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Non-alcoholic fatty liver disease: Insights from sphingolipidomics

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a major clinical concern and its treatment consumes abundant resources. While accumulation of lipids in hepatocytes initiates the disease, this in itself is not necessarily harmful; rather, initiation of inflammation and subsequent fibrosis and cirrhosis are critical steps in NAFLD pathology. Mechanisms linking lipid overload to downstream disease progression are not fully understood; however, bioactive lipid metabolism may underlie instigation of proinflammatory signaling. With the advent of high-throughput, sensitive, and quantitative mass spectrometry-based methods for assessing lipid profiles in NAFLD, several trends have emerged, including that increases in specific sphingolipids correlate with the transition from the relatively benign condition of simple fatty liver to the much more concerning inflamed state. Continued studies that implement sphingolipid profiling will enable the extrapolations of candidate enzymes and pathways involved in NAFLD, either in biopsies or plasma from human samples, and also in animal models, from which data are much more abundant. While most data thus far are derived from targeted lipidomics approaches, unbiased, semi-quantitative approaches hold additional promise for furthering our understanding of sphingolipids as markers of and players in NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the liver component of metabolic syndrome and is associated with chronic over-exposure of the liver to free fatty acid (FFA) that occurs in metabolic syndrome [1]. While a healthy hepatocyte has evolved to process, metabolize, and/or re-package free fatty acid (FFA) for storage, utilization, or distribution to other tissues, the overload of FFA can disrupt these pathways leading to aberrant FFA handling resulting in deleterious outcomes. The initiating insult involves excessive accumulation of lipid droplets, but the actual events linking lipid oversupply to hepatocyte toxicity could include disruption of lipid bilayer integrity, overloading endogenous lipid metabolic pathways, accumulation of oxidized lipids, activation of ER stress and the unfolded protein response, and/or deleterious production of bioactive lipids [2]. Hepatic injury, thought to be the major driving event behind disease progression, drives injured or dying hepatocytes to release proinflammatory factors. Subsequent activation and infiltration of immune cells, such as neutrophils and lymphocytes, lead to inflammation [3], and activation of hepatic stellate cells leads to fibrosis [4], furthering disease progression [5,6].

The interplay between free fatty acid (FFA) toxicity to hepatocytes, signaling between hepatocytes and immune cells, and the relationship between inflammation and fibrosis are of major interest in NAFLD research [1,7]. While the contributions of FFAs to mechanisms of hepatocyte injury and NAFLD progression are not completely understood, free palmitic acid (PA) is a particularly potent mediator of lipotoxicity [7]. PA is the product of fatty acid synthase and is the most abundant FFA in mammalian circulation. It is widely found esterified at the sn-1 position of glycerophospolipids and is also the substrate for the first step of sphingolipid synthesis [8]. Therefore, excess FFA-induced alterations to intracellular sphingolipid metabolism and sphingolipid profiles may play roles in NAFLD, as has been suggested by studies from our group and others [9–12].

Sphingolipids comprise a highly dynamic, diverse, and complex class of molecules that serve as both structural components of cellular membranes and signaling molecules in mammalian cells. The relatively recent application of sphingolipid lipidomics, or "sphingolipidomics", has uncovered numerous potential relationships between sphingolipids and NAFLD. Instrumentation and

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methods technology for lipidomics analysis has been available for nearly two decades, but its direct application to disease models like NASH has become more widespread over the past five years or so. This is likely a result of increased accessibility due to instrument availability and standardization of methodology. Here we highlight some of the insights gained into NAFLD mechanisms by the application of sphingolipidomics, followed by a discussion of key technical considerations for lipidomics approaches.

2. Sphingolipid metabolism

Sphingolipids arise from condensation of a fatty acyl CoA with an amino acid, giving rise to a sphingoid base, the amino alcohol upon which all sphingolipids are subsequently built (for a detailed depiction of sphingolipid pathways and structures see Fig. 1). Canonical substrates for this reaction are serine and palmitoyl-CoA, though recent studies have identified aberrant lipids synthesized using glycine, alanine, and/or myristoyl- or stearoyl-CoA [13,14], giving rise to alternative sphingolipid species whose biology is just beginning to be deciphered. However, those derived from serine and palmitoyl-CoA are by far the most abundant species. This reaction is catalyzed by serine palmitoyltransferase and is thought to be rate-limiting and highly sensitive to palmitoyl-CoA levels [15]. The product of this reaction, 3-ketodihydrosphingosine, is short lived and rapidly converted to dihydrosphingosine (also called sphinganine) by 3-ketodihydrosphingosine reductase. Dihydrosphingosine bears an amino group, which serves as the site of CoA-dependent acylation with a range of fatty acyl-CoA substrates by a family of six ceramide synthases (CerS1-6) (Fig. 2A). This enzyme family shows high homology yet also demonstrates partial acyl-CoA selectivity, which leads to a diverse and partially distinct product profiles for each CerS. For example, CerS6, implicated in liver pathology in murine high fat diet obesity models [16], generates ceramides using a long chain acyl-CoA, such as palmitoyl-CoA; in contrast, CerS2, whose depletion is implicated in liver pathology [17], utilizes acyl-CoAs of 20 carbons and longer (Fig. 2B). Therefore, determining the intracellular ceramide distribution (in terms of N-acyl chain length) provides hints as to enzymes that might underlie pathology.

While the actual products of de novo ceramide synthesis are dihydroceramides, these are desaturated by dihydroceramide desaturase 1 to yield ceramide. Ceramide regulates numerous cell processes including apoptosis, senescence, insulin signaling, signaling from membrane receptors, and many others [18]. Ceramide also gives rise to two key proinflammatory lipid mediators: 1) it can be directly phosphorylated by ceramide kinase to yield ceramide-1-phosphate, a regulator of iPLA2 and subsequent driver of proinflammatory eicosanoid production [19]; or 2) it is catabolized to sphingosine, a single-chain molecule that is then phosphorylated by sphingosine kinase 1 or 2 to yield sphingosine-1-phosphate, a ligand for a family of G protein-coupled receptors that has known roles in proinflammatory cytokine signaling and immune cell chemotaxis [20]. Apart from these fates, ceramides are the essential building block for all complex sphingolipids, including sphingomyelin and glycosphingolipids (Fig. 1). Importantly, catabolic enzymes exist that catalyze the reverse of many of these reactions. Therefore, there is potential for continuous flux within the pathway, where total sphingolipid content may remain constant, but profiles (i.e. distribution of total content between specific sphingolipid species and pools) may change drastically.

While links between sphingolipids and NAFLD are continuing to emerge, NAFLD at various stages involves alterations in multiple biological processes including lipid metabolism, lipotoxicity, ER stress, apoptosis, inflammation, and fibrosis. Because sphingolipids are implicated in these processes in other disease contexts, it seems likely they play similar roles in NAFLD. Measuring and quantification of sphingolipids in the NAFLD context, therefore, will be essential for further research to deepen our mechanistic understanding of sphingolipids in NAFLD [21,22].

3. Sphingolipid profiling in NAFLD

Despite the wealth of sphingolipidomics data available from patient serum and urine, there are few studies on patient liver biopsies. However, these, as well as additional insight gained from complementary approaches including animal models, studies from primary hepatocytes, and hepatocyte-like cell lines, commonly support a role for sphingolipids in the interplay between hepatic injury and inflammation, which is emerging as a central focus of lipidomics and NASH.

3.1. Patient samples

To date, two studies of patient liver biopsies have provided data on multiple species of sphingolipids. In Gorden et al. [23], a random group of patient biopsies were scored histologically and categorized as normal liver, livers showing steatosis, livers with non-alcoholic steatohepatitis (NASH), or cirrhotic liver, creating the opportunity to compare and contrast lipidomics trends among these patient groups. The focus of the paper was to identify circulating lipid biomarkers from among all lipid classes that correlated with NAFLD disease progression. To this end, lipid profiles of pre-classified healthy controls and NASH and cirrhosis patients were analyzed from both liver biopsies and patient serum. Lipid species were then identified that differed significantly between disease states in both liver tissue and plasma. Of the 48 species meeting this criterion, 14 were sphingolipids. Many sphingolipid classes were represented, including ceramides, 1-deoxyceramides, dihydroceramides, hexosylceramides, and sphingoid bases. Importantly, two deoxysphingolipids were identified that distinguished steatosis from NASH; these are sphingoid bases derived from glycine (rather than serine) and are increasingly implicated in toxicity in other systems [24,25], suggesting that aberrant metabolism through serine palmitoyltransferase may play a role in the development of NASH from steatosis.

The second study using patient samples partially corroborated these findings. Using slightly different approaches, Luukkonen et al. [26] collected biopsies from patients classified as low or high homeostatic assessment of insulin resistance (HOMA-IR) as an indirect readout of metabolic syndrome and also from patients carrying a mutation in the allele for the triacylglycerol lipase ($PNPLA3^{I148M}$), which is associated with exacerbation of NASH. Biopsies were scored by NASH severity. The main conclusions were that NASH associated both with high HOMA-IR and $PNPLA3^{I148M}$, as well as with an increase in TAGs containing polyunsaturated fatty acids. Consistent with the first study, NASH patients (but not necessarily patients with the mutant allele) also exhibited accumulations of ceramides and dihydroceramides, though other sphingolipids were not identified as correlates of disease.

While the methods of disease scoring were similar between these two studies, Gordon et al. presented absolute levels of a variety of lipid species while Luukkonen et al. reported relative values. Thus, it is difficult to directly compare lipid accumulation across the two studies. The lipidomics methodologies differed between the studies as well: the former employed reverse phase LC-MS/MS and multiple reaction monitoring, while the latter used LC-MS with a total ion scan. Importantly, despite these differences, both studies showed a general increase in ceramides and dihydroceramides. Also, due to application of mass spectrometry, N-acyl chain lengths of ceramides were determined to be specific. This

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