CHAPTER 6

SPHINGOLIPIDS AND HEPATIC STEATOSIS

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Abstract: The development of a fatty liver predisposes individuals to an array of health problems including diabetes, cardiovascular disease and certain forms of cancer. Inhibition or genetic ablation of genes controlling sphingolipid synthesis in rodents resolves hepatic steatosis and in many cases wards off the health complications associated with excessive hepatic triglyceride accumulation. Examples include the pharmacological inhibition of serine palmitoyltransferase or glucosylceramide synthase or the genetic depletion of acid sphingomyelinase, which dramatically reduce hepatic triglyceride levels in mice susceptible to the development of a fatty liver. The magnitude of the effects on triglyceride depletion in these models is impressive, but the relevance to humans and the mechanism of action is unclear. Herein we probe into the connections between sphingolipids and triglyceride synthesis in an attempt to identify causal relationships and opportunities for therapeutic intervention.

INTRODUCTION

Closely mirroring the meteoric rise in obesity levels is an array of health problems known collectively as the metabolic syndrome. The features of this syndrome include abdominal obesity, dyslipidemia, hypertension, insulin resistance and chronic low-grade inflammation.[1] While the metabolic syndrome is not in itself a disease, the presence of this cluster of risk factors predisposes individuals to heart disease and Type 2 diabetes, which account for roughly 36 percent of all deaths worldwide.[2-4] Given the liver's place in lipoprotein metabolism and the control of nutrient homeostasis,[5] it is noteworthy that these risk factors have a tremendous impact on hepatic lipid infiltration and the pathogenesis of nonalcoholic fatty liver disease (NAFLD). On one hand the liver suffers from the factors

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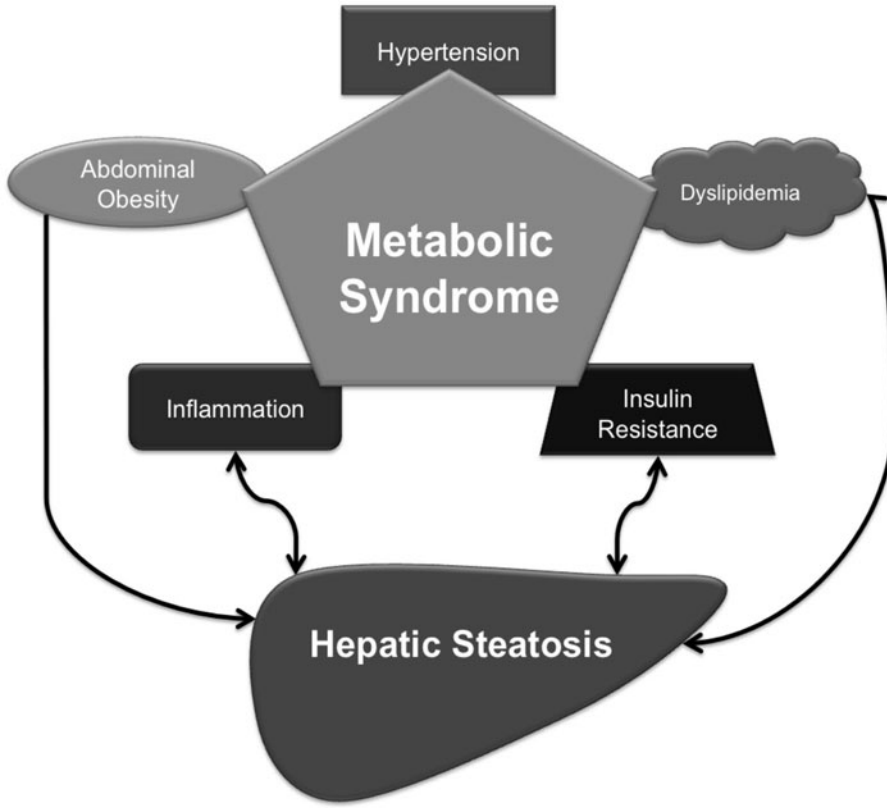

Figure 1. Schematic depicting various features of metabolic diseases.

of the metabolic syndrome, such as elevated circulating lipids and inflammation. But it also exacerbates the metabolic syndrome by releasing cytokines and LDL-bound fats, which can induce insulin resistance and inflammatory events. Thus, a fatty liver may be either a consequence of or a contributor to metabolic disease (Fig. 1).

NAFLD refers to a condition where fat accumulation in the liver is in excess of 5% to 10% of total tissue weight.[6] In the early stages the steatosis is largely benign, but as lipids continue to accrue the condition worsens, developing into steatohepatitis with damage resulting from cellular inflammation, cirrhosis and even hepatocellular carcinoma. As the condition progresses, the liver will ultimately fail.[7-8] Roughly 90% of the morbidly obese have NAFLD,[9] which is the most common cause of liver dysfunction in the US, affecting 20-30% of adults.[7,10] However, since the steatosis is often clinically silent, many more cases likely exist undiagnosed.

Triglycerides are produced via the Kennedy pathway, which starts with the conjugation of fatty acids onto a glycerol backbone. Surprisingly, recent studies in rodents reveal that pharmacological or genetic inhibition of enzymes in a parallel, but distinct, sphingolipid synthesis pathway substantially reduces hepatic triglyceride levels in rodents. Herein we probe into the relationship between these disparate events.

SPHINGOLIPID LEVELS IN THE STEATOTIC LIVER

Several animal models of obesity or various metabolic diseases demonstrate elevated hepatic sphingolipid levels.[11-14] Employing an agnostic lipidomic methodology to characterize the livers of the *ob/ob* mouse, Yetukuri et al[15] determined that hepatic levels of ceramide, which is a biosynthetic intermediate in the sphingolipid synthesis pathway and a precursor of all complex sphingolipids (e.g., sphingomyelin and glucosylceramides), strongly correlated with the degree of steatosis. Studies in humans have not been as exhaustive or definitive. Liver fat correlates strongly with transcripts encoding genes that drive sphingolipid metabolism, but not with ceramides themselves.[16-17] However, Kolak et al[18] found a correlation between *adipose* ceramide content and liver triglyceride in humans independent of obesity, suggesting a possible enterohepatic relationship through which adipose ceramides could contribute to NAFLD.

MODULATION OF SPHINGOLIPID SYNTHESIS IMPACTS HEPATIC STEATOSIS

Despite a recent surge in attention to the enzymes regulating the rate of sphingolipid synthesis and degradation (Fig. 2),[19] relatively little interest has been devoted to the tissue-specific characteristics of the enzymes in the liver. Those that have been studied have yielded noteworthy results.

Serine Palmitoyltransferase

Serine palmitoyltransferase (SPT) is the initial and rate-limiting step in de novo sphingolipid synthesis, condensing palmitoyl-CoA and serine to produce 3-ketosphinganine (Fig. 2). Memon et al[20] explored the effects of inflammatory mediators, including lipopolysaccharide, interleukin-1 and tumor necrosis factor-α, on SPT activity and gene expression in hamsters and HepG2 cells. LPS treatment increased hepatic sphingolipid levels (i.e., sphingomyelin (up 75%) and ceramide (up 200%) and doubled hepatic SPT gene expression and enzyme activity. Similarly, IL-1 injection increased hepatic SPT transcript levels and activity in vivo and both IL-1 and TNFα induced SPT transcript levels in HepG2 cells. In these studies, cytokine and LPS treatment increased sphingolipid release (i.e., ceramides, sphingomyelin and glucosylceramides) from the liver into the circulation via lipoproteins.

Myriocin is a potent SPT inhibitor and has been used frequently in rodent studies to slow rates of sphingolipid synthesis. Yang et al[21] determined the effects of myriocin treatment on hepatic steatosis in three mouse models of obesity. In all cases [(a) *ob/ob* mice, (b) C57BL/6J mice treated with myriocin at the onset of high-fat diet and (c) C57BL/6J mice treated with myriocin after eight weeks of high-fat diet], treatment with myriocin significantly reduced hepatic triglycerides.[21]

Glucosylceramide Synthase

Glycosphingolipids are produced by the addition of carbohydrate moieties to ceramide. The initial product is glucosylceramide, which is produced by

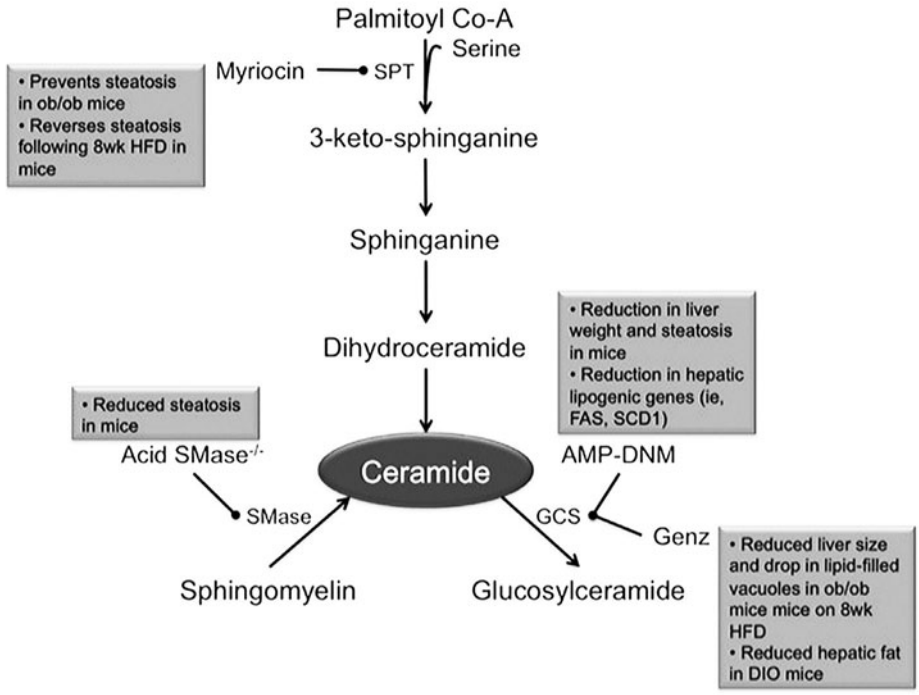

Figure 2. Shematic summarizing interventional studies showing how the modulation of sphingolipid synthesis impacts steatosis.

glucosylceramide synthase (GCS) and is the precursor of complex glycosphingolipids and gangliosides. Identification of a role for glucosylceramides in hepatic steatosis and other features of metabolic disease derives from the identification of two distinct GCS inhibitors: N-(5′-adamantane-1′-yl-methoxy)-pentyl-1-1deoxynojirimycin (AMP-DNM) and Genz-123346 ((1R,2R)-nonanoic acid[2-(2′,3′-dihydro-benzo [1,4] dioxin-6′-yl-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-1-tartaric acid salt).

Aerts et al[14] administered AMP-DNM to 6-week-old C57Bl/6J mice and observed a 41% drop in hepatic glucosylceramide levels. Treatment of *ob/ob* mice with AMP-DNM reduced hepatic glucosylceramide content to a similar extent and significantly diminished hepatic triglyceride levels. The compound improved insulin signal transduction in the liver and markedly decreased hepatic glucose production. Bijl et al[22] reported that a similar treatment in control and ob/ob mice led to a marked reduction in liver weight and steatosis.[22] AMP-DNM treatment was associated with a reduction in a variety of lipogenic genes, including Fatty Acid Synthase (FAS) and Stearoyl-CoA Desaturase-1 (SCD1). The disparate hepatic transcript levels of several chemokines, like monocyte chemoattractant protein-1 and macrophage inflammatory protein and their receptors observed in the ob/ob and control mice were resolved following prolonged GCS inhibition.

Zhao et al[23] performed a parallel study of the role of GCS in hepatic steatosis using Genz-123346. Drug treatment reduced hepatic GC content in ob/ob mice by roughly 40% compared with control (water) treatment, a change that was associated with improved

whole-body glucose homeostasis as evident by reduced blood glucose and HbA1c levels. In exploring the effect of treatment on liver pathology, the livers of the animals treated with Genz-123346 had decreased size and number of lipid-filled vacuoles and a substantial reduction in hepatic triglyceride levels. Comparable findings were observed in another model of steatosis—C57BL/6 mice fed a high-fat diet for 8 weeks. Finally, to determine whether GCS inhibition could reverse preexisting steatosis, older mice that had been high-fat fed for 31 weeks were treated with vehicle or Genz-123346 for 17 weeks. At the conclusion of the treatment period, the livers of the older high-fat-fed mice receiving Genz-123346 exhibited a reduced ratio of fat to lean mass compared with controls.

Sphingomyelinase

Sphingomyelin (SM) is the most prevalent sphingolipid in mammalian cells, found abundantly in plasma membranes and lipoproteins. The sphingolipid can be converted back into ceramide by a family of spingomeylinases (SMase) distinguished by their pH optima.[24] Deevska et al[25] reported the deletion of acid sphingomeylinase from LDL-receptor knockout mice resolved diet-induced hepatic steatosis and improved insulin sensitivity. The improvement in steatosis and insulin sensitivity was associated with a paradoxical elevation in hepatic ceramides and sphingomyelin and a marked increase in de novo synthesis.

Numerous cell stimuli regulate SMase activity and the enzyme is implicated in responses to a diverse number of cellular agonists (e.g., cytokines and oxidative stress). TNFα and IL-1β increase ceramide levels by stimulating at least two different SMase classes—neutral and acidic.[26-32] Moreover, the ability of TNF to induce SMase has been established across various human and rodent cell lines.[33-36] Among its many activities, TNFα induces SMase by binding the p55 TNFR.[37] These findings are interesting in light of the evidence that mice genetically deficient in the p55 TNFα receptor (also known as TNF Type 1 receptor) are resistant to diet-induced steatosis and liver injury.[38]

Oxidative stress also regulates SMase activity.[24] Alessenko et al[39] demonstrated that a SMase-mediated elevation in hepatic ceramides positively correlated with peroxide products in response to endotoxic stress. Additionally, they observed that administration of nitric oxide releasing compounds resulted in reduced hepatic SMase activity and ceramide levels and lipid peroxide oxidation.[39] In exploring the mechanisms behind bile-salt induced hepatocyte apoptosis, Reinehr et al[40] treated primary rat hepatocytes with taurolithocholate-3-sulfate (TLCS), a substance known to induce a potent oxidative stress response. In addition to rapidly inducing oxidative stress, TLCS treatment was also shown to stimulate ceramide synthesis via elevated SMase activity. When cells were treated with desipramine, a SMase inhibitor, prior to TLCS exposure, ceramide levels and downstream oxidative stress were both reduced.[40]

FACTORS ASSOCIATED WITH NAFLD INDUCE SPHINGOLIPID SYNTHESIS

The pathogenesis of NAFLD has been referred to as a ‘double-hit’ process, with hepatocellular lipid accumulation presenting the first insult and the second as a result of inflammation-induced hepatic injury.[41-43] Both lipid oversupply and inflammation are likely to drive sphingolipid production (Fig. 3).

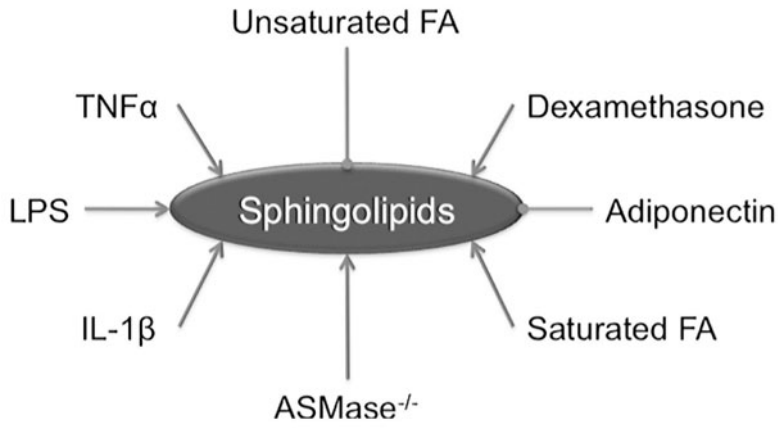

Figure 3. Schematic depicting factors discussed herein which impact hepatic sphingolipid levels.

Saturated Fatty Acids Induce Hepatic Sphingolipids

Fatty acids used in the production of hepatic triglyceride can derive from a number of distinct sources including plasma FFAs (accounting for ~59% of hepatic triglyceride), fatty acids made de novo within the liver (~26%) and dietary fatty acids (~15%), which can enter the liver by either spillover into plasma FFA or through chylomicron remnants.[44-45] We previously demonstrated that saturated fatty acids drove sphingolipid synthesis in the liver, as hepatic ceramide levels were elevated by roughly 60% in rats following a six-hour infusion of lard oil when compared with animals receiving a control (i.e., glycerol) infusate.[12] To highlight the necessity of saturated fatty acids in this effect, there was no increase in hepatic ceramide levels in the animals receiving a soy-based infusate comprised predominantly of unsaturated fatty acids.[12]

Inflammatory Cytokines Induce Hepatic Sphingolipids

The discovery of the interaction of inflammatory and metabolic pathways over 10 years ago provided a novel and important perspective in understanding the origin of many metabolic disorders.[46] While the defining characteristic of NAFLD is excessive hepatic deposition of fatty acids, the latter and more advanced stages are distinguished by an increasing inflammatory tone.[47-48] Several groups provide evidence supporting a correlation between varying degrees of NAFLD and circulating and tissue cytokine levels and expression.[38,49-57] In particular, Jarrar et al[58] observed both elevated TNFα levels (proinflammatory cytokine) and reduced adiponectin (anti-inflammatory) in patients with NAFLD compared with obese and nonobese controls. Moreover, within the NAFLD group, a further contrast was observed—those suffering from advanced stages of NAFLD, including NASH, displayed similar significant differences when compared with those suffering from simple steatosis. Similar trends are observed with hepatic TNFα gene expression and TNF receptors—both are increased with NAFLD when compared with healthy livers and the expression is further elevated in those with advanced NASH.[59-61]

The relationship between cytokines and sphingolipids has been explored for over 15 years and evidence attesting to the liver's ability to produce sphingolipids in response to dyslipidemia is even older.[62-64] Since those early discoveries, emerging evidence suggests a noteworthy relationship between sphingolipids and cytokines in various tissues.[65-68] Further, in support of the adipose-liver relationship, subjects with NAFLD exhibit significantly greater adipose-derived cytokines and ceramides.[18] Unsurprisingly, many studies have revealed a positive correlation between TNFα and sphingolipids.[19,21,69] Rodent models of obesity have shown elevated levels of both hepatic sphingolipids and pro-inflammatory cytokines.[13-14,51,70]

Central to inflammation and cytokine release throughout the body is the macrophage and the resident hepatic macrophages, known as Kupffer cells, represent the majority of all tissue macrophages in the body and as much as 10% of the total cell population within the liver itself.[71-72] Kupffer cells both respond to and release cytokines into the liver and circulation and their numbers vary greatly in response to inflammatory stimuli as a result of hyperplasia and infiltration of bone marrow progenitors.[73] Mediating the Kupffer cells response to pathogens are the pattern recognition receptors (PRR),[74-75] among which are the family of toll-like receptors (TLRs)(Fig. 3).[75-77] In particular, TLR4 plays a key role in mediating inflammatory signals throughout the liver by triggering Kupffer cells to produce an array of proinflammatory cytokines (TNFα, IL-1β, IL-6, etc.), which are known to stimulate certain enzymes of sphingolipid metabolism (see above).[77-80]

The best-characterized agonist of TLR4 is lipopolysaccharide (LPS), which is present on Gram-negative bacteria. The liver's ability to respond to LPS via TLR4 plays an important role in maintaining homeostasis by activating host immunity and acting as a final barrier to toxins before they enter the systemic blood stream.[81-82] Indeed, due to the liver's place as a first responder to toxins and ability to activate a potent immune response, it is tempting to view the Kupffer cells as a central regulator of inflammation-induced sphingolipid synthesis. In addition to eliciting an immune response, LPS treatment has been shown to induce sphingolipid accrual in hepatic and extrahepatic tissues, though whether this effect is mediated by TLR4 is unknown.[20,83]

Adiponectin

While the thrust of cytokine-induced steatosis research has focused on TNFα and other proinflammatory cytokines, an emerging area of research has explored the relationship between adiponectin, the most prominent and abundant anti-inflammatory cytokine, in regulating hepatic sphingolipid metabolism. In an assessment of patients with simple steatosis and NASH, Jarrar et al[58] saw that NASH patients have significantly lower adiponectin levels than those with simple steatosis and obese controls without NAFLD. Moreover, in analyzing the livers of obese, nondiabetic subjects, Kolak et al[18] revealed that adiponectin transcript expression in higher-fat livers was negatively associated with hepatic ceramides and sphingomyelinase transcript levels. In a recent study of adiponectin and its receptors, Peng et al[84] showed that serum adiponectin decreased in mice fed a high-fat diet when compared with control-fed animals and that this change was associated with dramatically reduced hepatic expression of adipoR2, the predominant adiponectin receptor in the liver. Similar examinations in humans have revealed comparable results—a reduction in hepatic adiponectin and adipoR2 expression with advancing steatosis.[56,85]

Interventional studies support the idea that adiponectin may deplete hepatic triglycerides. In particular, Yamauchi et al[86] discovered that obese mice treated with adiponectin had reduced intra-hepatic triglycerides. Morever, Stefan et al[87] demonstrated that adiponectin

receptor polymorphisms, which prevent normal adiponectin signal transduction, are associated with insulin resistance and high liver fat in obese mice. While the direct effects of adiponectin on *hepatic* sphingolipids have yet to be explored, recent evidence suggesting that adiponectin receptors contain intrinsic ceramidase activity[88-90] provides exciting insight into a possible mechanism.

POSSIBLE MECHANISMS

The precise mechanism of how sphingolipids contribute to steatosis remains undefined. The most central question is whether the sphingolipid effect is due to a cell autonomous role in the regulation of triglyceride synthesis, as is suggested by the studies showing that inhibition of ASMase in cultured hepatocytes inhibits triglyceride synthesis.[25] However, since sphingolipid levels were elevated in livers following ASMase depletion, the study seems incongruous with the finding that SPT inhibition, which lowers hepatic sphingolipids, also depletes hepatic triglycerides.[21] Thus, the details of such a cell autonomous mechanism are difficult to envision. An alternative possibility is that depleting ceramides improves peripheral insulin sensitivity, leading to the appropriate deposition of nutrients in peripheral tissues (i.e., skeletal muscle) and a preservation of the liver. Whether insulin resistance precedes steatosis or whether steatosis leads to insulin resistance is an area of considerable interest and debate.

CONCLUSION

The metabolic syndrome is a major health concern throughout the world and evidence indicates the liver occupies an increasingly prominent role in the etiology of this multifaceted condition. As worldwide prevalence continues to rise in parallel with obesity rates, research involving nonalcoholic fatty liver disease and its various manifestations has produced irrefutable evidence connecting the disease with the lipotoxic milieu generated in a high-lipid, insulin-resistant state. Despite years of correlational data, only recently has research begun to directly explore the role of sphingolipids in aggravating NAFLD. Given the promising results thus far, future efforts will likely continue to provide evidence implicating sphingolipids in the pathogenesis of NAFLD and as we come to a better understanding of its origins, we will be in a better position to uncover better treatments.

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