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PHYSIOLOGICAL AND NUTRITIONAL REGULATION OF ENZYMES OF TRIACYLGLYCEROL SYNTHESIS

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■ **Abstract** Although triacylglycerol stores play the critical role in an organism's ability to withstand fuel deprivation and are strongly associated with such disorders as diabetes, obesity, and atherosclerotic heart disease, information concerning the enzymes of triacylglycerol synthesis, their regulation by hormones, nutrients, and physiological conditions, their mechanisms of action, and the roles of specific isoforms has been limited by a lack of cloned cDNAs and purified proteins. Fortunately, molecular tools for several key enzymes in the synthetic pathway are becoming available. This review summarizes recent studies of these enzymes, their regulation under varying physiological conditions, their purported roles in synthesis of triacylglycerol and related glycerolipids, the possible functions of different isoenzymes, and the evidence for specialized cellular pools of triacylglycerol and glycerolipid intermediates.

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INTRODUCTION AND PERSPECTIVES

Living organisms gained a significant metabolic advantage when they became able to convert existing pathways of fatty acid and phospholipid synthesis toward the synthesis of triacylglycerol (TAG), a molecule in which surplus energy could be stored, thereby enhancing survival in the face of limited exogenous fuel. Although we think of TAG as an energy storage pool, its initial role may have been to serve as a precursor for the synthesis of phospholipids or acetate-derived antibiotics, as occurs in *Streptomyces* (110) and tetrahymena (13).

TAG synthesis begins with the activation of long-chain fatty acids to their coenzyme A (CoA) thioesters. Acyl-CoAs are then esterified to glycerol-3-phosphate in two steps to form first lysophosphatidic acid, then phosphatidic acid. Phosphatidic acid is hydrolyzed to form diacylglycerol (DAG), which undergoes a final acylation step to form TAG (Figure 1). This pathway, largely identified by Kennedy and his coworkers in the 1950s, is comprised of the initial steps in glycerophospholipid synthesis as well (69, 150). Progress in understanding the regulation of TAG synthesis has been hampered, because most of the enzymes of TAG synthesis are integral membrane proteins that have resisted purification, and because most of the substrates are hydrophobic or amphipathic molecules that are difficult to deliver to membrane-bound enzymes. Only in the past few years have acyl-CoA synthetase, mitochondrial glycerol-3-phosphate acyltransferase, lysophosphatidate acyltransferase, and diacylglycerol acyltransferase been cloned. Availability of these cloned enzymes will help us to understand how the synthesis of components of secreted products such as bile, very-low-density lipoproteins (VLDL), and milk are coordinately regulated. Construction of knockout and transgenic mice will help us to answer questions concerning the specific functions of the multiple isoforms of the enzymes involved in TAG synthesis, and why these isoenzymes are present in different subcellular membranes. And if we solve the crystal structures of the acyltransferases, we will identify the mechanisms of action, clarify seemingly conflicting information about regulation, and determine the role of the membrane in enzyme activation, substrate specificity and orientation, and product channeling.

This review focuses on recent molecular approaches taken toward understanding the regulation of enzymes of TAG synthesis in mammals. Readers are referred

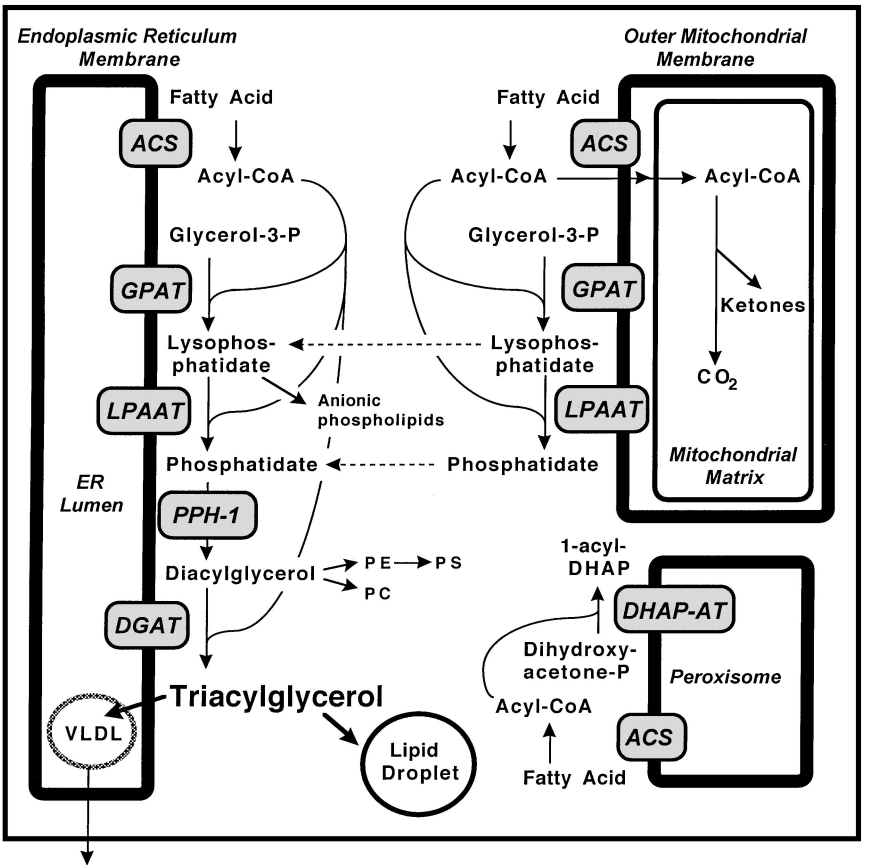

Figure 1 Major routes of triacylglycerol synthesis in hepatocytes. ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone-phosphate; DHAPAT, dihydroxyacetone-phosphate acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PPH-1, phosphatidate phosphohydrolase; PS, phosphatidylserine.

to earlier reviews for additional information about enzyme kinetics, substrate specificity, and descriptive physiological changes (5, 6, 83), related phospholipid synthetic enzymes (60), transcriptional regulation (135), positional asymmetry of fatty acids (153), and lipid synthesis in plants (105).

ENZYMES OF TRIACYLGLYCEROL SYNTHESIS

Acyl-CoA Synthetase

Long-chain acyl-CoA synthetase (ACS), the initial enzymatic step required for oxidation, elongation, and desaturation of fatty acids, and for the synthesis of

complex lipids and acylated proteins, adds a CoA thioester to fatty acids of 10–20 carbons to form long-chain acyl-CoAs (15, 147). The ACS active site lies on the cytosolic surfaces of the peroxisomal, endoplasmic reticulum, and outer mitochondrial membranes (28, 59, 79, 91). The relative distribution of ACS activity in human liver is 16% in peroxisomes, 21% in mitochondria, and 60% in microsomes (18). Although tissues like liver and adipocytes express as many as three different ACS isoforms, it remains uncertain as to which ACS isoforms are present in different subcellular organelles (74, 98, 113, 147). Localization is a critical point if different ACS isoforms associate with specific pathways (see below).

The five cloned ACSs range in size from 670 to 720 amino acids, and each contains two domains similar to that of luciferase. The second luciferase region and the C-terminal region are the most highly conserved in the different ACSs and may form the catalytic site (67). Each of the five cloned ACSs has a wide tissue distribution, but ACS1, ACS4, and ACS5 are prominent in liver and adipocytes, ACS2 and ACS3 in brain, ACS4 in adrenal and other steroidogenic organs, and ACS5 in intestine (45, 64, 67, 109, 136). ACS isoform 1 (ACS1), ACS2, and ACS5 make up a subfamily with about 60% homology, and ACS3 and ACS4 make up a second subfamily that has about 70% homology with each other and about 30% with ACS1. Each isoform can activate a wide range of saturated and unsaturated fatty acids of 12–20 carbons, but ACS4 is most active with 20:4, 20:5, and 22:6. The mechanism for fatty acid preferences has not been identified; mutational analysis suggests that seven amino acids located in the second luciferase region modulate fatty acid substrate specificity in mammalian and yeast ACSs and in *Escherichia coli* FadR (12). Little work has been done to identify any ACS structure, active site, or mechanism of action.

Changes in ACS enzyme activity under different physiological conditions suggest that ACS plays a critical role in regulating the entry of fatty acids into synthetic or oxidative pathways. The 100-fold increase in ACS activity when 3T3-L1 preadipocytes differentiate into adipocytes (34) is due to expression of ACS1 (109) and suggests an association of ACS1 with TAG synthesis. It will be useful to identify the isoforms associated with the alterations in ACS activity that occur during different physiological states, such as hibernation (102). ACS1, the best-studied isoform, is discussed later.

Relatively little information is available on ACS2–5 or the very-long-chain ACS (VLACS). The mRNA levels of ACS2, ACS3, and ASC4 have not been reported under different nutritional conditions (45, 46, 67). ACS5 mRNA in liver decreases 50% with fasting or with fasting followed by refeeding with a high-cholesterol diet, and it increases twofold after refeeding with a fat-free diet that is high in sucrose (109). Unlike ACS1, ACS5 does not increase in liver after refeeding with a high-fat diet. Although ACS5 mRNA abundance is highest in small-intestinal mucosa, diet has no effect on expression in this tissue (109). ACS1 mRNA is expressed in 3T3-L1 cells only after differentiation into adipocytes, but ACS5 mRNA is present at the same level before and after differentiation (109), which suggests that ACS5 is needed to activate fatty acids destined for phospholipid synthesis and that ACS1

is critical for TAG synthesis. VLACS, purified from rat liver peroxisomes and cloned (139, 140), differs in amino acid sequence from the long-chain ACSs and has a 40% amino acid identity and 72.9% similarity with FATP, a putative fatty acid transport protein (120). Because conversion of fatty acid to acyl-CoAs effectively traps fatty acids within cells, both ACS and VLACS may enhance the rate of fatty acid transport into cells by making the process unidirectional (39), and thereby increasing the rate of TAG synthesis.

Glycerol-3-Phosphate Acyltransferase

The initial and committed step in the de novo synthesis of TAGs and all cellular phospholipids except sphingomyelin is the acylation of *sn*-glycerol-3-phosphate to form 1-acyl-*sn*-glycerol-3-phosphate [lysophosphatidic acid (LPA)] by glycerol-3-phosphate acyltransferase (GPAT) (6). GPAT activity in mammalian tissues is comprised of two isoenzymes, one in the endoplasmic reticulum and the other in the outer mitochondrial membrane. The active sites of both isoforms face the cytosol (24, 25). In tissues other than liver, microsomal GPAT activity is approximately 10 times that of the mitochondrial fraction; in liver, GPAT activity is equal in these two organelles (6). Mitochondrial GPAT preferentially acylates glycerol-3-phosphate with saturated rather than unsaturated fatty acyl-CoA donors, possibly contributing to the asymmetric distribution of fatty acids in phospholipids (6). Even though mitochondrial GPAT activity decreases during starvation, the liver's capacity to synthesize TAG remains unimpaired when β-oxidation is inhibited (94). Responsiveness to starvation may therefore relate primarily to competition with carnitine palmitoyltransferase-1 for acyl-CoAs. It is not known whether microsomal and mitochondrial GPAT isoforms have different functions. Associations with enhanced TAG synthesis are suggested for both isoforms because the activity of the microsomal GPAT increases 70-fold in differentiating 3T3-L1 cells (27) and neonatal liver (31), and the mitochondrial GPAT mRNA and activity increase about 10-fold in differentiating 3T3-L1 cells (160).

Mitochondrial GPAT was cloned from both mice and rats (11, 106, 160) and purified to homogeneity from rat liver (143). The mouse enzyme was identified by its 30% identity and additional 42% similarity to a 300–amino acid region of *E. coli* GPAT (86, 160). Recombinant mouse and rat GPAT with an open reading frame of 827 amino acids have been functionally expressed (11, 38). The suggestion that rat mitochondrial GPAT is a 772–amino acid protein with a later initiating methionine (106) awaits confirmation by functional expression.

Four amino acid regions of strong homology (blocks I–IV) were identified in GPAT, lysophosphatidic acid acyltransferase (LPAAT), and dihydroxyacetonephosphate acyltransferase (DHAPAT) sequences from bacteria, yeast, nematodes, and mammals, which suggests that these regions comprise a catalytically important site and that the acyltransferase mechanism is similar (84). The consensus sequence for each conserved region is as follows: block I, [NX]-H-[RQ]-S-X-[LYIM]-D; block II, G-X-[IF]-F-I-[RD]-R; block III, F-[PLI]-E-G-[TG]-R-[SX]-[RX]; and block IV, [VI]-[PX]-[IVL]-[IV]-P-[VI]. Site-directed mutagenesis of

E. coli GPAT showed that the histidine and aspartate in block I, the glycine in block III, and the proline in block IV all play a role in GPAT catalysis, and that the phenylalanine and arginine in block II and the glutamate and serine in block III are important in binding the glycerol-3-phosphate substrate (84). The invariant histidine in block I may act as a general base to abstract a proton from the hydroxyl group at the *sn*-1 position of glycerol-3-phosphate and facilitate the nucleophilic attack on the thioester of palmitoyl-CoA, and the invariant aspartate in block I may act in a charge relay system with the histidine residue to increase the nucleophilicity of the glycerol-3-phosphate hydroxyl group (57). The critical residue for glycerol-3-phosphate binding is the invariant arginine in block II, which probably coordinates with the phosphate group in the glycerol-3-phosphate substrate (84). High-resolution analysis of protein crystals is needed for an accurate picture of the acyltransferase mechanism.

Lysophosphatidic Acid Acyltransferase

LPAAT acylates *sn*-1-acyl-glycerol-3-phosphate at the *sn*-2 position to form phosphatidic acid. Two human isoforms, α and β, with 46% homology have been cloned (41, 151). LPAAT-α is present in all tissues, predominantly in skeletal muscle, and LPAAT-β is found primarily in heart, liver, and pancreas. LPAAT-β mRNA may be regulated by alternative splicing (134). Human LPAAT-α has been localized to the endoplasmic reticulum (1). A third protein called endophilin I has LPAAT activity and is associated with clathrin-mediated endocytosis (122). By activity measurements, LPAAT activity is predominantly in microsomes, but it is also present in mitochondria. When mitochondria are incubated with glycerol-3-phosphate and palmitoyl-CoA, the major product is phosphatidic acid unless an LPA acceptor such as liver-FABP is present (142). In a reconstitution assay with liver fatty acid binding protein, LPA synthesized in mitochondria was transported to microsomes and converted to phosphatidic acid (53). LPAAT activity increases 2.5-fold postnatally in liver (31) and 59-fold during 3T3-L1 adipocyte differentiation (34), presumably via increased gene expression. Interleukin-1 acutely activates LPAAT in human mesangial cells (20). Changes of mRNA in response to diet have not been reported. When LPAAT-α was overexpressed in 3T3-L1 adipocytes, TAG synthesis increased and isoproterenol-stimulated lipolysis was suppressed, which suggests that the TAG synthetic pathway can be driven by increased availability of phosphatidic acid (118). Similarly, overexpression of yeast LPAAT (SLC1 gene product) in *Arabidopsis* and *Brassica napus* resulted in transgenic plants with 8%–48% increases in seed oil content and increases in very-long-chain fatty acids in the *sn*-2 position of the TAG (164). These experiments in plants and in cultured cells suggest the possibility that overexpressing any one of the enzymes in the synthetic pathway might increase TAG synthesis when acyl-CoA is not limiting. Alternatively, overexpression of an acyltransferase may act not to increase an enzyme-limited rate of esterification per se, but to increase substrate availability by regenerating CoA and allowing ACS to trap additional fatty acids in the cell.

Phosphatidate Phosphohydrolase

Phosphatidate phosphohydrolase (PPH-1) is unique among the enzymes of TAG synthesis in that it is amphipathic and probably hydrolyzes phosphatidic acid to form DAG only when it associates with the endoplasmic reticulum. Study of this enzyme has been neglected in recent years. Although it has not been purified or cloned, PPH-1 has been considered an important regulatory enzyme because its activity is low relative to other TAG synthetic enzymes, and because diet and hormone-induced changes in activity are more dramatic than changes in other pathway enzymes (138). Incubation of hepatocytes with fatty acid increases TAG biosynthesis with a concomitant increase in PPH-1 activity, possibly because long-chain fatty acids cause PPH-1 to translocate to the endoplasmic reticulum membrane. There is also a strong correlation between the activity of membrane-associated PPH-1 and the rate of TAG synthesis (138). Independent isoforms, termed PPH-2 or lipid phosphate phosphohydrolases, have been cloned and are part of DAG signaling pathways (17).

Dihydroxyacetone-Phosphate Acyltransferase

Microsomal GPAT can acylate both glycerol-3-phosphate and dihydroxyacetone-phosphate at the *sn*-1 position, but a separate and independent DHAPAT is located in peroxisomes (6). In addition to catalyzing the first reaction in the synthesis of ether lipids, peroxisomal DHAPAT can also provide an alternate route for LPA synthesis via the acylation of dihydroxyacetone-P and the subsequent reduction of the 1-acyl-DHAP product to LPA (6, 37). It is not known whether this route provides any significant amount of LPA for TAG synthesis, but in yeast that lack GPAT activity, the DHAP route ensures normal synthesis of phospholipids and normal yeast viability (2). Absence of peroxisomal DHAPAT in humans causes severe neurological impairment and skeletal deformities, but no alteration in TAG synthesis has been reported (108).

Monoacylglycerol Acyltransferase

Monoacylglycerol acyltransferase (MGAT) activities in liver and intestinal mucosa differ in substrate specificity, thermolability, and inhibition by amino acid modifying reagents, consistent with the existence of separate isoforms (30), each of which has been partially purified (8, 89, 90). The intestinal MGAT provides an alternate route for TAG synthesis by reacylating diet-derived *sn*-2-monoacylglycerols. Intestinal MGAT has not been reported to be regulated by diet. Because MGAT has a preference for *sn*-2-monoacylglycerols that contain polyunsaturated fatty acids, the liver isoform may prevent excess degradation of polyunsaturated fatty acids when β-oxidation rates are high. Thus, the monoacylglycerol pathway is active in liver during the neonatal period (157), with streptozotocin diabetes (100), and in hibernating animals (102) and is active in avian adipose tissue during migration (101). Conversely, in rat pups artificially reared on a high-carbohydrate diet,

the normal neonatal rise of hepatic MGAT activity is blunted and by day 12 is 80% lower than in mother-fed pups (33). The liver isoform, studied in mixed micelles, is activated by anionic phospholipids and can be fully activated by as few as seven molecules of phosphatidic acid (9). Stimulation either by its product *sn*-1,2-DAG (9) or by low concentrations of long-chain fatty acids (35) is highly cooperative, and fatty acids alter the enzyme's dependence on acyl-CoAs (29). MGAT is also inhibited by sphingosine (10) and by high concentrations of long-chain fatty acids (35). These studies suggest that hepatic MGAT may be directly regulated by several lipid second messengers, as well as by the influx of fatty acids from high-fat diets or from the hydrolysis of adipocyte TAG during fasting or diabetes.

Diacylglycerol Acyltransferase

DAG acyltransferase (DGAT), the enzyme unique to TAG synthesis, acylates DAG at the *sn*-3 position using either long- or medium-chain acyl-CoAs, and its highest activity is in tissues that specialize in TAG biosynthesis: adipose, liver, lactating mammary gland, small intestinal mucosa, and adrenal (6). Although the activity in the presence of ethanol is activated by taurocholate at concentrations that permeabilize membranes (111), the extremely low activity measured was not inhibited by the proteases that had previously been used to localize the active site of DGAT to the cytosolic surface (25, 28). Thus, the presence of a second DGAT isoform on the lumenal side of the endoplasmic reticulum remains an open question. Human (107) and mouse (23) DGAT were cloned because of homology to a yeast acyl-CoA:sterol acyltransferase. In mammalian cells, DGAT forms a family with acyl-CoA:cholesterol acyltransferases-1 and -2. The cDNA predicts a 498–amino acid protein with nine possible transmembrane domains and a conserved serine that is required for acyl-CoA:cholesterol acyltransferase activity (23). DGAT mRNA expression is high in human heart, liver, skeletal muscle, testis, small intestine, and colon. DGAT mRNA abundance increases eightfold during differentiation of 3T3-L1 adipocytes (23), but because DGAT activity increases 60-fold in these cells (34), the enzyme may also be regulated posttranscriptionally. Indirect evidence has been reported for inactivation by phosphorylation (see below).

Other Routes of Triacylglycerol Synthesis

A 52-kDa DAG transacylase, purified to homogeneity from rat intestinal microsomes, synthesizes TAG from two DAGs in a CoA-independent manner. Enzyme activity is 15% as high as that of DGAT (81) and could provide a significant amount of TAG. The transacylase uses both *sn*-1,2- and *sn*-1,3-DAGs to synthesize TAG, and the second product, *sn*-2-monoacylglycerol, is also a substrate for intestinal TAG synthesis. It is not known whether this transacylase is present in other tissues or how it is regulated. Phospholipid:DAG transacylases have not been identified in mammalian tissues.

NUTRITIONAL CONTROL

Transcriptional Regulation by Hormones

Enzymes involved in fatty acid and TAG synthesis are coordinately regulated through the counter-regulatory hormones insulin and glucagon. Insulin increases and the diabetic state reduces the activities of lipogenic enzymes. Streptozotocin-induced diabetes decreased fatty acid synthase activity 39%, mitochondrial GPAT activity 62%, microsomal GPAT activity 32%, and PPH-1 activity 37% in rat epididymal fat pads, and insulin administration restored these enzyme activities (119). Although diabetes decreases TAG synthetic enzymes in white adipocytes, it has no effect on these activities in brown adipocytes, despite an 80% decrease in the flux of [^{14}C]glucose into TAG (3). In perfused rat livers, insulin increases mitochondrial GPAT activity 34% and microsomal GPAT activity 9% (6). In addition, when fasted rats are refed a high-carbohydrate, low-fat diet, hepatic mitochondrial GPAT activity increases sixfold, with little change in the microsomal activity (38). Thyroid hormone may also play a role in regulating enzymes of TAG synthesis, as hypothyroidism increases the activity of microsomal GPAT and DGAT and decreases mitochondrial GPAT and PPH-1 (3).

Refeeding fasted animals a high-carbohydrate diet increases both fatty acid synthase and mitochondrial GPAT mRNA levels approximately 20-fold because of enhanced transcription rates (38, 135). Administering dibutyryl-cAMP to mice at the time of refeeding abolished the increase in FAS and mitochondrial GPAT transcription, which suggests that glucagon, which stimulates adenylate cyclase and increases cAMP levels, decreases mitochondrial GPAT transcription. In streptozotocin-diabetic mice, the increase in GPAT mRNA was not observed after refeeding unless insulin was administered.

Transcriptional Regulation by SREBP

Mitochondrial GPAT is also positively regulated by the cholesterol-responsive transcription factor SREBP-1, which up-regulates the synthesis of mRNAs for key regulatory enzymes of cholesterol and fatty acid synthesis (19). Mitochondrial GPAT expression increases 6.7-fold following ectopic expression of SREBP-1c in 3T3-L1 adipocytes (42). In liver, overexpression of SREBP-1c increases fatty acid synthase mRNA threefold, and overexpression of SREBP-1a results in a tenfold increase in mRNAs of both fatty acid synthase and mitochondrial GPAT (61). The murine mitochondrial GPAT promoter sequence contains three SREBP-1 sites and one NF-Y site, which are responsible for SREBP-1a- and NF-Y-mediated transactivation (42).

SREBP and insulin-stimulated transcription of fatty acid synthase and mitochondrial GPAT mRNA may be linked. SREBP-1c expression is itself regulated by an animal's nutritional state and by insulin, such that fasting decreases mRNA levels of SREBP-1c and fatty acid synthase 70%–90% in adipose and liver, and

refeeding increases them (72). Consistent with the proposed action through the insulin receptor, only 10 nM insulin was required to increase SREBP-1c expression in differentiated 3T3-L1 adipocytes. These data agree with reports showing that refeeding a high-carbohydrate/low-fat diet to fasted mice results in increases in nuclear SREBP-1c protein levels that parallel those of the mRNAs for lipogenic enzymes (61). Instead of using an SRE motif, however, SREBP-1c activates the fatty acid synthase promoter via an E-Box that lies within the previously identified insulin-responsive region (72, 135). Thus, SREBP-1c may act as an insulin-responsive transcription factor that regulates fatty acid synthase, other genes involved in cholesterol metabolism, and perhaps additional genes in the TAG synthetic pathway.

Role of Peroxisome Proliferator–Activated Receptor

Dietary polyunsaturated fatty acid (PUFA) decreases the transcription of hepatic genes encoding such lipogenic enzymes as fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CA desaturase, and malic enzyme (66), while concurrently increasing transcription of genes that encode enzymes required for fatty acid oxidation, including carnitine palmitoyltransferase 1 (14, 71). The nuclear transcription factor peroxisome proliferator–activated receptor alpha (PPARα) up-regulates oxidative gene transcription, but the regulator of the lipogenic enzymes appeared less certain because PUFA suppresses lipogenic gene transcription by a mechanism that does not directly involve PPARα (71, 117). However, administering a diet rich in PUFA or administering the PPARα-specific activator WY 14,643 reduces the hepatic nuclear content of SREBP-1c more than 50%; this decrease in nuclear SREBP-1c parallels a decrease in fatty acid synthase gene transcription and mRNA abundance (158). These data suggest that PPARα suppresses lipogenic genes by directly decreasing the amount of SREBP-1 and -2 present in liver nuclei, and thereby decreasing SREBP-activated lipogenic genes (154).

PPARγ, whose natural ligands are prostaglandin J_2 metabolites, is primarily found in adipocytes. Activation of PPARγ in preadipocytes causes adipocyte differentiation (131) with a concomitant induction of activities of the enzymes in the pathway of TAG synthesis (34). The PPARγ ligand BRL 49653 induces ACS1 in rat adipose tissue and muscle but not in liver or heart (92). Expression of PPARγ may be controlled by fatty acid activation of PPARδ, such that subsequent activation of PPARγ can induce terminal adipocyte differentiation (4). Another nuclear transcription factor that regulates a number of genes involved in energy metabolism, including proteins involved in cholesterol and lipoprotein metabolism, is hepatic nuclear factor 4α, whose high-affinity ligands are acyl-CoAs (58). No information is available on the regulation of enzymes of TAG synthesis by hepatic nuclear factor 4α.

Nutritional and Developmental Regulation of ACS1 Of the enzymes of TAG synthesis, only ACS1 is known to be regulated directly by PPARα, and it is speculated that fatty acids may be the physiological regulators that increase ACS1 mRNA

during physiological states that either induce fatty acid synthesis (136, 137) or release fatty acids from adipose stores (114). Further work is needed to determine whether PPARα causes ACS1 to redistribute to the mitochondria, where activated fatty acids can be channeled toward oxidation, and whether PPARα might reciprocally down-regulate the enzymes in the TAG synthetic pathway. When PPARα null mice were fasted for 24 h, TAG accumulated in their livers and they became hypoglycemic, hypothermic, and hypoketonemic, demonstrating the importance of PPARα in activating β-oxidation, which provides ATP for gluconeogenesis (71). Because starvation decreases activities of adipose ACS and mitochondrial GPAT, but not microsomal GPAT or PPH-1 (78), it will be of interest to determine whether other enzymes of TAG synthesis are also coordinately regulated by PPARα.

ACS1 mRNAs have 5′ heterogeneity, and PPARα independently regulates alternative transcription from three promoters after activation by drugs that induce peroxisomal proliferation and fatty acids (123, 137). One assumes that the increased fatty acids released from adipose stores during fasting would have the same effect. It would be interesting to know whether these different mRNA transcripts alter the subcellular distribution of ACS protein, because ACS1 mRNA increases during both TAG synthesis and β-oxidation. In this light it has been shown that, although liver mRNA values are similar for *ob/ob* and normal mice, a greater percentage of the ACS activity in *ob/ob* mice is present in microsomes, which again suggests possible enhancement of fatty acid esterification (96). Identification of protein isoforms in the organelles would have been useful in evaluating these changes in enzyme activity. Administration of lipopolysaccharide, tumor necrosis factor, or interleukin-1 to Syrian hamsters causes ACS1 mRNA to decrease more than 50% in liver, adipose tissue, heart, and skeletal muscle, with redistribution of greater than 50% of the activity from mitochondria to microsomes, consistent with the concomitant decrease in β-oxidation and increase in TAG synthesis (95). These differences would have been more convincing had markers for the organelles been shown.

Effects of Leptin

The peptide hormone leptin, which is expressed and secreted by adipocytes and acts via brain and peripheral receptors, regulates body weight by decreasing food intake and increasing energy expenditure (44). Serum leptin concentrations reflect adipose tissue mass but change in response to immediate alterations in adipocyte fuel metabolism, perhaps ongoing TAG synthesis (32, 40). Leptin regulates lipid homeostasis in adipose tissue, liver, muscle, and pancreas by upregulating mRNA expression of oxidative enzymes, and by stimulating fatty acid oxidation (103, 124, 141, 162). In addition to opposing TAG accumulation by favoring fatty acid catabolism, TAG biosynthesis might be a direct leptin target. In lean ZDF rats, adenovirus-mediated hyperleptinemia markedly decreases adipose tissue mRNA expression of acetyl-CoA carboxylase, fatty acid synthase, and

mitochondrial GPAT (163). Furthermore, in isolated islets from lean ZDF rats, leptin decreases mitochondrial GPAT and acetyl-CoA carboxylase mRNA expression 75%–80% (162), and adding leptin to rat adipocytes decreases fatty acid synthase mRNA 90% and increases PPARα mRNA twofold (148). In exteriorized fat pads, hyperleptinemia caused visible fat to disappear, indicating that neurotransmitters are not required to deplete TAG (149). These studies suggest that leptin acts directly on peripheral tissues to suppress TAG synthesis, which is consistent with the findings that in vitro, leptin depletes TAG in adipocytes, pancreatic islets, and muscle (103, 129, 141, 148). Collectively, these data suggest that leptin may regulate TAG homeostasis directly in both adipose and nonadipose tissues by promoting oxidation and suppressing biosynthesis.

Acute Regulation

In addition to long-term regulation of TAG synthesis at the level of gene transcription, acute mechanisms are needed to trigger responses to sudden changes in energy demand or supply. For example, during exercise and starvation, increased channeling of fatty acyl-CoAs into the mitochondria is critical to meet the energy requirements of muscle contraction or nutrient deprivation. Alternatively, after a high-carbohydrate meal, glucose oxidation increases, β-oxidation decreases, and fatty acids are preferentially esterified.

Hormonal Regulation Several enzymes of TAG synthesis are believed to be acutely regulated by phosphorylation and dephosphorylation, but direct proof remains elusive. cAMP-dependent protein kinase has been reported to inactivate GPAT in rat adipocyte microsomes, liver microsomes, and liver mitochondria (6), and phosphatidylinositol-specific phospholipase C mimics the insulin stimulation of microsomal GPAT in adipocytes and myocytes (145, 146). Adding NaF blocks the activation by insulin, which suggests involvement of a phosphatase. Other kinases have also been implicated in GPAT regulation. A partially purified tyrosine kinase from adipose cytosol inactivated microsomal GPAT, tyrosine kinase inhibitors prevented inactivation, and liver phosphatase reactivated GPAT (77).

There is evidence that other enzymes in the glycerolipid synthesis pathway are regulated acutely. Although incubating rat adipocytes with norepinephrine, ACTH, glucagon, or dibutyryl-cAMP decreases ACS activity, cAMP-dependent protein kinase did not affect ACS activity in adipocyte microsomes, which suggests that a nonmicrosomal ACS might be responsive (54). Glucagon and cAMP treatment of hepatocytes results in translocation of PPH-1 to the cytosol, where it is inactive (16, 138). This translocation is reversed when fatty acid is added (138), similar to the activation of CDP-choline cytidylyltransferase (70).

DGAT may also be regulated acutely. In rat liver microsomes, activity decreases 46% after incubation with cytosol, $MgCl_2$, and ATP (52). Similar results were observed in rat adipose tissue (77). Studies conflict as to whether glucagon or cAMP inhibits DGAT in rat hepatocytes and liver microsomes (138). DGAT

activity in rat liver and adipose microsomes is decreased in the presence of ATP, Mg^{2+}, and 105,000 $\times$ g supernatant. However, in hamster fibroblasts and other cultured cell lines, cAMP activates DGAT (83), and in guinea pig parotid gland lobules, isoproterenol and carbachol promote incorporation of acyl-CoAs into DAG and TAG, perhaps via phosphorylation of LPAAT and DGAT by cAMP-dependent or Ca^{2+}/calmodulin-dependent protein kinases (130). These conflicting results suggest that additional studies are needed with the now-cloned DGAT.

Coordinate Regulation at the Outer Mitochondrial Membrane Partitioning of fatty acids between degradative and biosynthetic fates appears to be acutely regulated via reciprocal modulation of the outer mitochondrial membrane enzymes, carnitine palmitoyltransferase-1 (CPT1) and mitochondrial GPAT (104). Coordinated regulation of CPT1 and mitochondrial GPAT is mediated by AMP-activated protein kinase (AMPK), which protects cells against the consequences of ATP depletion by inhibiting biosynthetic pathways and stimulating energy-generating pathways (55). In response to increases in the cellular AMP/ATP ratio, AMPK phosphorylates and inactivates liver acetyl-CoA carboxylase (21, 161) and decreases its product, malonyl-CoA, a potent inhibitor of CPT1 and β-oxidation. By decreasing malonyl-CoA, AMPK relieves the inhibition of CPT1 and thereby increases fatty acid oxidation (97, 144). In both muscle and liver, activated AMPK also inhibits fatty acid esterification into TAG by AMPK-dependent inactivation of mitochondrial GPAT, but not DGAT or microsomal GPAT (104). It is not known whether AMPK inhibits mitochondrial GPAT by directly phosphorylating the enzyme or by phosphorylating a regulatory protein. Because AMPK is activated by physiological stresses such as exercise and starvation, and inactivated by high sucrose feeding (55), AMPK-mediated regulation of mitochondrial GPAT could provide a mechanism whereby acute changes in energy status produce immediate adjustments in cellular TAG synthesis. Thus, when cellular ATP levels are compromised, not only does AMPK-mediated inactivation of mitochondrial GPAT conserve energy by inhibiting lipid biosynthesis, but by relieving competition with CPT1 for acyl-CoA substrates, it might also promote fatty acid entry into mitochondria. The precise role of mitochondrial GPAT in regulating TAG synthesis presents an intriguing question because mitochondria lack the enzymatic machinery necessary to synthesize glycerolipids beyond phosphatidic acid (6, 60).

PHYSIOLOGICAL CONTROL

Exercise

Exercise training decreases adiposity (93) and regulates whole-body TAG metabolism by increasing several of the key enzymes and proteins that regulate fatty acid oxidation, and by tissue-specific regulation of lipoprotein lipase (36). In rats, training decreases intraabdominal adipose tissue ACS activity and ACS1 mRNA levels and increases muscle ACS activity and mRNA expression (128). When rats

are fed diets containing fructose or sucrose, training decreases liver activities of acetyl-CoA carboxylase, fatty acid synthase, and mitochondrial GPAT (37%–60%) but does not affect the activities of liver microsomal GPAT or muscle mitochondrial GPAT (51). These limited data suggest that tissue-specific regulation of lipogenic enzymes might serve to divert fatty acids away from TAG biosynthesis in adipose tissue and liver, and to promote fatty acid delivery to muscle, where it is oxidized during contraction. Further studies are needed on the role of exercise training in modulating TAG biosynthesis.

Obesity

In obesity, TAG accumulation in both adipose and nonadipose tissues is closely associated with several metabolic comorbidities. TAG accumulation in muscle and pancreas occurs with insulin resistance (50, 75) and islet cell dysfunction (141), respectively, and in type 2 diabetes, both hepatic TAG synthesis in liver and VLDL secretion increase (85). These data have suggested that intracellular lipid accumulation might be causally linked to the pathogenesis of disordered energy homeostasis (141). Although mechanisms remain unclear, the close association between increased tissue TAG content and metabolic dysregulation indicates a pressing need to uncover the pathophysiological basis for this relationship.

During the development and maintenance of the obese state, overnutrition promotes TAG synthesis from increased substrate supplies, but there is also evidence that TAG biosynthetic enzymes are themselves up-regulated both physiologically and genetically. For example, in obese ZDF rats (*fa/fa*), the activities of adipose tissue ACS, mitochondrial GPAT, microsomal GPAT, PPH-1, and DGAT are 4- to 14-fold greater than in lean littermates (65, 127), and ACS1 mRNA abundance is increased two- to fourfold in liver, adipose tissue and pancreatic islets (80, 128). ACS1 mRNA is similarly increased in adipose tissue from leptin-deficient obese *ob/ob* mice compared with control littermates (96). Liver ACS1 mRNA values are similar for *ob/ob* and lean mice, but in *ob/ob* mice, a greater percentage of the ACS activity is present in microsomes than in mitochondria, which suggests that fatty acids are preferentially channeled toward esterification (96). In pancreatic islets of obese ZDF rats, mitochondrial GPAT activity and mRNA abundance is nine- and twofold greater, respectively, than in normal control rats (80). In rats made obese by lesioning the ventromedial hypothalamus, ACS1 mRNA increased 4.9-fold in mesenteric but not in subcutaneous fat on day 1, and in all adipose tissues by day 5, whereas ACS-specific activity increased about 10-fold. Thus, like increases in lipoprotein lipase and GLUT-4, adipose ACS1 expression is altered early in the course of obesity development (126). In differentiated 3T3-L1 adipocytes, insulin and T_3 induce twofold increases in ACS1 mRNA levels, whereas the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine down-regulates ACS1 mRNA (68), which suggests that, in addition to the acute effects of glucagon on fat cell lipolysis, there might be longer-term reciprocal effects of insulin and glucagon. Furthermore, treatment of obese ZDF rats with the antidiabetic drug troglitazone decreases islet mRNA

expression of ACS1 and mitochondrial GPAT by 38% and 57%, respectively, and concomitantly decreases islet cell TAG content (87, 125). Taken together, these findings indicate that increased activity and mRNA expression of several lipogenic enzymes accompanies obesity in rodents and probably contributes to TAG accumulation. Drug strategies that target these enzymes might provide important therapeutic benefits and deserve further investigation in both animals and humans.

REGULATION AT BRANCHPOINTS

Cells must maintain a balance between the synthesis of TAG and the synthesis of phospholipids for membranes, signal transduction, and lipoproteins. Two key regulatory branchpoints lie at the level of phosphatidate, the precursor of anionic phospholipids, and DAG, the precursor for phosphatidylcholine, phosphatidylethanolamine and TAG. These branchpoints are regulated by cellular ATP and CTP concentrations, which maintain the proportional synthesis of phospholipids and TAG. Increased cellular ATP concentrations in yeast favor phospholipid synthesis at the expense of TAG synthesis and vice versa via regulation of PPH-1 activity (22).

CTP is required for the biosynthesis of all phospholipids. When cell CTP concentrations decrease in H9C2 cells to less than 10% of normal levels, phospholipid biosynthesis is attenuated and DAG and TAG content increases (56). Reduced CTP levels did not affect the activities of phosphatidate: CTP cytidylyltransferase, cardiolipin synthase, or in vitro synthesis of phospholipids from glycerol-3-phosphate and CDP-DAG (56), consistent with data from yeast and mammalian cell lines in which phosphatidylcholine synthesis is elevated when CTP concentrations are high and decreased when cellular CTP concentrations are lowered. In general, it appears that cellular CTP concentrations are a universal signal for all phospholipid biosynthesis in eukaryotic cells.

Regulation at the DAG branchpoint appears to depend on the availability of the CDP-choline, CDP-ethanolamine, DAG, and acyl-CoA substrates (132, 138). Adding CDP-choline to permeabilized hepatocytes stimulates phosphatidylcholine synthesis and suppresses TAG synthesis through diversion of DAG (132). The availability of CDP-choline limits the rate of phosphatidylcholine biosynthesis, but because DGAT and choline phosphotransferase draw from the same pool of DAG, CDP-choline indirectly determines the rate of TAG synthesis as well. In the presence of sufficient CDP-choline, the affinity of DAG choline phosphotransferase for DAG is severalfold higher than that of DGAT, favoring phosphatidylcholine biosynthesis at low DAG concentrations (132). However, an increase in CDP-choline without increasing DAG does not result in an increase in phosphatidylcholine biosynthesis (138). DAG availability also affects DGAT activity. When DAG levels are elevated because of an abundant fatty acid supply, DGAT activity and TAG synthesis in rat hepatocytes increase (133). This result and other studies show that TAG biosynthesis is sensitive to changes in DGAT activity (138), and they suggest that DGAT may be a major control point for regulation of TAG synthesis.

PROTEIN AND LIPID REGULATORS

Both acyl-CoA binding protein and fatty acid binding protein stimulate several glycerolipid synthetic enzymes, presumably by binding and sequestering either long-chain fatty acids or fatty acyl-CoAs, which would otherwise inhibit enzymes either by product inhibition or by disruption of membranes (116). Because albumen can serve a similar function, the physiological function of these proteins remains an open question. Disruption of the yeast acyl-CoA binding protein blocked conversion of C18:0-CoA to C18:1-CoA, providing direct evidence that the acyl-CoA binding protein transports newly synthesized C18:0-CoA to the desaturase (121). TAG synthesis was not investigated in this study.

Altering specific lipids has been shown to affect several glycerolipid synthetic enzymes, including MGAT (see above) (9, 10, 29, 35) and ACS (99), which suggests that nutrient-induced changes in membrane phospholipid composition may regulate TAG synthesis. Diets containing different types of oil alter ACS activity in mitochondria and microsomes, but the question of changes in activity versus amount of protein was not addressed (7). It would be useful to repeat these measurements using antibodies against purified ACS isoforms and probes for the appropriate mRNAs.

LIPID POOLS AND CHANNELING

Organization of Pathway Enzymes

Investigations of cellular lipid dynamics during nutritional and physiological changes suggest that lipid molecules are functionally compartmentalized into distinct pools, thereby implicating a mechanism of regulation that is totally obscure at this time. Separate intracellular pools probably exist for each of the intermediates in the TAG synthetic pathway. These intermediates, DAG, LPA, and phosphatidic acid, are also lipid second messengers whose signals are believed to originate from the hydrolysis of existing membrane phospholipids rather than from the de novo synthetic pathway. How lipid pools are compartmentalized and channeled is not known, but it is likely to be critical in the regulation of TAG synthesis. The synthesis of glycerolipids could occur in a multienzyme complex so that hydrophobic intermediates become channeled within specific pathways. Partial copurification of ACS, MGAT, DGAT, and an acyl-CoA acyltransferase from rat intestinal mucosa is consistent with this view, although these proteins may have copurified because of nonspecific physical similarities (82).

Evidence for Acyl-CoA Channeling

Rather than having equal access to the many pathways in which they participate, evidence from a number of studies suggests that long-chain acyl-CoAs are functionally channeled toward specific metabolic fates, such as β-oxidation, esterification

and storage in endogenous lipid droplets, or esterification and secretion in VLDL. Channeling of acyl-CoAs toward synthetic or oxidative pathways by specific ACS isoforms has been shown genetically in yeast (73). How channeling can be accomplished remains a mystery because although acyl-CoAs synthesized on the cytosolic surface of membranes cannot flip across membrane bilayers, it is generally assumed they move freely within cell membrane monolayers and have access to the cytosolic active sites of all the acyltransferases present in those membranes (28, 43). In addition, long-chain acyl-CoAs are relatively water soluble and might also move through the cytosol either free or bound to acyl-CoA binding protein (115) or liver fatty acid binding protein (88). One possibility is that each ACS isoform contains structural binding sites that allow it to couple to an independently regulated pathway or channel the acyl-CoAs generated toward mutually exclusive products.

In mammalian cells, indirect evidence for acyl-CoA channeling is provided by studies using triacsin, which is a competitive inhibitor of ACS and a potent inhibitor of TAG synthesis, but not of cholesterol esters (155). In human fibroblasts, triacsin C eliminated de novo glycerolipid synthesis but did not alter lysophospholipid reacylation (63). Similar results were reported for the sulfo-conjugate of troglitazone, a noncompetitive inhibitor of ACS (47). Thus, when ACS is inhibited, acyl-CoA entry into some metabolic pathways is inhibited, whereas acyl-CoA entry into other pathways continues (63). The relevant acyltransferases have similar apparent K_ms, making it unlikely that these acyltransferases would function differentially when the amount of acyl-CoA is limiting (63).

Specialized TAG Pools Within Cells

Evidence suggests that specialized pools of TAG exist and that fatty acids mobilized from these pools are also targeted toward specific pathways. For example, in perfused liver and primary hepatocytes, extracellularly derived fatty acids appear to provide only a minor portion of the fatty acids that are used to synthesize the TAG that is directly incorporated into VLDL (48, 152). Instead, 60%–70% of the fatty acid esterified into VLDL-TAG is derived from lipolysis of a cytosolic TAG pool, regardless of the availability of extracellular fatty acid (48, 152), and agents that suppress lipolysis inhibit VLDL secretion (152). In HepG2 cells, the inability to secrete mature TAG-rich VLDL has been attributed to an inherent defect in a lipolysis/reesterification cycle (49). Thus, it appears that intact cytosolic TAG molecules are not incorporated into VLDL-TAG but, instead, must be partially hydrolyzed and then reesterified. Differences in the stereospecific distribution of acyl groups in intrahepatic TAG and VLDL-TAG support this view (159).

Not only does it appear that fatty acids of exogenous and endogenous origin are functionally compartmentalized, but within cells, specialized TAG pools may function as fatty acid reservoirs for distinct metabolic pathways. In liver, TAG is present in at least two distinct pools, cytosolic and microsomal. Studies using HepG2 cells suggest that only the microsomal pool is tightly coupled to VLDL

secretion (156). Incubating HepG2 cells with 0.2 mM oleate increases both TAG synthesis and secretion of apoB. However, 30–40 min after oleate is removed from the medium, stimulation of apolipoprotein (apo) B secretion is abolished, cytosolic TAG remains relatively unchanged, and microsomal TAG returns to baseline. Thus, changes in apoB secretion correspond with changes in microsomal TAG content.

During hepatic TAG lipolysis, excess fatty acid not targeted for secretion either returns to the cytosolic TAG pool or is oxidized. Dual labeling techniques in cultured rat hepatocytes reveal that not all fatty acid hydrolyzed from cytosolic TAG is available for β-oxidation (76). Conversely, in rat liver, glucagon and cAMP accelerate lipolysis, and the mobilized fatty acids are directed toward β-oxidation rather than into the secretory or reesterification pathways (152). These data might be explained by the existence of separate cytosolic TAG pools from which hydrolyzed fatty acids are specifically targeted to distinct metabolic pathways (e.g. biosynthesis versus oxidation).

The hepatic lipases responsible for hydrolyzing intracellular TAG have yet to be identified, but liver lipases might play a role in channeling fatty acids. When hormone-sensitive lipase was overexpressed in HepG2 cells, TAG lipolysis increased and TAG accumulation decreased, but none of the fatty acid derived from TAG was released into the medium either as unesterified fatty acids or as a component of VLDL-TAG (112). Instead, the hydrolyzed fatty acid was oxidized. Unless PPARα was up-regulated in this experiment, the data suggest that fatty acids mobilized by hormone-sensitive lipase, as occurs in muscle, are targeted specifically toward degradative pathways.

DAG Pools

In addition to providing substrates for β-oxidation and VLDL production, intracellular TAG also functions as a reservoir that supplies DAG moieties for phospholipid synthesis. Surprisingly, although fibroblasts from patients with the genetic disorder neutral lipid storage disease can synthesize phospholipids de novo via the glycerol-3-phosphate pathway, they are unable to recycle DAG hydrolyzed from cell TAG stores back to phospholipid (62). These data suggest that independent DAG pools exist in cells.

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