CHAPTER 4

BLOOD SPHINGOLIPIDS IN HOMEOSTASIS AND PATHOBIOLOGY

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Abstract: Sphingolipids have emerged as key signaling molecules involved in the regulation of a variety of cellular functions including cell growth and differentiation, proliferation and apoptotic cell death. Sphingolipids in blood constitute part of the circulating lipoprotein particles (HDL, LDL and VLDL), carried by serum albumin and also present in blood cells and platelets. Recent lipidomic and proteomic studies of plasma lipoproteins have provided intriguing data concerning the protein and lipid composition of lipoproteins in the context of disease. Sphingolipids have been implicated in several diseases such as cancer, obesity, atherosclerosis and sphingolipidoses; however, efforts addressing blood sphingolipidomics are still limited. The development of methods to determine levels of circulating bioactive sphingolipids in humans and validation of these methods to be a routine clinical laboratory test could be a pioneering approach to diagnose disease in the population. This approach would probably evolve to be analogous in implication to determining "good" and "bad" cholesterol and triglyceride levels in lipoprotein classes.

INTRODUCTION

Sphingolipids, once deemed mainly structural components of cell membranes, have emerged as key signaling molecules involved in the regulation of a range of cellular functions including cell growth and differentiation, proliferation and apoptotic cell death.[1-4] Ceramide (Cer), the central molecule in sphingolipid metabolism, is generated by either de novo synthesis or through the action of sphingomyelinases (SMases), a family of phospholipases.[5] The Cer formed from sphingomyelin (SM) turnover might be hydrolyzed by ceramidases to liberate sphingosine (Sph). The latter can be re-acylated to Cer or phosphorylated to sphingosine 1-phosphate (S1P) by sphingosine kinase (SK).[1,4,6] Several

Sphingolipids and Metabolic Disease, edited by L. Ashley Cowart.

sphingolipid metabolites, particularly Cer, Sph, S1P and ceramide 1-phosphate (Cer1P), have been recognized as bioactive signaling molecules that regulate cell growth and death.[1-4,7-12] In general, cellular accumulations of Cer and Sph, which occur in response to stress such as exposure to tumor necrosis factor alpha (TNFα) or oxidative stress, are associated with apoptotic responses.[13] In contrast, accumulation of S1P is usually a modulator of cell growth[14] and can protect cells from apoptosis.[1] Studies addressing the sphingolipidome of blood are limited. This chapter reviews current literature on blood sphingolipids under normal and abnormal conditions and highlights efforts addressing the importance of determining blood sphingolipid levels as biomarkers of disease.

BLOOD SPHINGOLIPIDS IN HOMEOSTASIS

Interest in blood sphingolipids has been broadened by the development and clinical application of the immunosuppressive drug FTY720, which targets S1P receptors resulting in lymphocyte sequestration.[15] It has since become increasingly essential to explore the metabolomic profile of blood sphingolipids under normal and abnormal conditions and to determine the mechanisms by which sphingolipid biosynthesis and turnover regulate cell function and pathobiology.

Sphingolipids are the most structurally diverse as well as complex category of lipids. In addition to numerous variations in the sphingoid bases, N-acyl linked fatty acids and head groups, sphingolipids contain long-chain hydrocarbon groups.[7] Furthermore, sphingolipids are insoluble in water and have both hydrophobic and hydrophilic properties.[7] Detection of sphingolipids has been hampered by their lack of chromophores necessary for traditional UV and fluorescence detection of high performance liquid chromatography (HPLC) techniques. The application of HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) has recently provided an effective analytical tool for the determination of sphingolipidomic profiles of varied biological materials;[16] however, studies addressing the sphingolipidome of blood are still scarce.

In a recent study using HPLC-MS/MS we have analyzed a comprehensive sphingolipid profile in "normal" human serum and plasma in an effort to establish a reference range for circulating sphingolipid species in blood of healthy humans.[17] We simultaneously analyzed the sphingoid bases (C18:1, C18:0) Sph and dihydrosphingosine (dhSph); sphingoid base phosphates S1P and dihydrosphingosine 1-phosphate (dhS1P); molecular species of Cer, dihydroceramides (dhCer), Cer1P, SM, hexosylceramide (HexCer) and lactosylceramide (LacCer) covering a basic metabolomic profile.[17] We found that the sphingolipids SM, LacCer, HexCer, Cer and Cer1P constitute 87.7%, 5.8%, 3.4%, 2.8% and 0.15% of total sphingolipids, respectively. The abundant circulating SM, LacCer, HexCer and Cer are C_{16}-SM, C_{16}-LacCer, C_{24}-HexCer and C_{24}-Cer, respectively. Interestingly, under fasting conditions, levels of C_{16}-SM and the very long-chain LacCers ($C_{24:1}$, $C_{26:1}$) increased, whereas levels of the long-chain Cers (C_{16}-C_{20}) and C_{26}-Cer1P decreased compared to fed state. The study also revealed gender differences and showed that levels of C_{18}- and $C_{18:1}$-SM, C_{18}-Cer1P and total dhCers are higher in females than males under fasting conditions.[17] This gender difference could probably be due to differences in the lipoprotein profile such as the higher number of HDL particles in females.[18] Future studies should be able to provide more information about the effects of starvation and overfeeding on levels of bioactive sphingolipids, as well as expand our knowledge of sphingolipid profiles under disease conditions.

Sphingolipids in the blood are found in circulating lipoprotein particles (VLDL, LDL and HDL) and in blood cells and platelets and also bound to serum albumin.[19] The information about the location and distribution of sphingolipid classes and species in lipoprotein particles are still obscure. In our recent study, HPLC-MS/MS was also used to analyze the level of sphingoid bases and their 1-phosphates, as well as levels of sphingolipid species of Cer and SM in VLDL, LDL and the subclasses HDL, HDL2 and HDL3.[17] The major carrier of Cer and dhSph in the circulation is LDL with 39.9% and 40.6% of total lipoprotein-associated Cer and dhSph, respectively.[17] In an analysis using fast performance liquid chromatography (FPLC), Weisner et al showed that the major SM in lipoprotein classes is SM 34:1 followed by SM 42:2,[20] which corresponds to C_{16}- and $C_{24:1}$-SMs containing C18:1 sphingoid backbone, respectively. In VLDL, LDL, HDL2 and HDL3 particles whether isolated by FPLC or preparative ultracentrifugation, C_{16}-SM was found to be the major SM species in the blood, followed by $C_{24:1}$-SM.[17,20] It has been also found that C_{24}-Cer is the most abundant Cer species in all lipoprotein classes including subclasses of HDL.[17,20] The concentration of SM and Cer species per lipoprotein particle reflects the size of the particle, with the larger size particle containing higher content of SM and Cer species.

It has been established that extracellular S1P binds members of the S1P receptor family (S1P1-5) on target cells inducing differentiation, migration and mitogenesis.[21,22] Numerous studies showed that S1P regulates various functions of cells involved in vascular remodeling, including endothelial cells, smooth-muscle cells, lymphocytes, monocytes and platelets.[12,23-27] It was determined that more than 60% of the S1P in blood is associated with LDL, VLDL and HDL particles.[19] Results from our recent HPLC-MS/MS analyses of lipoproteins showed that 78.6% of lipoprotein-associated S1P is carried on HDL3 particles, which are also the major carriers of dhS1P and Sph, with 63.5% and 47.9% of total lipoprotein-associated dhS1P and Sph, respectively.[17] Others have also shown that the smallest lipoprotein particles, HDL3, are enriched in S1P but poor in SM compared to the larger HDL2 particles.[28]

An array of superlative studies suggested that HDL-associated S1P mediates many of the beneficial effects of HDL on the cardiovascular system, including the synthesis of potent anti-atherogenic and anti-thrombotic molecules (e.g., nitric oxide and prostacyclin).[23,29,30] There is emerging literature, however, to suggest that S1P may also be pro-inflammatory[30,31] and pro-atherogenic[32] and was even considered a biomarker of obstructive coronary artery disease.[33] In concurrence, we have recently demonstrated that HDL3, which contains higher amounts of S1P than HDL2, significantly increases plasminogen activator inhibitor-1 secretion from adipocytes and thus, may negatively modulate fibrinolysis in vivo.[34] Because of the role of HDL in the reverse cholesterol transport from peripheral tissues to the liver, the larger diameter HDL2 particles viewed as more atheroprotective compared to the smaller sized HDL3 particles.[35] Moreover, the modification of the core lipid content of HDL particles was shown to alter the conformation of apolipoprotein AI domains that are critical for HDL to act as lipid acceptors.[36,37] More recently, it has been shown that the stability of the N-terminal helix bundle domain of apolipoprotein AI and the hydrophobicity of its C-terminal domain are important determinants of both nascent HDL particle size and rate of its formation.[38] It is intriguing then to hypothesize that the location of S1P is as critical as its amount in determining its beneficial characteristics. Recent studies suggest that more elaborate analyses of lipoprotein subclasses may lead to further improvements in cardiovascular disease risk evaluation and importantly in identification of appropriate targets for therapeutic intervention (reviewed in ref. 39).

BLOOD SPHINGOLIPIDS IN PATHOBIOLOGY

In a recent well-designed clinical study, Sattler et al demonstrated that the S1P content of HDL is negatively associated with plasma levels of S1P which is not bound to HDL in healthy controls, but not in patients with myocardial infarction (MI) or stable coronary artery disease (CAD).[40] The authors provided evidence that plasma levels of HDL-bound S1P are lower and those of non-HDL-bound S1P are higher in patients with MI and stable CAD compared to healthy controls. They suggested therefore that non-HDL-bound S1P may serve as a novel biomarker for CAD. Intriguingly, they also showed that MI patients with symptom duration of less than 12 h had the highest levels of plasma S1P, as well as the highest levels of S1P in isolated HDL. They concluded that CAD-associated defects in S1P "uptake" by HDL could potentially allow deleterious effects of free S1P.[40] Alternatively, CAD-related shifts in the distribution of HDL sub-fractions might cause the attenuated binding of S1P to HDL in patients with CAD.

Red blood cells,[41,42] vascular endothelial cells[43] and platelets[44,45] all contribute to blood S1P. Platelets lack the enzyme S1P lyase, which is responsible for degradation of S1P; but maintains a highly active Sph kinase,[45] which converts Sph to S1P. Therefore, platelets are able to store and release S1P upon stimulation.[44,45] Thus, higher levels of S1P are constantly found in serum than plasma due to platelet activation and release of S1P during clotting.[17] S1P can be generated from membrane sphingolipids and their metabolites, Cer, Sph and SM.[46] Our recent finding of SK1 release from activated macrophages in response to modified lipoprotein immune complexes could significantly influence studies addressing mechanisms mediating S1P formation extracellularly.[47] This novel mechanism could have a significant impact on studies related to inflammation and inflammation-related diseases.

Lipoprotein particles consist of hydrophobic lipids located within the core and amphipathic molecules in the surface. Complex sphingolipids such as SM exist predominantly in the outer leaflet of the bilayer of cell membrane as well as the hydrophobic outer layer of the lipoprotein particle with free cholesterol and phospholipids.[48] The surface of the LDL particle for instance contains approximately 200 molecules of SM.[49] The use of techniques capable of revealing detailed lipid interactions, including molecular particle dynamics, is hampered by the large molecular dimensions and complex thermodynamic properties of lipids in addition to the structural complexity of lipoprotein particles.[50] Kumpula et al used a structural model to optimize the lipid distributions within lipoprotein particles based on the total molecular volumes of the core and surface.[48] This model was applied for compositional data on eleven lipoprotein subclasses for optimizing the distribution of the hydrophobic lipids, triglyceride and cholesterol ester molecules. The model revealed that particle size-dependent proportion of the core lipids may locate in the surface of lipoprotein particles. Additionally, they showed that the composition of the particles influences the molecular content of the surface.[48] Such structural models seem to provide a logical structural rationale for metabolic cascades in lipoprotein metabolism with the activity of catalytic enzyme and the molecular binding of transport proteins at the surface of the particles.

To link lipidomic profiles measured in serum to those identified in major lipoprotein classes, a hierarchical Bayesian regression model was developed by a group from the VTT Technical Research Center of Finland.[51] They found that the amount of a lipid in serum can be adequately described by the amounts of lipids in the lipoprotein classes.[51]

The applied approach if used widely could eventually facilitate dynamic modeling of lipid metabolism at the individual molecular species level.

It is established that sphingolipids interact with cholesterol to form membrane lipid microdomains that mediate signal transduction. Complex membrane sphingolipids such as SM and glycosphingolipids modulate the function of growth factor receptors and extracellular matrix proteins and serve as binding sites for micro-organisms and toxins.[7] Accordingly, maintenance of the membrane structure is crucial for mechanical stabilization and any shift in lipids asymmetry can induce a variety of unfavorable cellular responses. Alterations in sphingolipids have been implicated in several diseases.[10,11,52-57] For example, elevated plasma SM levels were shown to be closely related to the development of atherosclerosis[9] and plasma Cers were also proposed to serve as biomarkers for atherosclerosis.[8]

It has been shown that SM and phosphatidylcholine modulate the function of lipoproteins and serve as precursors for a variety of regulatory molecules, including lysophosphatidylcholine[58,59] and Cer.[60] Typically, the breakdown of complex sphingolipids results in the formation of Cer through the action of sphingomyelinases or glycosidases and Cers in turn can serve as precursors for major sphingolipids such as SM and glucosylceramide.[61] Ceramide was therefore proposed as a "coordinator" of stress responses in eukaryotes.[2,62,63] In accordance with this concept, it has been shown that plasma levels of the most abundant Cer, C_{24}-Cer, increased in coronary artery disease and stroke patients compared to levels in control subjects, without considerable changes of other Cer species.[64] Farber and Niemann-Pick diseases, which are triggered by dysfunction of acid ceramidase and acid sphingomyelinase, are associated with elevated levels of Cer and SM.[10] Furthermore, abnormalities of glycolipid metabolism in Gaucher disease, Fabry disease and metachromatic leukodystrophy have led to an interest in the composition, metabolism and function of the glycosyl ceramides in human blood.[11]

Gaucher disease is a glycolipid storage disorder characterized by the accumulation of glucosylceramide. Using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE-MALDI-TOF-MS), a study analyzed sphingolipids in pericardial fluid, peritoneal fluid and serum from two patients with Gaucher disease.[65] The results showed that in pericardial fluid, peritoneal fluid and serum, the ceramide monohexoside/SM ratio was increased in the Gaucher disease patients compared to controls. The same group analyzed the sphingolipids in the cardiac valves from a 49-year-old male patient with Fabry disease who suffered from congestive cardiac failure.[66] Fabry disease is a glycolipid storage disorder caused by a defect of alpha-galactosidase A and characterized by the systemic deposition of glycosphingolipids with terminal alpha-galactosyl moieties, mainly globotriaosylceramide, in tissues. Using this semi-quantitative analysis of DE MALDI-TOF-MS, it was revealed that ceramide trihexoside species clearly accumulated in the cardiac valves from the patient.[66]

It was documented that all blood cells can remove plasma Sph, which is harmful or suppressive to cellular functions and convert it into plasma S1P.[44] Plasma S1P may play diverse important roles in blood vessels. We have recently shown that normal physiological levels of circulating S1P play a key role in vascular stability and permeability.[23] Recent work in our laboratory also showed that S1P can be generated in response to monocyte/macrophage activation[47] and S1P can also stimulate the secretion of key inflammatory markers including COX2, TNFα and PGE2.[12] In a study designed to test the ability of serum sphingolipids to predict obstructive coronary artery disease, serum S1P was proposed to be a remarkably strong and robust predictor of both the occurrence and severity of coronary stenosis.[33]

Sphinganine and Sph, the two sphingoid base backbones of sphingolipids, are highly bioactive compounds that are of increasing interest to nutritionists because they occur in food and their metabolism can be altered by fungal toxins. A cross-sectional study of 265 subjects in Linxian, China, showed significant differences in Sph among strata of age, menstruation status, serum cholesterol, carotenoids, retinol, tocopherols, fresh and dried vegetable and fresh fruit consumption.[67] For sphinganine, no significant differences were found.[67] Ecologic studies of esophageal squamous cell carcinoma (ESCC) showed an association with consumption of maize contaminated with the fungus *Fusarium verticillioides*. This fungus produces the toxin fumonisin, which disrupts sphingolipid metabolism. Abnet et al studied the relationship between serum sphinganine and Sph and the incidence of ESCC.[68] They found no significant association between sphingolipid levels and risk of ESCC.[68] However, in a group of Type 2 diabetic patients it was found that the concentrations of plasma Sph and sphinganine were elevated compared with the healthy control subjects (by 55 and 45%, respectively), which indicated that the rate of Cer metabolism in the cells of diabetic patients was elevated.[69] To determine the concentrations and ratios of sphinganine and Sph in the serum and urine of healthy individuals, as a basis for the normal value range, another study found that serum but not urine concentrations of sphingoid bases could be used as a sensitive indicator in the diagnosis of the diseases associated with sphingolipid metabolism impairment.[70] Sphinganine and Sph in those studies were determined by HPLC. Lieser et al developed a methodology for quantification of Sph and sphinganine based on an HPLC/MS/MS separation using extracts from cultured cells.[71]

Given that SM is a key constituent of plasma lipoproteins, along with cholesterol and triglyceride, it has been associated with lipid risk factors of coronary artery disease. Using an enzymatic method to measure plasma SM, Schlitt et al showed that SM is particularly enriched in remnant lipoproteins, which are not present at high concentrations in fasting plasma.[72] In an epidemiological case-control study Jiang et al showed that the plasma SM level is positively and independently correlated with age, body mass index and systolic blood pressure.[73] They also showed lower mean plasma SM levels in men compared with women, in smokers compared with nonsmokers and in Caucasians compared with other ethnic groups.[73] Nelson et al then investigated whether plasma SM is an early atherogenic risk factor and examined the association between plasma SM level and carotid intimal-medial wall thickness, ankle-arm blood pressure index and the Agatston coronary artery calcium score in asymptomatic adults.[74] They concluded that plasma SM is associated with subclinical atherosclerotic disease.[74] Recently, Park et al[75] and Hojjati et al[76] independently showed that inhibition of SM synthesis reduces atherogenesis in apolipoprotein E-knockout mice. It has been also shown that adenovirus-mediated overexpression of SM synthase 1 and 2 increases the atherogenic potential in mice.[77] Thus, abnormal sphingolipid metabolism could conceivably be involved with accelerated atherosclerosis and alterations in certain sphingolipid levels in the blood could be evaluated as possible markers for CVD.

In a study examining the role of sphingolipids in the pathophysiology of sepsis, Drobnik et al provided data from 102 sepsis patients showing that plasma levels of Cer and lysophosphatidylcholine have a highly predictive power in respect to sepsis-related mortality.[78] In addition to the absolute concentrations, they analyzed the molar ratios with their respective precursor molecules reflecting the enzymatic reactions responsible for the generation of both lipids.[78]

CONCLUSION

In summary, the lipoprotein field was limited for decades to measurements of major lipid classes, namely phospholipids, cholesteryl esters, free cholesterol and triglycerides. The application of the emerging proteomic and lipidomic techniques to total plasma lipids and isolated plasma lipoproteins have provided notable detailed characterization of metabolic pathways involved in lipoprotein metabolism in both health and disease states, including the effect of dietary regimens and lipid-modifying treatments.[79-81] Although efforts addressing the importance of determining blood sphingolipid levels as biomarkers of disease are still at their infancy, currently available sphingolipidomic methodologies have facilitated further characterization of lipid molecular species present in plasma as well as in lipoprotein fractions.[17,20] Sphingolipidomics of plasma lipoproteins will eventually provide molecular details of lipoprotein composition, which will be integrated into our knowledge of the structure, metabolism and function of lipoproteins in health and disease.

ACKNOWLEDGEMENTS

Special thanks to Dr. A. Bielawska and Dr. Y.A. Hannun and their teams at MUSC for stimulating discussions and contribution of ideas and information to this chapter. Special thanks also to present and former members of the Hammad laboratory for their work on the human sphingolipidomics project. Special acknowledgement is for the Lipidomics Shared Resource facility at MUSC. SMH was supported by NIH grant HL079274, NIH (ARRA) grant R01 HL079274-04S1, The Southeastern Clinical and Translational Research Institute (SCTR, formerly GCRC) and the South Carolina COBRE in Lipidomics and Pathobiology (P20 RR17677 from NCRR).

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