 capsule protein (23, 24), and vimentin (23). Other proteins, such as sterol carrier protein-2 (15) and hormone-sensitive lipase (25), have been shown to translocate to lipid droplets of steroidogenic cells or steroidogenic cells from animals upon stimulation with lipolytic hormones. However, it is only recently that the function of proteins associated with the lipid droplet surface capsule have begun to be resolved. Upon lipolytic stimulation, the intracellular hormone-sensitive lipase is phosphorylated and translocated to the lipid droplet surface where it catalyzes the rate-limiting step in the release of fatty acids from interior core triacylglycerides and cholesteryl esters (25). However, accessibility of the triacylglycerols and cholesteryl esters for lipolysis by hormone-sensitive lipase/cholesteryl esterase is closely correlated with decreased activity of perilipin (26, 27) and of P200 capsule protein (24) with the lipid droplet surface. In contrast, ADRP expression appears to be linked to the conversion of small to larger lipid droplets (3, 5). ADRP also binds fatty acids (28) and enhances their transport (20). SCP-2 has high affinity for not only fatty acids (29–31) and fatty acyl-CoA (32) but also for other lipid droplet-associated lipids such as cholesterol (32–34) and phospholipids (35, 36). SCP-2 accelerates the intracellular transfer of fatty acids (37, 38) and differentially targets lipid droplet cholesterol away from HDL-mediated efflux through the plasma membrane (21) while enhancing lipid droplet cholesterol transfer toward the mitochondria for steroidogenesis (7, 16). Although these data suggest an intricate balance of protein association with the lipid droplet, relatively little is known regarding either the lipid composition of lipid droplets (7) or if their lipid composition may be determined by the expression of the lipid droplet-associated proteins.

The nature of lipid droplets from cell types other than adipocytes and steroidogenic cells is essentially unknown. The purpose of the present investigation was 2-fold as follows: first, to examine the protein and lipid structure of such lipid droplets; second, to determine the effect of SCP-2 expression on modulating the lipid and protein components of the lipid droplets. t. cells provided a useful model for these purposes based on the following data. (i) t. cells have substantial lipid droplets (21, 22, 23). (ii) SCP-2 expression, transport of cholesterol from lipid droplets (for efflux to cell surface bound HDL), and cellular levels of a lipid droplet-specific protein (adipose differentiation related protein, ADRP) are interrelated in t. cells (21, 22). (iii) t. cells express low levels of SCP-2 (0.008 ± 0.001% of total protein) (39) comparable to most peripheral tissues (i.e. <0.01% of soluble protein) (reviewed in Refs. 17 and 34). (iv) Immunofluorescence confocal microscopy revealed that the intracellular localization of endogenous SCP-2 in t. cells (40, 41) reflected that observed in other cells and tissues (reviewed in Refs. 17 and 34). (v) t. cells posttranslationally process SCP-2 to SCP-2 gene products (38, 39, 42, 43) in a similar manner as in animal tissues (reviewed in Ref. 17). Transfection of t. cells with a plasmid construct containing the cDNA encoding 15-kDa pro-SCP-2 (the normal SCP-2 gene translation product is the 15-kDa pro-SCP-2 precursor protein) resulted in overexpression and complete posttranslational processing to the 13-kDa SCP-2. This was in contrast to transfected Chinese hamster ovary cells (44) and hepatoma cells (45) where incomplete posttranslational processing of 15-kDa pro-SCP-2 to the mature 13-kDa SCP-2 was observed. (vi) The intracellular distribution of SCP-2 in transfected t. cells exhibited a pattern similar to that of SCP-2 in untransfected or mock transfected t. cells (40, 41, 46, 47) as well as in animal tissues (reviewed in Refs. 17 and 34). In summary, these data suggested that t. cells provide a useful model to study the protein and lipid components of lipid droplets as well as the effect of increased SCP-2 expression thereon in intact cells.

In the present work, cellular subfractionation together with confocal and multiphoton laser scanning microscopy as well as environmental scanning electron microscopy showed the following. 1) The lipid droplets from t. cells exhibited properties intermediate to those of lipid droplets from adipose and steroidogenic cells. 2) SCP-2 was not tightly associated with lipid droplets. 3) The expression of SCP-2 significantly altered the lipid and fatty acyl composition of purified lipid droplets, the association of several proteins (important to lipid droplet metabolism) with purified lipid droplets in vitro as well as in intact cells, the targeting of fatty acids and cholesterol to lipid droplets, and/or the association of these lipids with ADRP in intact cells. The results presented herein provide new insights into the structure and regulation of lipid storage droplet lipids on a molecular and functional level.

MATERIALS AND METHODS

Materials Sources—Lab-Tek chamber coverglass slides and organic solvents including petroleum ether, diethyl ether, glacial acetic acid, and methanol were from Fisher; silica gel G plates were purchased from Analtech (Newark, DE); lipid standards were purchased from Nu-Chek Prep (Elysian, MN) and Avanti Polar Lipids, Inc. (Alabaster, AL). Complete Mini Protease Inhibitor Mixture was from Roche Molecular Biochemicals. Rat and rabbit polyclonal anti-human SCP-2 were obtained and purified as described earlier (40). Rabbit polyclonal antisera to ADRP was prepared as described (21). Rabbit polyclonal antibodies against perilipin A and B and hormone-sensitive lipase were a generous gift from Drs. A. S. Greenberg, Tufts University (Boston, MA), and Dr. F. Kraemer, Stanford University Medical Center (Stanford, CA), respectively. Monoclonal anti-vimentin was from Accurate Chemical and Scientific Corp. (Westbury, NY). The F(ab′)2 fragment of Alexa Fluor 594 goat anti-rabbit IgG (H + L chain-specific), Alexa Fluor 488 goat anti-rat IgG (H + L chain-specific), and NBD-steareate were purchased from Molecular Probes (Eugene, OR). Data obtained on the environmental scanning electron microscope was obtained with the technical support of Dr. Helga Sittertz-Bhatkar (Electron Microscopy Center, Texas A & M University, TX). Visualization of protein bands on the SDS-PAGE gel was accomplished using the Silver Stain Plus kit from Bio-Rad. All reagents and solvents used were of the highest grade available and were cell culture tested as necessary.

L Cell Culture—Cells were grown to confluency in Higuchi medium (48) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) as described previously (39). Murine t. cell fibroblasts (L arpt: tk-52) were obtained and stably transfected with the cDNA encoding the 15-kDa pro-SCP-2 as described (42). As expected, Western blotting showed that the 15-kDa pro-SCP-2 was completely and posttranslationally processed to the mature 13.2-kDa SCP-2 protein. SCP-2 composed 0.036 ± 0.002% of the total proteins in stably overexpressing cells (34). In contrast, SCP-2 expression was very low (0.008 ± 0.001% of total protein) in control cells (i.e. untransfected and mock-transfected) (39). Thus, the levels of SCP-2 expression in both SCP-2 overexpressing and in control cells were in the range of those reported for murine tissues (0.01–0.08% of cytosolic proteins) (49). For immunocytochemistry experiments, cells were seeded at a density of 50,000 cells/60 mm trays (Nunc, Naperville, IL).

Isolation of Lipid Droplets from Mouse L Cell Fibroblasts—Lipid droplets were isolated from control and SCP-2-expressing cells as described by Chanderban et al. (7). To avoid potential proteolytic degradation during homogenization and isolation of lipid droplets, Complete Mini Protease Inhibitor Mixture (Roche Molecular Biochemicals) was included in the isolation buffer as recommended by the manufacturer. Briefly, cells scraped from 8–10 confluent trays were homogenized in 50 mM NaH2PO4 buffer, pH 7.4, containing 154 mM NaCl, 5 mM MgCl2, and protease inhibitor mixture, followed by centrifugation at 800 g for 10 min. In order to sediment mitochondria the resulting supernatant was centrifuged at 5,000 × g for 20 min, followed by a second centrifugation step at 35,000 rpm in a SW 41.1 rotor for 2 h at 4 °C.
lipid droplet fraction, forming a distinct white band on the surface of the preparation, was removed for lipid analysis. Details of the purity of the lipid droplets are provided under "Results."

**Lipid Mass Determination**—Lipid droplets, isolated from control and SCP-2-expressing cells, were extracted with n-hexane/2-propanol 3:2 (v/v) in a 1:1 ratio for 2 h at 60 °C. The lipid droplets were resolved by thin-layer chromatography plates developed in the following solvent system: petroleum ether/diethyl ether/methanol/acetic acid 90:7:2:0.5:0.5 (v/v). Each sample was divided into two portions for mass and fatty acid composition determination. Total cholesterol, free fatty acid, triglyceride, and cholesteryl ester content were determined by the method of Marder et al. (51). Total phospholipid contents were determined by hydrolysing the phospholipid fraction in deionized water and perchloric acid for 1 h at 180 °C followed by addition of ammonium molybdate and ascorbic acid (52). The sample was further heated for 5 min in a boiling water bath and cooled, and the absorbance read at 797 and 660 nm to quantify total phosphorous. Proteins were determined by the method of Bradford (53) from the dried protein extract residue digested overnight in 0.2 M KOH. Lipids were stored under an atmosphere of N₂ to limit oxidation, and all glassware was washed with sulfuric acid-chromate before use.

**Transsterification**—Base-catalyzed transsterification was performed on one-half of each lipid fraction (i.e. cholesteryl esters, triglycerides, and phospholipids), isolated from the above lipid extraction procedure, to convert the lipid acyl chains to fatty acid methyl esters (FAME) (54). FAME were extracted into n-hexane and were separated by gas chromatography (GLC) on a GLC-14A (Shimadzu, Kyoto, Japan) equipped with a SP-2332 capillary column (0.32 mm inner diameter × 30 m length, Supelco, Bellefonte, PA). The injector and detector temperatures were set at 220 °C with the column temperature maintained at 185 °C. A Dionex U120 analytical-to-digital interface was used to collect the peak area data that were converted to peak area using Dionex PeakNet software.

**SDS-PAGE and Western Blot Analysis**—Cell homogenates and lipid droplets from control and SCP-2-expressing cells were subjected to SDS-PAGE using 12% Tricine gels (20 g per lane) analyzed by Western blot analysis to determine the content of specific proteins such as SCP-2, ADRP, perilipin, hormone-sensitive lipase, and vimentin. The cellular level of ADRP was shown previously to be decreased 70% in the SCP-2, ADRP, perilipin, hormone-sensitive lipase, and vimentin. Immunoblot analysis to determine the content of specific proteins such as SCP-2, ADRP, perilipin, hormone-sensitive lipase, and vimentin. Alkaline-phosphatase conjugates of goat anti-rabbit or mouse IgG and Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma) were used to visualize the bands of interest. After washing with Hanks' solution, the cells were blocked with 2% BSA in Hanks' at room temperature using a SCP-2 (rat polyclonal anti-human SCP-2 also purified by subtractive Tricine gel method as described earlier (39)) and anti-human SCP-2, ADRP, perilipin, hormone-sensitive lipase, and vimentin. A SABRE argon ion laser operating at 12 watts provided the pump source for a femtosecond pulsed MIRA-906-F Ti:Sapphire laser (Coherent, Inc., Sunnyvale, CA) that was used to provide multiphoton excitation at 875 nm. Fluorescence of Nile Red was detected through an external detector system (Bio-Rad) using a HQG75/150 bandpass filter (Chroma Technology Corp., Brattleboro, VT). A Zeiss Axiovert 135 inverted microscope equipped with a Peltier cooling stage and micro-manipulator/injector at 15 kV and pressures of 5 torr. Multiphoton laser scanning microscopy was performed on isolated lipid droplets incubated with Nile Red (0.05%) on an MRC-1024MP multiphoton laser scanning system (Bio-Rad) in combination with a Axiovert 135 inverted microscope fitted with a 63 × oil-immersion objective with a numerical aperture of 1.4. A SABRE argon ion laser operating at 12 watts provided the pump source for a femtosecond pulsed MIRA-906-F Ti:Sapphire laser (Coherent, Inc., Sunnyvale, CA) that was used to provide multiphoton excitation at 875 nm using a PC1 Photon Counting Fluorometer (ISS Instruments, Champaign, IL). The data were fitted using a simple, single binding site model (29).

**Statistics**—All values were expressed as the mean ± S.E. with n and p indicated under "Results." Statistical analyses were performed using Student's t test (GraphPad Prism, San Diego, CA). Values with p < 0.05 were considered statistically significant.

**RESULTS**

**Lipid Droplet Purification**—Although the lipid composition of very large lipid droplets (up to 15 μm diameter) from steroi
dogenic or adipose tissue has been examined (5, 7), almost nothing is known regarding the lipid content/composition of the smaller lipid droplets (near 1 μm diameter) more typical of most other mammalian cells. i cell fibroblasts contain small lipid droplets in this size range (21, 22). Furthermore, confocal microscopy studies indicate that intact cells the lipid (chole
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tion, these results were extended to examine the structure of isolated lipid droplets from i cell fibroblasts.

The relative purification of lipid droplets was assessed by comparison of lipid composition and protein content markers of isolated lipid droplets versus cell homogenate. Whereas lipid droplets do not contain the specific lipid species unique to lipid droplets, as compared with cell homogenate, lipid droplets are relatively deficient in surface lipids (cholesterol and phospho
lipids) and rich in core lipids (triacylglycerol and cholesterol) (7). As shown in Table I, lipid droplets had 29.4-, 647-, 30.1-
and 10.8-fold lower cholesterol, phospholipid, surface lipid, and total lipid mass (nmol/mg protein), respectively, than the cell homogenate. The lipid droplets also exhibited 20.9-, 6.8-, and 17.9-fold lower molar ratios of phospholipid/cholesterol, choles
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branes would have led to increased levels of cholesterol and phospholipid in the isolated lipid droplet fractions. In sum-

| Component | Control | SCP-2 | p-value |
|-----------|---------|-------|---------|
| Cholesterol | 30.1 nmol/mg | 6.8 nmol/mg | <0.05 |
| Phospholipids | 10.8 nmol/mg | 2.9 nmol/mg | <0.05 |
| Surface Lipids | 20.9 nmol/mg | 0.3 nmol/mg | <0.05 |

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mary, these data showed that lipid droplets were highly purified as compared with cell homogenate.

**Lipid Droplet Lipid Mass and Composition in L Cell Fibroblasts**—Two classes of lipids were resolved from purified L cell lipid droplets as follows: hydrophobic core lipids and more polar surface lipids. The hydrophobic core lipids, triacylglycerols and cholesteryl esters, represented 68% of the total lipids in L cell lipid droplets (Fig. 1). L cells exhibited higher triacylglyceride (10.6 ± 2.1 nmol/mg protein) than cholesteryl ester (5.30 ± 0.47 nmol/mg protein) content, representing 45.2 ± 8.8 and 22.2 ± 2.0 mol % of total lipids, respectively. The surface lipids (fatty acids, cholesterol, and phospholipids) represented 32% of total lipids in the lipid droplets (Fig. 1). Fatty acids were present at 5.2 ± 1.7 nmol/mg lipid droplet protein and represented 21.6 ± 6.9 mol % of total lipids in the lipid droplet. In contrast, the fatty acid content of cell homogenate, 13.9 nmol/mg protein, represented only 5.4 ± 1.9 mol % of the lipids in the cell homogenate. Cholesterol and phospholipids were present at 2.2 ± 0.3 and 0.23 ± 0.03 nmol/mg lipid droplet protein, respectively, and represented 9.2 ± 1.1 and 0.1 ± 0.02 mol % of total lipids in the lipid droplet, respectively.

In summary, the smaller lipid droplets isolated from L cells exhibited a unique lipid composition. The proportion of triacylglycerol and cholesteryl ester was intermediate between that of the larger triacylglyceride-rich lipid droplets from adipose tissue and the cholesteryl ester-rich lipid droplets from steroidogenic tissue. Finally, the data showed for the first time that the lipid droplet was 4-fold enriched in unesterified fatty acids as compared with the cell homogenate.

**Effect of SCP-2 Expression on the Lipid Composition of Lipid Droplets Isolated from L Cell Fibroblasts**—As indicated in the Introduction, relatively little is known regarding regulation of the lipid composition of intracellular lipid droplets. SCP-2 binds many of the lipids detected in lipid droplets (fatty acids, cholesterol, and phospholipids) (reviewed in Ref. 34), influences the uptake, intracellular transport, and/or metabolism of these lipids (reviewed in Refs. 34 and 37), and is significantly localized in the cytoplasm (40, 41, 54). Therefore, it was important to establish whether increased intracellular levels of SCP-2 could influence the composition of core and surface lipids in lipid droplets isolated from L cell fibroblasts.

As with the control cells, the predominant hydrophobic lipid core lipids in lipid droplets isolated from SCP-2-expressing L cells were triacylglyceride and cholesteryl esters, 13.65 ± 1.53 and 2.20 ± 0.75 nmol/mg protein, respectively (Fig. 1). Lipid droplets isolated from SCP-2-expressing cells more closely resembled those of adipocyte-type lipid droplets with an increased triacylglyceride content and a 2.4-fold decrease in cholesteryl ester, p < 0.05 n = 3–5 (Fig. 1). The trend toward a more adipocyte lipid droplet was also reflected in nearly 3-fold increased relative ratio of triacylglycerides/cholesterol esters in the core lipids.

SCP-2 expression equally dramatically altered the surface lipids of the lipid droplets. SCP-2 expression resulted in 11.8-fold reduction of surface lipid mass from 7.6 ± 1.7 to 0.65 ± 0.04 nmol/mg lipid droplet protein (Fig. 1). This reduction in surface lipid mass was selective. Unesterified fatty acids were decreased 5.2-fold, below the level of detection (p < 0.01, n = 3–5). Since SCP-2 expression did not significantly decrease the unesterified fatty acid content of L cell homogenate (15.3 ± 8.6 versus 14.0 ± 4.9 nmol/mg protein, n = 4–5), this suggested that the majority of unesterified fatty acids were redistributed away from the lipid droplet in SCP-2-expressing cells. SCP-2 expression also decreased the lipid droplet, but not cell homogenate, cholesterol content (nmol/mg lipid droplet protein) by 6.6-fold (p < 0.01, n = 3–5), which resulted in a redistribution of the majority of cholesterol away from the lipid droplet (Fig. 1). Although SCP-2 expression did not significantly alter the lipid droplet phospholipid mass, the cell homogenate phospholipid mass was decreased nearly 2-fold from 148.8 ± 16.5 to 73.6 ± 12.4 (nmol/mg protein).

In summary, SCP-2 expression differentially altered the lipid composition of isolated lipid droplets as compared with the cell homogenate. The relative loss of fatty acids and cholesterol from lipid droplets of SCP-2-expressing cells suggested that, due to its high affinity for fatty acids (29–31) and cholesterol (31, 34, 55), SCP-2 may partition these lipids away from the lipid droplets. In contrast, the lower affinity of SCP-2 for phospholipids (36, 56) may account for the lack of change in lipid droplet phospholipid mass.

**Fatty Acid Composition of Surface and Core Lipids**—Since SCP-2 binds fatty acyl-CoAs with high affinity (32, 57) as well as triacylglycerols (34), the effect of SCP-2 expression on fatty acid composition was examined (Table 1). Lipid droplets isolated from SCP-2-expressing L cells were extracted and resolved into separate lipid classes to determine lipid mass and composition. Values represent the means ± S.E., n = 3–5 separate isolations. Surface lipids (SL) include cholesterol (C), phospholipids (PL), and free fatty acids (FFA). Core lipids (CL) include triacylglycerol (TG) and cholesteryl esters (CE). * indicates significance as compared with the control, p < 0.05.

| Lipid marker            | Cell homogenate (nmol/mg protein) | Lipid droplet (nmol/mg protein) | -Fold decrease |
|-------------------------|-----------------------------------|---------------------------------|----------------|
| Cholesterol             | 64.6 ± 9.8                         | 2.2 ± 0.3                       | 29.4           |
| Phospholipid            | 148.8 ± 16.5                       | 0.68 ± 0.1                      | 218.8          |
| Surface lipids          | 227.4 ± 19.8                       | 8.0 ± 1.7                       | 28.4           |
| Total lipids            | 253.7 ± 20.3                       | 23.9 ± 2.7                      | 10.6           |
| Phospholipid/cholesterol| 2.3 ± 0.4                          | 0.31 ± 0.05                     | 7.4            |
| Cholesterol/cholesterol ester | 2.8 ± 0.2                  | 0.41 ± 0.06                     | 6.8            |
| SL/CL                   | 8.6 ± 0.3                          | 0.5 ± 0.12                      | 17.2           |

**FIG. 1.** Effect of SCP-2 expression on lipid droplet lipid mass. Lipid droplets isolated from transfected L cells were extracted and resolved into separate lipid classes to determine lipid mass and composition. Values represent the means ± S.E., n = 3–5 separate isolations. Surface lipids (SL) include cholesterol (C), phospholipids (PL), and free fatty acids (FFA). Core lipids (CL) include triacylglycerol (TG) and cholesteryl esters (CE). * indicates significance as compared with the control, p < 0.05.
as differentially stimulates fatty acyl-CoA utilization for microsomal phosphatidic acid and phospholipid synthesis in vitro and in intact cells (41, 52), the possibility that SCP-2 expression may alter the fatty acids esterified to lipids from lipid droplets was examined.

Four major fatty acids (16:0, 18:0, 18:1(n-9), and 22:4(n-6)) were associated with the phospholipid component of lipid droplets (Fig. 2). SCP-2 expression significantly decreased only the 16:0 content in phospholipids from 35.3 nmol/nmol phospholipid to below the level of detection ($p < 0.01, n = 3–5$) (Fig. 2). This was consistent with the greater ability of SCP-2 to stimulate in vitro microsomal incorporation of unsaturated fatty acyl-CoAs into phosphatidic acid as compared with 16:0-CoA (41).

Six fatty acids (16:0, 18:0, 18:1(n-9), 20:1(n-7), 22:4(n-6), and 22:6(n-3)) represented the major fatty acid species esterified to cholesterol (Fig. 3). Four of the six were unsaturated fatty acids with the 18:1(n-9) being in largest concentration in control cells. SCP-2 expression significantly increased the levels of 16:0, 18:1, and 20:1 in the cholesteryl ester fraction of the lipid droplets. Concomitantly, the mass of 22:6(n-3) was increased 10.5-fold ($p < 0.05, n = 3–5$) in the SCP-2-expressing cells (Fig. 3). Overall, the saturated fatty acids were significantly decreased (2-fold, $p < 0.05, n = 3–5$) in the lipid droplet cholesteryl esters from SCP-2-expressing cells, and polyunsaturated fatty acids were increased 5-fold ($p < 0.5, n = 3–5$), and the ratio of unsaturated to saturated fatty acids was increased 2-fold ($p < 0.025, n = 3–5$). These results were consistent with the ability of SCP-2 to stimulate cholesterol esterification in vitro (43, 58) as well as cholesterol esterification and cholesterol cycling in transfected cells (43, 45).

Seven fatty acids (16:0, 18:0, 18:1(n-9), 18:3(n-6), 20:3(n-6), 22:4(n-6), and 22:6(n-3)) were detected at significant levels in the triglyceride fraction of lipid droplets (Fig. 4). The 18:0, 16:0, and 22:6(n-3) fatty acids represented the largest components. Except for the absence of 18:3(n-6) in the triglycerides from lipid droplets of SCP-2-expressing cells, no significant difference in the rest of the fatty acids isolated from the triglyceride fraction of lipid droplets was observed between the control and SCP-2-expressing cells.

In summary, a comparison of the fatty acids detected in the triglycerides, cholesteryl esters, and phospholipids showed that polyunsaturated fatty acids were 12- and 4.5-fold higher in concentration in the lipid droplet phospholipid fraction ($p > 0.05, n = 3–5$) than in the cholesteryl esters and triglycerides fractions, respectively. SCP-2 expression differentially targeted unsaturated/polyunsaturated fatty acids toward phospholipids and cholesterol esters but had little effect on the fatty acids esterified to triglycerides.

**Cholesteryl Ester, Triglyceride, and Phospholipid Fatty Acid Mass of Fetal Bovine Serum**—In order to determine if the fatty acids targeted to the different lipid fractions of the lipid droplet simply reflected those of the corresponding lipids in the fetal bovine serum added to the cell culture medium, the fatty acid masses from the cholesteryl ester, triglyceride, and phospholipid fractions isolated from fetal bovine serum were determined. Whereas the same fatty acids esterified in the lipid fractions of lipid droplets were also qualitatively present in the fetal bovine serum, important quantitative differences were evident in all three fractions.

The major fatty acids present in serum phospholipids were 18:0 > 18:1 > 16:0 > 22:0 > 24:1 > 22:6. In contrast, the major fatty acids in phospholipids from lipid droplets were 18:1 > 18:0 > 16:0 > 22:4 (Fig. 2). The ratio of serum and lipid droplet unsaturated/saturated fatty acids in the phospholipid fraction, 0.91 ± 0.04 and 0.96 ± 0.67 (Fig. 2), respectively, did not differ. However, the ratio of serum and lipid droplet phospholipid

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**Fig. 2.** Effect of SCP-2 expression on L cell lipid droplet phospholipid fatty acid mass. Four fatty acids from the phospholipid class of lipid droplets isolated from transfected L cells were resolved as FAME by GLC as described under “Materials and Methods.” Values represent the means ± S.E., $n = 3–5$ separate isolations. * indicates significance as compared with the control, $p < 0.01$.

**Fig. 3.** Effect of SCP-2 expression on L cell lipid droplet cholesteryl ester fatty acid mass. Six fatty acids from the cholesteryl ester class of lipid droplets isolated from transfected L cells were resolved as FAME by GLC as described under “Materials and Methods.” Values represent the means ± S.E., $n = 3–5$ separate isolations. * indicates significance as compared with the control, $p < 0.01$.

**Fig. 4.** Effect of SCP-2 expression on L cell lipid droplet triglycerol fatty acid mass. Seven fatty acids from the triglycerol class of lipid droplets isolated from transfected L cells were resolved as FAME by GLC. Values represent the means ± S.E., $n = 3–5$ separate isolations. * indicates significance as compared with the control, $p < 0.01$. 

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polysaturated/monounsaturated fatty acids, 0.37 ± 0.02 and 0.15 ± 0.08 (Fig. 2), respectively, differed by 2.5-fold.

The major fatty acids present in serum cholesteryl esters were 18:1 > 16:0 > 20:4 > 16:0 = 18:2 > 16:1 > 18:0. In contrast, the major fatty acids in cholesteryl esters from lipid droplets were 18:1 > 18:0 > 20:1 = 22:4 > 22:6 (Fig. 3). The ratio of serum and lipid droplet unsaturated/saturated fatty acids in the cholesteryl ester fraction, 4.36 ± 0.32 and 3.50 ± 1.19 (Fig. 3), respectively, did not differ significantly. The ratio of serum and lipid droplet in the cholesteryl ester fraction polyunsaturated/monounsaturated fatty acids, 0.59 ± 0.05 and 0.22 ± 0.11 (Fig. 3), respectively, differed 2.7-fold.

The major fatty acids present in serum triacylglycerols were 18:1 > 20:4 > 16:0 > 18:2 > 20:2 > 18:0. In contrast, the major fatty acids in triacylglycerols esters from lipid droplets were 18:0 > 16:0 > 22:6 > 18:1 > 22:4 > 20:3 (Fig. 4). The ratio of serum and lipid droplet unsaturated/saturated fatty acids in the triacylglycerol fraction, 3.48 ± 0.40 and 0.61 ± 0.21 (Fig. 4), respectively, differed 5.7-fold. The ratio of serum and lipid droplet triacylglycerol polyunsaturated/monounsaturated fatty acids, 1.54 ± 0.20 and 4.04 ± 1.61 (Fig. 4), respectively, differed severalfold.

In summary, the pattern of fatty acid esterification to phospholipids, cholesteryl esters, and triacylglycerols of intracellular lipid droplets differed substantially from those of the same lipid components in the serum lipids.

Effect of SCP-2 Expression on Cholesterol to Phospholipid Ratio—Although cells closely regulate the phospholipid/cholesterol mass ratio (nmol/nmol) of their surface membranes (reviewed in Ref. 59), it is not known if this is also true for the surface monolayer of lipid droplets. Because of the paucity of phospholipids in the polar surface of the lipid droplet, the ratio of cholesterol/phospholipid was very low, 0.11 ± 0.018 (Fig. 5), and much lower than that typical of cell surface and intracellular membranes (59). Due to the 6.6-fold decrease in cholesterol mass, the phospholipid/cholesterol ratio was increased 8.7-fold (p < 0.01, n = 3–5) to 0.96 ± 0.13 in SCP-2-expressing cells.

In summary, SCP-2 expression dramatically increased the phospholipid/cholesterol ratio in the surface of lipid droplets. In bilayer membranes such alterations in phospholipid/cholesterol ratio are typically accompanied by significant fluidization and altered function of membrane proteins (reviewed in Refs. 60–63).

**Effect of SCP-2 Expression on the Relative Proportion of Esterified Cholesterol in the Lipid Droplet**—It is important to note that SCP-2 expression induced a loss of cholesterol not only from the surface lipid but also from the interior core wherein the mass of cholesteryl esters was decreased 2.4-fold (Fig. 1). The relatively greater loss of cholesterol than cholesteryl ester in lipid droplets of SCP-2-expressing cells (Fig. 1) decreased the ratio of cholesterol/cholesterol esters 2.7-fold from 0.41 ± 0.06 to 0.15 ± 0.05 nmol/nmol (Fig. 5). Thus, SCP-2 expression dramatically mobilized/shifted cholesterol, and less so cholesteryl esters, away from the lipid droplets.

**Effect of SCP-2 Expression on Surface to Core Lipid Ratio**—Since the ratio of surface area to volume determines particle size, the ratio of surface to core lipid (surface lipids/core lipids) ratio in lipid droplets may be a predictor of the average size of lipid droplets. Surface lipids include cholesterol, phospholipids, and free fatty acids, whereas the core lipids are the triglycerides and cholesteryl esters. Although no significant difference was observed in the amount of core lipids, the 11.6-fold decrease (p < 0.01, n = 3–5) in surface lipids in lipid droplets isolated from SCP-2-expressing cells resulted in an 11.7-fold decrease (p < 0.01, n = 3–5) in the surface lipids/core lipids ratio (Fig. 5). If the lipids components were the exclusive determinants of the surface/volume relationship, these data suggested that the lipid droplets from SCP-2-expressing cells were smaller or that the amount and/or type of proteins associated with these lipid droplets was different.

**Effect of SCP-2 Expression on Lipid Droplet Morphology**—A random sampling of lipid droplets derived from control and SCP-2-expressing cells was measured from confocal images obtained as described under “Materials and Methods” to determine lipid droplet size. SCP-2 expression did not significantly alter the mean diameter of droplets (1.47 ± 0.037 μm, n = 70, versus 1.57 ± 0.037 μm, n = 100) that ranged in size from 0.5 to 3.0 μm. Since the decreased ratio of surface/interior core lipid in the lipid droplets noted in SCP-2-expressing cells did not result in increased lipid droplet size, these data suggested that SCP-2 expression also altered the protein components of the lipid droplets.

In order to characterize further the lipid droplets, environmental scanning electron microscopy (Fig. 6A) and multiphoton laser scanning microscopy (Fig. 6B) were performed on lipid droplets isolated from control and SCP-2-expressing cells. Environmental scanning electron microscopy allowed a high resolution, three-dimensional view of isolated lipid droplets in solution. Whereas some dehydration of sample occurred resulting in flattened lipid droplets with concave centers, the size distribution of the purified lipid droplets ascertained by environmental scanning electron microscopy was within that observed by confocal microscopy within intact cells (0.5–3.0 μm). Multiphoton laser scanning microscopy was performed on isolated lipid droplets labeled with Nile Red as described under “Materials and Methods.” The solution was allowed to partially dry on a coverslip before viewing. This resulted in a concentrated collection of lipid droplets with concave centers similar to those seen by environmental scanning electron microscopy. Although both techniques allowed good resolution of the droplets and showed good agreement in size and overall shape, the results from the two microscopy techniques revealed that the lipid droplets isolated from the control (Fig. 6) and SCP-2-overexpressing cells (data not shown) were not morphologically different.

**Effect of SCP-2 Expression on Lipid Droplet Protein Content and Profile**—As indicated in the Introduction, relatively little is known regarding the function of the protein capsule of the lipid droplet surface. To avoid potential proteolytic degradation
during isolation of lipid droplets, a protease inhibitor mixture (Complete Mini protease inhibitor mixture) was included in the homogenization and lipid droplet isolation buffer as described under “Materials and Methods.” SCP-2 expression increased the mass ratio of protein/lipid 1.4-fold in the lipid droplets. Furthermore, the profile/composition of the proteins associated with the lipid droplet surface was significantly altered in the SCP-2-expressing cells (Fig. 7). When 20 μg of proteins from lipid droplets isolated from control cells were loaded on the SDS-PAGE gel and stained using Silver Stain Plus (Bio-Rad), less than 10 prominent bands ranging from 27 to 100 kDa molecular mass were observed (Fig. 7, lane 2), including an unknown protein of >94 kDa as well as bands in the molecular weight range of hormone-sensitive lipase (84 kDa), perilipin A (57 kDa), vimentin (58 kDa), ADRP (53 kDa), and perilipin B (46 kDa). Several minor additional bands are as yet unidentified. When equal amounts (20 μg) of proteins from lipid droplets isolated from SCP-2-overexpressing cells were concurrently loaded on the SDS-PAGE gel and stained using Silver Stain Plus (Bio-Rad) (Fig. 7, lane 3), the relative proportion of the above proteins was clearly different. There was some loss of the higher molecular weight protein (i.e. >94 kDa) as well as significant loss of protein in the molecular weight range of hormone-sensitive lipase. Concomitantly, there was a significant increase in proteins located just below the 67-kDa protein marker (i.e. proteins with molecular weights in the range of perilipin A, vimentin, and ADRP).

In summary, the reduced surface/core lipid ratio in lipid droplets of SCP-2-expressing cells was at least in part compensated by more protein associating with the lipid droplet surface. However, this increase in proteins was selective. The lipid droplets from SCP-2-expressing cells exhibited a relative paucity of high molecular weight protein (>94 kDa), with some increases in middle molecular weight proteins (53–58 kDa). It should be noted that lipid droplet proteins are tightly associated with the lipid droplets and are not dislodged by alkaline carbonate or the mild techniques used herein to isolate lipid droplets (5).

**Western Blotting to Determine the Effect of SCP-2 Expression on the Levels of Specific Lipid Droplet-associated Proteins**—Although the silver-stained SDS-PAGE gels in the preceding section were informative, they did not identify specific proteins associated with the lipid droplets. Therefore, the effect of SCP-2 expression on the level of several functionally important proteins associated with lipid droplets was quantitated by Western blotting. The level of ADRP, a 53-kDa lipid droplet-specific protein (5, 64), was reduced 1.8-fold from 952 ± 34 to 538 ± 23 ng/mg protein (n = 4) in SCP-2-expressing cells as compared with controls. Since ADRP expression in intact cells was similarly reduced 1.7-fold in SCP-2-expressing cells, this difference in isolated lipid droplets was not due to differential loss of ADRP upon lipid droplet isolation. Perilipins A and B, 57- and 46-kDa lipid droplet-associated proteins (27), were differentially affected by SCP-2 expression. Western analysis showed that perilipin A (Fig. 8B, band 1) was increased by 3.1-fold in lipid droplets isolated from SCP-2 expression cells (p < 0.0008, n = 3). Perilipin B (Fig. 8B, doublet band 2) was unchanged. Hormone-sensitive lipase, a 84-kDa protein whose active form is associated with lipid droplets, was detected in lipid droplets isolated from the control cells but not from SCP-2-expressing cells. Finally, vimentin, a 58-kDa protein associated with lipid droplets of cells undergoing adipose conversion (23), was found to be decreased by 4.5-fold in SCP-2-expressing cells (Fig. 8D). These results were consistent with SCP-2 expression altering protein composition of lipid droplet-associated proteins.

**Is SCP-2 Directly Bound to the Surface of Isolated Lipid Droplets?**—The possibility that the above effects of SCP-2 expression were due to direct association of SCP-2 with lipid droplets was examined. First, Western blots showed that SCP-2 was detectable in cell homogenates (Fig. 8A, lanes 1 and 3) but not in isolated lipid droplets (Fig. 8A, lanes 2 and 4). Second, SCP-2-expressing L cells were simultaneously immunolabeled with antisera to SCP-2 and ADRP, a protein closely associated with lipid droplets (Fig. 9A). A punctate pattern (green) indicative of peroxisomes (where SCP-2 primarily re-

**Fig. 6.** Environmental scanning electron microscopy and multiphoton laser scanning microscopy of lipid droplets isolated from L cells. A, high resolution secondary images of lipid droplets isolated from control L cells were taken on an Electroscan E3 Environmental Scanning Electron Microscope equipped with a Peltier cooling stage and micro-manipulator/injector at 15 kV and pressures of 5 torr. B, multiphoton laser scanning microscopy was performed on lipid droplets isolated from control cells and incubated with Nile Red (0.05%). Nile Red in lipid droplets was excited at 875 nm with a Bio-Rad MRC-1024MP Multiphoton Laser Scanning Microscopy system as described under “Materials and Methods.” Fluorescence emission of Nile Red was detected at 63× (objective) through an external detector system using a HQ575/150 bandpass filter.

**Fig. 7.** Effect of SCP-2 expression on lipid droplet protein profile. A silver-stained Tricine gel (12%) was loaded as follows: lane 1, lipid droplets (20 μg) isolated from control cells; lane 2, lipid droplets (20 μg) isolated from transfected L cells overexpressing SCP-2; and lane 3, molecular weight marker. The approximate molecular masses where hormone-sensitive lipase, perilipin A, vimentin, ADRP, and perilipin B are expected to appear are indicated from top to bottom.
ADRP bound both with nearly equal affinity. Finally, ADRP binds NBD-cholesterol and NBD-stearic acid with high affinity (2.0 and 145 nM, respectively) (22, 28). However, it is not known if these lipids bind to ADRP simply because of the unique properties of the NBD fluorophore attached to the cholesterol and fatty acid. Likewise, it is not known if ADRP can bind the activated forms of fatty acids, fatty acyl-CoAs, and cholesterol, i.e., fatty acyl-CoAs. Therefore, a displacement assay was developed, and the ability of stearic acid, cholesterol, oleic acid, and oleoyl-CoA to displace NBD-labeled stearic acid bound to ADRP was determined (Fig. 10). Inhibition constants ($K_i$) were calculated from nonlinear fits to an exponential decay curve of NBD-stearate fluorescence versus the competitor concentration. The $K_i$ values for displacement by stearic acid, oleic acid, oleoyl-CoA, and cholesterol were $41.7 \pm 6.9$, $23.4 \pm 1.2$, $8.6 \pm 0.5$, and $10.2 \pm 0.9$, respectively (Table II).

In summary, the displacement assay clearly showed that all of these naturally occurring lipids displaced the ADRP-bound NBD-stearic acid. The displacement assay indicated that ADRP bound the unsaturated oleic acid (18:1) with nearly 2-fold higher affinity than the saturated stearic acid (18:0). Comparison of cholesterol and oleoyl-CoA binding showed that ADRP bound both with nearly equal affinity. Finally, ADRP bound both with nearly equal affinity.
ADRP and NBD-stearic acid were found in 69.8 ± 4.9 and 23.8 ± 4.4% of lipid droplets, respectively. Colocalization of both ADRP and NBD-stearic acid in control cells was very low, 12.6 ± 1.8% (Table III). SCP-2 expression dramatically altered this pattern by decreasing the percentage of lipid droplets containing ADRP by 34% (p < 0.01). Concomitantly, the percentage of lipid droplets stained with only with NBD-stearic acid was increased 2-fold (p < 0.05). Interestingly, SCP-2 expression increased the colocalization of ADRP and NBD-stearic acid by 3-fold (p < 0.05).

In summary, these data suggest that SCP-2 expression may reduce, at least in part, the association of ADRP with the lipid droplet surface by stripping away ADRP-bound ligands (fatty acids and cholesterol) for which SCP-2 competes. The data further suggest that ADRP-containing bound ligand (NBD-stearic acid) is more closely/tightly associated with the lipid droplet. Consistent with these possibilities, SCP-2 expression dramatically reduced the content of unesterified fatty acid (Fig. 1), cholesterol (Fig. 1), and ADRP (see above) associated with the lipid droplet.

**DISCUSSION**

Although much progress has been made in our understanding of the structure, function, and regulation of serum lipoproteins, relatively little is known regarding the processes governing intracellular lipid droplets. Cells store lipids as lipid droplets composed of a surface monolayer (cholesterol, phospholipids, fatty acids, and proteins) encasing a neutral lipid core (cholesterol esters and triglycerides) (5). At least three types of lipid droplets are recognized as follows. (i) Large (up to 15 μm diameter) triacylglyceride-rich lipid droplets, found in adipose tissue (adipose and mammary), contain almost no esterified cholesterol (reviewed in Ref. 5). These triacylglycerol-
rich lipid droplets provide an energy storage site for secretion in milk (mammary tissue) or for release of fatty acids (from triacylglycerides by hormone-sensitive lipase) to be transported to and oxidized in peroxisomes and mitochondria. (ii) Small (~1 μm diameter) lipid droplets, found in steroidogenic tissues (adrenal, Leydig, and luteal) (15, 65), are cholesteryl ester-rich (81% of core lipids), and almost all cholesteryl (97%) is esterified (7). Cholesterol and cholesteryl esters (hydrolyzed by hormone-sensitive lipase) stored in the lipid droplet provide a ready source of cholesterol for transfer, via the plasma membrane (66), to mitochondria for steriodogenesis (6, 16, 65, 68). (iii) Almost all other mammalian tissues examined contain small (near 1 μm diameter), less well characterized lipid droplets (5, 69, 70). One potential function of these smaller lipid droplets was suggested by a recent report showing that plasma membrane caveolar SRB1 mediates reversible uptake and efflux of unesterified cholesterol between HDL and intracellular lipid droplets in non-adipose, non-steroidogenic cells (21, 22). Thus, the smaller lipid droplets common to nearly all cells may be essential for “reverse cholesterol transport” (10). However, almost nothing is known of the structure, composition, or function of the ubiquitous smaller lipid droplets found in nearly all mammalian cells. The results presented herein demonstrate for the first time several new observations characterizing these smaller lipid droplets from l. cells and examine potential roles of SCP-2 in regulating their lipid and protein composition.

First, the lipid composition of these lipid droplets was unique. Key differences from lipid droplets of adipose and steroidogenic tissues are as follows. (i) The core lipid profile, 66% triacylglycerol and 33% cholesteryl ester, was intermediate between that of the large lipid droplets found in adipose tissues (5) and that of the small lipid droplets typical of steroidogenic cells (7). (ii) The ratio of surface lipids/core lipids of the lipid droplets (0.50), while much lower than that of adipose lipid droplets, was in the range of the reported for lipid droplets of steroidogenic cells, i.e. 0.70 (7), possibly reflecting the similarity in size of these lipid droplets. (iii) The surface monolayer of the l. cell lipid droplets exhibited a very high cholesteryl/ phospholipid ratio, almost 19-fold higher than that of lipid droplets from steroidogenic cells (7). This ratio was also 3–6-fold higher than ratios typical of plasma membrane bilayers, normally containing the highest level of cholesterol in the cell (59). Consistent with the high degree of localization of cholesterol to lipid droplets, fluorescent sterols are rapidly taken up (NBD-cholesterol) and preferentially targeted (NBD-cholesterol, dehydroergosterol) to lipid droplets in living cells (21, 22, 70). Thus, the data presented herein indicate that the high localization of NBD-cholesterol and dehydroergosterol to lipid droplets of living cells reflects the high proportion of lipid droplet cholesterol. (iv) The surface lipids of the isolated lipid droplets contained significant amounts of unesterified fatty acids, i.e. 5.2 nmol/mg protein (21.6% of total lipid droplet lipids). This represented a 4-fold enrichment of unesterified fatty acids in the lipid droplet as compared with the cell homogenate. The appearance of unesterified fatty acid in the lipid droplet surface lipids was consistent with confocal microscopic imaging of living cells which showed that fluorescent fatty acids were rapidly targeted to the lipid droplet (69). Equally important, it demonstrated that the targeting of the fluorescent labeled fatty acids (e.g. NBD-stearic acid) to the lipid droplets was not an artifact induced by the insertion of the NBD group into the fatty acid. (v) The fatty acids esterified to the lipid droplet lipid species (phospholipids, triacylglycerols, and cholesteryl esters), while qualitatively similar to those of the serum lipoproteins in the culture medium, exhibited distinct quantitative differences. Lipid droplet phospholipids, cholesteryl esters, and triacylglycerols were enriched 2–3-fold in polyunsaturated fatty acids as compared with the corresponding serum lipoprotein lipids. This suggests that the lipid droplets may be important not only for storage of fatty acids in esterified form to be used as future energy sources but also for storage of polyunsaturated fatty acid signaling molecules or precursors thereof (2).

Second, overexpression of SCP-2 significantly altered the lipid composition of lipid droplets by decreasing their cholesteryl ester, cholesterol, and unesterified fatty acid content. The loss of lipid droplet sterol was consistent with observations showing that SCP-2 enhances cholesterol transfer from lipid droplets to mitochondria for oxidation (7) as well as reports that SCP-2 expression may play an important role in cholesterol secretion into bile (71–73). Overexpression of SCP-2 in mice increased hepatic cholesterol content and increased enterohepatic circulation of bile (74), whereas treatment of rats with SCP-2 antisense reduced and delayed the appearance of biliary cholesterol (75). Contrary to expectation, SCP-2/SCPx gene ablation caused biliary cholesterol hypersecretion and gallstone formation, an effect due to concomitant up-regulation of liver fatty acid-binding protein, another cholesterol transport molecule (76). The relatively smaller loss of cholesteryl ester and lower unesterified fatty acid levels were consistent with the reduced association of hormone-sensitive lipase with lipid droplets of SCP-2-expressing cells (Fig. 7). Taken together with the high affinity of SCP-2 for cholesterol (33, 34, 40) and fatty acids (29, 33), these data suggested that SCP-2 expression enhanced the efflux of cholesterol as well as fatty acids from the lipid droplets.

Third, the protein profile of the lipid droplets was intermediate with that of triglyceride-rich and cholesteryl-ester-rich lipid droplets. Tricine/SDS-PAGE gels of the isolated lipid droplet proteins resolved over 27 protein bands, some of which were in the high molecular weight range of P200, a lipid droplet surface capsule protein that prevents access to hormone-sensitive lipase (77). Western blotting showed that the isolated lipid droplets contained ADRP, perilipin A, perilipin B, vimentin, hormone-sensitive lipase, but not SCP-2. Immunofluorescence confocal microscopy also detected ADRP, but not SCP-2, in the lipid droplets. The distribution of several of these proteins was intermediate with that of triglyceride-rich and cholesteryl ester-rich lipid droplets. ADRP is absent from large triglyceride-rich lipid droplets of mature adipocytes (3). Perilipins are present in high amounts in adipocytes and steroidogenic cells (reviewed in Ref. 3). Both ADRP and perilipins were present in l. cell lipid droplets. These data suggested that many of the proteins thought to be important for lipid droplet function are detected in isolated l. cell lipid droplets.

Fourth, SCP-2 expression dramatically altered the proteins associated with the lipid droplet. Lipid analysis showed that SCP-2 expression increased the protein/lipid ratio in the lipid droplet. However, the profile of lipid droplet proteins was significantly altered. The isolated lipid droplets were reduced in the proportion of high molecular weight proteins with concomitant slight increases in intermediate molecular weight proteins. Loss of the high molecular weight capsular proteins such as P200 makes the lipid droplet accessible to hormone-sensitive lipase (24). Consistent with this possibility, the lipid droplet of cholesteryl ester content was reduced in SCP-2-expressing cells. Furthermore, levels of perilipin A, but not perilipin B, was increased 3.1-fold, whereas levels of ADRP and vimentin were decreased. An inverse relationship between perilipins and ADRP has also been reported for the lipid droplets of differentiating adipocytes (3).

Fifth, although a direct interaction of SCP-2 tightly associated with lipid droplets was not observed in the present study,
a link between the two has nevertheless been suggested from data reported herein and earlier by this and other laboratories as follows. (i) The observation that SCP-2 can affect transfer of cholesterol from lipid droplets in intact cells (16, 18, 21), without SCP-2 being detected in Western blots of isolated lipid droplets has precedent. SCP-2 enhanced the transfer of cholesterol from plasma membranes for esterification in the endoplasmic reticulum in intact cells, even though SCP-2 was not detected in purified plasma membranes (43, 76). (ii) SCP-2 expression in transfected cells redirected the transfer of cholesterol from lipid droplets away from the plasma membrane (i.e. inhibited efflux to HDL) (21) and toward intracellular sites, especially mitochondria and endoplasmic reticulum (reviewed in Refs. 17, 47, 78–80). (iii) Since SCP-2-mediated intermembrane cholesterol transfer required interaction of SCP-2 with a donor membrane surface (81), it seems likely that a similar, albeit transitory requirement may exist for SCP-2-mediated cholesterol transfer from lipid droplets. Indeed, the small lipid droplets of L cells present a surface ideal for electrostatic interactions with SCP-2 (67, 82). The lipid droplet surface is rich in anionic lipid (unesterified fatty acids), exhibits a high cholesterol/phospholipid ratio, and has a high radius of curvature. (iv) Microinjection of steroidogenic cells with antisera to SCP-2 inhibited cholesterol transfer from lipid droplets for oxidation to steroids in mitochondria (16, 18). (v) Immunogold electron microscopy showed a close association of SCP-2 with lipid droplets in luteal cells derived from hormone-stimulated, but not control, rats (15). As indicated in the Introduction, endocrine stimulation alters the pattern of multiple proteins (e.g. hormone-sensitive lipase) associated with lipid droplets. In summary, the above data suggest that SCP-2 may interact with lipid droplets through electrostatic interactions that are weak, transient, and/or require endocrine stimulation.

Sixth, since levels of ADRP, a protein closely associated with lipid droplets, were decreased in transfected L cells expressing SCP-2, the ability of ADRP to affect lipid content was further investigated. Several lines of evidence suggest ADRP has a regulatory role in lipid metabolism as follows. (i) Indirect immunofluorescence imaging studies have shown that ADRP is localized to the surface of lipid droplets (3, 21). (ii) In the present study, the surface lipid content of transfected L cells was observed to be significantly lower (7.7-fold, p < 0.01) than in control cells. Surface lipid to core lipid ratios were also decreased (7.2-fold, p < 0.01). (iii) A concomitant decrease (1.7-fold) in ADRP levels was observed in transfected cells (21). The above observations suggest that ADRP functions as a regulatory protein, governing the deposition and release of lipid stores from droplets. Results from several binding and displacement studies help support this theory. Although ADRP doesn’t bind cholesterol esters, it exhibited saturable binding of NBD-labeled cholesterol and stearic acid to high affinity (2.0 and 145 nM, respectively) (22, 28). Inhibition constants (Ki) calculated from displacement data in the present study were in agreement with the cholesterol and stearic acid binding values, indicating that cholesterol binds tightly to ADRP and is able to displace readily fatty acids. The results from the binding and displacement data were further confirmed by immunofluorescence imaging studies where colocalization of ADRP and NBD-labeled stearic acid in transfected L cells showed up to three populations of lipid droplets. One population was rich in ADRP; another set had both ADRP and the fatty acid colocalized, and the last retained only the fatty acid probe. Although it is possible the last set might not be lipid droplets, morphologically it appeared the fatty acid probe localized in storage droplets. Evidence for different populations of lipid droplets have been reported elsewhere where changing levels of ADRP and perilipins, another lipid droplet-associated protein, were found when differentiation was induced in 3T3-L1 adipocytes (3). Consistent with the binding and displacement data and the fact that SCP-2 also binds both fatty acids and cholesterol with affinities very similar (22, 29, 34) to those of ADRP, the lipid droplets from SCP-2-expressing cells lost much less cholesterol than unesterified fatty acid. Furthermore, the data suggested that the liganded ADRP was preferentially retained in the lipid droplets. In summary, given the results from the binding, displacement, and imaging experiments, along with the concomitant low levels of surface unesterified fatty acid, cholesterol, and ADRP in SCP-2 transfected cells, the results presented herein point to a potential functional role for SCP-2 and ADRP in regulating lipid content in lipid storage droplets.

In conclusion, the data presented here demonstrate for the first time that expression of SCP-2 in transfected L cells affects the lipid content, esterified fatty acid composition, and protein content/profile of lipid storage droplets. This is not surprising given the huge body of evidence supporting a role for SCP-2 in cholesterol, fatty acid, and phospholipid metabolism and intracellular trafficking (reviewed in Ref. 34). While the interaction was shown not to be due to permanent association of SCP-2 with the lipid droplet, clearly the cellular expression of SCP-2 alters the structure of the lipid droplet. A concomitant displacement and immunolocalization imaging study of ADRP, whose levels were decreased in the presence of SCP-2, provided evidence for regulatory roles for both SCP-2 and ADRP in maintaining lipid stores. In summary, the results presented herein provide new insights into the functional significance of these proteins associated with lipid droplets.

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