Regulation of Triglyceride Metabolism.

I. Eukaryotic neutral lipid synthesis: “Many ways to skin ACAT or a DGAT”

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Esterification of sterols, fatty acids and other alcohols into biologically inert forms conserves lipid resources for many cellular functions. Paradoxically, the accumulation of neutral lipids such as cholesteryl ester or triglyceride, is linked to several major disease pathologies. In a remarkable example of genetic expansion, there are at least eleven acyltransferase reactions that lead to neutral lipid production. In this review, we speculate that the complexity and apparent redundancy of neutral lipid synthesis may actually hasten rather than impede the development of novel, isoform-specific, therapeutic interventions for acute, type 2 diabetes, obesity, hyperlipidemia, fatty liver disease, and atherosclerosis.

IN MAMMALS, THE CYTOPLASMIC storage of fatty acids, sterols, and alcohols as neutral lipids provides reservoirs for steroidogenesis, bile acid synthesis, lipoprotein trafficking, membrane formation and maintenance, and epidermal integrity. Cholesteryl ester, triglyceride (TG), and wax ester synthesis provides a method for detoxification of fatty acids and alcohols. TG, a fatty acyl ester derivative of glycerol, is the major energy depot of all eukaryotic and some bacterial cells. The energy of oxidation of the alkyl chains of TG (38 kJ/g) is more than twice the same weight of carbohydrate or protein and, unlike polysaccharide, TG carries no extra weight as water of solvation. Moreover, TG is segregated into cytoplasmic lipid droplets and thus has no effect on the osmolarity of the cytosol.

Given such critical roles in cellular function, it is not surprising that many distinct pathways and genes exist for the production of neutral lipids (Fig. 1) and that they are conserved across kingdoms (17). In this review, we will focus on this enzyme redundancy and the concept that this may permit manipulation of the individual reactions as therapeutic targets in several disease pathologies.

Molecular Aspects of Sterol Esterification

In mammals, sterols are esterified with fatty acids but are biologically active (and potentially cytotoxic) in the nonacylated free form, where they contribute to membrane stability and act as substrates for steroid hormones or bile acids. In all eukaryotic cells, stereryl esters are stored in cytoplasmic lipid droplets. In mammalian liver or intestine, cholesteryl ester is packaged into lipoprotein particles in the endoplasmic reticulum for redistribution via the circulation. The esterification reaction thus represents a critical, evolutionary conserved step in sterol homeostasis. However, the accretion of cholesteryl ester in macrophages or smooth muscle cells leads to the formation of foam cells along the arterial wall and has been implicated in the development of atherosclerotic lesions. This has sparked interest in the elucidation of the mediators and mechanisms of these reactions, with hopes of treating atherosclerosis and/or hypercholesterolemia.

Two acyl-CoA cholesterol acyltransferase (ACAT) genes (1 and 2) have been identified and characterized in mammals (for reviews, see Refs. 4, 9, 17). ACAT1 is widely expressed with the highest levels in macrophages, steroidogenic tissues, and sebaceous glands as well as atherosclerotic lesions. ACAT2, on the other hand, is limited in its expression to the liver and small intestine (4, 17). These findings suggest that ACAT1 primarily incorporates cholesteryl esters into cytoplasmic lipid droplets, whereas ACAT2 is involved with lipoprotein assembly in the enterohepatic system. However, it remains unclear which ACAT predominantly operates in the human liver. Immuno-depletion experiments (7) indicate that ACAT1 accounts for more than 90% of ACAT activity in adult liver autopsy tissue although it is the less abundant transcript. By contrast, in the intestine the majority of ACAT activity is determined by ACAT2.

The ACATs are regulated by translational and posttranslational mechanisms (for review, see Ref. 4). ACAT1 mRNA is increased in the liver and aorta following a cholesterol-rich diet and after exposure to free fatty acids in human HepG2 cells. For the most part, however, ACAT1 is allosterically activated by cholesterol and oysterolys but not fatty acids. It seems likely, given the degree of sequence conservation, that ACAT2 is also allosterically regulated by cholesterol although transcriptional regulation of this isoform is observed.

Conservation of the ACAT Reaction and Consequences of the Loss of Sterol Esterification

Esterification of sterols has been conserved throughout eukaryotic evolution. In yeast, ACAT-related enzymes 1 and 2 (ARE1 and ARE2) are the orthologs of mammalian ACATs. Although deletion of ARE1 and ARE2 eliminates sterol esterification in yeast, the cells are viable because of downregulation of ergosterol synthesis. Deletion of ARE2 leads to a marked reduction in ergosterol esterification, reflecting its substrate preference for the major free sterol in yeast. ARE1 esterifies a wider range of sterols and thus likely serves to detoxify other sterols within the cell (17).

Further insight into the role of each of the ACATs has been gleaned from knockout models in mice. Mice with partial ACAT1 activity (Acact1−/−) are healthy with normal serum cholesterol, intestinal cholesterol absorption, and hepatic ACAT activity (owing to the presence of ACAT2). However, ACAT1-null mice (Acact1−/−) suffer from dry eyes due to...
ACAT inhibitors have long been sought as potential therapeutics for atherosclerosis. Several animal studies involving nonselective ACAT inhibitors (e.g., avasimibe, pactimibe) showed a promising reduction of atherosclerotic plaques (14). Unfortunately, human trials have proven to be disappointing, likely because generalized ACAT inhibition leads to an increase in free cholesterol levels, apoptosis, and destabilization of ATP binding cassette protein A1 (ABCA1), a crucial mediator of reverse cholesterol transport (14). However, ACAT2 specific inhibition may be a viable option for future pharmacological intervention. Atherosclerosis-susceptible mice that were given liver-specific antisense oligonucleotides targeting ACAT2 were resistant to diet-induced hypercholesterolemia and had fewer aortic atherosclerotic lesions (2).

Molecular Aspects of Diacylglycerol Esterification

Diacylglycerol, the obligate precursor to TG, is derived either from the glycerol-3-phosphate pathway or the monoacylglycerol pathway whereby 2-monoyacylglycerol, a lipolysis product of TG, is reesterified. Whatever its source in mammals, diacylglycerol is esterified to TG by an acyl CoA:diacylglycerol acyltransferase (DGAT) reaction (17). There are at least two independent mammalian enzymes known to catalyze this reaction, DGAT1 and DGAT2 (11). DGAT1 is closely related to the ACATs (Fig. 1A); the divergence in its amino acid sequence redefines its substrate specificity to diacylglycerol (5). DGAT2 defines an unrelated gene family (Fig. 1B). Both enzyme families are conserved across multiple organisms including yeast and plants (17).

In humans, DGAT1 is highly expressed in human small intestine, colon, testis, and skeletal muscle but has notably lower levels of expression in adipose and liver (5, 16). Mice lacking DGAT1 (Dgat1<sup><s>-/-</s></sup>) were found to have normal plasma TG levels, suggesting other mechanisms by which mammals synthesize TG (19). Subsequently, DGAT2, the original member of the second human DGAT family, was identified by sequence similarity to proteins purified from Mortierella ramanniana, an oleaginous fungus (6). Members of the DGAT2 family have no sequence homology to the ACAT family, including DGAT1. They are likely derived from an ancestor with lysoosphatidic acid acyltransferase (LPAAT) activity, required for the final steps in phosphatidic acid biosynthesis. This homology is maintained in the whole human DGAT2 gene family and includes the conservation of residues at the active sites of bacterial glycerol-3-phosphate acyltransferases.

When expressed in insect or yeast cells, DGAT2 produces robust DGAT activity and, like DGAT1, shows little preference in terms of the fatty acyl-CoA substrate (6). DGAT2 possesses widespread expression in humans, with particularly high levels in liver and adipose tissue (6). The expression patterns of the DGATs indicate that they may have different functions within different tissues. DGAT1 likely plays a role in intestinal repackaging of free fatty acids using the monoacylglycerol pathway, whereas DGAT2 may function primarily in TG synthesis and export from the liver and deposition in adipose tissue.

TG Biosynthesis Is Conserved in Yeast

Studies of neutral lipid biosynthesis in yeasts such as Saccharomyces cerevisiae have led to further major insights into the mechanisms and mediators of TG biosynthesis in eukaryotes. In yeast, three structurally different enzymes mediate diacylglycerol esterification (for review, see Ref. 17). By a novel (PDAT; phospholipid diacylglycerol acyltransferase) reaction, the LRO1 gene product, an ortholog of mammalian lecithin-cholesterol acyltransferase (LCAT), mediates TG synthesis up to 75% of the normal strain, depending on culture conditions. LRO1 utilizes phospholipids in an acyl-CoA-independent esterification of diacylglycerol, a reaction that predominates during the growth phase of yeast. PDAT (i.e., LRO1) orthologs have been identified in multiple plant species; however, mammalian LCAT specifically uses cholesterol as its substrate. Interestingly, under appropriate conditions mammalian LCAT is able to esterify diacylglycerol to TG. Presumably as serum lipoprotein trafficking systems evolved the substrate specificity of the LCAT orthologs shifted from diacylglycerol to sterols and from a subcellular to extracellular location.

The product of DGA1, the sole yeast ortholog of human DGAT2, mediates the majority of TG synthesis in LRO1 knockout strains (17). A yeast ortholog of ACAT and DGAT1, ARE2, plays a minor role in TG synthesis (17). Deletion of DGA1, LRO1, ARE1 and ARE2 completely abolishes the ability of yeast to synthesize any TG or sterol ester, thus defining the enzymes responsible for neutral lipid synthesis in yeast. Surprisingly, under standard conditions, the quadruple deletion strains (are1Δ are2Δ dga1Δ lro1Δ) are healthy and have no...
growth defects (17). It appears from these studies that, under stress-free conditions, neutral lipid storage is not necessary for survival. Neutral lipids probably play more of a role during periods of nutrient deprivation or cellular stress. In view of this, utilization of yeast strains devoid of background neutral lipid synthetic abilities has facilitated the study of mammalian mediators of neutral lipid metabolism (23).

Consequences of the Loss of Diacylglycerol Esterification: Is the DGAT Reaction a Therapeutic Target for Obesity?

Aberrant DGAT expression in any of several tissues or organ systems may play a role in disorders such as obesity and nonalcoholic fatty liver disease. DGAT1 mRNA levels increase sevenfold when 3T3L1 cells are induced with insulin and dexamethasone to differentiate into adipocytes, leading to a 90-fold increase in DGAT1 protein and DGAT activity (26). However, when DGAT1 is overexpressed in undifferentiated 3T3L1 cells, a 20- to 40-fold increase in mRNA is associated with a modest 2- to 3-fold increase in DGAT activity. TG turnover remained stable, and thus cellular TG mass was doubled, suggesting that DGAT1 is rate limiting in TG synthesis. However, mice that lack DGAT1 (Dgat1−/−) have normal serum TG levels, indicating that DGAT1 does not play an essential role in the export of TG from the liver (19). DGAT2 expression increases 30-fold upon differentiation of 3T3L1 cells and yet further when treated with glucose and insulin (13). Glucose preferentially enhances DGAT1 mRNA expression, whereas insulin increases the level of DGAT2 mRNA. However, when fasted mice are fed a high-carbohydrate meal, DGAT2 but not DGAT1 mRNA is increased in liver, adipose, and small intestine.

Mice lacking DGAT1 (Dgat1−/−) exhibit 50% less body fat but are otherwise healthy and fertile with normal serum TG (19). Their observable phenotypes include poor milk productivity but are otherwise healthy and fertile with normal serum TG and thermogenesis (8, 19). This suggests that factors secreted from adipocytes in a DGAT1 deficiency state are responsible for the improved body fat, glucose disposal, and energy expenditure. Adiponectin, an adipocyte secreted factor that stimulates energy expenditure, was ruled out in that mice lacking both adiponectin and DGAT1 continue to be protected from obesity and hepatosteatosis (22).

These approaches were complemented by studies in which DGAT1 was overexpressed in cell culture or in specific tissues (15). Transgenic mice that overexpress DGAT1 in white adipose tissue become obese owing to adipocyte TG deposition but surprisingly are insulin sensitive and have normal glucose disposal. Overexpression of DGAT1 in adipocytes of obese-resistant FVB mice prompted hepatic steatosis in association with obesity resistance, elevated plasma free fatty acids, and insulin and leptin resistance.

Little is known about DGAT expression and regulation within the liver. Two topological types of DGAT activities have been described biochemically in studies using rat liver microsomes: an overt DGAT activity associated with cytosolic droplet TG synthesis, and a latent DGAT activity in the lumen of the endoplasmic reticulum that may be responsible for TG secretion in lipoprotein particles (18). However, the expression of DGAT1 and DGAT2 does not correlate with either of these DGAT activities. Overexpression of human DGAT1 in rat hepatoma McA-RH7777 cells increases synthesis, cellular accumulation, and secretion of TG (12). This is associated with decreased intracellular degradation of newly synthesized apolipoprotein B. In support of these findings, mice overexpressing DGAT1 via an adenoviral-mediated gene transfection exhibit increased hepatic very low-density lipoprotein secretion. The precise role of DGAT1 in the development of fatty liver syndromes such as nonalcoholic fatty liver disease (NAFLD) remains to be determined. However, in a small study, DGAT1 expression was found to be upregulated in the livers of individuals with this syndrome (15).

Mice deficient in DGAT2 (Dgat2−/−) are severely depleted of TGs in their tissues and plasma, leading to neonatal death from metabolic disarray and poor skin barrier function (21). DGAT1 was unable to compensate for the loss of DGAT2, suggesting different roles for the two enzymes, and that DGAT2 is the enzyme responsible for the majority of TG synthesis in mice and may not be an ideal target for treatment of obesity disorders. Conversely, a marked reduction in hepatic TG content and steatosis arose when hepatic DGAT2 expression was reduced with antisense oligonucleotides in wild-type and ob/ob obese mice on high-fat diets (25). Several studies found that, when hepatic DGAT2 is overexpressed in mice, liver TG mass increases without a concomitant change in very low-density lipoprotein secretion. Thus, DGAT2 may be a legitimate target for dyslipidemic-specific disorders such as NAFLD.

The DGAT2 Gene Family: Many More Neutral Lipids?

As described here, eukaryotic cells have evolved at least three independent mechanisms to neutralize and/or store alcohols such as sterols and diacylglycerols. In humans, the DGAT2 gene family is even more complex, comprising six additional members, all of which synthesize TG in vitro (Fig. 1B). Three members of this family are autosomally encoded acyl-CoA:monoacylglycerol acyltransferases (MGATs 1–3, re-
viewed in Ref. 9) that direct the synthesis of diacylglycerol by esterification of monoacylglycerol. Two encode acyl-CoA wax alcohol acyltransferases (AWATs 1–2) that synthesize wax esters by esterification of long-chain alcohols (23). The latter reaction appears to be particularly important in the sebocyte, where wax esters comprise a significant component of sebaceous gland secretions. The final member of this gene family (termed hDC3) remains to be characterized with regard to substrates other than diacylglycerol or wax monoalcohols.

The MGAT reaction provides an alternative to the Kennedy pathway for the synthesis of diacylglycerol that is particularly important for dietary fat absorption at the intestinal enterocyte, where TG is lipolysed to monoacylglycerol and free fatty acids, and then resynthesized and secreted in chylomicrons. However, why are there three MGATs in humans? It has been postulated that the existence of multiple MGATs may be related to specific tissue expression patterns. For example, mouse MGAT1 is expressed in most tissues but not in the intestine. MGATs 2 and 3 are primarily expressed in the intestine and likely are the key contributors to TG repackaging within the enterocyte. Interestingly, MGAT3 transcripts are most abundant in the ileum, distal to the regions of maximum lipid absorption. The MGATs are also partially defined by their substrate specificity in that MGAT3 prefers 2-monocacylglycerol, the predominant product of TG lipolysis in the intestinal tract, but MGATs 1 and 2 do not have such substrate specificity. The MGATs also exhibit some DGAT activity, and so the precise physiological roles of these members of the DGAT2 gene family remain to be determined.

Interestingly, the remaining members of the human DGAT2 gene family belong to an X-linked cluster (Xq13.1) of ~200 kbp (Fig. 1B). All three enzymes are highly expressed in the skin and possess significant DGAT activity; however, two of these (AWAT 1 and 2) were found to predominantly mediate the esterification of various fatty alcohols and fatty acids into wax esters via an acyl-CoA wax alcohol acyltransferase reaction (23). A murine wax synthase ortholog of AWAT2 with similar fatty acyl-CoA and fatty alcohol preferences was also reported (10). Wax esters are abundant in the cuticle of plants, insect exoskeleton coating, and mammalian sebum where they likely act as permeability barriers.

The existence of two AWATs can be explained by their different expression and substrate specificity patterns. Within the sebaceous gland, the peripheral layer of sebocytes is comprised of undifferentiated cells deficient in lipid droplets. As cells shift toward the center of the gland, they mature and accumulate lipid droplets (20). AWAT expression is limited to the sebaceous gland in a differentiation specific manner (23). AWAT2 is restricted to undifferentiated peripheral sebocytes, whereas AWAT1 is expressed in more mature, centrally located cells. The expression and substrate specificity pattern of the AWATs suggests that wax ester metabolism reflects and may play an important role in sebocyte differentiation. It is possible that as immature sebocytes differentiate, the wax esters undergo hydrolysis and reesterification to optimize the fatty acid and alcohol saturation and chain length within the sebaceous milieu, thus providing maximum hydrophobicity and protection for the skin. One may postulate, then, that aberrant expression of the AWATs may lead to any number of disorders in which the lipid composition of the skin is awry, such as acne vulgaris or ocular rosacea.

Conclusions: “Many Means to an End”

There are multiple pathways to the formation of neutral lipids in eukaryotes. These reactions provide a critical resource for many distinct cellular processes and their loss is often catastrophic but not immediately fatal. The importance of these reactions is further demonstrated by the fact that they are conserved across many billions of years of evolution and have arisen independently at least three times. This apparent redundancy in neutral lipid synthesis may be advantageous; marked changes in lipid homeostasis arise when expression of the DGATs is altered in mammals, but embryonic lethality is not a consequence. Further clarification of the metabolic pathways of neutral lipid synthesis may hasten the development of therapeutic interventions for several diseases. It is interesting to speculate that isoform-specific inhibition of DGAT1, DGAT2, ACAT2, or the AWATs may be effective and nontoxic therapeutics for type 2 diabetes and obesity, NAFLD, hyperlipidemia and atherosclerosis, or acne, respectively.

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