Expression of fatty acid binding proteins inhibits lipid accumulation and alters toxicity in L cell fibroblasts

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Received 10 December 2001; accepted in final form 18 April 2002

Expression of fatty acid binding proteins inhibits lipid accumulation and alters toxicity in L cell fibroblasts. Am J Physiol Cell Physiol 283: C688–C703, 2002. First published April 24, 2002; 10.1152/ajpcell.00586.2001.—High levels of saturated, branched-chain fatty acids are deleterious to cells and animals, resulting in lipid accumulation and cytotoxicity. Although fatty acid binding proteins (FABPs) are thought to be protective, this hypothesis has not previously been examined. Phytanic acid (branched chain, 16-carbon backbone) induced lipid accumulation in L cell fibroblasts similar to that observed with palmitic acid (unbranched, C16): triacylglycerol >> free fatty acid > cholesterol > cholesteryl ester >> phospholipid. Although expression of sterol carrier protein (SCP)-2, SCP-x, or liver FABP (L-FABP) in transfected L cells reduced [3H]phytanic acid uptake (57–87%) and lipid accumulation (21–27%), nevertheless [3H]phytanic acid oxidation was inhibited (74–100%) and phytanic acid toxicity was enhanced in the order L-FABP >> SCP-x >> SCP-2. These effects differed markedly from those of [3H]palmitic acid, whose uptake, oxidation, and induction of lipid accumulation were not reduced by L-FABP, SCP-2, or SCP-x expression. Furthermore, these proteins did not enhance the cytotoxicity of palmitic acid. In summary, intracellular FABPs reduce lipid accumulation induced by high levels of branched-chain but not straight-chain saturated fatty acids. These beneficial effects were offset by inhibition of branched-chain fatty acid oxidation that correlated with the enhanced toxicity of high levels of branched-chain fatty acid.

sterol carrier protein-2; sterol carrier protein-x; liver fatty acid binding protein; fatty acids; phytanic acid; palmitic acid; lipid mass; toxicity

Although high levels of branched-chain (6, 52, 64, 94, 95, 97) or straight-chain (8, 14, 29, 35, 49, 66, 86, 87, 92, 101) saturated fatty acids induce lipid accumulation and toxicity in cultured cells and animals, the potential role of intracellular fatty acid binding proteins in modulating these effects is unknown. The existing data do not allow a priori prediction of whether the intracellular fatty acid binding proteins may be protective.

An early hypothesis is that intracellular fatty acid binding proteins bind fatty acids in the cytoplasm and thereby prevent their nonspecific detergent effects on disrupting membrane functions (61). Indeed, liver fatty acid binding protein (L-FABP) (57) and sterol carrier protein (SCP)-2 (77) have high affinity [dissociation constant (Kd) in nM range] for both branched-chain and straight-chain fatty acids. L-FABP and SCP-2 also bind branched- and straight-chain saturated fatty acyl CoA esters (20, 33, 81, 88) with high affinity (Kd in nM range). Furthermore, intracellular fatty acid binding protein levels are in the 0.2–0.4 mM range in tissues (liver, intestine, heart) active in fatty acid metabolism. However, if fatty acids were not rapidly metabolized, then intracellular fatty acid binding proteins would quickly become saturated with ligand and would not protect against toxic effects of fatty acids. On the contrary, because of the rapid metabolism of fatty acids, the intracellular free fatty acid (reviewed in Ref. 47) and fatty acyl CoA (reviewed in Refs. 18, 28) concentrations are normally maintained at constant, very low levels. Thus it is unlikely that fatty acid binding proteins would be protective simply by binding fatty acids.

A considerable body of evidence suggests that intracellular fatty acid binding proteins actually enhance, rather than prevent, the formation of lipids that are typically induced by high levels of fatty acids. However, these experiments were performed under conditions of low fatty acid concentrations. L-FABP, intestinal fatty acid binding protein (I-FABP), and SCP-2 stimulate microsomal incorporation of fatty acyl CoAs into glycerides in vitro (10, 38–40, 76, 85) and in transfected cells (3, 47, 85). Both SCP-2 and L-FABP also stimulate microsomal incorporation of fatty acyl CoA into cholesteryl esters in vitro (12, 51, 69, 70, 91) and in transfected cells (36, 37, 56). Thus the data obtained with low concentrations of fatty acids indicate that fatty acid binding proteins are likely to induce rather than inhibit accumulation of fatty acids in esterified...
lipids. However, the effect of intracellular fatty acid binding proteins on lipid accumulation and toxicity in the presence of high fatty acid levels is not known. In fact, some data suggest that induction of lipid accumulation may not necessarily be coupled to toxicity of the fatty acid (25, 26).

Although this has not been demonstrated directly in intact cells, in vitro experiments are consistent with a role for intracellular fatty acid binding proteins in enhancing oxidation of low levels of fatty acids. Branched-chain saturated fatty acids exclusively undergo α-oxidation and partial β-oxidation in peroxisomes (reviewed in Ref. 94). SCP-2 and, even more so, SCP-x are present in highest concentrations in peroxisomes (4, 23, 59, 71). Importantly, SCP-x is a peroxisomal enzyme that exclusively exhibits 3-ketoacyl-CoA thiolase activity with branched-chain substrates (1, 2, 96). In contrast, straight-chain fatty acids are β-oxidized primarily in mitochondria (32). Fatty acid binding proteins enhance transport of saturated fatty acids in intact cells (45, 47, 53, 54, 100). In addition, fatty acid binding proteins enhance transport of fatty acyl CoA to isolated mitochondria (63) and stimulate their mitochondrial β-oxidation (62, 65) in vitro. Low levels of L-FABP are associated with the outer envelope of mitochondria (10) and SCP-2 has also been detected in mitochondria (23, 24, 41). Although these in vitro data are consistent with a role for the intracellular fatty acid binding proteins in oxidation of low levels of fatty acids, their effect in the presence of high levels of fatty acids remains to be determined.

Finally, studies with gene-targeted mice provide additional evidence for fatty acid binding protein functioning in the oxidation of low levels of branched-chain and straight-chain fatty acids. For example, disruption of the heart fatty acid binding protein (H-FABP) gene in mice results in 90% inhibition of fatty acid oxidation by cardiomyocytes exposed to low levels of straight-chain saturated fatty acid (i.e., palmitic acid) (9, 67). Branched-chain saturated fatty acids such as phytanic acid accumulate in sera of SCP-x/SCP-2 gene-ablated mice fed normal chow or high levels of phytol (81). Although high levels of phytol are more toxic in SCP-x/SCP-2 gene-ablated mice, lipid accumulation is reduced rather than increased as expected (81). Although the latter studies represent the first report on the effect of fatty acid binding proteins on lipid accumulation and toxicity of high levels of branched-chain fatty acids, it must be noted that multiple fatty acid binding proteins are missing (i.e., both SCP-x and SCP-2) and, concomitantly, L-FABP is upregulated fourfold in these gene-targeted mice (81). Thus the individual contributions of SCP-x, SCP-2, and L-FABP in modifying the lipid accumulation and toxicity induced by high levels of branched-chain, much less straight-chain, saturated fatty acids remain to be resolved.

The present investigation used transfected L cell fibroblasts individually overexpressing L-FABP, SCP-2, or SCP-x to address the hypothesis that these proteins protect the cell from lipid accumulation and toxicity induced by branched- and straight-chain saturated fatty acids. Phytanic acid (16-carbon backbone with 4 methyl branches) was chosen as a model because it is the most prevalent dietary branched-chain saturated fatty acid. Furthermore, phytanic acid has the same carbon chain length as the unbranched saturated palmitic acid, one of the most prevalent dietary straight-chain saturated fatty acids.

**MATERIALS AND METHODS**

**Materials.** Calcein AM and ethidium homodimer-1 were purchased from Molecular Probes (Eugene, OR); phytanic acid, palmitic acid, bovine serum albumin (BSA; essentially fatty acid free), and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO); [2,3-3H]phytanic acid (specific activity of 50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA); [9,10-3H]palmitic acid (specific activity of 36.3 Ci/mmol) was purchased from Dupont New England Nuclear (Boston, MA); silica gel G plates were purchased from Analtech (Newark, DE); neutral lipid and fatty acid standards were purchased from Nu-Chek Prep (Elysian, MN); and phospholipid standards were purchased from Avanti (Alabaster, AL). Rabbit polyclonal antisera to recombinant human SCP-2, mouse SCP-x, mouse acyl CoA binding protein (ACBP), and rat L-FABP were prepared as described previously (4). Antiserum against CD-36 (also called fatty acid translocase; FAT) was purchased from Research Diagnostics (Flanders, NJ). Rabbit polyclonal anti-fatty acid transport protein (FATP) was a generous gift from Dr. J. Schaffer (Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO). Sheep anti-bovine catalase was purchased from Biodiagnos (Kennebunk, ME). Rabbit anti-mouse peroxisomal membrane protein-70 (PMP-70) was purchased from Affinity Bioreagents (Golden, CO). All reagents and solvents used were of the highest grade available and were cell culture tested.

**L cell fibroblast culture.** Murine L cells (L arpt−/−t−) were grown to confluence at 37°C and 5% CO2 in Higuchi medium (31) supplemented with 10% FBS (Hyclone, Logan, UT). The cell lines used were derived as follows: the mock-transfected cells (designated as control) (37, 50) and cells overexpressing L-FABP (4, 37) were developed as described previously. Because the SCP-x/SCP-2 gene encodes for two proteins (15-kDa pro-SCP-2 and 58-kDa SCP-x), it was necessary to prepare clones transfected with the cDNAs separately encoding the 15-kDa pro-SCP-2 and the 58-kDa SCP-x.

It is important to note that although the gene encodes for 15-kDa pro-SCP-2, only the mature 13-kDa SCP-2 is detected in tissues and almost all transfected cells (reviewed in Ref. 23). In contrast to 15-kDa pro-SCP-2, transfection of cells with the cDNA encoding 13-kDa SCP-2 does not efficiently target the expressed protein to peroxisomes (72). Therefore, L cells were transfected with the cDNA encoding the 15-kDa pro-SCP-2 cDNA to appropriately overexpress and target the mature 13-kDa SCP-2 (50).

To develop the SCP-x expression cell line, the entire coding region of mouse 58-kDa SCP-x cDNA was cloned into the eukaryotic expression vector PkJ1ΔF, a generous gift from Dr. M. McBurney (Department of Medicine and Biology, University of Ottawa, Ottawa, Canada). The resultant expression vector was cotransfected with SV2neo (a vector containing neomycin resistance) into mouse L cell fibroblasts with the LipofectAMINE reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD). All clones resistant to G418 were selected and screened by Western blotting for the respective proteins basically as described previously (4).
Presence of mitochondria and peroxisomes in L cells. The presence of mitochondria and peroxisomes was determined by Western blotting and/or electron microscopy. L cells were homogenized, and fractions enriched in organelles involved in fatty acid oxidation, i.e., mitochondria and peroxisomes, were obtained by differential centrifugation and discontinuous sucrose gradient centrifugation as described previously (24). A 12,000 g pellet from L cell homogenates was run over a sucrose gradient to produce two bands (24): one at 37% sucrose containing both mitochondria and peroxisomes and one at 33% sucrose identified by Western blot analysis as consisting of mostly peroxisomes (see RESULTS). L cell homogenates and peroxisomal enriched fractions were resolved with tricine SDS-PAGE gels (16%), transferred to nitrocellulose membranes, and probed against affinity-purified antisera against peroxisomal markers PMP-70 and catalase basically as described previously (68). The peroxisome-enriched bands were also collected and processed for electron microscopy as described previously (7).

Supplementation of L cells with branched- and straight-chain saturated fatty acids: preparation of fatty acid-BSA complexes. Fatty acid-BSA complexes were prepared with the method of Spector and Hoak (84), in which palmitic acid (dissolved in hexane) and phytic acid (dissolved in dimethylformamide: DMF) were added to celite particles (Sigma, St. Louis, MO) with 1 μmol of fatty acid per 10 mg of solid phase. After the solvent was removed under a N2 stream, the fatty acid-coated particles were mixed with lipid-free albumin in serum-free medium for 45 min at room temperature. Celite particles were removed by centrifugation, and the resultant supernatant was adjusted to pH 7.4. The concentration of fatty acid and BSA in the complexes was determined by the methods of Dole and Meinertz (15) and Bradford (11), respectively.

Cells from the respective cell lines were plated at ~1.4 × 10^6 cells/10-cm dish in serum-containing medium. After 24 h, the cells were washed extensively with PBS and the medium was changed to serum-free medium to limit the presence of serum-derived fatty acids. BSA or palmitic acid-BSA or phytic acid-BSA complexes (25 μM fatty acid equivalents, ~90 μM BSA) were then added to the cells cultured in serum-free medium followed by incubation for 6 h at 37°C before lipid extraction. These concentrations of fatty acid-BSA complexes were similar to those found in the serum-containing medium used to culture cells (42, 60), and the ratios of fatty acid to BSA in the complexes were similar to those reported previously (15). Under these conditions not only was cell viability >95%, but both palmitic acid-BSA and phytic acid-BSA complexes induced lipid accumulation in all three cell lines (see RESULTS).

Measurement of lipid accumulation and analysis. Cells incubated with BSA or fatty acid-BSA complexes as described above were extracted with n-hexane-2-propanol (3:2 vol/vol) (30, 37). Lipid classes were resolved on silica gel G thin-layer chromatography plates developed in petroleum ether-diethyl ether-methanol-acetic acid (90:7:2:0.5 vol/vol) (30, 37). Total cholesterol, free fatty acid, triacylglyceride, and cholesteryl ester content were determined by the method of Marzo et al. (46). Total phospholipid content was determined by digesting the phospholipid fraction in deionized water and perchloric acid for 1 h at 180°C followed by addition of ammonium molybdate and ascorbic acid. After further heating for 5 min in a boiling water bath, the sample was cooled and the absorbance was read at 797 nm to quantify total lipid phosphorus. Lipids were stored under an atmosphere of N2 to limit oxidation. All glassware was washed with sulfuric acid-chromate before use. Protein concentration was determined from the dried protein extract residue digested overnight in 0.2 M KOH.

Uptake of radiolabeled fatty acids by cultured L cells. To determine the extent of fatty acid uptake, cells were incubated with BSA-fatty acid complexes (25 μM fatty acid equivalents, ~90 μM BSA) in the presence of trace amounts of [3H]palmitic acid or [3H]phytanic acid (2 μCi/35-mm dish) for 6 h at 37°C. The medium was then removed, and lipids were extracted from the medium and from the cells as described previously (19). The residual, nonlipid residue (remaining after lipid extraction of the cell monolayer) was dissolved in 0.2 M KOH overnight and assayed for protein and radiolabel content. Radiolabeled lipids were quantified in a liquid scintillation cocktail (Scinti Verse, Fisher Scientific, Pittsburgh, PA) on a Packard 1600TR liquid scintillation counter (Meridian, CT).

Oxidation of radiolabeled fatty acids by cultured L cells. To determine the extent of fatty acid oxidation, cells were incubated with fatty acid-BSA complexes (25 μM fatty acid equivalents, ~90 μM BSA) in the presence of trace amounts of [3H]palmitic acid and [3H]phytanic acid (2 μCi/35-mm dish) for 6 h at 37°C. Oxidation was measured from the formation of water-soluble tritiated degradation products released into the culture medium after the medium was removed and extracted by the method of Folch et al. (19) with modifications as described previously. Radioactivity was quantified in a liquid scintillation cocktail (Scinti Verse) on a Packard 1600TR liquid scintillation counter.

Determination of saturated fatty acid cytotoxicity in L cells: flow cytometry. To determine the cytotoxicity of saturated fatty acids, an assay kit (Molecular Probes) composed of calcein AM and ethidium homodimer-1 was used. The assay was based on membrane-permeant calcein AM, which, after cleavage by intracellular esterases, yielded a green cytoplasmic fluorescence (with a blank nucleus) in live cells. Ethidium homodimer-1 was used as a membrane-impermeant fluorophore that penetrated membrane-compromised (dead) cells to label nucleic acids in the nucleus and yield a red fluorescence. By using both dyes simultaneously, the ratio of live to dead cells was detected and quantitated by flow cytometry. Briefly, cells were washed with PBS to remove serum before addition of the fatty acid-BSA complexes. At various timed intervals (from 12 to 96 h) the cells were prepared for analysis by removing the medium from each plate and washing once with PBS, followed by incubation in trypsin (0.25%, 1 mM EDTA-4Na). After 5 min, trypsinization was stopped by addition of complete medium containing 10% FBS, and the cell count was determined. The trypsinized cell suspensions and all culture medium and PBS washes removed from the dishes were combined by centrifuging at 1,000 rpm for 6 min, and the resulting cell pellets were then resuspended in Puck’s buffer (in mM: 1 Na2HPO4, 0.9 H2PO4, 5.0 KCl, 1.8 CaCl2, 0.6 MgSO4, 6 glucose, 138 NaCl, and 10 HEPES) to give a cell density of ~9 × 10^4 cells/ml. To limit artifacts associated with potential toxicity and/or saturation of live/dead dyes in the assay, dye concentrations were individually titered over a broad range to obtain an optimal dye solution: 0.1 μM calcein AM-0.8 μM ethidium homodimer-1. This dye solution was added to the cell suspension and incubated as indicated in the assay kit. Each sample was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at a low flow rate (~670 cells/s) and simultaneous excitation of both dyes at 488 nm. Fluorescence emission was detected through two separate filters, calcein (green fluorescence at 530 nm) and ethidium homodimer-1 (red fluorescence at 585 nm). As a result, the live
and dead cells were resolved into two bivariate populations by flow cytometry.

Measurement of plasma membrane fatty acid transport proteins and intracellular acyl CoA binding protein. Cell homogenates of control and SCP-2-, SCP-x-, and L-FABP-expressing cells were analyzed by Western blot analysis to determine whether expression levels of plasma membrane fatty acid transport proteins associated with fatty acid transport were altered. Cell homogenates (10 μg) were run on tricine gels (16%) before transfer to 0.45-μm nitrocellulose paper (Sigma) by electrophoresis in a continuous buffer system at 0.8 mA/cm² for 2 h. After transfer, blots were treated as described previously (4) with affinity-purified polyclonal anti-L-FABP (A) or polyclonal anti-SCP-2 (B and C) as described in MATERIALS AND METHODS.

RESULTS

Expression of L-FABP, SCP-2, and SCP-x in transfected L cell fibroblasts. Expression of L-FABP, SCP-2, and SCP-x in transfected L cell fibroblasts was determined by Western blot analysis of cell homogenates probed with specific antisera to the proteins as described in MATERIALS AND METHODS. Although levels of L-FABP, SCP-2, or SCP-x were not detected in control mock-transfected cells, quantitative analysis of the transfected clones indicated that protein expression levels were 0.4% (Fig. 1A, lane 2), 0.036% (Fig. 1B, lane 2), and 0.01% (Fig. 1C, lane 3) of soluble protein, respectively, consistent with earlier findings. Furthermore, partial posttranslational cleavage of the 58-kDa SCP-x protein resulted in detectable 43-kDa protein and up to a twofold increase in level of 13-kDa SCP-2 (Fig. 1C, lane 3, and Fig. 1B, lane 3, respectively). The partial posttranslational cleavage of 58-kDa SCP-x confirmed previous suggestions (4, 59).

Presence of fatty acid oxidative organelles in L cells: mitochondria and peroxisomes. To confirm that L cell fibroblasts have the basic machinery necessary for fatty acid oxidation, Western blot analysis was performed on L cell homogenates and subcellular fractions enriched in peroxisomes and mitochondria (Fig. 2). The peroxisomal markers PMP-70 (Fig. 2A, lane 1) and catalase (Fig. 2B, lane 2) were both present in L cell
homogenates. The level of catalase was about fivefold higher in the peroxisome-enriched fraction (Fig. 2B, lane 3) compared with the L cell homogenate (Fig. 2B, lane 2). Electron micrographs of the peroxisome-enriched fraction showed that L cells contain mitochondria, the primary site of fatty acid ß-oxidation (Fig. 2C), as well as peroxisomes, the site of ß-oxidation of branched-chain and very long fatty acids (Fig. 2C).

Effect of acyl chain branching on saturated fatty acid-induced lipid accumulation in L cell fibroblasts. Although straight-chain saturated fatty acids such as palmitic acid are known to induce lipid accumulation in cultured cells (e.g., L cell fibroblasts) and animals (reviewed in Ref. 26), the effect of fatty acid acyl chain branching on this lipid accumulation is not known. To resolve this issue, control L cells were supplemented with phytanic acid (16-carbon backbone with 4 methyl branches) presented as an albumin complex as described in MATERIALS AND METHODS. For comparison with the effects of straight-chain saturated fatty acid, cells were also supplemented with palmitic acid (16-carbon chain length fatty acid without branching). The cells were incubated with the respective fatty acid-albumin complexes in serum-free medium for 6 h followed by washing, lipid extraction, and analysis as indicated in MATERIALS AND METHODS.

Supplementation of L cells with phytanic acid-BSA complex induced a 1.7-fold increase in total lipid mass (nmol lipid/mg protein) compared with supplementation with BSA only (Table 1). However, this increase was selective among the major lipid classes in the following order: 6.5-fold for triacylglycerol > 3.0-fold for free fatty acid > 2.0-fold for cholesterol > 1.3-fold for cholesteryl ester. No significant change for phospholipid mass. To determine whether these effects were due to the presence of methyl branching in phytanic acid or simply the presence of saturated fatty acid, this experiment was compared with that of cells supplemented with palmitic acid-BSA-complex.

Culturing L cells with palmitic acid-BSA also induced lipid accumulation, as indicated by a 1.9-fold increase in total lipid mass (nmol lipid/mg protein) compared with cells supplemented with BSA only (Table 1). Examination of individual lipid classes revealed that this increase was selective in the order 7.6-fold for triacylglycerol > 3.3-fold for free fatty acid > 1.9-fold for cholesterol > 1.6-fold for cholesteryl ester. No significant increase for phospholipid mass. Comparison of the fold increases in total lipid as well as each lipid class induced by phytanic acid-BSA vs. palmitic acid-BSA revealed no significant differences.

In summary, compared with straight-chain saturated fatty acid (palmitic acid), the presence of methyl branches in phytanic acid (also 16-carbon chain length) 1) did not further exacerbate lipid accumulation in L cells and 2) did not alter the pattern of specific lipid fractions that accumulated. Both phytanic and palmitic acid preferentially induced the accumulation of lipids primarily comprising lipid storage droplets, i.e., triacylglycerol and cholesteryl ester, and less so cholesterol, fatty acid, and phospholipid, which are minor constituents of L cell lipid droplets (5).

Fatty acid binding protein expression differentially alters saturated fatty acid-induced lipid accumulation. To examine the effect of fatty acid binding proteins on protection of the cells against saturated fatty acid-induced lipid accumulation, the above experiments were repeated with transfected L cells individually overexpressing SCP-2, SCP-x, or L-FABP at levels in the range of those reflecting their respective physiological values. Control, untransfected L cells did not differ from mock-transfected L cells. Three types of experiments were performed. First, the effect of SCP-2, SCP-x, and L-FABP expression on basal (i.e., in the absence of added palmitic or phytanic acid) lipid mass was examined (Fig. 3). Only SCP-x expression signifi-

Table 1. Saturated fatty acid-induced lipid accumulation in L cell fibroblasts

<table>
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<tr>
<th>Lipid</th>
<th>Fold Increase in Lipid Mass</th>
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<tr>
<td></td>
<td>Palmitic acid-BSA</td>
</tr>
<tr>
<td>Total lipid</td>
<td>1.90 ± 0.04*</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>3.27 ± 0.40*</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>7.56 ± 0.80*</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>1.63 ± 0.09*</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>1.19 ± 0.13</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.92 ± 0.20*</td>
</tr>
<tr>
<td>Cholesterol/phospholipid</td>
<td>1.66 ± 0.15*</td>
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Values are means ± SE (n = 4–8). The fold increase in lipid mass (nmol/mg protein) induced by palmitic acid-BSA alone or as described in MATERIALS AND METHODS. *Significance (P < 0.05) compared with cells supplemented with BSA only.
cantly altered basal lipid mass (nmol lipid/mg protein) to elicit a 1.6-fold decrease ($P < 0.01; n = 3–6$) compared with the control cells (Fig. 3). In contrast, expression of SCP-2 or L-FABP had no effect on basal lipid mass.

Second, the effect of SCP-2, SCP-x, and L-FABP expression on palmitic acid-BSA-induced lipid accumulation was determined (Fig. 3). Overexpression of fatty acid binding proteins in L cells did not prevent the palmitic acid-BSA-induced lipid accumulation. Supplementation with palmitic acid-BSA increased total lipid mass (nmol lipid/mg protein) of control and SCP-2-, SCP-x-, and L-FABP-expressing cells 1.9-, 2.0-, 2.1-, and 2.0-fold ($P < 0.01; n = 3–6$), respectively, compared with the corresponding cell line supplemented with BSA only (Fig. 3). Comparison within the palmitic acid-BSA supplementation group showed that the total lipid mass (nmol lipid/mg protein) was 30% lower ($P < 0.01$) in the SCP-x-expressing cells compared with the control cells supplemented with palmitic acid-BSA, similar to the result observed with the basal lipid mass in SCP-x-expressing cells (Fig. 3).

Third, the effect of SCP-2, SCP-x, and L-FABP expression on lipid accumulation induced by supplementation with the methyl-branched phytanic acid-BSA was determined (Fig. 3). As with palmitic acid-BSA supplementation, phytanic acid-BSA also induced lipid accumulation in transfected L cells, regardless of which type of fatty acid binding protein was expressed. Phytanic acid-BSA supplementation increased total lipid mass (nmol lipid/mg protein) of control and SCP-2-, SCP-x-, and L-FABP-expressing cells 1.7-, 1.3-, 2.0-, and 1.3-fold ($P < 0.01$), respectively, compared with control cells supplemented with BSA only. However, comparison within the phytanic acid-BSA supplementation group showed that SCP-2, SCP-x, and L-FABP expression significantly reduced the lipid accumulation induced by supplementation with BSA-phytanic acid complex compared with control cells supplemented with phytanic acid-BSA (Fig. 3).

In summary, expression of SCP-x partially protected L cells from lipid accumulation in the basal state as well as with palmitic acid-BSA or phytanic acid-BSA supplementation. In contrast, SCP-2 or L-FABP expression was protective only in phytanic acid-BSA-fed cells.

Effect of methyl branching on saturated fatty acid-induced free (unesterified) fatty acid accumulation in L cells. As indicated above, cellular levels of fatty acids in esterified form (e.g., triglycerides, phospholipids, cholesterol esters) are high and depend on available dietary fatty acids. In contrast, because of their potent detergent as well as metabolic regulatory effects, cellular levels of free (unesterified) fatty acids are more tightly regulated and are maintained at very low concentrations (reviewed in Ref. 61). Basal levels of free fatty acids in control L cells (Fig. 4) comprised only a small proportion (i.e., $<5\%$) of total basal lipids (Fig. 3). In contrast, supplementation of control L cells with palmitic acid-BSA or phytanic acid-BSA increased the cellular levels of free fatty acids by 3.3-fold (Fig. 4) and

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Fig. 4. Effect of BSA-palmitic and BSA-phytanic acid supplementation on free fatty acid content in transfected L cells expressing SCP-2, SCP-x, and L-FABP proteins. Values are means ± SE ($n = 3–6$). Significance difference: *$P < 0.01$ compared with control cells supplemented with BSA only; $^{*}P < 0.05$ compared with control cells within the same supplementation group.

These data indicate that saturated fatty acid supplementation significantly elevated cellular levels of free fatty acids. However, this effect was not exacerbated by the presence of methyl branching (phytanic acid) in the fatty acyl chain.

Expression of intracellular fatty acid binding proteins differentially alters cellular levels of free (unesterified) fatty acids. Because the intracellular fatty acid binding proteins (SCP-2, SCP-x, and L-FABP) all interact with fatty acids, the possibility that expression of these proteins may increase the free fatty acid pool size was examined (Fig. 4). With one exception (i.e., SCP-2 overexpression and palmitic acid-BSA supplementation), expression of SCP-2, SCP-x, or L-FABP did not significantly increase the cellular level of free fatty acids under basal (BSA only), palmitic acid-BSA-, or phytanic acid-BSA-supplemented conditions (Fig. 4). On the contrary, expression of SCP-2, SCP-x, or L-FABP either had no effect or decreased the cellular level of free fatty acids (Fig. 4). Expression of SCP-x or L-FABP significantly (5.3- to 12-fold) decreased free fatty acid levels under basal (BSA only) or phytanic acid-BSA-supplemented conditions but not in palmitic acid-BSA-supplemented cells.

In summary, the expression of intracellular fatty acid binding proteins differentially decreased cellular levels of free fatty acids under basal conditions and in phytanic acid-BSA (but not palmitic acid-BSA)-supplemented cells in the following order: SCP-x $\geq$ L-FABP $>$ SCP-2. SCP-x expression reduced the free fatty acid mass to undetectable levels in phytanic acid-BSA-supplemented cells. These results showed that 1) expression of fatty acid binding proteins generally did not
increase free fatty acid pool size and 2) expression of SCP-x or L-FABP decreased the free (more toxic) forms of phytanic acid but not palmitic acid.

Supplementation of L cells with either branched- or straight-chain saturated fatty acids increased molar ratio of cholesterol to phospholipid. Culturing control L cells in the presence of palmitic acid-BSA or phytanic acid-BSA resulted in 1.6- and 2-fold (P < 0.05) increased molar ratio of cholesterol to phospholipid, respectively, compared with control cells fed BSA only (Fig. 5A). This effect was not due to altered cellular phospholipid mass (Fig. 5B). Instead, supplementation with palmitic acid-BSA or phytanic acid-BSA increased the cellular cholesterol mass 1.9- and 2.0-fold (P < 0.05, n = 4–5; Fig. 5C), respectively, compared with control cells supplemented with BSA (no fatty acid) only.

Although phospholipids are primarily membrane constituents, cholesterol is not only a component but also a constituent of the surface monolayer of intracellular lipid storage droplets (5). Whereas accumulation of excess cholesterol in membranes and/or in crystal-line form is deleterious to cellular function (reviewed in Refs. 22, 73, 74, 82, 83), incorporation into the surface monolayer of lipid droplets is important for their formation (reviewed in Refs. 5, 44).

Intracellular fatty acid binding protein expression differentially alters saturated fatty acid-induced increase in cholesterol-to-phospholipid ratio. Under basal conditions, control L cells exhibited a cholesterol-to-phospholipid molar ratio near 0.4 (Fig. 5A). In addition, under basal conditions this ratio was not significantly affected by expression of intracellular fatty acid binding proteins because neither the cholesterol nor the phospholipid content was significantly altered (Fig. 5, B and C). Likewise, expression of intracellular fatty acid binding proteins did not prevent the palmitic acid-BSA-induced increase in molar ratio of cholesterol to phospholipid compared with control cells fed BSA only (Fig. 5A). In contrast, expression of intracellular fatty acid binding proteins significantly prevented the phytanic acid-BSA-induced increase in molar ratio of cholesterol to phospholipid compared with control cells fed BSA only (Fig. 5A). In fact, the molar ratio of cholesterol to phospholipid in phytanic acid-BSA-supplemented cells expressing SCP-2, SCP-x, or L-FABP (Fig. 5A) did not significantly differ from the corresponding cells in the basal state (Fig. 5A). Expression of intracellular fatty acid binding proteins protected the cell from phytanic acid-BSA-induced elevation in cholesterol-to-phospholipid ratio primarily by preventing cholesterol accumulation (Fig. 5C) and, in the case of SCP-x, also by lowering cellular phospholipid content (Fig. 5B).

In summary, expression of intracellular fatty acid binding proteins differentially protected L cells from phytanic acid-BSA-, but not palmitic acid-BSA-, induced increase in cellular cholesterol content and cholesterol-to-phospholipid ratio.

Effect of methyl branching on saturated fatty acid-induced accumulation of neutral storage lipids of L cells: triacylglycerols and cholesteryl esters. Because saturated fatty acid supplementation increased the cellular cholesterol content (Fig. 5C), it was important to determine whether this increase was associated with increased formation of intracellular neutral storage lipids such as triacylglycerols and cholesteryl esters. Triacylglycerols and cholesteryl esters represent
a major storage depot for fatty acids in control L cells (56).

Under basal conditions the triacylglycerols (Fig. 6A) comprised only ∼5% of total lipids (Fig. 3). In contrast, supplementing the cells with palmitic acid-BSA (Fig. 6A) increased the triacylglycerol mass by 7.6-fold ($P < 0.001$) compared with control cells supplemented with BSA only ($P < 0.05$). In contrast, expression of SCP-2, SCP-x, and L-FABP proteins did not prevent formation of triacylglycerols for storage compared with control cells supplemented with the same fatty acids (Fig. 6A). Only in the case of SCP-x expression was a partial reduction in triglyceride accumulation observed on palmitic acid-BSA (but not BSA-phytanic acid) supplementation. In general, fatty acid binding protein expression appeared more effective in reducing the accumulation of cholesteryl esters (Fig. 6B) than of triacylglycerols (Fig. 6A) induced by palmitic acid-BSA or phytanic-BSA supplementation.

Roles of fatty acid methyl branching and intracellular fatty acid binding protein expression in saturated fatty acid uptake in L cells. Together, the data in the preceding sections suggested that SCP-2, SCP-x, and L-FABP expression differentially modulated the palmitic acid- and palmitic acid-induced lipid accumulation in part by decreasing uptake of phytanic, but not palmitic, acid. To determine whether this is the case, total uptake of [3H]palmitic and [3H]phytanic acid was examined in control and SCP-2-, SCP-x-, and L-FABP-expressing cells. In control cells, the uptake of straight-chain saturated fatty acid (i.e., [3H]palmitic acid) was essentially the same as that of the methyl-branched chain saturated fatty acid (i.e., [3H]phytanic acid) (Fig. 7). However, expression of intracellular fatty acid binding proteins differentially affected [3H]palmitic and [3H]phytanic acid uptake (Fig. 7). None of the fatty acid binding proteins inhibited [3H]palmitic acid; in fact, expression of L-FABP stimulated [3H]palmitic acid uptake by ∼30% ($P < 0.05$). In contrast, expression of SCP-2, SCP-x, and L-FABP significantly ($P < 0.05$) inhibited the uptake of [3H]phytanic acid by 2.8-, 7.5-, and 2.3-fold, respectively, compared with control cells (Fig. 7). In summary, only L-FABP expression enhanced uptake of [3H]palmitic acid, whereas the expression of all three intracellular fatty acid binding proteins (SCP-2, SCP-x, and L-FABP) inhibited uptake of [3H]phytanic acid. Thus reduced [3H]phytanic acid uptake (Fig. 7) was consistent with the reduced total lipid accumulation exhibited in SCP-2-, SCP-x-, and L-FABP-expressing cells supplemented with phytanic acid-BSA (Fig. 3). Furthermore, the lack of inhibition of [3H]palmitic acid uptake (Fig. 7) in the fatty acid binding protein-expressing cells was consistent in general with the lesser ability of intracellular fatty acid
binding protein-expressing cells to prevent the palmitic acid-induced lipid accumulation (Fig. 3).

Differential oxidation of branched- vs. straight-chain saturated fatty acids: effects of intracellular binding protein expression. As indicated in the introductory paragraphs of this article, both in vitro studies and some animal studies suggested that intracellular fatty acid binding proteins (SCP-2, SCP-x, and L-FABP) may function in the oxidation of straight-chain fatty acids and, at least in the case of SCP-x, in peroxisomal oxidation of branched-chain fatty acids (2, 80). However, neither the role of SCP-x in straight-chain (palmitate) oxidation nor the role of SCP-2 and L-FABP in either type of fatty acid oxidation has been directly addressed in any cell type or animal. The differential protective effects of intracellular fatty acid binding proteins on phytic acid, but not palmitic acid, induced lipid accumulation noted in the preceding sections may, at least in part, be due to these fatty acid binding proteins differentially facilitating the oxidation of phytic acid compared with that of palmitic acid.

Significant differences were observed between the total oxidation of straight- and branched-chain fatty acids in control L cells. The total oxidation of \(^3\)Hpalmitic acid was nearly fivefold \((P < 0.001)\) greater than that of \(^3\)Hphytanic acid (Table 2). Although expression of intracellular fatty acid binding proteins did not significantly affect the total oxidation of \(^3\)Hpalmitic acid, total oxidation of \(^3\)Hphytanic acid was 3.8-, 9.3-, and 100-fold slower \((P < 0.01)\) in SCP-2-, SCP-x-, and L-FABP-expressing cells, respectively, compared with total \(^3\)Hphytanic acid oxidation in control cells (Table 2).

When fatty acid oxidation was expressed as percent (of total fatty acid taken up) oxidized, >20% of \(^3\)Hpalmitic acid taken up was oxidized compared with only 5% of \(^3\)Hphytanic acid taken up and oxidized under the conditions of the experiment (Table 2). The expression of SCP-x and L-FABP slightly reduced the percentage of total \(^3\)Hpalmitic acid taken up and oxidized by 27% and 35% \((P < 0.01)\), respectively, compared with control (Table 2). In contrast, expression of all SCP-2, SCP-x, and L-FABP reduced the percentage of total \(^3\)Hphytanic acid taken up and oxidized by 1.5-, 13.5-, and >100-fold, respectively, compared with control cells (Table 2).

In summary, only a small proportion of \(^3\)Hpalmitic acid and \(^3\)Hphytanic acid taken up was oxidized, 21% and 5%, respectively, when control L cells were supplemented with palmitic acid-BSA and phytic acid-BSA. Expression of the three fatty acid binding proteins (SCP-2, SCP-x, and L-FABP) did not stimulate the oxidation of either straight-chain (palmitic acid) or branched-chain (phytanic acid) fatty acid. On the contrary, SCP-x and L-FABP (but not SCP-2) modestly inhibited rather than stimulated the oxidation of palmitic acid. Even more striking, expression of all three fatty acid binding proteins (SCP-2, SCP-x, and L-FABP) resulted in severalfold less oxidation of the methyl-branched, straight-chain saturated fatty acid (i.e., phytic acid).

Effect of SCP-2, SCP-x, and L-FABP expression on incorporation of radiolabeled palmitic acid and phytic acid into esterified lipids. Because only a small proportion of \(^3\)Hpalmitic acid and \(^3\)Hphytanic acid

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**Table 2. \(^3\)Hpalmitic acid and \(^3\)Hphytanic acid oxidation in transfected L cells expressing SCP-2, SCP-x, and L-FABP**

<table>
<thead>
<tr>
<th>Cells</th>
<th>(^3)Hpalmitic acid</th>
<th>(^3)Hphytanic acid</th>
<th>%Oxidation of Total Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{dpm} \times 10^{13} \mu \text{g protein})</td>
<td>(\text{dpm} \times 10^{13} \mu \text{g protein})</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.22 ± 0.21(8)</td>
<td>0.463 ± 0.029(8)*</td>
<td>20.99 ± 0.74(8)</td>
</tr>
<tr>
<td>SCP-2</td>
<td>1.98 ± 0.09(6)</td>
<td>0.123 ± 0.034(4)*†</td>
<td>23.08 ± 0.22(4)*</td>
</tr>
<tr>
<td>SCP-x</td>
<td>1.58 ± 0.22(4)*</td>
<td>0.05 ± 0.035(3)*†</td>
<td>15.27 ± 1.21(4)*</td>
</tr>
<tr>
<td>L-FABP</td>
<td>1.86 ± 0.04(6)*</td>
<td>ND†</td>
<td>13.69 ± 0.16(6)*</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of experiments indicated in parentheses. Cells were supplemented with fatty acid-BSA complexes as described in MATERIALS AND METHODS. Total oxidation was measured as the formation of water-soluble tritiated degradation products released into the culture medium. % Oxidation refers to the % of total \(^3\)Hpalmitic acid or \(^3\)Hphytanic acid taken up that was oxidized. SCP, sterol carrier protein; L-FABP, liver fatty acid binding protein; ND, not detected. *Significance \((P < 0.01)\) of the fatty acid oxidation or % oxidation compared with the control cells supplemented with the same fatty acid-BSA; † significance \((P < 0.001)\) of the \(^3\)Hpalmitic acid oxidation or % oxidation compared in the same cell line.
taken up was oxidized in the control cells, i.e., 21% and 5%, respectively (Table 2), it was important to determine whether the remainder was esterified or remained as free fatty acids in the cells. Only 14% of [3H]palmitic acid taken up was esterified in control L cells supplemented with palmitic acid-BSA (Fig. 8). Similarly, only a small portion (i.e., 8.6%) of [3H]phytanic acid taken up was esterified in control L cells supplemented with phytanic acid-BSA (Fig. 8). However, this was 39% less \( (P < 0.05) \) than observed with [3H]palmitic acid that was taken up. Expression of SCP-x and L-FABP (but not SCP-2) increased the portion of [3H]palmitic acid taken up that was esterified 1.6- and 1.3-fold, respectively (Fig. 8). In contrast, expression of SCP-2, SCP-x, and L-FABP decreased the percentage of [3H]phytanic acid taken up that was esterified 2.4-, 5.4-, and 1.8-fold, respectively (Fig. 8).

In summary, these data from phytanic acid-BSA-supplemented cells demonstrated that fatty acid binding expression inhibited [3H]phytanic acid esterification (Fig. 8). This observation was in contrast to the increase in [3H]palmitic acid esterification noted when cells expressing these fatty acid binding proteins (SCP-x, L-FABP) were supplemented with palmitic acid-BSA.

**Effect of SCP-2, SCP-x, and L-FABP expression on palmitic acid-BSA- and phytanic acid-BSA-induced cytotoxicity in L cell fibroblasts.** The above differential effects of intracellular fatty acid binding proteins on palmitic acid- vs. phytanic acid-induced lipid accumulation as well as uptake and oxidation of the respective fatty acids suggested that SCP-2, SCP-x, or L-FABP might also alter the toxicity of these fatty acids, especially that of phytanic acid. In all of the preceding studies on palmitic acid-BSA and phytanic acid-BSA effects (i.e., lipid accumulation, uptake, oxidation, esterification) the cells were incubated with complexes made of 25 \( \mu \text{M} \) fatty acid and \( \sim 90 \mu \text{M} \) BSA for a period of 6 h. Under these conditions, no overt cytotoxicity (based on protein/dish, cell number/dish, live/dead) was noted for any of the cell lines examined (data not shown). However, at longer incubation times (\( \geq 12 \) h) and/or higher concentrations of saturated fatty acids significant cytotoxicity was observed.

Cytotoxicity was detected by staining the cells with calcein AM and ethidium homodimer-1 and detecting the proportion of live to dead cells by flow cytometry as described in MATERIALS AND METHODS. Control cells supplemented only with BSA were almost 100% viable up to 80 h of incubation (Fig. 9). Although high concentrations of either palmitic acid-BSA or phytanic acid-BSA were extremely cytotoxic after 12 h of incubation, at 100 \( \mu \text{M} \) palmitic acid-BSA the control cells exhibited >98% viability over the same time frame and thereafter viability decreased to <10% by 70 h of incubation (Fig. 9). In contrast, supplementation with 100 \( \mu \text{M} \) phytanic acid-BSA resulted in <10% viability after 12 h of incubation, which decreased to <1% viability by 20 h of incubation (Fig. 9). Thus phytanic acid appeared more cytotoxic than palmitic acid.

To determine whether SCP-2, SCP-x, or L-FABP expression altered the toxicity of the straight-chain saturated fatty acids, cells were supplemented for 12 h with palmitic acid-BSA and phytanic acid-BSA at fatty acid concentrations of 50 and 25 \( \mu \text{M} \) each (Table 3). At 50 \( \mu \text{M} \) palmitic acid-BSA or 25 \( \mu \text{M} \) phytanic acid-BSA, the control cells showed >95% viability after 12 h. In contrast, expression of the three fatty acid binding proteins (SCP-2, SCP-x, L-FABP) dramatically enhanced the cytotoxicity of 50 \( \mu \text{M} \) phytanic acid-BSA such that <1% of cells remained viable (Table 3). This potentiation of phytanic acid cytotoxicity by intracellular fatty acid was observed even at lower concentra-
Table 3. Effect of SCP-2, SCP-x, and L-FABP expression on palmitic and phytanic acid cytotoxicity

<table>
<thead>
<tr>
<th>Cells</th>
<th>Palmitic acid (50 μM)</th>
<th>Phytanic acid (50 μM)</th>
<th>Phytanic acid (25 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96.2 ± 0.3</td>
<td>1.06 ± 0.07†</td>
<td>94.0 ± 0.2</td>
</tr>
<tr>
<td>SCP-2</td>
<td>96.2 ± 0.1</td>
<td>0.76 ± 0.20†</td>
<td>32.4 ± 1.0††</td>
</tr>
<tr>
<td>SCP-x</td>
<td>95.0 ± 0.1</td>
<td>0.42 ± 0.07**†</td>
<td>23.4 ± 1.0††</td>
</tr>
<tr>
<td>L-FABP</td>
<td>96.2 ± 0.3</td>
<td>0.71 ± 0.20†</td>
<td>2.3 ± 0.2††</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cells were supplemented with fatty acid-BSA complexes as described in MATERIALS AND METHODS. Cytotoxicity was determined by flow cytometry after cells were cultured for 12 h with the indicated fatty acid-BSA complex as described in MATERIALS AND METHODS. *Significance (P < 0.01) compared with the control cells labeled with the same fatty acid-BSA; † significance (P < 0.001) compared with the respective cell line supplemented with palmitic acid-BSA.

DISCUSSION

Almost nothing is known regarding potential effects of intracellular fatty acid binding proteins on lipid accumulation and toxicity of high levels of branched- and straight-chain fatty acids. To investigate these possibilities in detail, three intracellular fatty acid binding proteins (SCP-2, SCP-x, L-FABP) were individually overexpressed in transfected L cells. L cell
fibroblasts were an appropriate model because protein levels of L-FABP, SCP-2, SCP-x (Fig. 1), or other known members of the intracellular fatty acid binding protein family were below the level of detection (37). The three transfected L cell lines overexpressed SCP-x, SCP-2, and L-FABP at levels (0.01%, 0.036%, and 0.40% of cytosol protein, respectively) and proportions (10- to 40-fold more L-FABP than SCP-2 or SCP-x) found in animal tissues (reviewed in Refs. 23, 37, 47, 71). The transfected L cells overexpressing 58-kDa SCP-x partially posttranslationally processed the 58-kDa SCP-x to yield detectable 43-kDa thiolase and 13-kDa SCP-x partially posttranslationally processed the 58-kDa SCP-x to yield detectable 43-kDa thiolase and 13-kDa SCP-2 (~2-fold increased), consistent with earlier data (4). Examination of a variety of transfected clones expressing different levels of intracellular fatty acid binding proteins showed that the direction of their effect on lipid metabolism was similar and dose responsive (4, 37, 50, 55). The highest-expression clone for each intracellular fatty acid binding protein with levels closest to physiological significance was used for the studies presented here. This allowed analysis of the effect of the individually expressed proteins on lipid accumulation and toxicity induced by high levels of branched- and straight-chain fatty acids, independent of the presence and/or concomitant upregulation of other fatty acid binding proteins. It should be noted that L cells contain the appropriate machinery for fatty acid uptake and esterification (reviewed in Ref. 47) and fatty acid oxidation (e.g., mitochondria and peroxisomes; Fig. 2, Refs. 24, 68, 72, 78) and have been used to examine fatty acid-induced lipid accumulation and toxicity (25, 26). Fibroblasts have been used by many laboratories to study human defects of fatty acid oxidation (reviewed in Refs. 93, 98). The data presented here contributed significantly to our understanding of the roles of intracellular fatty acid binding proteins on branched- vs. straight-chain saturated fatty acid-induced cellular lipid accumulation, uptake, oxidation, and cytotoxicity.

First, it was shown that the methyl-branched saturated fatty acid (phytanic acid) was much more cytotoxic than the straight-chain saturated fatty acid (palmitic acid), even when L cells were exposed to much lower concentration and/or exposure time with phytic acid than palmitic acid. Interestingly, the greater cytotoxicity of phytic acid in L cells did not correlate with any differences in total lipid accumulation or specific lipid class accumulation. Similar lack of correlation of lipid accumulation with cytotoxicity has also been reported for saturated vs. unsaturated fatty acids in L cells in that both similarly induce lipid accumulation but only the saturated fatty acids are cytotoxic (25). Because the greater cytotoxicity of phytic acid was not due to any differences in uptake of [2,3-3H]phytanic acid vs. [9,10-3H]palmitic acid, two factors appeared to be consistent with the greater cytotoxicity of phytic acid. 1) [2,3-3H]phytanic acid oxidation was more than fourfold slower than that of [9,10-3H]palmitic acid. 2) [2,3-3H]phytanic acid esterification was nearly 40% slower than [9,10-3H]palmitic acid. The lower oxidation and esterification of phytic acid vs. palmitic acid may be accounted for by the much lower activity of fatty acyl CoA synthases with branched- vs. straight-chain fatty acids (99). Together with the fact that the total mass of unesterified free fatty acid in the palmitic acid- and phytanic acid-supplemented cells did not differ in control cells (Fig. 4), these data suggest that a greater proportion of phytic acid remained unesterified in the free fatty acid fraction, where it was apparently more cytotoxic than the unesterified palmitic acid. Interestingly, high levels of saturated fatty acids inhibit cell growth and cause cell death by disruption of cellular metabolism because of excessive lipid accumulation of triglycerides and free fatty acids to the endoplasmic reticulum (ER), where they crystallize (25). In contrast, high levels of unsaturated fatty acids typically accumulate in cytoplasmic lipid droplets, a storage depot (25). The incorporation of branched-chain saturated fatty acids into membranes induces the formation of abnormal, inverted nonbilayer phases (79) and increases membrane fluidity (17, 21, 43). Both factors may contribute significantly to altered membrane functions (43). Although their uptake was similar under the conditions examined, the fourfold lower oxidation and 40% lower esterification of phytic acid vs. palmitic acid would suggest that higher levels of branched-chain fatty acids accumulated in L cell membranes and may therefore be more damaging to the membranes and their functions.

Second, under conditions in which fatty acid-induced lipid accumulation was observed, the expression of the intracellular fatty acid binding proteins did not protect L cell fibroblasts from the cytotoxicity of saturated branched-chain phytic acid. Although expression of these proteins (SCP-2, SCP-x, and L-FABP) reduced the phytanic acid-induced lipid accumulation, these proteins concomitantly enhanced the cytotoxicity of phytic acid. The enhanced cytotoxicity was not due to increased [2,3-3H]phytanic acid uptake or to increased levels of fatty acid transporters in the plasma membrane. Instead, increased toxicity correlated with
significant inhibition of [2,3-3H]phytanic acid oxidation (in the order L-FABP >> SCP-x >> SCP-2) and inhibition of [2,3-3H]phytanic acid esterification (in the order SCP-x > SCP-2, L-FABP). These effects of intracellular fatty acid binding protein expression on phytanic acid were in contrast to those observed with palmitic acid: unaltered (or slightly increased) [9,10-3H]palmitic acid uptake, unaltered (or only modestly decreased) [9,10-3H]palmitic acid oxidation, and unaltered (or increased) [9,10-3H]palmitic acid esterification. Thus expression of intracellular fatty acid binding protein increased the proportion of [2,3-3H]phytanic acid present in unesterified form more than that of [9,10-3H]palmitic acid in unesterified form. Increased cytotoxicity with cells overexpressing these proteins would be expected if the unesterified branched-chain fatty acids accumulated in membranes, leading to the complications described above.

Third, the present studies for the first time directly examined the effect of intracellular fatty acid binding protein expression in intact cells on fatty acid oxidation in intact cells. At levels (i.e., 25 μM) at which both these saturated fatty acids induce intracellular lipid accumulation, expression of all three fatty acid binding proteins inhibited total phytic acid, but not palmitic, acid oxidation by 35–99%. Even when expressed as a percentage of fatty acid taken up and oxidized, expression of all three proteins much more strongly inhibited oxidation of phytic acid. In contrast, at low concentrations (i.e., 50 nM phytic acid or palmitic acid) expression of fatty acid binding protein did not inhibit phytic or palmitic acid oxidation (data not shown). Thus, in contrast to studies with stimulatory effects of fatty acid binding proteins on palmitic acid oxidation by isolated mitochondria in vitro (62), expression of these proteins in L cells did not enhance palmitic or phytic acid oxidation in intact cells. These fatty acid binding proteins did not inhibit phytic acid oxidation by decreasing phytic acid uptake or the levels of plasma membrane transport proteins (Fig. 10) or other intracellular fatty acid/fatty acyl CoA binding proteins (Fig. 10). Furthermore, the inhibition was specific for the branched-chain compared with the straight-chain saturated fatty acid. The molecular mechanism accounting for this specificity is not yet known. Future studies are needed.

Fourth, the observation that the L-FABP expression elicited the highest cytotoxicity of phytic acid in L cells may help explain recent observations of phytic acid toxicity in SCP-x/SCP-2 gene-ablated mice (81). In that study, it was observed that phytic acid toxicity was dramatically increased after feeding a diet high in phytic acid to SCP-x/SCP-2 gene-ablated mice but not control mice (81). The latter finding, at first glance, apparently contradicted the observations presented here with control L cells (no SCP-x or SCP-2) vs. transfected L cells overexpressing SCP-x or SCP-2. However, this apparent discrepancy may be readily explained by the concomitant fourfold upregulation of L-FABP in livers of the SCP-x/SCP-2 gene-ablated mice. L-FABP upregulation to near millimolar levels was much greater than the loss of micromolar amounts of SCP-x/SCP-2 in the knockout mice and could therefore account for the increased phytic acid toxicity, consistent with the enhanced cytotoxicity of phytic acid in L-FABP-overexpressing L cells reported here.

Several additional factors contribute to the enhanced phytic acid cytotoxicity in cells overexpressing intracellular fatty acid binding proteins. 1) Expression of intracellular fatty acid binding proteins decreased the ratio of cholesterol to phospholipid in the presence of phytic, but not palmitic, acid. Decreasing the plasma membrane cholesterol-to-phospholipid ratio significantly increases membrane fluidity and inhibits Na+-K+-ATPase activity (34). Agents that fluidize plasma membranes significantly inhibit Na+-K+-ATPase activity (89, 90). Fatty acid-induced cytotoxicity is observed in L cells when membrane fluidity is outside an optimal range (75). 2) Expression of intracellular fatty acid binding proteins increased the levels of unesterified, free phytic acid much more than free palmitic acid. Branched-chain fatty acids are much more membrane disruptive than straight-chain saturated fatty acids (17, 21, 25, 26, 43, 48). 3) The preferential accumulation of free branched-chain fatty acids in intracellular fatty acid binding protein-expressing cells can differentially alter intracellular signaling pathways. Branched-chain saturated fatty acids alter intracellular signaling pathways (16), but in a manner different from the effect of straight-chain saturated fatty acids (reviewed in Ref. 13). 4) The increase in free phytic acid compared with free palmitic acid induced by intracellular fatty acid binding protein expression can differentially affect nuclear regulatory proteins. In contrast to straight-chain saturated fatty acid, phytic acid is a potent peroxisome proliferator-activated receptor (PPAR) ligand and transcriptional activator (102). SCP-2 expression was increased by phytic acid-BSA, but not palmitic acid-BSA, supplementation in all the cell lines examined (data not shown). Interestingly, an imperfect DR1 element, which is potentially a binding site for PPAR, was reported in the promoter region of SCP-2, making this a distinct possibility (58). These data suggest the possibility that phytic acid could inappropriately affect PPAR transcriptional activity to upregulate additional fatty acid binding proteins that enhance phytic acid transport to peroxisomes. Consistent with this possibility, phytic acid induced L-FABP expression fourfold in SCP-x/SCP-2 gene-ablated mice (81).

In summary, the studies presented here for the first time directly examined whether expression of intracellular fatty acid binding proteins (SCP-2, SCP-x, L-FABP) could protect cells from lipid accumulation and/or cytotoxicity induced by high levels of branched- or straight-chain saturated fatty acids. It was shown that, although SCP-x expression partially protected the cells against straight-chain saturated fatty acid (palmitic acid)-induced lipid accumulation, neither SCP-2 nor L-FABP did so. In contrast, although expression of all three proteins (SCP-2, SCP-x, and L-FABP) partially protected the cells against branched-
chain saturated fatty acid (phytanic acid)-induced lipid accumulation, all three intracellular binding proteins enhanced the cytotoxicity of phytanic acid but not palmitic acid. The enhancement of phytanic acid cytotoxicity appeared to be due primarily to inhibition of palmitic acid. The enhancement of phytanic acid cyto-
toxicity, enhanced the cytotoxicity of phytanic acid but not accumulation, all three intracellular binding proteins.

This work was supported in part by National Institutes of Health Grants DK-41402 and GM-31651.

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