Triacsin C blocks *de novo* synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipid: evidence for functionally separate pools of acyl-CoA

R. Ariel IGAL, Ping WANG and Rosalind A. COLEMAN

Departments of Nutrition and Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7400, U.S.A.

The trafficking of acyl-CoAs within cells is poorly understood. In order to determine whether newly synthesized acyl-CoAs are equally available for the synthesis of all glycerolipids and cholesterol esters, we incubated human fibroblasts with [¹⁴C]oleate, [³H]arachidonate or [³H]glycerol in the presence or absence of triacsin C, a fungal metabolite that is a competitive inhibitor of acyl-CoA synthetase. Triacsin C inhibited *de novo* synthesis from glycerol of triacylglycerol, diacylglycerol and cholesterol esters by more than 93%, and the synthesis of phospholipid by 83%. However, the incorporation of oleate or arachidonate into phospholipids appeared to be relatively unimpaired when triacsin was present. Diacylglycerol acyltransferase and lysophosphatidylcholine acyltransferase had similar dependences on palmitoyl-CoA in both liver and fibroblasts; thus it did not appear that acyl-CoAs, when present at

low concentrations, would be preferentially used to acylate lysophospholipids. We interpret these data to mean that, when fatty acid is not limiting, triacsin blocks the acylation of glycerol 3-phosphate and diacylglycerol, but not the reacylation of lysophospholipids. Two explanations are possible: (1) different acyl-CoA synthetases exist that vary in their sensitivity to triacsin; (2) an independent mechanism channels acyl-CoA towards phospholipid synthesis when little acyl-CoA is available. In either case, the acyl-CoAs available to acylate cholesterol, glycerol 3-phosphate, lysophosphatidic acid and diacylglycerol and those acyl-CoAs that are used by lysophospholipid acyltransferases and by ceramide *N*-acyltransferase must reside in two non-mixing acyl-CoA pools or, when acyl-CoAs are limiting, they must be selectively channelled towards specific acyltransferase reactions.

INTRODUCTION

Long-chain acyl-CoAs are substrates for *de novo* synthesis of glycerolipids and cholesterol esters, reacylation of lysophospholipids, and *N*-acylation of proteins and sphingosine. They are also precursors for fatty acid elongation and desaturation reactions and substrates for β -oxidation. Long-chain acyl-CoAs function in vesicle trafficking within cells, and evidence obtained *in vitro* suggests that they play regulatory roles by inhibiting fatty acid synthesis [1], activating protein kinase C [1], inhibiting the nuclear thyroid hormone receptor [2] and regulating the activation of O₂⁻· generation and degranulation of neutrophils [3]. These functions take place in different cellular compartments but nothing is known about the partitioning of acyl-CoAs within cells, although it has been hypothesized that acyl-CoAs that are synthesized on the outer mitochondrial membrane and on the peroxisomal membrane might be destined for oxidation, whereas those synthesized in the endoplasmic reticulum might play biosynthetic roles. This view fails to take into account the facts that: (1) in addition to carnitine palmitoyltransferase I, which channels acyl-CoAs towards β -oxidation, the outer mitochondrial membrane contains a glycerol-3-phosphate acyltransferase isoenzyme that is the committed step in glycerolipid biosynthesis [4]; (2) acyl-CoA-binding protein could transport acyl-CoAs to different sites within cells [5]; (3) peroxisomes can use acyl-CoAs both for oxidation [2] and for the synthesis of alkyl lipids [5]. Further, the problem of regulation of the numerous individual acyltransferase functions within the endoplasmic reticulum remains unresolved.

In studying human fibroblasts to elucidate the biochemical defect in neutral lipid storage disease [6], we used triacsin C, a

potent competitive inhibitor of long-chain acyl-CoA synthetase (ACS) [7,8] to block reuse of lipolysed fatty acids. Because our results suggested that the presence of triacsin C inhibited *de novo* synthesis of triacylglycerol and phospholipid from glycerol but did not inhibit the acylation of phospholipids, we initiated further studies to examine acylation in human fibroblasts. These studies provide evidence for functional partitioning of acyl-CoA within cells.

EXPERIMENTAL

Materials

Silica-gel G plates were from Whatman. [2-³H]Glycerol, [³H]palmitate, [1-¹⁴C]oleate and [5,6,8,9,11,12,14,15-³H]arachidonic acid were from Amersham Life Sciences Co. Glycerol, BSA (essentially fatty acid-free), phospholipase A₂ (*Naja naja*) and sodium oleate were from Sigma. Lipid standards, lysophosphatidylcholine and *sn*-1,2-diacylglycerol were from Serdary. Triacsin C (>95% pure) was from Biomol Research Laboratories. Tissue culture supplies and fetal bovine serum (FBS) were from Gibco. [6-(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid (C6-NBD-PC), where PC is phosphatidylcholine, was from Avanti Polar Lipids.

Cell culture

Normal human skin fibroblasts were obtained from the American Type Tissue Culture Collection (cell line CCD). Normal fibroblasts were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in minimum essential medium with Earle salts plus 1% non-

Abbreviations used: ACS, acyl-CoA synthetase; C6-NBD, [6-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid; DGAT, diacylglycerol acyltransferase; FBS, fetal bovine serum; LPC-AT, lysophosphatidylcholine acyltransferase; E-MEM, minimum essential medium with Earle salts plus 1% non-essential amino acids.

essential amino acids (E-MEM) and 10 % FBS that had been heat inactivated for 60 min at 56 °C. The concentration of oleic acid from FBS was 4 μ M. The medium was changed every 2–3 days. For each experiment, total cellular DNA content was measured fluorimetrically [9].

Dose–response of triacsin C on fatty acid incorporation into lipids

Normal fibroblasts were grown to near-confluence in 60 mm dishes. Cells were incubated for 9 h with 0.25 μ Ci of [14 C]oleic acid in the presence or absence of 100 μ M unlabelled sodium oleate. Monolayers were simultaneously treated with different concentrations of triacsin C (from 0.25 to 10 μ M) or its vehicle (DMSO (0.1 %, v/v)). The incubation was terminated by discarding the labelling medium, and washing the cells twice with 0.1 % BSA in PBS at 37 °C. Fibroblasts were rapidly scraped into two additions of 1 ml of methanol and one of 0.5 ml of water. Chloroform (1 ml) was added and total cell lipids were extracted [10] and concentrated using a SpeedVac concentrator (Savant, Hicksville, NY, U.S.A.).

Pulse radiolabelling

Fibroblasts were seeded in 60 mm dishes and grown to confluence and incubated with 0.5 μ Ci of [14 C]oleic acid, 2 μ Ci of [3 H]glycerol or 0.25 μ Ci of [3 H]arachidonic acid in 2 ml of 10 % FBS/E-MEM. The labelling medium contained 0.1 mM unlabelled sodium oleate or arachidonic acid to promote incorporation of label into triacylglycerol. Sodium oleate was dissolved in water at 65 °C and added to dry [14 C]oleate. Then 1 % BSA (final concentration) in E-MEM was added. Monolayers were treated with 5 μ M triacsin C in DMSO (0.1 %, v/v, final concentration). Control cells received the vehicle alone. After 1, 3, 6 or 24 h, the incubation was stopped by aspirating the medium. Residual radiolabel was removed by washing the cells twice with 0.1 % BSA in PBS at 37 °C. Lipids were extracted and concentrated by following the procedure described above.

[14 C]Oleate incorporation into the *sn*-2 position of phospholipid

Fibroblasts were grown in 100 mm dishes to near-confluence and incubated with 100 μ M [14 C]oleic acid (0.5 μ Ci/dish) complexed with 1 % BSA in the same growing medium in the presence or absence of 5 μ M triacsin C. After 24 h the medium was aspirated and the residual label was washed with 0.1 % BSA in PBS. Cells were scraped into methanol and lipids were extracted as described above. The polar and neutral lipid fractions were separated on silica-gel G plates in hexane/ethyl ether/acetic acid (80:20:1, by vol.). Polar lipids, which remained at the origin, were scraped from the plate and extracted twice from the silica gel with chloroform/methanol (1:1, v/v). The solvents were dried and the phospholipids were diluted in 100 μ l of DMSO. Half of each sample was treated with buffer alone (50 μ M Tris, pH 8.6, 25 mM CaCl₂, 1 mM EDTA) and half with 800 units of phospholipase A₂ in 100 μ l of buffer. The incubation was performed at 37 °C in an orbital shaker for 1 h. The reaction was stopped by adding 1.5 ml of chloroform/methanol (1:2, v/v), and lipids were extracted [10]. The lipid extracts were then chromatographed with hexane/ethyl ether/acetic acid (80:20:1, by vol.) in order to separate unesterified fatty acid from total polar lipids. The percentage of fatty acids released from phospholipid was calculated after scanning the plate in a Bioscan Image System. To determine whether the phospholipase A₂ hydrolysis was complete under these conditions and to check the specificity of the enzyme, we incubated C6-NBD-PC (which contains the fluorescent fatty acid analogue C6-NBD in the *sn*-

2 position) under the same conditions. The C6-NBD fluorophore was released completely and no fluorescent *sn*-2-lysophosphatidylcholine was detected after chromatography in a unidirectional two-solvent system [6]. These data indicate that the phospholipase A₂ acted specifically and completely on the *sn*-2 position.

Lipid analysis

Aliquots of the lipid extracts were spotted on 0.25 mm silica-gel G plates. Neutral and polar lipids were resolved in the same plate by a combination of a one-directional double-solvent system. First, phospholipids were separated by running the chromatoplate in chloroform/methanol/ammonium hydroxide (65:25:4, by vol.) to 8 cm from the top. Then the residual solvents on the plate were evaporated under a stream of N₂. To resolve neutral lipid species, the plate was rerun in the same direction to the top in heptane/isopropyl ether/acetic acid (60:40:4, by vol.), in order. Samples were chromatographed in parallel with authentic standards. Lipid products labelled with 3 H or 14 C were visualized using a BioScan Image 200 System (Washington, DC, U.S.A.). The 3 H-labelled spots were scraped into vials and counted in a liquid-scintillation counter. The 14 C-labelled spots were quantified by the BioScan 200 system.

Enzyme assays

In order to compare dependence on palmitoyl-CoA of diacylglycerol acyltransferase (DGAT) and lysophosphatidylcholine acyltransferase (LPC-AT) under the same conditions, we assayed each of these activities in 175 mM Tris/HCl, pH 8.0, containing 8 mM MgCl₂, 1 mg/ml BSA, 50 μ M lysophosphatidylcholine, 200 μ M *sn*-1,2-diacylglycerol in acetone (2.5 % of the final 200 μ l volume) and 30 μ g of microsomal protein for liver or 30 μ g of total particulate protein for fibroblasts. Forty-minute assays were started by adding 30 μ M [3 H]palmitoyl-CoA. For DGAT, the reaction product was extracted into heptane [11]. For LPC-AT, the reaction was stopped by adding 4 ml of cold chloroform/methanol (2:1, v/v), then water (800 μ l) to break the phases. The tubes were vortex-mixed and centrifuged at 1100 g for 10 min, and the upper phase was discarded. The lower phase was washed twice with 2 ml of water. An aliquot was counted and the remainder was evaporated in a Speed-Vac Concentrator and chromatographed on silica-gel G plates in hexane/ethyl ether/acetic acid (80:20:1, by vol.) with authentic standards. Phosphatidylcholine (> 90 % of the labelled phospholipid product) and other products were scraped off and counted and the specific radioactivity was calculated. Both assays measured initial rates.

Other methods

Near-confluent fibroblasts were scraped from the dishes and total particulate preparations were obtained and stored at –80 °C [6]. KCl-washed liver microsomes from 200 g Sprague–Dawley rats were obtained [12] and stored at –80 °C. ACS was assayed using 10–30 μ g of total particulate protein and 50 μ M [14 C]oleate as described [13]. [3 H]Palmitoyl-CoA was synthesized enzymically [14].

RESULTS

Triacsin inhibition of oleate incorporation into lipid

ACS activity was 0.92 nmol/min per mg of protein in total particulate preparations from human fibroblasts. This activity

Figure 1 Incorporation of [^{14}C]oleate into (A) triacylglycerol and phospholipid and (B) fatty acid and cholesterol ester in the presence of various concentrations of triacsin C

Preconfluent cells were incubated for 9 h with 100 μM [^{14}C]oleate in the presence of various concentrations of triacsin C, as indicated. Each data point represents the mean $\pm$ S.D. from triplicate samples. Some error bars are hidden by the symbol.

Figure 2 Incorporation of a low concentration of fatty acid into (A) triacylglycerol and phospholipid and (B) diacylglycerol and fatty acid in the presence of different concentrations of triacsin C

Confluent cells were incubated for 9 h with 2 μM [^{14}C]oleate plus concentrations of triacsin C as indicated. Each data point represents the mean $\pm$ S.D. from triplicate samples. Some error bars are hidden by the symbol.

was inhibited by 68 % in the presence of 5 μM triacsin C. In a 9 h incubation with 100 μM oleate, the ACS inhibitor triacsin C (0.25 μM) inhibited [^{14}C]oleate incorporation into total glycerolipid by 40 % (Figure 1A). Maximal inhibition of 57 % was observed when 10 μM triacsin C was present. Analysis of the glycerolipids formed demonstrated that incorporation of oleate into triacylglycerol was inhibited by 80 and 91 % in the presence of 0.25 and 10 μM triacsin C respectively (Figure 1A), and incorporation of [^{14}C]oleate into cholesterol ester was inhibited by 75 and 91 % by 0.25 and 10 μM triacsin respectively (Figure 1B). No change was observed in labelled fatty acid. In contrast, [^{14}C]oleate incorporation into phospholipid was not affected (Figure 1A). This experiment suggested that some acyl-CoA was formed in the presence of triacsin C, but that this acyl-CoA pool was available for the synthesis of phospholipid, but not of triacylglycerol or cholesterol esters.

In order to determine whether the relative distribution of esterified fatty acid would be different if only a small amount of fatty acid was available to the cells, the experiment was repeated with 2 μM [^{14}C]oleate. When the amount of fatty acid was limited, the incorporation of label into triacylglycerol relative to phospholipid was markedly inhibited (Figure 2A). Triacsin C at

Figure 3 Time course of glycerol incorporation into triacylglycerol (TG; top left), phospholipid (PL, top right) or diacylglycerol (DAG, bottom) in the presence and absence of triacsin C

Cells were incubated with 2 μCi of [^3H]glycerol and 100 μM oleate plus or minus 5 μM triacsin C for up to 6 h. Each data point represents the mean $\pm$ S.D. from triplicate samples. Some error bars are hidden by the symbol.

0.25 or 10 μM inhibited incorporation of label into triacylglycerol by 91 and 99 % respectively, but decreased incorporation into phospholipid by only 46 and 70 % respectively. The high specific radioactivity of the label allowed labelled diacylglycerol to be measured (Figure 2B). Incorporation of label into diacylglycerol decreased by 78 and 93 % in the presence of 0.25 and 10 μM triacsin respectively, whereas the amount of labelled cellular unesterified fatty acid did not change. Labelled monoacylglycerol was not detected. This degree of inhibition suggests that triacsin C blocks diacylglycerol and triacylglycerol formation almost totally whereas phospholipid synthesis is maintained selectively even with limited fatty acid availability. The experiment also suggests that either the *de novo* synthesis of diacylglycerol from glycerol 3-phosphate is severely impaired under these conditions or that any diacylglycerol formed is rapidly depleted.

Inhibition of *de novo* glycerolipid synthesis by triacsin C

We previously reported that when cells are incubated for 24 h, 5 μM triacsin C inhibits [^3H]glycerol incorporation into triacylglycerol by 99 % and into phospholipid by 83 % [6]. In order to examine the effect of triacsin C at early time points, we incubated cells with [^3H]glycerol in the presence or absence of 5 μM triacsin C for up to 6 h (Figure 3). Triacsin inhibited the incorporation of [^3H]glycerol into diacylglycerol and triacylglycerol by more than 95 % and into phospholipid by an average of 70 % over the 6 h period. These studies suggest that triacsin C blocks *de novo* synthesis of glycerolipids and the acylation of diacylglycerol to form triacylglycerol. With *de novo* synthesis of phospholipids blocked by 70–80 % during the first 3 hours of incubation, any

Figure 4 Time course of oleate incorporation into cell lipids in the presence and absence of triacsin C

(A) Phospholipid (PL) and triacylglycerol (TG); (B) phosphatidylcholine (PC) and phosphatidylethanolamine (PE); (C) sphingomyelin (Sph) and phosphatidylinositol (PI)/phosphatidylserine (PS); (D) fatty acid (FA) and diacylglycerol (DAG). Cells were incubated with 100 μ M [14 C]oleate plus or minus 5 μ M triacsin C for up to 24 h. Each data point represents the mean $\pm$ S.D. from triplicate samples. Some error bars are hidden by the symbol.

major incorporation of labelled fatty acid into phospholipid could occur only by reacylation of existing lysophospholipids.

This hypothesis, that exogenous fatty acid is incorporated into phospholipids by acylation of lysophospholipid species in the presence and absence of triacsin C, was checked by examining the incorporation of [14 C]oleate into the *sn*-2 position of phospholipids. When the fatty acid in the *sn*-2 position of cell phospholipids was released by phospholipase A_2 , 71.8 % of the [14 C]oleic acid was released from phospholipid from untreated cells and 73.9 % from phospholipids from cells treated with triacsin C. These data indicate that most of the labelled fatty acid incorporated into phospholipid by these near-confluent cells was used to reacylate *sn*-1-lysophospholipids and that the proportion of label in the *sn*-2 position was not altered in the presence of triacsin C.

Time course of inhibition by triacsin C

In order to determine whether triacsin C varied in its effects on the synthesis of different phospholipid species, confluent fibroblasts were incubated for up to 24 h with [14 C]oleate. Triacsin C inhibited oleate incorporation into phospholipid and triacylglycerol by 62 % at 1 h and by 65 % at 24 h (Figure 4A). Most of the decrease was the result of a complete inhibition of triacylglycerol synthesis from labelled oleate (Figure 4A). In contrast, incorporation of [14 C]oleate into phospholipid decreased by only 13 %. In the presence of the ACS inhibitor, incorporation

Table 1 Incorporation of [14 C]oleate or [3 H]arachidonate into cholesterol esters in the presence or absence of triacsin C

Cell lipids were extracted and analysed at 24 h. Each data point represents the mean $\pm$ S.D. from triplicate samples.

Fatty acid (100 μ M)	Cholesterol esters (nmol/ μ g of DNA)	
	No addition	5 μ M Triacsin C
18:1, <i>n</i> -9	0.446 $\pm$ 0.036	0.016 $\pm$ 0.001
20:4, <i>n</i> -6	0.304 $\pm$ 0.013	0.029 $\pm$ 0.002

Figure 5 Incorporation of [3 H]arachidonate into neutral lipid

Cells were incubated with 100 μ M [3 H]arachidonate in the presence or absence of 5 μ M triacsin C. At various time points cell lipids were analysed for (A) phospholipid (PL) and triacylglycerol (TG), (B) fatty acid (FA) and diacylglycerol (DAG), (C) phosphatidylcholine (PC) and phosphatidylethanolamine (PE) or (D) phosphatidylinositol (PI)/phosphatidylserine (PS). Each data point represents the mean $\pm$ S.D. from triplicate samples. Some error bars are hidden by the symbol.

of [14 C]oleic acid into phosphatidylcholine and phosphatidylethanolamine decreased by 30 and 60 % respectively (Figure 4B), whereas incorporation into sphingomyelin and phosphatidylserine/phosphatidylinositol increased 1.4-fold and 0.7-fold respectively, compared with non-treated control cells (Figure 4C). These data suggest that, in confluent human fibroblasts, turnover of sphingomyelin and PI/PS may either be more rapid than that of phosphatidylcholine and phosphatidylethanolamine or that independent lysophospholipid acyltransferases and acyl-CoAs exist for each phospholipid species.

The cellular content of labelled unesterified fatty acid did not change in the presence of triacsin C (Figure 4D), suggesting that when acyl-CoA synthesis is blocked, the amount of fatty acid

Figure 6 Dependence of DGAT and LPC-AT on palmitoyl-CoA

Activities were measured under identical conditions with various amounts of palmitoyl-CoA as indicated (A) liver KCl-washed microsomes, (B) and (C) human fibroblast total particulate fractions.

entering cells is equal to the amount leaving. The extremely low amount of label incorporated into diacylglycerol in control cells was inhibited completely in the presence of triacsin, again reflecting the block in *de novo* biosynthesis from glycerol 3-phosphate (Figure 4D). Likewise, triacsin C inhibited the incorporation of [14 C]oleate into cholesterol esters by 96 % at 24 h (Table 1).

Triacsin inhibition of arachidonate incorporation into glycerolipid

In HSDM $_1$ C $_1$ mouse fibrosarcoma cells, triacsin C inhibits activation of arachidonate by ACS less well than it inhibits oleate activation [15]. To determine whether this difference would also be observed into fibroblasts, we incubated cells for 24 h with 100 μ M [3 H]arachidonate. Unlike oleate incorporation which labelled triacylglycerol and phospholipid equally (Figure 1A), arachidonate incorporation into triacylglycerol reached a plateau at 6 h and the amount of arachidonate-labelled triacylglycerol was only 25 % of the amount of labelled phospholipid (Figure 5A). At 24 h, triacsin C inhibited the incorporation of [3 H]arachidonate into triacylglycerol (Figure 5A) and cholesterol ester (Table 1) by 95 and 90 % respectively, whereas incorporation into total phospholipid was unaffected (Figure 5A). Arachidonate incorporation into diacylglycerol was inhibited by 63 % by triacsin at 24 h (Figure 5B), suggesting that the block in *de novo* synthesis of diacylglycerol was less than with oleate or that the cells used arachidonate-labelled diacylglycerol poorly. Incorporation of arachidonate into total phospholipid was the same in the presence or the absence of triacsin C and no differences were observed in PI/PS (Figures 5A and 5D). Triacsin altered label incorporation into phosphatidylcholine and phosphatidylethanolamine only between 6 and 24 h (Figure 5C). With [3 H]arachidonate, no labelled sphingomyelin was observed.

If the K_m values for the lysophospholipid acyltransferase

activities were markedly lower than that for DGAT activity, reacylation of lysophospholipids would predominate over acylation of diacylglycerol if acyl-CoA availability were limiting. To determine whether our results might be due to differences in palmitoyl-CoA dependence rather than to separate acyl-CoA pools, we assayed DGAT and a representative lysophospholipid acyltransferase, LPC-AT, under identical conditions (Figure 6). In liver microsomes, maximal activity was observed with 40 μ M palmitoyl-CoA for both activities and dependence on palmitoyl-CoA was almost identical. In fibroblast total particulate preparations, however, similar lags in activity for both DGAT and LPC-AT were observed at low palmitoyl-CoA concentrations. Although the specific activity of LPC-AT in fibroblasts was 10-fold higher than that for DGAT, the activity ratios were similar at high and low concentrations of palmitoyl-CoA. These data strongly suggest that large differences do not exist in the affinities of these enzymes for their acyl-CoA substrates and that such differences cannot explain the preservation of [14 C]oleate incorporation into phospholipid at a time when incorporation into triacylglycerol has been inhibited almost entirely.

DISCUSSION

Long-chain fatty acyl-CoAs are amphipathic molecules that associate primarily with membranes [2,16], but it is not known whether the dispersal of acyl-CoAs within cell membranes is uniform or whether they accumulate in specific membranes or at specific membrane regions. Acyl-CoAs cannot permeate the endoplasmic reticulum and thus bind to acyltransferase active sites that face the cytosolic surface [17]. Movement of acyl-CoAs is probably unconstrained within the outer phospholipid monolayer. Thus it is generally assumed that newly synthesized acyl-CoAs can move freely within cell membrane monolayers and have free access to any of the acyltransferases present in that membrane. Although it is unknown whether an acyl-CoA that has been synthesized within a specific membrane can move to another membrane, long-chain acyl-CoAs are, in fact, water-soluble and could theoretically move through cytosol either free or bound to a protein such as the acyl-CoA-binding protein [5].

Our studies indicate that this view of acyl-CoA trafficking is incomplete. We have shown in human fibroblasts that, although 5 μ M triacsin C inhibits *de novo* synthesis of triacylglycerol by 99 % and phospholipid by 83 % from glycerol, the incorporation of labelled oleate into phospholipid is relatively unimpaired when triacsin is present. We interpret these data to mean that reacylation of lysophospholipids is not inhibited by triacsin when fatty acid is not limiting. Two explanations are possible: (1) different ACSs exist that vary in their sensitivity to triacsin; (2) an independent mechanism channels acyl-CoA towards phospholipid synthesis when little acyl-CoA is available. If incorporation occurred because of the presence of a specific uninhibited ACS, one might hypothesize that a triacsin-sensitive ACS synthesizes acyl-CoAs that can only be used to acylate cholesterol, glycerol 3-phosphate, lysophosphatidic acid and diacylglycerol, and that a second, triacsin-resistant, ACS synthesizes acyl-CoAs that can only be used by lysophospholipid acyltransferases and by ceramide *N*-acyltransferase. The two acyl-CoA pools would not mix. The alternative possibility is that one or all ACSs are inhibited only partially by triacsin C, thereby limiting the total amount of acyl-CoA produced, and that this acyl-CoA is used only to reacylate lysophospholipid; it is not available for the processes of *de novo* glycerolipid synthesis, the synthesis of triacylglycerol from diacylglycerol, or the synthesis of cholesterol esters from cholesterol. In either case, however, the data imply that acyl-CoAs are functionally channelled towards selected

acyltransferase reactions. Decreases in the synthesis of phosphatidylcholine and phosphatidylethanolamine and increases in phosphatidylinositol and sphingomyelin in the presence of triacsin imply that there are further constraints on the use of the limited amount of acyl-CoA. Such channelling into different lipid pathways could occur by means of transport proteins such as the acyl-CoA-binding protein [5], multienzyme complexes, or because lysophospholipid acyltransferases and a triacsin-resistant ACS are located together in a membrane other than the endoplasmic reticulum.

Existing information does not provide support at present for triacsin-sensitive and -resistant long-chain ACSs in mammalian tissues. Three different long-chain ACSs have been identified in rats, one associated with the endoplasmic reticulum, mitochondrial outer membrane, nuclear membrane and peroxisomal membrane that activates saturated fatty acids of 10–20 carbons and unsaturated fatty acids of 16–20 carbons [2,18], one cloned from rat brain, the location and substrate specificity of which have not been characterized [19] and one in the peroxisomal matrix that activates very-long-chain fatty acids [20]. Although they have multiple promoters that mediate expression [21,22], long-chain ACSs from rat liver mitochondria, endoplasmic reticulum and peroxisomes are identical immunologically and have the same molecular mass, amino acid composition and pH optimum [2]. It therefore seems unlikely that they would vary substantially in their sensitivity to triacsin C. The differential sensitivity to triacsin apparent in the ACSs that activate oleate and arachidonate does not explain the fact that both oleoyl-CoA and arachidonoyl-CoA are used primarily for phospholipid and not triacylglycerol synthesis.

The situation differs in yeast. There four separate ACSs have been purified and cloned, and the presence of an additional peroxisomal ACS has been inferred [23]. Extensive genetic studies have demonstrated varying susceptibility of the cloned ACSs to inhibition by triacsin C, different preferences for fatty acid chain length, and differences in the ability of the ACSs to activate exogenously added fatty acids [22,24,25]. Only two of these ACSs can activate exogenous fatty acids and overcome growth arrest after fatty acid synthesis is blocked by cerulenin, implying a high degree of compartmentalization of acyl-CoA metabolism [24]. Despite this information, it is not known in which membranes each of these ACSs is located or whether specific ACSs provide substrates for specific acyltransferase reactions.

Triacsin C is a competitive inhibitor of ACS with respect to its fatty acid substrate [7]. The IC_{50} appears to vary in different tissues [8], and cultured cells respond differently. In Raji cells incubated for 2 days with 1.0 μ M triacsin C, growth was inhibited by 50%, and the incorporation of [14 C]oleate into phosphatidylcholine, phosphatidylethanolamine and triacylglycerol was inhibited similarly during a 20 min incubation [8]. After 3–4 days of exposure to triacsin C at 4.8 μ M, Raji cells were killed. In contrast, 5 μ M triacsin C had no discernible effect on the survival of human fibroblasts for 4 days [6], and the present study shows that the drug differed in its effects on the incorporation of fatty acid into phospholipid and triacylglycerol, severely inhibiting the latter. Compared with our studies in fibroblasts, the Raji cells were exposed to lower concentrations of oleate, which may

protect cells exposed to the competitive ACS inhibitor. In addition, the effect of triacsin may be more toxic in rapidly growing cells. Human fibroblasts, which when dividing have a doubling time of more than 2 days (R. A. Coleman, unpublished work), were studied when confluent, whereas the proliferating Raji cells double their number in less than a day [8].

In HSDM₁C₁ mouse fibrosarcoma cells, triacsin C at 40 μ g/ml (190 μ M) for 1 h inhibited cell uptake of arachidonate and oleate; at 4.7 μ M, triacsin C inhibited long-chain ACS by 90% [15]. In intact HSDM₁C₁ mouse fibrosarcoma cells, 4.7 μ M triacsin inhibited ACS maximally by 2 min [15]. In liver, 12.5 μ M triacsin D (less than 20 times as potent an ACS inhibitor as triacsin C [8]) for 1 h inhibited very-low-density lipoprotein and oleate stimulation of triacylglycerol synthesis from glycerol but, unlike our studies, did not block the synthesis of cholesterol esters from [14 C]acetate [26]. Thus different tissues may have different complements of ACSs.

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