Acyl-CoAs are functionally channeled in liver: potential role of acyl-CoA synthetase

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Muoio, Deborah M., Tal M. Lewin, Petra Wiedmer, and Rosalind A. Coleman. Acyl-CoAs are functionally channeled in liver: potential role of acyl-CoA synthetase. Am J Physiol Endocrinol Metab 279: E1366–E1373, 2000.—Acyl-CoA synthetase (ACS) catalyzes the activation of long-chain fatty acids to acyl-CoAs, which can be metabolized to form CO₂, triacylglycerol (TAG), phospholipids (PL), and cholesteryl esters (CE). To determine whether inhibiting ACS affects these pathways differently, we incubated rat hepatocytes with [14C]oleate and the ACS inhibitor triacsin C. Triacsin inhibited TAG synthesis 70% in hepatocytes from fed rats and 40% in starved rats, but it had little effect on oleate incorporation into CE, PL, or β-oxidation end products. Triacsin blocked [3H]glycerol incorporation into TAG and PL 33 and 25% more than it blocked [14C]oleate incorporation, suggesting greater inhibition of de novo TAG synthesis than reacylation. Triacsin did not affect oxidation of prelabeled intracellular lipid. ACS1 protein was abundant in liver microsomes but virtually undetectable in mitochondria. Refeeding increased microsomal ACS1 protein 89% but did not affect specific activity. Triacsin inhibited ACS specific activity in microsomes more from fed than from starved rats. These data suggest that ACS isoforms may be functionally linked to specific metabolic pathways and that ACS1 is not associated with β-oxidation in liver.

Rather than being equally accessible to all enzymes that use acyl-CoA, acyl-CoAs may be channeled into distinct acyl-CoA pools that are functionally linked to specific pathways. This hypothesis is mainly supported by studies that have evaluated the metabolism of long-chain fatty acids in cells exposed to triacsin C, a family of competitive inhibitors of ACS (34). In fibroblasts, triacsin C completely blocks de novo synthesis of TAG and PL without inhibiting the reacylation of lysophospholipid (14), and in rat insulinoma cells, triacsin C inhibits de novo synthesis of cellular lipids by 83% (29).

In these studies, the majority of acyl-CoAs were esterified into TAG. In primary hepatocytes, however, acyl-CoA use is more equally distributed among diverse pathways, including β-oxidation and the synthesis of lipids secreted in very low-density lipoproteins (VLDL) (37). Furthermore, in liver, acyl-CoA partitioning among these pathways is regulated nutritionally. Starvation increases the rates of β-oxidation and ketogenesis (26), whereas refedding a high-sucrose diet stimulates TAG synthesis and VLDL secretion (11). Thus primary hepatocytes provide an ideal model in which to study regulated trafficking of acyl-CoAs into oxidative and storage pathways. In the present study, we investigated the effects of triacsin C on long-chain fatty acyl-CoA metabolism in hepatocytes that were isolated from either fed or starved rats. These studies provide strong evidence that, in liver, acyl-CoAs are functionally channeled toward specific pathways, and they suggest that one mechanism responsible for selective trafficking of acyl-CoAs might involve regulated expression of ACS1.

METHODS

Materials. Silica gel G plates were from Whatman. [2-3H]glycerol, [9,10-3H]palmitate, and [1-14C]oleate were from Amersham Life Sciences. Glycerol, BSA (essentially fatty acid-free), and sodium oleate were from Sigma. Lipid
standards and sn-1,2-dioleoylglycerol were from Serdary. Triacsin C (>95% pure) was from Biolom Research Lab. Tissue culture supplies and fetal bovine serum (FBS) were from Life Technologies. Rat-tail collagen was from Sigma. Western blots were performed using polyvinylidene fluoride (PVDF) membranes from Bio-Rad, horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G and a Super signal chemiluminescence detection kit from Pierce, and 125I-labeled protein A from ICN Pharmaceuticals.

Hepatocyte isolation and incubations. Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200–250 g) were maintained on a 12:12-h light-dark cycle with free access to Purina rat chow. Before experiments, animals were given free access to food overnight, and hepatocytes were isolated at 0900 or 1700 so that rats were deprived of food for 8 or 24 h, respectively, before hepatocytes were isolated at 1500. Hepatocytes were isolated by collagenase perfusion (3). Cell viability, determined by trypsin blue exclusion, exceeded 95%. Hepatocytes were seeded at a density of 200,000 cells per well into 24-well dishes pretreated with 0.01% rat-tail collagen, 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. After cells had been attached for 3–4 h, medium was replaced with serum-free MEM plus 10 nM triacsin C.

Effects of triacsin C on hepatocyte fatty acid partitioning. When hepatocytes from rats starved for 8 h were incubated with 5 or 10 µM triacsin C for 24 h, the incorporation of [14C]oleate into total lipid decreased 55% (Fig. 1A). Triacsin’s effect differed markedly for individual lipid species. The presence of 10 mM triacsin had only a negligible effect on CE and inhibited DAG only 50%, but it was similar to the effect of 10 µM triacsin C on all other lipid metabolites.
synthesis from glycerol, reacylation of lysophospholipids, esterification of cholesterol, and the DAG and monoacylglycerol (MAG) products of TAG lipase, as well as reactivation and use of hydrolyzed fatty acid.

To examine de novo glycerolipid synthesis alone, hepatocytes were incubated with [3H]glycerol for 1 h. Hepatocytes isolated from the same liver were simultaneously incubated with [14C]oleate for direct comparison. Triacsin blocked glycerol incorporation into TAG and PL 33 and 25% more than it blocked oleate incorporation (Fig. 2), suggesting less inhibition of reacylation and/or that the activation of fatty acids hydrolyzed from labeled TAG or PL stores might be less sensitive to triacsin C. Although we cannot exclude the possibility that triacsin-induced differences in incorporation of [14C]oleate and [3H]glycerol resulted from different effects on label recycling and tracer dilution, recycling of 14C should have been minimized by the short (1-h) incubation period.

To examine the effects of triacsin on oleate recycling and TAG hydrolysis, hepatocytes were prelabeled for 24 h with [14C]oleate. Then, after treatment for 24 h in unlabeled medium with or without triacsin C, cells and media were analyzed to determine how much [14C]oleate was hydrolyzed and recycled to glycerolipids, CE, ASM, and CO2 (Fig. 3). After 24 h, 75.1% of the intracellular label was present in TAG, 17.1% in PL, 4.7% in DAG, and 3.1% in CE. Compared with time 0 in the chase medium, a 24-h chase in the absence of oleate decreased the amount of label recovered from intracellular TAG 84%, PL 55%, DAG 67%, and CE 57%. The presence of 200 μM oleate in the chase medium attenuated the loss of label from TAG but increased loss of labeled PL. In contrast, adding triacsin C to the chase medium increased losses from TAG but decreased loss of radiolabeled PL. These data indicate that most of the newly synthesized TAG turned over within 24 h and that only a small portion of the [14C]oleate that was hydrolyzed during the 24-h chase was reesterified to TAG. Furthermore, these results also suggest that when triacsin blocked reesterification to form TAG, the hydrolyzed [14C]oleate was more available for reesterification.
fication to PL. Additionally, the presence of triacsin did not appear to alter hydrolysis of intracellular TAG, and neither the presence of triacsin C nor the presence of fatty acid in the chase medium altered the amount of label recovered in ASM and CO₂. These data suggest that oxidation of endogenous fatty acid does not depend on the availability of extracellular fatty acid, and, furthermore, that triacsin C did not inhibit the activation of endogenous fatty acids that were channeled toward β-oxidation. The remaining 14C label not recovered in oxidation products or in intracellular lipid was present in media lipids, which were not further examined.

Effect of feeding status on triacsin’s inhibition of fatty acid metabolism. To determine whether feeding status affects the ability of triacsin C to inhibit fatty acid incorporation into different lipid species, we examined [14C]oleate incorporation in hepatocytes from fed and 24-h-starved rats (Fig. 4). In the presence of 0.125 mM oleate, hepatocytes from fed rats incorporated 57% more oleate into TAG (Fig. 4A) and ~47% less oleate into ASM (Fig. 4B), CE (Fig. 4C), and PL (Fig. 4D) than did cells from starved rats. Increasing oleate to 1.0 mM diminished nutrition-induced differences in hepatocyte synthesis of complex lipids and markedly enhanced oxidation in hepatocytes from fed rats. At all oleate

![Graphs showing incorporation of oleate into lipids and ASM in the presence or absence of triacsin C in hepatocytes from fed or 24-h-starved rats.](http://ajpendo.physiology.org/)

Fig. 4. Incorporation of oleate into lipids and ASM in the presence or absence of triacsin C in hepatocytes from fed or 24-h-starved rats. Hepatocytes were incubated for 24 h with different concentrations of [14C]oleate and 0 or 10 μM triacsin C, as indicated. Incorporation of [14C]oleate was measured into TAG (A), ASM (B), CE (C), and PL (D). E: ratio of dpm incorporated into TAG divided by dpm in ASM. Data are means ± SD from 3 samples.
concentrations, triacsin inhibited TAG synthesis in hepatocytes from fed and starved rats by 70 and 40%, respectively. In comparison, regardless of feeding status, triacsin C had little effect on incorporation of 0.125 mM oleate into CE or PL. Only when hepatocytes from starved rats were incubated with 1 mM fatty acid did triacsin inhibit incorporation into CE (by 44%) (Fig. 4C). In contrast to the oxidation of endogenous fatty acid, which was unaffected by medium fatty acid concentration (Fig. 3), the amount of exogenous fatty acid incorporated into ASM varied strongly with the oleate concentration in the media (Fig. 4B). In the presence of 0.25 and 0.5 mM oleate, triacsin actually increased incorporation into ASM in hepatocytes from starved rats, whereas in fed rats, triacsin decreased incorporation into ASM only at high oleate concentrations. Differences in partitioning between synthetic and oxidative pathways are shown as a ratio of label incorporation into TAG and ASM (Fig. 4E). When the fatty acid supply was low, acyl-CoAs were preferentially esterified to TAG in fed hepatocytes but were preferentially oxidized in starved hepatocytes. High concentrations of oleate stimulated a shift toward TAG synthesis in starved hepatocytes but stimulated production of ASM by fed hepatocytes. Thus, in the presence of high (1.0 mM) oleate, hepatocytes partitioned fatty acid equally between TAG and ASM. In the presence of triacsin C, changes in nutritional status and medium oleate concentration no longer altered partitioning between TAG synthesis and oxidation.

Analyses of ACS activity and protein abundance in mitochondria and microsomal fractions. Rat liver contains at least three ACS isozymes, two of which are known to be nutritionally regulated (30, 33). To determine whether some of the changes observed in partitioning of fatty acids between synthesis and oxidation might be related to differences in ACS activity and/or expression in different subcellular organelles, we measured total ACS activities and ACS1 protein abundance in mitochondrial and microsomal fractions (Table 1). In microsomes, the mitochondrial isoform of GPAT contributed only 9% to the total specific activity, thus showing little contamination of microsomes with mitochondrial outer membrane (2). Microsomal GPAT contributed 13% to the total GPAT specific activity in mitochondria, and the specific activity of DGAT, another microsomal marker (2), was 19-fold higher in microsomes than in mitochondria, thus indicating only a small degree of contamination of the mitochondrial fraction with endoplasmic reticulum membrane. The specific activity of ACS was 57% greater in microsomes than in mitochondria. By immunoblot analysis, ACS1 protein was abundant in microsomes but barely detectable in mitochondria, regardless of nutritional status (Fig. 5A). When 15% microsomal contamination was present, a faint ACS1 band could be observed in some mitochondrial preparations (data not shown). These results indicate either that ACS1 is not present in mitochondria or that it contributes only minimally to total mitochondrial ACS activity. In microsomes, refeeding increased ACS1 protein abundance 89% (Fig. 5, B and C), but neither fasting nor refeeding changed ACS activity in either microsomes or mitochondria. When analyzed in isolated subcellular fractions, triacsin’s relative inhibition of total ACS activity was greater in microsomes from fed compared with starved rats (Fig. 6), consistent with triacsin’s potent inhibition of hepatocyte TAG synthesis in hepatocytes from fed rats. Surprisingly, although triacsin did not inhibit β-oxidation in cultured hepatocytes (Fig. 4B), triacsin inhibited ACS activity similarly in isolated mitochon-

Table 1. Enzyme activities in isolated mitochondria and microsomes

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<th>ACS</th>
<th>Microsomal</th>
<th>Mitochondrial</th>
<th>DGAT</th>
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<tr>
<td>Microsomes</td>
<td>21.5 ± 3.89</td>
<td>1.38 ± 0.19</td>
<td>0.14 ± 0.01</td>
<td>3.34 ± 0.13</td>
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<tr>
<td>Mitochondria</td>
<td>13.7 ± 2.60</td>
<td>0.07 ± 0.03</td>
<td>0.44 ± 0.07</td>
<td>0.18 ± 0.02</td>
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Liver microsomes and mitochondria were assayed for the activity of acyl-CoA synthetase (ACS), and for the activities of microsomal marker enzymes: microsomal glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT), and the mitochondrial marker enzyme, mitochondrial GPAT.
Acyl-CoAs are synthesized from long-chain fatty acids by ACSs, a family of integral membrane proteins that vary in subcellular location, substrate preference, and tissue distribution (15, 16). Liver expresses at least three long-chain ACS isoenzymes, ACS1, ACS4, and ACS5, and may express others that have yet to be identified (16, 30, 33). It is not known whether different ACSs synthesize acyl-CoAs that comprise a common pool, which is available to all acyl-CoA-utilizing pathways, or separate acyl-CoA pools that are functionally distinct. If acyl-CoAs were equally available to all metabolic pathways, one would expect the ACS inhibitor triacsin C to block production of all acyl-CoA dependent end products to a similar degree. Instead, triacsin C preferentially inhibited hepatocyte TAG synthesis, whereas other acyl-CoA pathways, including β-oxidation, were either less inhibited or unaffected. Furthermore, the magnitude of triacsin's inhibition of acyl-CoA metabolism depended on the nutritional status of the rat from which hepatocytes were isolated.

These data provide strong evidence that, in liver, acyl-CoAs do not move freely within cellular compartments but instead are channeled toward specific pathways. This model of acyl-CoA channeling is supported by previous studies in other types of cells that also relied on the use of ACS inhibitors (10, 14, 29, 36). To account for these observations, we hypothesize that distinct ACS isozymes vary in their sensitivities to ACS inhibitors and are functionally complexed with specific metabolic pathways, a hypothesis which is consistent with in vitro data demonstrating that triacsin C differentially inhibits arachidonoyl-CoA synthetase compared with long-chain ACS (13). Additionally, this hypothesis predicts that the ACS isoform most tightly coupled to TAG biosynthesis is triacsin sensitive, whereas the ACS isoform closely associated with β-oxidation is triacsin resistant. If this hypothesis is true, it would explain why triacsin preferentially inhibits TAG synthesis. Additionally, it might also explain why triacsin's inhibition of TAG synthesis was more marked in hepatocytes isolated from fed than from starved rats, because we predict that feeding might be associated with preferential synthesis of acyl-CoAs by the ACS linked to TAG synthesis. We found that ACS1 protein is abundant in microsomes, the site of TAG synthesis, but not in mitochondria, the site of β-oxidation. These results, which are the first to report ACS1 protein abundance in different subcellular fractions, provide strong evidence that ACS1 is not the primary isoform that activates fatty acids in liver mitochondria. Additional indirect evidence that ACS1 is closely associated with TAG biosynthesis and that it might be triacsin sensitive is provided by ACS1's microsomal localization, refeeding-induced stimulation of microsomal ACS1 protein expression, and the high potency of triacsin C against TAG synthesis, but not β-oxidation.

The existence of triacsin-sensitive and triacsin-resistant ACS isozymes has been shown in yeast, which express at least four separate ACSs (faa1-faa4) (18). Genetic studies suggest that yeast ACSs have specific functions. Only FAA1 and FAA4 can activate exogenous fatty acids, and a yet unidentified ACS specifically activates endogenously synthesized fatty acids (17). When faa1/faa4 null yeast are grown in the presence of cerulenin, which inhibits de novo fatty acid synthesis and thus kills the cells, rat ACS1 complements and rescues the cells (17). Triacsin C blocks complementation by ACS1 (17), consistent with our prediction that ACS1 is triacsin sensitive.

Contrary to our expectation, triacsin inhibited ACS similarly in microsomes and mitochondria, suggesting the presence of at least one mitochondrial ACS that is triacsin sensitive but perhaps not directly linked to β-oxidation. Additionally, despite increased ACS1 protein abundance in liver microsomes from fed compared with starved rats, microsomal ACS specific activity was unaffected by nutritional status. Others have reported similar inconsistencies between ACS1 mRNA abundance and enzyme activities in subcellular fractions from hamsters (24). These data demonstrate the difficulty in interpreting ACS specific activities, which reflect several ACS isoenzymes. Discrepancies between changes in ACS1 expression compared with changes in enzyme activity might be explained if distinct ACS isozymes are regulated in opposite directions. For example, whereas starvation decreased the abundance of ACS1 protein, expression of other ACS isoforms might have been increased, thereby negating measurable changes in ACS specific activity. Alternatively, because of the inherent limitations of measuring enzyme activities in vitro, the specific activity in isolated membrane fractions might not reflect regulation that occurs in vivo or in intact cells.
The alternative to our hypothesis that distinct ACS isoforms are functionally linked to specific pathways is that different ACSs synthesize acyl-CoAs that form a common pool. In this case, adding triacsin C should uniformly decrease the availability of acyl-CoA to all pathways. Use of a limited pool of acyl-CoAs would then be regulated by enzyme kinetics, such that the enzyme with the lowest Michaelis-Menten constant ($K_m$) for acyl-CoA would esterify more acyl-CoAs. In a previous study (14) we reported data that oppose this hypothesis. We found that in rat liver microsomes, DGAT, which catalyzes the final step in TAG synthesis, and lysophosphatidylcholine acyltransferase, which catalyzes the reacylation of lysophosphatidylcholine, have similar dependencies on palmitoyl-CoA. Thus differences in triacsin’s effect on oleate incorporation into TAG and PL (Fig. 4) probably did not result merely from differences in apparent $K_m$ values for the acyl-CoA substrates. Other enzymes that compete for acyl-CoA, such as carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme in β-oxidation, and mitochondrial GPAT, which catalyzes the initial step of glycerolipid synthesis, appear to be acutely regulated by allosteric and reversible phosphorylation (22, 27). Thus direct comparisons of $K_m$ values obtained from in vitro assays would be difficult to interpret.

A second argument against regulation by enzyme kinetics is based on reports that feeding decreases liver CPT1 activity and increases GPAT activity, suggesting that, in the fed state, acyl-CoAs would be preferentially esterified (1, 7, 23). Acyl-CoAs were, in fact, preferentially esterified in hepatocytes from fed rats; ~2.5 times more oleate was incorporated into TAG than was oxidized to ASM. Despite preferential esterification, however, adding triacsin inhibited TAG synthesis more than β-oxidation. Furthermore, if limited availability of acyl-CoA were critical in determining acyl-CoA partitioning, then increasing acyl-CoA availability should have produced a shift in acyl-CoA use. On the contrary, in triacsin-treated cells from both fed and starved rats, the TAG-to-ASM ratio remained constant at all concentrations of fatty acid (Fig. 4E). Together, these data argue that enzyme kinetics alone cannot explain our observation that acyl-CoAs were selectively channeled in hepatocytes treated with triacsin C. Definitive evidence supporting the hypothesis that acyl-CoA trafficking is regulated by coordinated expression of distinct ACS isozymes awaits further investigation of acyl-CoA metabolism in transgenic animals or in cells that selectively express specific ACSs.

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