Do Long-Chain Acyl-CoA Synthetases Regulate Fatty Acid Entry into Synthetic Versus Degradative Pathways?[1,2]

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ABSTRACT Recent studies suggest that the long-chain acyl-CoA synthetases (ACS) may play a role in channeling fatty acids either toward complex lipid synthesis and storage or toward oxidation. Each of the five members of the ACS family that has been cloned has a distinct tissue distribution and subcellular location, and is regulated independently during cellular differentiation and by diverse hormones and nuclear transcription factors including adrenocorticotropic hormone (ACTH), peroxisomal proliferator-activated receptor-α (PPARα) and sterol regulatory element binding protein. Taken as a whole, these features suggest that in liver, ACS1 and ACS5 may provide acyl-CoA destined primarily for triacylglycerol synthesis or for mitochondrial oxidation, respectively. ACS4 may provide acyl-CoA for both synthesis and peroxisomal oxidation, depending on whether the enzyme is associated with the mitochondrial-associated membrane or with peroxisomes. It should be emphasized that although the data for acyl-CoA channeling are strong, they are indirect. Rigorous testing of these predictions will be required. J. Nutr. 132: 2123–2126, 2002.

KEY WORDS: • diabetes • oxidation • triacylglycerol

Disposal of long-chain fatty acids into pathways of β-oxidation or triacylglycerol synthesis is regulated globally by energy intake and energy expenditure; within cells, however, fatty acid partitioning is often viewed as a function of the interplay between insulin and its counterregulatory hormones, the transcription of degradative enzymes such as carnitine palmitoyltransferase-1 (CPT-1)[4] and lipogenic enzymes such as fatty acid synthase and glycerolphosphate acyltransferase, and the acute regulation of CPT1 by malonyl-CoA. In contrast, the long-chain acyl-CoA synthetases (ACS) that catalyze the synthesis of acyl-CoA, the substrates for both synthetic and oxidative pathways, have been largely ignored. Absence of change in ACS activity under different physiologic conditions suggested that ACS played no role in the regulation of lipid metabolism or the partitioning of fatty acids. During the last 10 years, however, five rat long-chain ACS isoforms have been cloned, and several of their human homologs have been identified[5]. The differential expression of ACS mRNAs in different tissues and under varying nutritional regimens, their separate locations in different subcellular membranes, and the varying responses of synthetic and oxidative pathways to specific inhibitors now suggest that the fate of a particular acyl-CoA depends on which of the long-chain ACS catalyzed its synthesis.

Molecular Characterization of Rat ACS. Five rat ACS form two subfamilies (1). ACS1, ACS2 and ACS5 have ~60% amino acid identity with each other and ~30% with the two members of the second subfamily, ACS3 and ACS4. Although all five ACS use saturated and unsaturated fatty acids of chain lengths 8–22 carbons, ACS4 has a marked preference for 20:4 and 20:5 (2). Each of the ACS has a region that contains a putative AMP binding site and one that probably contains the fatty acid binding site (1). These sites have been used to define the members of the ACS superfamily specific for different fatty acid chain lengths (3). We will focus on the group that activates the most common fatty acids in the human diet, saturated and unsaturated fatty acids of 14–22 carbons. Despite a decade of active research on the cloned isoforms, a number of critical questions remain to be answered.

What is the Evidence for Independent Functions of ACS Isoforms? Acyl-CoA can enter numerous pathways including the de novo synthesis of triacylglycerol and phospholipids, reacylation pathways, β-oxidation, and cholesterol and retinal esterification (**Fig. 1**). Additionally, acyl-CoA are cell-signaling molecules and may be ligands for transcription factors. As signals, acyl-CoA alter insulin secretion, apoptosis, and glucose transport and metabolism (4). But why are multiple isoforms required to synthesize these acyl-CoA? Studies with triacsin C provided the initial evidence for ACS-catalyzed channeling of acyl-CoA in mammalian cells. When ACS is inhibited by triacsin, a fungal-derived competitive inhibitor, some pathways of lipid metabolism are completely blocked, whereas other pathways continue at near normal rates. For example, in human fibroblasts, triacsin C inhibits the incorporation of [^{3}H]glycerol into phospholipid and triacylglycerol 80 and 99%, respectively, indicating severely impaired acylation of glycerol-3-phosphate, lysophosphatidic acid and diacylglycerol via the de novo synthetic pathway from glycerol-3-phosphate (**Figs.** 1, **2**) (5). Incorporation of [^{14}C]oleate into triacylglycerol is also blocked 95%, consistent

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[4] Abbreviations used: ACS, acyl-CoA synthetase; ACTH, adrenocorticotropic hormone; CPT-1, carnitine palmitoyltransferase-1; ER, endoplasmic reticulum; GLUT, glucose transporter; MAM, mitochondrial-associated membrane; PG, prostaglandin; PKC, protein kinase C; PPAR, peroxisomal proliferator-activated receptor.

[5] The acyl-CoA synthetase family that prefers long-chain fatty acids has been designated acyl-CoA synthetase (ACS) in rodents and fatty acid CoA ligase (FACL) in humans, but is also termed fatty acyl-CoA synthetase (FACS), and fatty acid CoA synthase.

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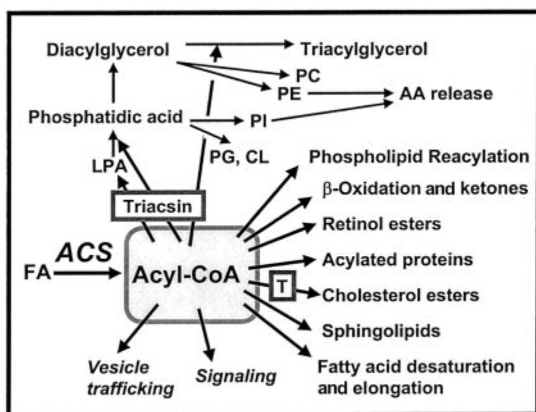

FIGURE 1 Pathways of acyl-CoA metabolism and triacsin inhibition. AA, arachidonic acid; ACS, acyl-CoA synthetase; CL, cardiolipin; FA, fatty acid; PA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; T, triacsin.

with impaired acylation via the de novo pathway. In contrast, incorporation of [^{14}C]oleate into phospholipids is not impaired, suggesting that the reacylation pathway is functionally separate from de novo glycerolipid synthesis, and that separate pools of acyl-CoA exist. Troglitazone, a thiazolidinedione formerly used to treat type 2 diabetes, also selectively inhibits some, but not all acyl-CoA pathways (Fig. 3) (6). We hypothesized that triacsin and troglitazone must inhibit an ACS isoform that provides acyl-CoA for de novo lipid synthesis, and that a second ACS isoform, insensitive to the inhibitors, would provide acyl-CoA used to reacylate lysophospholipids.

The ability to express purified recombinant ACS isoforms allowed us to test this hypothesis. Thiazolidinediones were found to be potent inhibitors only of ACS4, and triacsin inhibited ACS1 and ACS4, but not ACS5 (7). These studies suggest that ACS1 and ACS4 provide acyl-CoA destined primarily for triacylglycerol synthesis and the de novo synthesis of phospholipids, but that phospholipid reacylation and oxidation must rely on acyl-CoA synthesized by other isoforms.

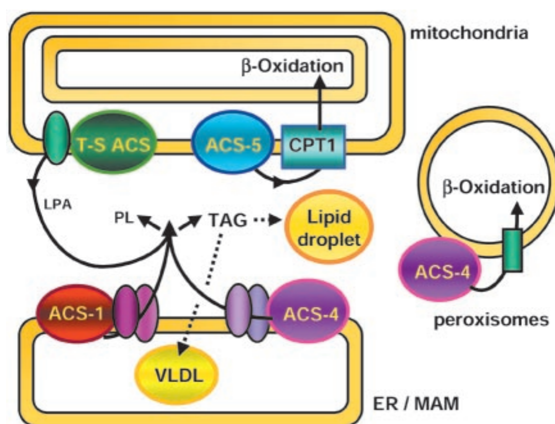

FIGURE 2 Associations of ACS isoforms with specific subcellular organelles in hepatocytes and possible associated pathways. A, mitochondrial glycerol-3-phosphate acyltransferase; B, C, D, and E, enzymes in the pathway of glycerolipid biosynthesis; E, peroxisomal acyl-CoA transporter. ACS, acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; LPA, lysophosphatidic acid; PL, phospholipid; TAG, triacylglycerol; T-S ACS, triacsin-sensitive ACS present in mitochondria.

Why are the Different ACS Isoforms Located on Different Cellular Membranes? ACS activity is prominent in microsomes, mitochondria and peroxisomes, comprising ~73, 20 and 7%, respectively, of total cellular palmitoyl-CoA synthetase activity (8). The ability to distinguish between the different isoforms allowed us and others to raise noncross-reacting peptide antibodies and show that in rat liver, ACS1 is an intrinsic membrane protein of the endoplasmic reticulum (ER) and of the mitochondrial-associated membrane (MAM), a locus that may be involved in the biogenesis of ER-mitochondrial membranes and/or VLDL secretion (1). ACS1 is also present in adipocyte glucose transporter (GLUT) 4 vesicles and the plasma membrane. ACS4, the only isoform that is a peripheral membrane protein, is located in the MAM fraction and on the peroxisomal membrane. ACS5 is the only ACS identified to date that is located on the mitochondrial outer membrane (9). These separate locations for ACS1, 4 and 5 are consistent with the idea that each ACS isoform catalyzes the synthesis of acyl-CoA that enter independent pathways (**Fig. 2**). For example, the initial esterification steps in the synthesis of triacylglycerol and phospholipids occur in both ER and mitochondrial outer membrane, and although reesterification for remodeling of most phospholipids occurs in the ER, synthesis and remodeling of cardiolipin take place primarily in the mitochondria. Further, both mitochondria and peroxisomes are sites of fatty acyl-CoA oxidation.

It is difficult to conceptualize how the ACS are organized to have overlapping functions at different sites and separate functions at the same sites. The active site of each ACS faces the cytosol (1), and acyl-CoA are fairly water-soluble, amphipathic molecules. Thus, they can theoretically partition into either membranes or cytosol. In liver cytosol, they may move freely or bind to acyl-CoA binding protein and to fatty acid binding protein. How then would an acyl-CoA be channeled into a specific pathway? One can envision physical interactions of the ACS with specific acyltransferases such as glycerolphosphate acyltransferase or CPT-1, but as yet, no evidence exists for such interactions.

Why Do ACS Message Levels Vary in Different Tissues? The mRNA abundance of each of the five rat ACS isoforms differs markedly in different tissues, suggesting possible tissue-specific functions for each ACS. ACS1, which is prominent in liver, adipose tissue and differentiated 3T3-L1 adipocytes, seems likely to provide acyl-CoA for triacylglycerol synthesis. Although the presence of ACS1 in GLUT4 vesicles was linked to the requirement for palmitoyl-CoA in

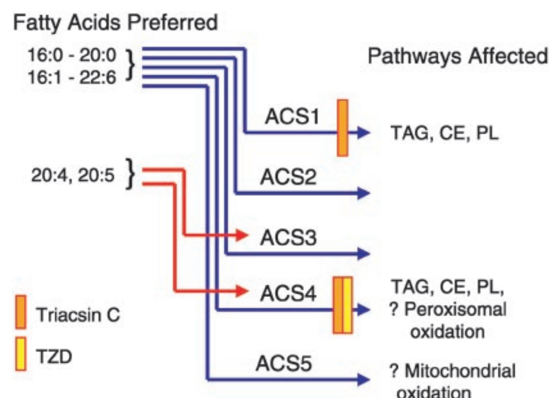

FIGURE 3 Inhibitors suggest possible products of acyl-CoA produced by ACS isoforms. ACS, acyl-CoA synthetase; CE, cholesteryl ester; PL, phospholipid; TAG, triacylglycerol; TZD, thiazolidinedione.

vesicle trafficking, ACS1 might also facilitate fatty acid entry into cells in response to insulin stimulation (1). ACS2 and ACS3 were both cloned from a rat brain library and their mRNAs are expressed predominantly in brain and neural cell lines, although their developmental expression differs (10,11). ACS4, which prefers 20:4 and 20:5 fatty acids, is expressed at the highest levels in the adrenal gland (2) where arachidonate comprises 40% of membrane fatty acids (12) and 22% of fatty acids esterified to cholesterol (13). Because arachidonate is part of a signaling cascade initiated by adrenocorticotropic hormone (ACTH) (14), ACS4 might function to terminate the arachidonate signal, thereby diminishing cortisol synthesis. ACS5 mRNA expression is greatest in intestinal epithelial cells (1), but its regulation has not been studied. ACS may also increase the vectorial transport of fatty acid into cells; whether exogenous fatty acids require a protein transporter or can cross the plasma membrane by diffusion, activation to acyl-CoA renders cell entry unidirectional.

ACS1: Nutritional and Developmental Regulation Correlates with Triacylglycerol Synthesis. Before the ACS isoforms were cloned, the 100-fold increase in total ACS activity observed when 3T3-L1 preadipocytes differentiated into adipocytes, suggested expression of an ACS required for triacylglycerol synthesis; this isoform is now known to be ACS1 (1). ACS1 mRNA also increases in liver, heart and adipose tissue with refeeding after food deprivation, with obesity and diabetes in rodent models, and in adipose tissue after peroxisomal proliferator-activated receptor-γ (PPARγ) stimulation (1,15). ACS1 mRNA in liver and adipose tissue is higher in Zucker *fa/fa* rats than in their lean littermates (16), in adipose tissue from rats with obesity caused by lesions in the ventromedial hypothalamus (17) and in insulin-treated 3T3-L1 adipocytes (18). In contrast, exercise decreases ACS1 activity and mRNA in rat visceral fat (19). These studies are all compatible with the hypothesis that ACS1 synthesizes acyl-CoA that are used primarily for the synthesis of triacylglycerol.

Adipocyte ACS Activity is Inhibited by a cAMP Pathway. Because adipocyte triacylglycerol synthesis and lipolysis are reciprocally regulated via insulin and cAMP-dependent hormones, ACS might be regulated by a phosphorylation/dephosphorylation mechanism. Norepinephrine, ACTH, glucagon or dibutyryl cAMP decrease rat adipocyte ACS-specific activity, whereas insulin or propranolol reverse the norepinephrine effect. Phorbol esters also decrease ACS in rat adipocytes, suggesting a protein kinase C (PKC)-mediated inhibition. Thus, cAMP and PKC-dependent phosphorylation may inhibit ACS activity, and insulin-dependent dephosphorylation may activate ACS (1). ACS1, the isoform expressed only in differentiated 3T3-L1 adipocytes, would be a good candidate for this type of regulation; however, the evidence is indirect because the studies that suggested regulation by phosphorylation were performed before the ACS isoforms were cloned. These studies should now be performed with the cloned ACS isoforms.

Are Specific ACS Associated with Oxidation? Surprisingly, despite the associations of ACS1 with triacylglycerol biosynthesis outlined above, ACS1 mRNA expression is also induced by ligands for PPARα (20–22), suggesting that ACS1 may also synthesize acyl-CoA that are destined for oxidation. Upregulation is controlled by multiple promoters that result in three different mRNAs (A, B and C) differing in their 5′-untranslated regions (20). The A type of ACS1 mRNA is normally expressed in heart, adipose, and liver. In PPAR$\alpha^{-/-}$ mice, the constitutive level of hepatic ACS1 protein decreases, showing that PPARα is required for both normal as well as activated expression of ACS1 (23). The C type is expressed in heart and liver. Peroxisomal proliferators increase both A and C in liver, but the B type is observed in liver only after peroxisomal proliferators are given. Although no functional data or changes in relative subcellular localization were presented, Suzuki et al. (20) hypothesized that the A type of ACS1 mRNA was linked to lipogenesis, and the B and C types to peroxisomal β-oxidation. Because each of these different promoters results in the translation of the same open reading frame, it is difficult to understand how the ACS proteins produced would be associated with different functions unless the mRNA itself targeted newly synthesized ACS to specific membranes or allowed interaction with specific pathways. Although these authors attributed the increase in ACS1 with PPARα activation to a mitochondrial form related to β-oxidation, our studies show that ACS1 protein is not present in mitochondria or peroxisomes (24,25). Thus, the function of ACS1 remains unknown.

ACS4 and ACS5 are located in peroxisomes and mitochondria, respectively; thus, they might provide activated fatty acids for oxidation. Unlike ACS1, the expression of ACS5 mRNA does not change during adipocyte differentiation of 3T3-L1 cells, suggesting a function unrelated to triacylglycerol synthesis (1,9,26). However, ACS5 mRNA increases after food deprivation (9) and parallels β-oxidation. Further, in rats treated with a PPARγ activator, ACS5 mRNA in brown adipose tissue increases about sixfold (15). High ACS4 is observed in liver peroxisomes of rats treated with the PPARα activator gemfibrizol and might thereby provide some polyunsaturated fatty acyl-CoA for peroxisomal oxidation (25). Consistent with a deficiency in arachidonoyl-CoA metabolism, female ACS4$^{+/-}$ mice have increased uterine prostaglandin E_2 (PGE_2), 6-keto $PGF_{1\alpha}$, and $PGF_{2\alpha}$ (27). Taken as a whole, ACS4 and 5 seem less likely to be linked to triacylglycerol synthesis than ACS1, and their locations and responses to physiological stimuli suggest that they may activate fatty acids required for β-oxidation.

Is Acyl-CoA Partitioning Involved in Lipid-Mediated Toxicity in Type 2 Diabetes? Triacylglycerol stores in muscle, heart and pancreatic β-cells are associated with manifestations of insulin resistance and diabetes. In islets from Zucker diabetic fatty rats, lipogenic capacity is markedly increased compared with islets from lean rats, and there is no concomitant decrease in oxidation (28). The resulting large increases in islet triacylglycerol content are accompanied by β-cell apoptosis, which increases after islets are incubated with fatty acids and is preceded by an 82% rise in ceramide content (29). The metabolism of fatty acids to acyl-CoA seems essential for apoptosis because both apoptosis and the rise in ceramide are blocked by the ACS-1 and -4 inhibitor, triacsin C (29). In addition, triacsin C increases the low content of the antiapoptosis factor Bcl-2 in these β-cells, indicating that changes in Bcl-2 are mediated by acyl-CoA or their metabolites. High circulating fatty acids also inhibit insulin-stimulated glucose transport into muscle (30). In obese Zucker *fa/fa* rats, the heart becomes dilated and has reduced contractility; troglitazone therapy decreased the elevated myocardial triacylglycerol and ceramide levels and the increased DNA laddering (a measure of apoptosis), and prevented the decline in contractile function (31). Thus, acyl-CoA in β-cells, skeletal muscle and heart are heavily implicated in both the etiology and complications of obesity, insulin resistance and diabetes. The ACS isoforms present in β-cells and other tissues are imperfectly characterized and their subcellular locations have not been determined. More information is required to assess the effect of acyl-CoA partitioning on apoptosis.

Is There a Relationship Between Acyl CoA Partitioning and Insulin Resistance? Increased cell contents of triacylglycerols and fatty acid metabolites are associated with increased ACS1 mRNA expression in adipose tissue (16), impaired insulin secretion from β-cells (29,32) and impaired muscle glucose uptake. Transgenic mice that lack fat have severe insulin resistance, defects in IRS1 and IRS2 activation of PI3-kinase in muscle and liver, and a doubling of liver and muscle triacylglycerol content, suggesting that insulin resistance develops when fat partitioning is altered between adipocytes and other tissues (33). A current model proposes that accumulation of lipid metabolites leads to PKC activation and phosphorylation of insulin receptor substrates, which then fail to activate PI3-kinase, thereby impairing glucose transport; it was suggested that insulin resistance would be induced when fatty acyl-CoA or their metabolites accumulate in muscle and liver (34).

Perspective. We are far from understanding the roles of the individual ACS isoforms. If, as we have hypothesized, each isoform channels its acyl-CoA products into specific pathways, there must be protein-protein interactions that facilitate this vectorial function. We do not know how the ACS are targeted to specific membranes or whether a peripheral protein such as ACS4 moves from ER to peroxisomes. We have to identify the active site of ACS, its transmembrane domains and their roles, if any, in orienting substrates for catalysis. We also must determine the mechanism that underlies fatty acid preference, and whether acute regulation occurs by phosphorylation. With their overlapping substrate specificities, can ACS1, 4 and 5 substitute for each other? Can they substitute for medium-chain and very-long-chain ACS? Why are acyl-CoA thioesterases present in cytosol, mitochondria and peroxisomes and why are these induced by PPARα activators (35), which simultaneously up-regulate ACS1? Is ACS1 an essential component of the cellular fatty acid import machinery and, if so, why are additional ACS present on internal cell membranes? The answers to these questions should be forthcoming in the next few years and will likely lead to a search for therapeutic agents that target individual ACS associated with triacylglycerol synthesis or with β-oxidation.

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