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THE TWISTS AND TURNS OF SPHINGOLIPID PATHWAY IN GLUCOSE REGULATION

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Abstract

Palmitic acid is a saturated fat found in foods that lead to obesity, cardiovascular disease, and Type II diabetes. It is linked to the development of resistance to insulin stimulation in muscle, liver and other organs involved in glucose metabolism, which, in turn, underlines the onset of Type II diabetes. The cellular and molecular mechanisms of this insulin resistance are complex and not completely understood. This article is focused on the role of palmitic acid as a precursor in the synthesis of sphingolipids, a class of lipid molecules that participate in cellular stress response. Recent evidence had indicated that increased dietary supply of palmitate can stimulate the rate of sphingolipid synthesis in “lean” tissues and generate excessive amounts of sphingolipid metabolites that have a negative effect on the insulin signaling cascade. Many experimental results point to the existence of a causative link between sphingolipid synthesis, insulin response, and hyperglycemia. It is not yet clear, however whether ceramides or glycosphingolipids are involved as both have been implicated to be inhibitors of the insulin signaling cascade. Evidence for a coordinated regulation of sphingolipid and tri/diacylglycerol metabolism complicates further the delineation of a single mechanism of sphingolipid effect on glucose homeostasis.

Keywords

ceramide; liver; triacylglycerols; serine palmitoyltransferase; acid sphingomyelinase

Introduction

“Metabolic syndrome” is a cluster of metabolic disorders including obesity, insulin resistance, hyperglycemia, dyslipidemia, hypertension, and cardiovascular disease. The consumption of food with high fat content and the resulting pathology in lipid metabolism is causatively linked to metabolic syndrome. In a healthy state, the excess fatty acids accumulate in the adipose tissues in the form of triacylglycerol (TAG); however, in conditions characterized by the metabolic syndrome, TAG droplets form in the “lean” organs such as liver, muscles, and pancreas. The accumulation of excessive fat, mostly neutral lipids, in the parenchymal cells of these organs is known as steatosis. The development of hepatic steatosis in particular, is a strong predictor for the onset of insulin resistance and hyperglycemia in obese individuals [1]. The precursor of TAG synthesis,

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diacylglycerol (DAG), has been identified as the bioactive metabolite that can inhibit insulin signaling pathway in liver and muscle, thus leading to a decreased cellular response to insulin stimulation and to the onset of hyperglycemia; however it is hard to accept that this is the sole or even the main mechanism, as increasing number of studies provide evidence that the different types of fats have complex effects on cell lipid homeostasis. Insulin resistance in particular is associated with high intake of saturated and monounsaturated fats and low intake of polyunsaturated fats. Epidemiological studies consistently suggest that the fatty acid pattern that is associated with insulin resistance includes high proportion of palmitic acid (a saturated fat) and palmitoleic acid (a monounsaturated fat), but low content of linoleic acid (a polyunsaturated fat) [2]. These specific fatty acids can induce changes at different level of cellular lipid metabolism and properties, including but not limited to changes in membrane fluidity and biophysical characteristics, the lipid microenvironment of integral membrane proteins, the rate of fatty acid oxidation, and gene expression via PPAR or other receptors that bind lipids (i.e. zTLR-4)[3].

A growing body of evidence however, shows that steatosis is also associated with an increased synthesis of sphingolipids and an accumulation of ceramide, a bioactive sphingolipid metabolite [4–7]. These findings had outlined a novel concept for the development of insulin resistance in lean tissues because the accumulation of sphingolipids and ceramide, in particular, during the consumption of high fat diet is linked solely to the increased up-take of palmitate, but not other fats.

Sphingolipids are class of lipids that contain a characteristic 18-carbon amino-alcohol, sphingosine, or its saturated analog, sphinganine. The rate-limiting step in the synthesis of sphingolipids is catalyzed by serine palmitoyltransferase (SPT) [8], which exhibits a high degree of specificity for the CoA-thioester of palmitic acid [9,10]. The flux through the sphingolipid synthetic pathway depends upon the availability of palmitate [11,12]. Substantial increases in sphingolipid synthesis have been observed in C₂C₁₂ myotubes, adipocytes, and hepatocytes when the culture medium is supplemented with palmitate, as well as in muscles and liver of mice, when the animals are fed food with elevated content of saturated fats, mainly palmitate. These additions have also led to the accumulation of ceramide, a bioactive lipid molecule, and glycosphingolipids and sphingomyelin (SM), ceramide metabolites that are the major components of the lipid rafts. The latter are specialized microdomains on the plasma membrane that seem to play an essential role in cell signaling by facilitating activation of various receptors by their respective ligands. The membrane microdomains have been recognized as critical for proper compartmentalization of insulin signaling [13].

A number of studies have suggested that palmitate-induced accumulation of ceramide might be responsible for the onset of insulin resistance in lean tissues [14,15]. This evidence can be briefly summarized as follows:

- i. Palmitate-independent increases in ceramide in various cells are sufficient to blunt cellular insulin response in vitro. Cell-permeable ceramide analogs have been shown to antagonize insulin signaling in myotubes [7,16] and adipocytes [17]. The mechanisms of these effects of ceramide involve an inhibition of Akt-1, key mediator of the insulin signaling cascade. Ceramide efficiently blocks the translocation of Akt-1 to the plasma membrane [7] in muscle cells [16,18,19], in adipocytes [19] and in the cells of the vasculature [20,21]. Increases in cellular ceramide content induce Akt dephosphorylation in C₂C₁₂ myotubes [14], adipocytes [22,23], and PC12 cells [24] through the activation of protein phosphatase 2A.

- ii. Myriocin, cycloserine, or fumonisins B1 that specifically inhibit the *de novo* synthesis of sphingolipids are shown to promote normal phosphorylation and translocation of Akt-1 in response to insulin, even in the presence of excess palmitate. Inhibition of SPT in animals similarly blocks the flux of palmitate through the *de novo* pathway, leading to less lipotoxicity, improved insulin response, and better glucose regulation [25,26]. Together, these data indicate that elevation in ceramide biosynthesis is required for the onset of insulin resistance; it is less certain however, whether ceramide effects are direct or mediated by other bioactive lipids.

Mechanisms of stimulation of *de novo* ceramide synthesis by palmitic acid

The sphingolipid biosynthesis (Fig. 1) starts in the endoplasmic reticulum with the condensation of L-serine and palmitoyl CoA, a reaction catalyzed by SPT. The product, 3-ketosphinganine, is first reduced to sphinganine and then it is acylated at the amide group by dihydroceramide synthase (CerS) forming dihydroceramide [27]. Over the past few years, six mammalian CerS (formerly known as Lass (longevity assurance)) genes were described, with each generating dihydroceramides with specific acyl chain lengths [28,29]. Dihydroceramides however are biologically inactive and are converted to the bioactive form, ceramides, after the introduction of a characteristic 4,5-double bond in the sphingoid portion of the molecule.

A rate-limiting step in this pathway (often referred to as the *de novo* pathway) is the activity of SPT (reviewed in [8]) which is affected by the concentration of the available palmitate [11,12]. Increases in the amount of palmitate (but not other free fatty acids) stimulate the formation of sphingolipids in a variety of cells. In cultured primary hepatocytes, for example, the addition of palmitic acid at a concentration as low as 0.4 mM induces sphingolipid biosynthesis within 4 h [30]. Similarly, HepG2 cells exhibit 3 to 6 fold higher rate of incorporation of radiolabeled palmitate when the latter is added at high (1 mM), as compared to low (0.1 mM) concentration [31]. Rat hepatoma H4IIE cells synthesize more ceramide when incubated with 0.5 mM palmitate but not when treated with oleate instead [32]. Increased biosynthesis of ceramide as a consequence of supplementation with palmitic acid has been shown also in long-term cultures of isolated islets [33], in muscle cells including C₂C₁₂ myotubes [14,15,34], L6 skeletal muscle cells [18,35,36], human vastus lateralis myoblasts [37], cardiac myocytes [38] and isolated rat soleus muscle [26], in human umbilical vein endothelial cells (HUVECs) [39], in cultured bovine retinal pericytes [40] and in astroglia [41]. Therefore, the upregulation of ceramide by elevated palmitate appears to be a common response in mammalian cells in culture.

Increased dietary intake of palmitate has similar effect on sphingolipid biosynthetic pathway *in vivo*. Rodents maintained on experimental diets enriched in saturated fats (with palmitate being the most abundant saturated fatty acid) have higher levels of ceramide in the plasma [42], liver [31], heart [43], soleus muscle and in the red, but not the white, section of gastrocnemius muscle [44]. Dihydroceramide and sphinganine, precursors of ceramide generation via the *de novo* pathway, are also elevated [31], consistent with a role of SPT in the diet-induced increases in ceramide content.

The mechanisms for this stimulation of the *de novo* sphingolipid synthesis have been extensively studied and seem to be more complex than anticipated. In addition to increasing the flux of the substrate palmitate through the pathway, diets enriched in saturated fats seem to affect the activity, the transcription, and the post-translational processing of SPT. An increase in mRNA levels of SPT1, one of the two subunits of SPT, was observed in developing hypothalamus [45], liver, [46], and adipose tissues of C57BL/6J mice fed diets enriched in fats [47]. mRNA levels of SPT1 and SPT2 are markedly elevated in adipose

tissues [4] and myocardium of obese patients [48], and in different animal models of obesity such as ob/ob mice [4] and fa/fa rats [49]. Our recent study found no change in SPT mRNA levels in the liver of C57/B16 mice fed saturated fat-enriched diet, although SPT1 protein levels, as well as SPT specific activity were markedly increased [31].

At least in liver, the flux through the *de novo* pathway is also regulated by a negative-feedback mechanism reflecting the rate of sphingolipid clearance in the lysosomes. Our recent study found that the *de novo* sphingolipid synthesis is stimulated by palmitate more potently in mice and cells lacking acid sphingomyelinase, the enzyme that catalyzes the first step in the turnover of sphingomyelin in the endosomal/lysosomal subcellular compartment, as compared to animals and cells with functional acid sphingomyelinase [31]. The differences were abrogated by the SPT inhibitor, myriocin, which supports the existence of a negative-feed back mode of regulation of the *de novo* synthesis of sphingolipids.

Palmitate-induced stimulation of *de novo* ceramide synthesis is required, but not sufficient, for the onset of insulin resistance

Myriocin (ISP-1) is an atypical amino acid antibiotic derived from fungal strains such as *Mycelia sterilia* and *Isaria sinclairii*, which inhibits specifically SPT [50]. Since its discovery, it has been routinely used to test the role of the *de novo* sphingolipid synthesis in a variety of experimental systems. Myriocin treatment prevents palmitate-induced accumulation of ceramide in human myoblasts and C₂C₁₂ myotubes [37] [51] [14]. More importantly, it also seemed to restore normal insulin sensitivity of these cells evidenced by an improved phosphorylation of Akt-1 and GSK3 β .

Administration of inhibitors of the *de novo* sphingolipid synthesis in animals reveals a diverse role of the pathway in the development of metabolic syndrome. Application of L-cycloserine, another SPT inhibitor, completely blocks the formation of ceramide in isolated pancreatic islets and β -cell lipoapoptosis in prediabetic Zucker fa/fa rats [49]. Treatment with myriocin lowers the levels of TAG and cholesterol in the plasma of ApoE knockout mice, a model of atherosclerosis [52] and decreases ceramide levels in plasma, liver and soleus muscle of Zucker fa/fa rats while improving overall glucose homeostasis [26]. Decreases in plasma ceramide levels, improved glucose homeostasis, and restoration of insulin stimulated Akt-1 phosphorylation in the liver and muscle were also achieved in myriocin-treated high-fat diet fed or ob/ob mice [42]. However, the above studies did not provide data in regards to the levels of sphingolipids in insulin sensitive tissues, which may not correlate with the plasma sphingolipid content [4]. Therefore, it is uncertain whether the improved whole body insulin sensitivity was a direct result of the blocked ceramide production in the liver and muscle or a general effect.

Recent reports began to suggest that although required, palmitate-induced stimulation of ceramide biosynthesis might not be the only mechanisms for the onset of insulin resistance. Several studies have showed restoration of insulin sensitivity in diet-induced hyperglycemic animals while ceramide content remained elevated. These data had suggested either that ceramide is not the direct lipid regulator, or that its metabolism is coordinately regulated with that of another bioactive metabolite. These two options are discussed below.

Possible role of the complex sphingolipids, glycosphingolipids and gangliosides

Once ceramide is synthesized in the endoplasmic reticulum, it is carried to the Golgi apparatus and converted to SM or glycosphingolipids (glucosphingolipids and gangliosides) by the addition of a head group to its primary hydroxyl. Ceramide is transferred in a non-

vesicular manner by ceramide transfer protein CerT1, which contains an N-terminal plextrin homology (PH) domain and a C-terminal steroidogenic acute regulatory protein (StAR)-related (START) domain [53]. Some recent studies seem to indicate that CerT1-transferred ceramide is utilized more efficiently in the SM rather than glucosylceramide synthesis. According to recently proposed model, CerT1 extracts newly synthesized ceramide from the endoplasmic reticulum (ER), depending on its START domain and targets the Golgi apparatus, depending on its PH domain recognizing phosphatidylinositol-4-phosphate (PI4P). After the release of ceramide at the Golgi apparatus, CERT might bind diacylglycerol generated during SM synthesis and transfer it back to ER. SM synthase 1 and 2 (SMS1) and (SMS2) function as the key Golgi- and plasma membrane-associated SM synthases by catalyzing the transfer of phosphorylcholine head group from a phosphatidylcholine to the primary hydroxy group in ceramide [54]. In turn, glucosylceramide synthase (GCS) is a Golgi-localized membrane bound glycosyl transferase, which catalyzes the glycosylation of ceramide using UDP-glucose as a donor to produce glucosylceramide. The latter is a key player in the biosynthesis of more complex sphingolipid that are generated through stepwise addition of galactose, glucose, N-acetylglucosamine, or N-galactosamine at either the 3-O-position or the 4-O-position of the sugar moiety [55]. Golgi-synthesized complex glucosphingolipids and SM undergo vesicular trafficking to the plasma membrane, where they are localized mainly at the outer leaflet. In the liver, SM and glucosphingolipids can also be incorporated into lipoprotein particles and secreted in the plasma.

The synthesis of these complex sphingolipids increases following palmitate-induced stimulation of ceramide generation. The resulting accumulation of glucosylceramide and complex glucosphingolipid metabolites including ganglioside GM3 is proposed to have a deleterious effect on insulin response and glucose regulation. Patients with type I Gaucher disease, an inheritable glucosphingolipid storage disorder caused by mutations in glucosylceramidase gene, accumulate glucosylceramide in the lysosomes of many tissues and organs and have impaired peripheral insulin sensitivity [56]. The levels of GM3 synthase mRNA in adipose tissue of obese Zucker *fa/fa* rat and *ob/ob* mouse are higher than in tissues from their lean counterparts [57]. Moreover, the addition of exogenous GM3 to adipocytes has been found to suppress insulin-stimulated tyrosine phosphorylation of the insulin receptor, the binding of insulin receptor substrate-1, and respectively, insulin-stimulated glucose uptake [57], suggesting that diet-induced elevation in glucosphingolipids might be causatively linked to the onset of insulin resistance. Indeed, an increase in glucosylceramide content in the retina of diabetic rats has been shown to accelerate local insulin resistance resulting in neuronal cell death and the development of diabetic retinopathy [58]. Pharmacological inhibition of GCS however restores insulin sensitivity in cultured retinal neurons [58] and improves the overall glucose homeostasis and insulin sensitivity in *ob/ob* mice, Zucker *fa/fa* rats and mice fed diet high in fat [59,60]. The treatment also prevents the loss of insulin secretion by the pancreatic β -cells, and restores insulin signaling in the muscles of Zucker *fa/fa* rats [60]. Suppression of glucosphingolipid biosynthesis in *ob/ob* mice by feeding with AMP-DNM iminosugar, restores the *ex vivo* measured insulin signaling in adipocytes, improves the overall adipogenesis, GLUT-4 translocation, and lessens inflammation in adipose tissue [61]. The same GCS inhibitor prevents hepatomegaly, decreases the development of steatosis and dramatically improves insulin signaling in the liver of *ob/ob* mice [62]. Oddly enough, these beneficial effects take place while ceramide levels are not changed, or even in some occasions are increased [60,62]. In a very recent study, glucosylceramide biosynthesis in hepatocytes was effectively blocked using the Cre/loxP system to delete GCS in liver [63]. However, no alteration in glucose tolerance after intraperitoneal application of glucose and insulin nor protection against high-fat diet steatosis appeared in the mutant animals [64]. Seemingly, the dilemma

of which bioactive metabolite, ceramide or a higher glycosphingolipid, is directly involved in the onset of insulin resistance remains unsolved.

Coordinated partitioning of exogenous palmitate into sphingolipid and TAG metabolic pools

Several recent studies have suggested that palmitate-induced ceramide accumulation may play a more diverse and complex role in the onset of insulin resistance. These studies have provided compelling evidence that the *de novo* sphingolipid pathway is metabolically linked to TAG synthesis and may have adverse effect on DAG and TAG accumulation in tissues.

The accumulation of fat in the cells of lean tissue, mainly liver and muscle is obesity-related pathology referred to as steatosis which correlates with the development of insulin resistance. The excess fatty acids can enter three lipid pools that are metabolically interconnected but have different impact on cellular responsiveness to insulin. DAG, the common precursor for TAG and glycerophospholipid synthesis is considered the culprit that impairs insulin signaling during steatosis. However, evidence have indicated that the different DAG pools play a different role in impairing insulin responsiveness. Two DGATs, DGAT1 and DGAT2 can convert DAG into TAG for storage, or into glycerophospholipids for membrane biogenesis. Deletion of DGAT1 has only a minor effect on TAG synthesis. Its overexpression is sufficient to elevate synthesis of TAG however, surprisingly, it also reduces that of glycerophospholipids, suggesting that the overexpressed enzyme uses a pool of DAG initially designed for phospholipid synthesis [65]. In contrast, overexpression of DGAT2 has much greater effect on TAG accumulation than DGAT1, and its silencing inhibits hepatic TAG synthesis [66]. Notably, suppression of DGAT2, but not DGAT1, can reverse diet-induced hepatic steatosis and insulin resistance [67]. Thus, the two DGAT forms seem to use two distinct and competitive pools of DAG that are specifically designated for TAG or phospholipids synthesis while only DGAT2-accessible pool seems to impair insulin signaling [65].

Recently published work has suggested that similar interaction exists between TAG and sphingolipid metabolic pathways. Initial indications to that effect have emerged from the intriguing observation that only short-term inhibition of SPT ameliorated palmitate-induced insulin resistance of L6 muscle cells, while sustained loss of SPT activity brought about by gene silencing or prolonged pharmacological inhibition failed to do so. Experiments aimed at understanding the reason of this bimodal effect revealed that in the cells with constitutively reduced *de novo* sphingolipid synthesis, the flux of exogenous palmitate was redirected preferentially towards TAG synthetic pathway leading to overaccumulation of TAG and the TAG precursor, DAG [68]. Apparently, in spite of the successful elimination of palmitate-induced ceramide increases, TAG/DAG overproduction is sufficient to impair insulin signaling. This observation also suggests that the sphingolipid and TAG synthetic pathways most likely compete for the available palmitate.

Further evidence for competitive partitioning of exogenous palmitate between TAG and sphingolipid pools had come from work in our lab. Deletion of the gene for acid sphingomyelinase, whose activity seems to inhibit the *de novo* sphingolipid synthesis, in mice leads to chronic up-regulation of the pathway and excessive hepatic accumulation of sphinganine, ceramide and more complex sphingolipids, which is especially pronounced when the animals were placed on diet rich in saturated fats for up to 12 weeks. Notably, this is paralleled by a complete elimination of diet-induced TAG accumulation and by an improved insulin response *in vivo*. Apparently, in this experimental model, dietary palmitate again partitioned into TAG and sphingolipid pool in a competitive manner, dependent on the rate of sphingolipid synthesis.

The interaction(s) between TAG and sphingolipid metabolic pathways during diet-induced obesity however may be quite complex. This is implied by the somewhat underappreciated role of SM synthase (SMS1) as generator of DAG in an unique compartment of the cell, the Golgi apparatus [69]. The activity of SM synthase results in the generation of one molecule of DAG for each molecule of SM. Increases in the synthesis of ceramide, including those ensued from a diet, cause reciprocal stimulation of synthesis of SM, and consequently leads to an increased generation of DAG in the Golgi. The fate of the SMS-generated DAG is unclear. It is of interest that neither of the two DAG-Acyl transferases, DGAT1 and DGAT2, are localized in the Golgi [70], and one possible explanation is that excessive stimulation of SM synthase may cause re-distribution of DAG pools away from TAG synthesis and towards phospholipid synthesis.

Concluding Remarks

It is well known that accumulation of TAG in lean tissue is a strict predictor of the development of insulin resistance; however, it is also clear that TAG is not the active mediator. Instead, the TAG precursor, DAG, is considered the culprit because it stimulates PKC, which in turn down-regulates the insulin signaling cascade. Data discussed in the review summarize some of the published evidence that consumption of diet rich in saturated fats, palmitate in particular, causes not only TAG accumulation but also stimulation of the *de novo* synthesis of ceramide, SM and complex sphingolipids that is causatively linked to the off-set of insulin responses in muscle, liver, and adipose tissue. In contrast to the TAG metabolic pathway, which exhibits no preference for a particular fatty acid in the diet, the stimulation of sphingolipid synthesis is specifically linked to the presence of palmitic acid, the most deleterious fat for the pathophysiology of obesity. The flux through the sphingolipid pathway seems also to regulate the extent of TAG and may be DAG accumulation, suggesting interplay between the two pathways, and respectively the two bioactive lipids, DAG and ceramide, in the onset of insulin resistance and hyperglycemia.

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Abbreviations

| | |
|---------------|-----------------------------|
| DAG | diacylglycerol |
| DGAT | DAG acyltransferase |
| CerS | Dihydroceramide synthase |
| ER | endoplasmic reticulum |
| GlcCer | glucosylceramide |
| GCS | glucosylceramide synthase |
| PA | palmitic acid |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PS | phosphatidylserine |
| SPT | serine palmitoyltransferase |
| SM | sphingomyelin |

| | |
|------------|------------------------|
| SMS | sphingomyelin synthase |
| TAG | triacylglycerides |

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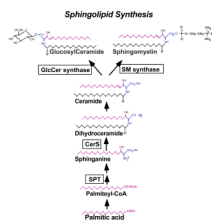


Fig. 1.
Schematic representation of the de novo synthesis of sphingolipids.

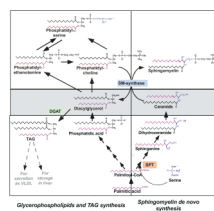


Fig. 2.

Proposed interplay between TAG, glycerophospholipids and sphingolipid metabolism. *De novo* synthesis of sphingolipids occurs via SPT in the endoplasmic reticulum. A ceramide transfer protein transports ceramide to the Golgi apparatus, where complex sphingolipids, like SM and glycosphingolipids (not shown) are synthesized. Complex sphingolipids are then transferred to the plasma membrane, which contains more than 75% of the total cellular sphingolipids.

The synthesis of both TAG and glycerophospholipid from activated fatty acids follows a common pathway to the formation of DAG. DAG can be used either for TAG synthesis, storage, and secretion or for synthesis of the two main glycerophospholipid classes, phosphatidylcholine (PC) or phosphatidylserine (PS). Metabolically, the sphingolipid, glycerophospholipid and TAG pathways are linked through SM synthase, shown in the middle of the diagram. Exogenous palmitic acid partitions in a competitive fashion between sphingolipid and TAG pools.

DAG and ceramide (boxed in the middle) are bioactive metabolites implicated in down-regulation of insulin signaling cascade. As the diagram indicates, they are in the crossroad of multiple pathways and their levels are subject to complex regulation.