Advances in Experimental Medicine and Biology 721

L. Ashley Cowart *Editor*

Sphingolipids and Metabolic Disease

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Volume 721
SPHINGOLIPIDS AND METABOLIC DISEASE
L. Ashley Cowart

Sphingolipids and Metabolic Disease

Edited by

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Springer Science+Business Media, LLC

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Springer Science+Business Media, LLC
Landes Bioscience

Printed in the USA.

Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, USA
http://www.springer.com

Please address all inquiries to the publishers:
Landes Bioscience, 1806 Rio Grande, Austin, Texas 78701, USA
Phone: 512/ 637 6050; FAX: 512/ 637 6079
http://www.landesbioscience.com

The chapters in this book are available in the Madame Curie Bioscience Database.
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Sphingolipids and Metabolic Disease, edited by L. Ashley Cowart. Landes Bioscience / Springer Science+Business Media, LLC dual imprint / Springer series: Advances in Experimental Medicine and Biology.

ISBN: 978-1-4614-0649-5

Library of Congress Cataloging-in-Publication Data

Sphingolipids and metabolic disease / edited by L. Ashley Cowart.
p. ; cm. -- (Advances in experimental medicine and biology ; v. 721)
Includes bibliographical references.
ISBN 978-1-4614-0649-5
1. Sphingolipidoses. 2. Sphingolipids--Pathophysiology. I. Cowart, Lauren Ashley. II. Series: Advances in experimental medicine and biology ; v. 721. 0065-2598
[DNLM: 1. Metabolic Diseases--pathology. 2. Sphingolipids--metabolism. W1 AD559 v.721 2011 / WD 200]
RC632.S67S64 2011
616.3'9--dc23
2011021157

PREFACE

Recent years have witnessed an explosion in the incidence of obesity and its sequelae including Type 2 diabetes and the metabolic syndrome. While originally confined to developed countries, increasing prosperity in developing countries has broadened the worldwide incidence of these disorders. Moreover, while diagnoses of Type 2 diabetes were originally almost exclusively confined to adult populations, the rise in childhood obesity has precipitated a marked increase of this disorder in children. Thus, it is crucial to explore all possible therapeutic and preventive methods to attenuate pathological processes associated with these disorders.

Current thinking holds that obesity derives primarily from overnutrition (though compelling arguments for other mechanisms, for example, endocrine disruption by environmental pollutants, also gain support from the literature). In animals, overnutrition is initially handled by adipose tissue expansion; however, exhaustion of this route of lipid sequestering results in oversupply of lipid to other tissues including skeletal muscle, heart, liver, and others. Failure of these tissues to clear excess lipids through either metabolism or sequestration into putatively inert triacylglycerols results in perturbation of bioactive lipid metabolism in cells. In particular, aberrant generation of bioactive sphingolipids is implicated in a multitude of pathological outcomes of metabolic disease including insulin resistance, inflammation, cardiomyopathy, and others. This volume addresses not only the fundamentals of sphingolipid metabolism and analysis, but also the roles of sphingolipids in these disease processes.

Chapter 1: *Sphingolipid Metabolism and Analysis in Metabolic Disease*, by Sarah E. Brice and L. Ashley Cowart. This chapter presents an overview of sphingolipid metabolism and its regulation, followed by caveats and technical considerations for sphingolipid measurement.

Chapter 2: *Sphingolipids and Cardiovascular Diseases,* by Xian-Cheng Jiang, Ira J. Goldberg, and Tae-Sik Park. This chapter addresses current knowledge of the roles of sphingolipids in dysfunction of the cardiovascular system including lipoprotein metabolism, atherosclerosis, and cardiomyopathy.

Chapter 3: *Heart Sphingolipids in Health and Disease,* by Marcin Baranowski and Jan Górski. This chapter continues the cardiovascular theme by addressing novel

mechanisms of regulating sphingolipid biosynthesis in the heart in diabetes as well as the protective role of sphingolipids in ischemia/reperfusion injury.

Chapter 4: *Blood Sphingolipids in Homeostasis and Pathobiology,* by Samar M. Hammad. This chapter addresses the clinical assessment of blood sphingolipids for diagnostic purposes.

Chapter 5: *Adipose Tissue and Ceramide Biosynthesis in the Pathogenesis of Obesity*, by Fahumiya Samad, Leylla Badeanlou, Charmi Shah, and Guang Yang. This chapter discusses changes in sphingolipids that occur as a result of obesity and how these changes mediate inflammation and cardiovascular risk.

Chapter 6: *Sphinoglipids and Hepatic Steatosis,* by Benjamin T. Bikman and Scott A. Summers. This chapter discusses how manipulation of sphingolipid metabolism influences triacylgylerol metabolism in the context of fatty liver.

Chapter 7: *Glycosphingolipids and Insulin Resistance,* by Johannes M. Aerts and colleagues. This chapter discusses the roles of glycosphinoglipids in insulin signaling and how pharmacological reduction of glycosphinoglipid synthesis ameliorates symptoms of the metabolic syndrome.

Chapter 8: *Glycosphingolipids and Kidney Disease*, by Andrew R. Mather and Leah J. Siskind. This chapter continues a focus on glycosphingolipids in the context of kidney pathology. Although the implication of glycosphingolipids in kidney disease associated with diabetes is still conjectural, the role these lipids play in a spectrum of kidney disorders, as discussed in this chapter, justifies further investigation in this highly novel and underexplored area.

Chapter 9: *Sphingolipid Synthetic Pathways are Major Regulators of Lipid Homeostasis*, by Tilla S. Worgall. This final chapter presents compelling findings that sphingolipids may regulate cholesterol homeostasis through SREBP and lipid efflux. The cross-talk between sphingolipids and lipoprotein metabolism presents rich opportunities for therapeutics aimed at ameliorating dyslipidemia associated with metabolic syndrome that promotes atherosclerosis.

Our goal in this volume was to compile chapters presenting broad overviews of tissue-specific effects of sphingolipids, while emphasizing interrelatedness of cellular processes and cross-talk between organs.

We hope you enjoy the volume.

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ABOUT THE EDITOR...

L. ASHLEY COWART obtained her BS in Biology in 1995 from Furman University in Greenville, South Carolina, and her PhD in Biochemistry in 2001 from Vanderbilt University in Nashville, Tennesee. After postdoctoral work in the laboratory of Dr. Yusuf Hannun, she joined the faculty at the Medical University of South Carolina in Charleston, South Carolina, where she currently holds the position of Assistant Professor of Biochemistry and Molecular Biology. Dr. Cowart's main research focus is the regulation of sphingolipid metabolism in diabetes and obesity and the roles of sphingolipids in pathological outcomes associated with these disorders. In addition to research, she is involved with graduate admissions and education.

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CONTENTS

ACKNOWLEDGEMENTS

The Editor wishes to acknowledge funding from the Department of Veteran's Affairs and the NIH Cobre in Lipidomics and Pathobiology at the Medical University of South Carolina. She also wishes to acknowledge her PhD mentor, Dr. Jorge Capdevila, at Vanderbilt University, for his patience and adherence to the highest standards for science in lipid biochemistry. In addition, this work would not have been possible without the training, mentorship, and continued support from Dr. Yusuf A. Hannun and Dr. Lina M. Obeid.

CHAPTER 1

SPHINGOLIPID METABOLISM AND ANALYSIS IN METABOLIC DISEASE

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Abstract: Sphingolipids are an important class of structural and signaling molecules within the cell. As sphingolipids have been implicated in the development and pathogenesis of insulin resistance and the metabolic syndrome, it is important to understand their regulation and metabolism. Although these lipids are initially produced through a common pathway, there is no "generic" sphingolipid. Indeed, the biophysical and signaling properties of lipids may be manipulated by the subunit composition or isoform of their synthetic enzymes, via regulation of substrate integration. Functionally distinct pools of chemically-equivalent lipids may also be generated by de novo synthesis and recycling of existing complex sphingolipids. The highly integrated metabolism of the many bioactive sphingolipids means that manipulation of one enzyme or metabolite can result in a ripple effect, causing unforeseen changes in metabolite levels, enzyme activities, and cellular programmes. Fortunately, a suite of techniques, ranging from thin-layer chromatography to liquid chromatography-mass spectrometry approaches, allows investigators to undertake a functional characterization of all or part of the sphingolipidome in their systems of interest.

INTRODUCTION

Mammalian sphingolipid metabolism consists of a complex network of interlocking pathways (Fig. 1). The basic currency of sphingolipid metabolism is the sphingoid base (1,3-dihydroxy-2-amino-alkane and its derivatives); this base may be subject to the addition of a fatty acid, head group, or phosphate moiety (reviewed in refs. 1-3). This intricate

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

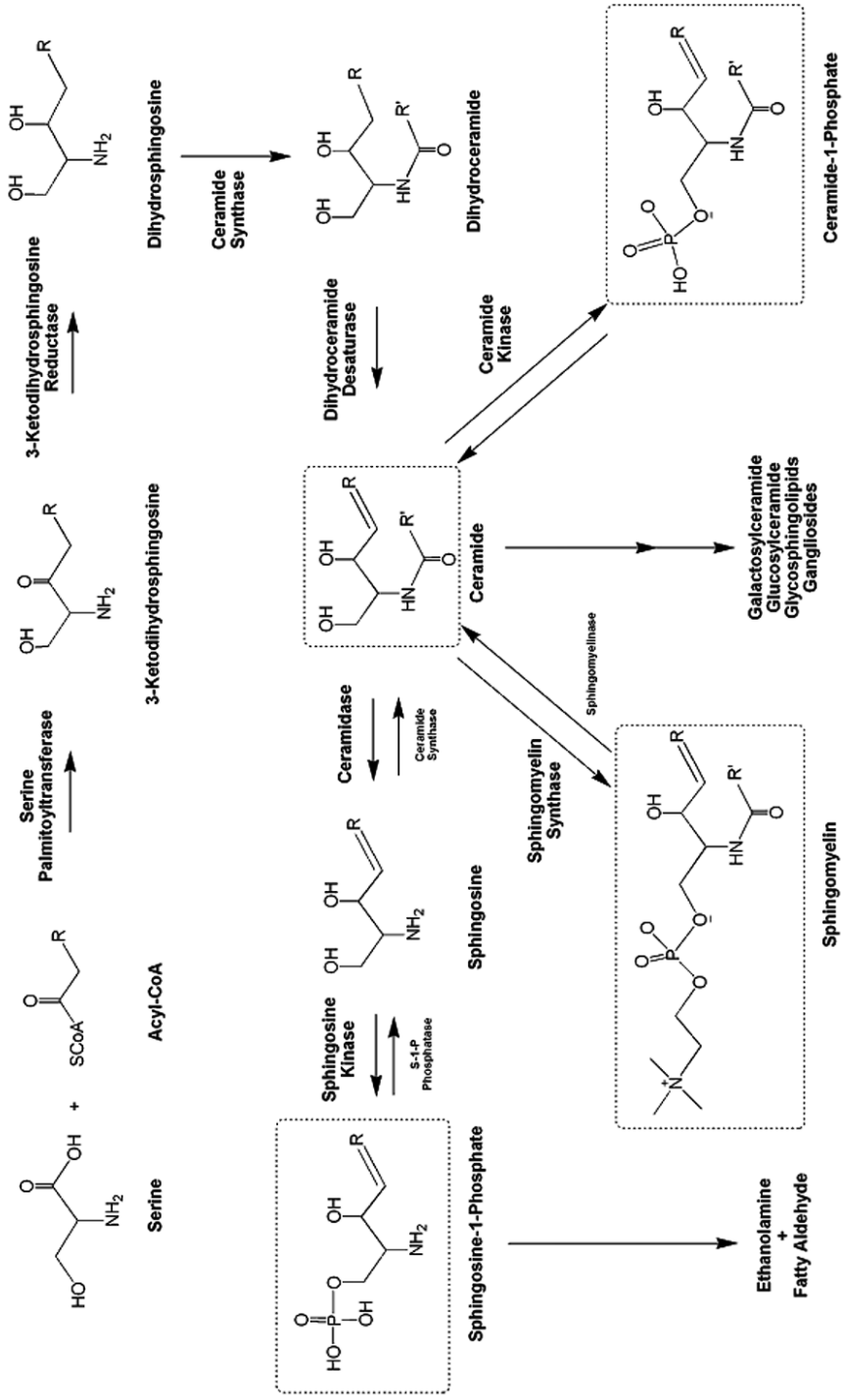

Figure 1. Metabolism of simple sphingolipids.

web of interactions provides the cell with numerous points of regulation, allowing precise control of these important bioactive metabolites.

Insulin resistance and the metabolic syndrome are two increasingly important public health concerns in Western nations. In these pathologies, excessive accumulation of adipose tissue combined with dysregulation of plasma free fatty acid levels leads to ectopic lipid accumulation in nonadipose tissues (steatosis).[4-6] Although cells can biochemically sequester some of the excess lipid as triglycerides, this mechanism is eventually overwhelmed and lipids spill over into nonoxidative pathways.[7-9] This can lead to aberrant cellular signaling, mitochondrial dysfunction and lipotoxic cell death.[10]

Fatty acid oversupply and oxidative stress are two points at which metabolic syndrome and diabetes may touch sphingolipid metabolism and much attention has been paid in recent years to potential modulation of sphingolipid metabolism in these disease states.[11] Some investigators have focused on the phenotypic effects of correcting dysregulation of sphingolipid levels, while others have attempted to resolve the molecular effects of aberrant sphingolipid metabolism in several cell systems relevant to diabetes and metabolic syndrome.[12-14] Systems of particular interest include the pancreatic β cells, skeletal muscle and the heart. Popular models for diabetes and obesity include the Zucker diabetic fatty (ZDF) mouse and rat, diet-induced obesity, toxicant-induced diabetes and various cell culture systems.[8,15-19] Interestingly, while commonalities occur across model systems, there are also some tissue-specific effects, as will be described below and in other chapters.

SPHINGOLIPID METABOLISM

Entry into sphingolipid metabolism begins with the condensation of serine and palmitoyl-coenzyme A (CoA) by the enzyme serine palmitoyltransferase (SPT) to form 3-ketodihydrosphingosine (Fig. 1).[20,21] This enzyme, which localizes to the endoplasmic reticulum, is thought to exist as a 480 kDa multimer composed of four sets of dimers.[22,23] Dimers may form between the SPTLC1 subunit and SPTLC2 and/or SPTLC3.[24] Additionally, binding of the either of the putative small subunits ssSPTa and ssSPTb has been shown to stimulate SPT activity, and ORM/ORMDL family proteins have recently been claimed to associate with the SPT complex and modulate sphingolipid homeostasis.[25-27] The subunit composition of the enzyme, including binding of small subunits, has been shown to modulate fatty acyl-CoA chain length specificities, as described below. Several compounds inhibit SPT; two that are commonly encountered in the literature are myriocin and L-cycloserine.[28] Myriocin (ISP-1/thermozymocidin) is typically the inhibitor of choice due to its high specificity for SPT.[29,30] L-cycloserine, which is less commonly used, efficiently inhibits SPT but also acts as a broad-spectrum inhibitor of pyridoxal 5'-phosphate-dependent enzymes.[31,32]

Significantly, recent studies have increased awareness of the synthesis of noncanonical sphingoid bases utilizing alternative amino acids or fatty acyl-CoAs. For example, although serine is the amino acid canonically considered to form a basis for sphingolipids, a recent study demonstrated that alanine could be incorporated in some cases.[33,34] In the hereditary sensory neuropathy Type 1 (HSAN1) mouse model, a mutation in SPTLC1 allows the SPT enzyme to use alanine and glycine, forming toxic sphingolipid metabolites.[34,35] Similarly, it has long been appreciated that, while palmitoyl-CoA is the preferred fatty acyl chain donor for SPT, microsomal SPT shows activity toward a number of physiologically-available fatty acids.[36-40] It has been demonstrated that total SPT in cellular extracts typically has its

Table 1. Chain length specificities of dihydroceramide synthase isoforms[44]

	CerS1	CerS2	CerS3	CerS4	CerS5	CerS6
Carbon Chain Length	18:0, 20:0	20:0, 22:0, 24:0, 24:1, 26:0	14:0, 22:0, 24:0	18:0, 22:0, 24:0	14:0, 16:0, 18:0, 18:1	14:0, 16:0

greatest activity toward palmitoyl-CoA (C_{16}), with fatty acids with chain lengths of more than the optimal sixteen carbons being utilized more readily than those with shorter chain lengths.[36,39] Additionally, *cis* double bonds, found in naturally occurring unsaturated fatty acids, have been shown to inhibit SPT activity in vitro and in vivo, while activities toward *trans* unsaturated fatty acids were more similar to maximal activity for a given chain length.[38] Specifically, SPTLC1-SPTLC2-ssSPTa demonstrated maximal activity toward palmitoyl CoA (C_{16}), while substitution of ssSPTb for ssSPTa shifted this preference toward stearoyl-CoA (C_{18}).[27] Complexes composed of SPTLC1-SPTLC3-ssSPTa, on the other hand, utilized myristoyl-CoA (C_{14}) somewhat in preference to palmitoyl-CoA, whereas inclusion of ssSPTb reduced overall activity compared to the SPTLC1-SPTLC3-ssSPTa complex but broadened the substrate specificity to include all saturated fatty acids of even-numbered chain lengths between fourteen and twenty carbons.[27] Thus, although all combinations of subunits retain activity toward palmitoyl-CoA, changes in the subunit profile of cellular SPT could potentially allow incorporation of alternative fatty acids.

After its formation by SPT, 3-ketodihydrosphingosine is reduced to form dihydrosphingosine.[41] This compound is then N-acylated by one of six dihydroceramide synthases (CerS, formerly known as Longevity Assurance Genes, or LASSes, prior to their functional characterization), which act on dihydroceramide in a chain length-dependent manner (Table 1).[42-48] Indeed, while the predominant acyl chain lengths of mammalian ceramide are C_{16}- and C_{24}-saturated fatty acids, investigators have encountered fatty acids of chain lengths ranging from C_{14} to C_{32}.[1,42] Ceramides are mostly saturated and may contain α and/or ω-hydroxyl groups.[1,42] This provides an additional point of control where the cell may exert specific manipulations of the chain length profile of sphingolipid species. The maintenance and differential regulation of numerous genes encoding CerS with different substrate preferences supports the speculation that modification of specific chain lengths may be an important cellular process.[42]

After synthesis by one of the CerS, dihydroceramide is reduced by dihydroceramide desaturase (DEGS1/DES1) to form ceramide, the first lipid in this pathway known to have important signaling functions.[49] Ceramide has been implicated in stress responses, senescence, cell-cycle arrest, apoptosis and differentiation.[47,50-53] It acts through a number of target or effector proteins, including cathepsin D, JNK, KSR, p38, PKCζ, PP1, PP2A, Rac, Raf and Rb (reviewed in ref. 54). Induction of apoptosis, a key function of ceramide signaling, occurs through cell type-specific mechanisms. Many of these involve the activation of protein kinases or proteases or promotion of mitochondrial pore formation.[55-60] Notably, ceramide produced through de novo sphingolipid synthesis may evoke a somewhat different intracellular response than ceramide derived from salvage pathways, and these pathways may be differentially regulated.[61-63]

Ceramide acts as a building block for the other branches of sphingolipid metabolism, in which it may be modified with headgroups, phosphorylated, or degraded to sphingosine, which is an eighteen carbon amino alcohol with an unsaturated hydrocarbon tail.[64] Formation of sphingosine is mediated by several ceramidase enzymes.[65-67] Acid, neutral

Table 2. Intracellular localization, expression and specificities of mouse ceramidases[67,170]

Protein	Gene	Intracellular Localization	Substrate Specificity	Highest Expression
Acid Ceramidase	mAC/ASAH1	Lysosome	C_6-C_{16}-Cer	Kidney, lung, heart, brain
Neutral Ceramidase	mNC/ASAH2	Plasma Membrane	C_{14}-Cer and Longer	Kidney, liver, heart
Akaline Ceramidase 1	ACER1/ASAH3	Endoplasmic Reticulum	$C_{24:1}$-Cer and Longer, unsaturated	Skin
Alkaline Ceramidase 2	ACER2/ ASAH3L/ maCER1	Golgi	C_{14}-Cer and longer	Placenta
Alkaline Ceramidase 3	ACER3/PHCA/ aPHC	Endoplasmic Reticulum/ Golgi	$C_{20:1}$-Cer/DHCer and shorter (unsaturated)	Unknown

and alkaline ceramidases have distinct expression patterns, intracellular topologies and substrate preferences for acyl-CoA chain lengths and saturation (Table 2) (reviewed in ref. 67). Relative contributions of the hydrolytic and de novo pathways vary widely by cell type. For example, C2C12 myotubes rely very heavily on de novo sphingolipid synthesis, while other cell lines strongly prefer hydrolytic metabolism.[68]

Importantly, sphingosine may be phosphorylated by sphingosine kinase (SK) to form sphingosine-1-phosphate (S1P).[69] S1P is an important signaling molecule imputed with both intracellular and extracellular signaling properties.[70-72] The extracellular effects of S1P are mediated by interaction with a family of at least five G-protein-coupled receptors, named $S1P_1$-$S1P_5$ (reviewed in ref. 73).The S1P receptors signal through heterotrimeric G-proteins, with subtype-specific G-protein coupling leading to unique signaling pathways for each receptor subtype.[74] The intracellular targets of S1P have not yet been identified. Two isoforms of SK, SphK1 and SphK2, are responsible for production of S1P.[75,76] These isoforms are differentially expressed in various tissues in the body, and they may serve separate signaling functions and be subject to distinct modes of regulation.[76-79] S1P generally promotes cell proliferative and anti-apoptotic pathways.[80-82] Important signaling activities of S1P include stimulation of inositol trisphosphate-independent calcium mobilization, inhibition of caspase activity, activation of nonreceptor tyrosine kinases and the Raf/MKK/ERK signaling cascades and stimulation of PLD via MAPK and AP-1.[54,83]

In another important branch of the pathway, ceramide receives a phosphocholine head group via the action of sphingomyelin synthase, forming sphingomyelin and a biologically-active pool of diacylglycerol.[84,85] There are two known forms of sphingomyelin synthase (SMS1 and SMS2).[86] Sphingomyelin (choline phosphoceramide) comprises 5-10% of membrane phospholipids in mammalian cells.[87]

In another portion of the pathway, ceramide may be modified with a carbohydrate head group, forming glycosphingolipids such as glucosylceramide and galactosylceramide.[88] Over four hundred complex sugar-containing species may be generated in this manner. The ceramide portion of these lipids is embedded in the plasma membrane, while the sugar moieties extend into the extracellular space.[88] These lipids serve as precursors for synthesis of gangliosides and cerebrosides, which are more complex glycosphingolipids.[89-92]

Table 3. Localization of mouse sphingomyelinases with known in vivo activity[171,172]

Protein	Gene	Intracellular Localization/Secreted
Secretory sphingomyelinase (S-Smase)	SMPD1	Secreted
Lysosomal acid sphingomyelinase (L-SMase)		Lysosome, translocates to the plasma membrane under stress
Neutral sphingomyelinase 2 (nSMase2)	SMPD3	Plasma membrane
Neutral sphingomyelinase 3 (nSMase3)	SMPD4	Endoplasmic reticulum, Golgi

Ceramide-1-phosphate (C1P) and ceramide kinase (CERK), which are still being characterized, may form an important link between sphingolipids and eicosanoid biosynthesis.[93,94] CERK has high structural and amino acid homology to the sphingosine kinase (SPHK) enzymes; it is primarily expressed in heart, kidney, brain and hematopoetic cells.[95] Only one isoform of CERK has been identified, but elimination of this kinase does not eliminate C1P, suggesting the existence of an alternative isoform or synthetic pathway for this lipid. C1P has been implicated in allergic and inflammatory signaling, membrane fusion and prostaglandin synthesis.[93,94,96,97] C1P promotes $cPLA_2$ activation and production of arachidonic acid and it inhibits PP1 and PP2A in vitro.[93,94]

The products of these biosynthetic pathways may also be modified and degraded. S1P may be dephosphorylated by either specific S1P phosphatases or members of the lipid phosphate phosphohydrolase family.[98,99] Alternatively, the cell may shunt lipids out of the sphingolipid pathway by degrading S1P via S1P lyase, which cleaves the C2-C3 bond of S1P to yield hexadecanal and phosphoethanolamine.[100] Under stress conditions, sphingomyelin may be broken down to release ceramide and phosphocholine.[101,102] Intriguingly, ceramide produced by this pathway may have distinct functional roles from that produced by de novo synthesis and the subcellular localization of these enzymes may have functional importance (Table 3).[61-63] Finally, glycosphingolipids may be degraded by exohydrolases such as glucosidase and galactosidase; this degradation results in the stepwise release of terminal monosaccharides from the oligosaccharide chain.[103,104]

Up-regulation of sphingolipid metabolism occurs in many stress responses. Stimuli that can trigger de novo sphingolipid synthesis and ceramide formation include chemotherapeutic agents, oxidized LDL, excess substrate and heat stress.[105-110] Salvage-based sphingolipid metabolism is triggered by phorbol esters, which stimulate PKC, oxidative stress and TNF-α.[63,101,111-116] Additionally, the generation of ceramide from sphingomyelin can be stimulated by Fas ligand.[117] It has been proposed that differential activation of specific ceramide-generating pathways is facilitated by the distinct topology of the individual enzymes.

SPHINGOLIPIDS AND LIPOTOXICITY

Based on the work of numerous researchers, sphingolipids have been shown to act in the development and pathology of metabolic syndrome. Elevated ceramide has been mechanistically associated with the development of insulin resistance.[118-120] Currently, it is believed that, in scenarios of palmitate oversupply, ceramide activates PP2A.[121-123] This results in the dephosphorylation of Akt/PKB, subsequently reducing glucose transport

and activating glycogen synthase kinase.[124,125] Alternatively, ceramide may act to stabilize the repressive protein-protein interaction of PKCζ with Akt/PKB, preventing release and activation of Akt/PKB upon insulin stimulation.[126] Inhibition of de novo sphingolipid synthesis restored glucose uptake and phosphorylation of Akt/PKB in skeletal muscle of animals infused with lard oil.[127] Additionally, treatment with several inhibitors of de novo ceramide synthesis overcame the inhibitory effect of palmitate on Akt/PKB and GSK-3β phosphorylation in C2C12 myotubules, which are a model of human skeletal muscle.[128,129] Furthermore, heterozygous DES1 knockouts had enhanced insulin sensitivity and they developed less insulin resistance in response to treatment with synthetic glucocorticoids.[127]

Elevated sphingolipid levels also seem to play a role in the development of characteristic pathologies seen in the heart in metabolic syndrome. For example, mice overexpressing GPI-tagged lipoprotein lipase (LpL-GPI) had elevated cardiac ceramide levels as well as cardiac hypertrophy, increased left ventricular systolic diameter and decreased fractional shortening.[130] Furthermore, expression of the cardiac failure markers ANF and BNP was elevated in these mice. All of these pathologies were corrected by myriocin treatment. Additionally, the aberrant patterns of substrate utilization seen in these hearts, which mirror a significant pathology of the heart in metabolic syndrome, were corrected with myriocin treatment. Attenuating de novo sphingolipid synthesis also reduced phosphorylation of AKT and its downstream target GSK-3β, which acts to inhibit cardiac hypertrophy and glycogen synthesis in the heart.[12,130-132] Furthermore, elevated levels of ceramide have been shown to promote myocardial apoptosis in ZDF rats.[133] In particular, accumulation of ceramide is associated with inhibition of prosurvival signaling kinases, including Akt/PKB and PKCζ and stimulation of pro-apoptotic protein kinases, such as c-jun N-terminal protein kinase (JNK).[134-136] These actions result in suppression of Bcl2, activation of caspases and other pro-apoptotic events.

It has been shown in a variety of cell types that increasing concentrations of palmitate in the extracellular environment induces an increase in formation of ceramide or other sphingolipids (reviewed in ref. 54). This is attributable both to increased substrate supply and changes in gene expression. [17,130, 137,138] For example, cardiac ceramide levels were increased in rats fed a diet with a relatively higher proportion of calories derived from saturated fat, as compared to a low-fat diet and a diet with calories derived equally from saturated and unsaturated fats.[139] Intriguingly, the high fat diet incorporating equal contributions of saturated and unsaturated fats actually decreased ceramide levels, arguing for an additional level of regulation of ceramide levels beyond simple substrate supply. Further emphasizing the importance of altered transcriptional regulation, de novo ceramide production and mRNA levels of SPT were elevated in pancreatic islets from *fa/fa* ZDF rats, which have elevated plasma free fatty acid levels.[17] Heart muscle from LpL-overexpressing mice, which had increased uptake of fatty acids into cells, also contained excess ceramides; this was corrected after six weeks of treatment with myriocin, which inhibits serine palmitoyltransferase.[130] This response seems to be widespread among tissues in the body; for example, treatment of primary astrocytes with palmitate increased activity and protein levels of SPT.[140]

However, palmitate supplementation may increase sphingolipids other than pro-apoptotic ceramides, potentially acting to protect the cell. For example, it has been shown that treatment with palmitate results in an increase in S1P in C2C12 myotubes.[141] Further supporting this perspective, a drastic reduction in SPTLC1 expression in L6 myotubes, which are a model of skeletal muscle, actually sensitized cells to palmitate-induced cell death.[14] This may be mediated by increased routing of

fatty acids into diacylglycerol (DAG), which has potent signaling properties. Reducing expression of SPTLC1 in palmitate-treated cells also increased levels of PKCθ, which phosphorylates IRS1 at Ser[1,110].

Human studies have generally supported the findings in animal and cell culture systems. Skeletal muscle from obese, insulin-resistant humans contain increased levels of ceramide and exposure of human skeletal muscle to excess palmitate results in the development of insulin resistance and elevated ceramide levels.[11,142-145] A recent, carefully-controlled study demonstrated elevation of long-chain ceramides in skeletal muscle from insulin resistant patients.[143] In particular, significant increases were seen in C_{14}-, C_{16}- and $C_{18:0}$-ceramides as well as S1P and total ceramide levels.

Quantification of sphingolipid levels in human hearts has produced less conclusive results on differences between lean, obese and diabetic subjects.[146] However, obesity, alone or in combination with Type-2 diabetes, was associated with increased expression of the serine palmitoyltransferase subunits SPT1 and SPT2, the sphingosine kinase SPHK1, alkaline/neutral ceramidase, acid ceramidase and neutral sphingomyelinase in right atrial tissue from patients receiving elective coronary bypass graft surgery. Additionally, expression of these enzymes was higher in papillary (ventricular) muscle than in atrial muscle, suggesting that ceramide turnover is higher in ventricular tissue than in the atria. Intriguingly, overweight or obesity was associated with increased CPT1 and PDK4 in the human myocardium, while FAT/CD36 and LPL levels remained constant. This results in increased potential for fatty acid oxidation without a similar upregulation of uptake capacity. Based on these data, it has been proposed that upregulation of sphingolipid metabolic enzymes may represent a stabilizing mechanism that allows sphingolipid metabolism to compete for the available fatty acyl-CoA.

It has been hypothesized that elevated triglyceride (TG) stores are an important source of fatty acids for ceramide overproduction in metabolic syndrome and acute fatty acid oversupply.[143] Storage of excess fatty acids in TG is proposed to initially serve as a protective mechanism, sequestering lipids away from the synthesis of bioactive metabolites.[137,147,148] However, as cellular TG content peaks, fatty acids escape from futile TG cycling into other pathways, such as DAG or sphingolipid production. Although this relationship has never been definitively tested, the correlative data are highly suggestive. For example, TG content in fatty acid-treated pancreatic islets from the ZDF rat is about three times that in wild type islets treated with fatty acid and leptin; the magnitude of this change is similar to the two- to three-fold increase in the rate of ceramide formation in these islets.[5,17,149] Powerfully, overexpression of perilipin, which prevents breakdown of TG and thus flow of fatty acids into sphingolipid metabolism, attenuated palmitate-induced insulin resistance.[137] Additionally, elevation of ceramides in insulin-resistant skeletal muscle was accompanied by a significant increase in intramyocellular TG and downregulation of adipose triglyceride lipase and hormone-sensitive lipase, the first two enzymes of TG breakdown.[150] These data suggest that ceramides increase when bulk fatty acid uptake overwhelms the ability of the cell to store FFA as neutral lipid.

Intriguingly, the composition of fatty acids in the blood or media can profoundly impact the effect of fatty acid oversupply on cells.[151] In particular, oleate appears to attenuate the pathological effects of excess palmitate. For example, ceramide seems to inhibit insulin signaling through a PP2A-dependent mechanism in situations of palmitate, but not oleate, oversupply.[123] Additionally, infusion with lard oil, which is high in saturated fatty acids and is composed of 25-28% palmitate and 44-47% oleate,

disrupted glucose uptake and Akt/PKB phosphorylation in a manner that could be corrected by inhibition of serine palmitoyltransferase, while infusion with soy oil, which is high in unsaturated fatty acids and contains 10% palmitic acid and 23% oleic acid, did not.[129] Importantly, oversupply of unsaturated fatty acids results in buildup of DAG but not ceramides.[152] It has also been shown that treatment with palmitate resulted in increased SK1 message levels, which resulted in elevated S1P levels and SK enzyme activity, in C2C12 myotubes, an in vitro skeletal muscle model.[141] In contrast, cosupplementation with oleate attenuated the increase in SK1 mRNA.[141] Because of the different effects of exposure to specific fatty acids, results should be interpreted in the light of the fatty acid composition of the specific diet, infusion, or cell culture conditions used in any particular study.

APPROACHES TO THE STUDY OF SPHINGOLIPIDS IN LIPOTOXICITY

Sphingolipidomics, the study of the complete sphingolipid profile of a cell, flows naturally from the interconnectedness of sphingolipid pathways.[153] Because of the metabolic ties between all portions of sphingolipid metabolism, changes in regulation or flux in one part of the pathway can profoundly change the sphingolipidome as a whole.[47,154-156] This remodeling may act to amplify or to attenuate the effects of a treatment or mutation beyond the proximal point of contact with the sphingolipid pathway. Furthermore, although compounds may be lumped together in broad categories, e.g., ceramides, for purposes of discussion, distinct enzymes produce and act on sphingolipids containing different fatty acyl chain lengths.[43,44] Emerging evidence suggests that the cell distinguishes between chain lengths for signaling purposes. For example, C_{18}-ceramide was implicated as a key pro-apoptotic molecule in a head and neck squamous cell carcinoma model, while C_{16}-ceramide was found to protect against cell death.[157,158]

It has been suggested that differential regulation of ceramide chain lengths could be due to the influence of chain length on biophysical properties of the membrane bilayer or by direct interaction with downstream players in signaling pathways. The properties of specific lipids may also dictate their transport and routing.[159] For example, the sphingoid bases sphingosine and dihydrosphingosine are rapidly exchanged between adjacent membrane surfaces bordering the cytosol and across membranes; this is due to the partially charged amino group on the head group and the single anchoring acyl chain. In contrast, ceramide, which possesses two acyl chains, can both diffuse laterally along a membrane and move across its faces, but it cannot spontaneously move between membranes. Additionally, sphingolipids may be sorted at the Golgi for routing to the plasma membrane, as opposed to routing to the endoplasmic reticulum, and additional lipid-specific routing by proteins and physical chemical segregation both play important roles in cellular function, membrane composition and lipid routing (reviewed in see ref. 159). Furthermore, ceramide N-acyl chain length has been demonstrated to dictate its effects on membrane biophysical properties, including miscibility with the major membrane constituent sphingomyelin and consequent organization and stabilization of membrane domains.[160] As roles for sphingolipids of specific chain lengths become better understood, revealing answers may be gleaned from both a quantitative assessment of changes in levels of the major sphingolipid species as well as a finer resolution of changes in chain length distribution within species.

The choice of approach in determining sphingolipid levels can significantly impact results. Although there have been numerous exciting technological advances in sphingolipidomic analysis,[161,162] (also reviewed in refs. 153 and 163) not all labs have access to facilities to perform these analyses at their home institutions. In such situations, thin-layer chromatography and the DAG kinase assay are two relatively easy and commonly-employed options. However, the reliability of results is highly contingent on appropriate experimental design and a reasonable understanding of the pitfalls associated with the chosen approach, especially in the case of the DAG kinase assay.[164,165] Extraction methods and solvent systems for isolation of particular sphingolipid species by TLC have been reviewed in detail elsewhere and will not be discussed further.[162,166-169] However, the DAG kinase method merits further comment, as it is both relatively commonly used and can produce misleading results if conducted improperly.[164,165]

Briefly, the DAG kinase converts DAG to phosphatidic acid, and it was demonstrated that this reaction can be used to quantitatively measure DAG levels in crude cellular extract.[164,165] Later, it was discovered that DAG kinase will phosphorylate ceramide to C1P in a manner that is similarly amenable to quantitation. When performed carefully, the DAG kinase assay can rapidly and simultaneously quantify mass levels of ceramide and DAG, which are separated by TLC and identified by comparison to lipid standards. Results produced by this method have compared well with those generated by other methods. In contrast, failure to use an excess of enzyme or to allow the reaction to go to completion will skew data obtained from this assay; this is due to effects of reaction kinetics and competition between DAG and ceramide as substrates for the DAG kinase. Furthermore, it should be appreciated that mild alkaline hydrolysis, which is included in some protocols to eliminate diglycerides and glycerolipids, will also hydrolyze 1-*O*-acyl ceramides, which can reduce detected ceramides by ten to twenty percent. Additionally, the Bligh-Dyer extraction efficiently isolates C1P, dihydroceramide phosphates and phosphatidic acids but not short chain C1Ps, hydroxylated ceramides or lyso-PA. Finally, the choice of solvent system impacts the separability of distinct C1Ps by chain length. Thus, while the DAG kinase method can be an extremely useful tool, the results should be interpreted circumspectly, based on the specific protocol used.

Finally, the availability of knockout mice, siRNA and inhibitors has opened the door to studies examining the effects of reducing levels or activity of individual sphingolipid metabolic enzymes. However, the interconnectedness and metabolite-specific effects of sphingolipid metabolism present a significant caveat for such studies. While these models can be excellent tools, they should be used judiciously and the data must be interpreted with caution. This point is demonstrated by a recent study of CerS2 knockdown in a cancer cell line.[47] Downregulation of CerS2 protein levels and activity only produced a minor decrease in very-long chain ceramides, which are the main products of this enzyme. Additionally, CerS2 knockdown induced an increase in levels of long-chain ceramides due to reverse ceramidase activity as well as upregulation of other (dihydro)ceramide synthase isoforms. As stated above, long chain ceramides have been implicated in cell death signaling. Thus, perturbing one component of the sphingolipid biosynthetic pathway activated compensatory mechanisms, resulting in unforeseen changes in metabolite levels, enzyme activities and cellular programmes. For this reason, investigations of metabolic disturbances in sphingolipid biosynthesis should take advantage of sphingolipidomic approaches to examine the effects of these disturbances with a fine resolution.

CONCLUSION

Sphingolipids comprise a key component of the cellular signaling apparatus that responds to conditions of fatty acid oversupply. Ceramide and sphingosine-1-phosphate are two specific metabolites that have been implicated as critical mediators of this process, and disturbance of the delicate inner balance of sphingolipid metabolism can push a cell toward apoptosis or pro-inflammatory signaling. Because of the highly interconnected nature of this system, studies attempting to identify the specific agents promoting pathology in a situation of fatty acid oversupply must be designed and interpreted with care.

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CHAPTER 2

SPHINGOLIPIDS AND CARDIOVASCULAR DISEASES:

Lipoprotein Metabolism, Atherosclerosis and Cardiomyopathy

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Abstract: Heart disease is widely believed to develop from two pathological processes. Circulating lipoproteins containing the nondegradable lipid, cholesterol, accumulate within the arterial wall and perhaps are oxidized to more toxic lipids. Both lipid accumulation and vascular reaction to the lipids lead to the gradual thickening of the vascular wall. A second major process that in some circumstances is a primary event is the development of a local inflammatory reaction. This might be a reaction to vessel wall injury that accompanies infections, immune disease, and perhaps diabetes and renal failure. In this chapter, we will focus on the relationship between de novo synthesis of sphingolipids and lipid metabolism, atherosclerosis, and cardiomyopathy.

INTRODUCTION

Coronary artery disease is widely believed to develop from two pathological processes. Circulating lipoproteins containing the nondegradable lipid, cholesterol, accumulate within the arterial wall and perhaps are oxidized to more toxic lipids. Both lipid accumulation and vascular reaction to the lipids lead to the gradual thickening of the vascular wall. A second major process that in some circumstances is a primary event is the development

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

of a local inflammatory reaction. This might be a reaction to vessel wall injury that accompanies infections, immune disease and perhaps diabetes and renal failure.

Lipid accumulation is associated with a number of other diseases. These include nonalcoholic liver disease, Type 2 diabetes due to reduced insulin secretion[1] and skeletal muscle insulin resistance[2] and some forms of cardiomyopathy.[3] Several mechanisms have been proposed to explain how lipid accumulation leads to organ dysfunction: (1) direct toxic effects of neutral droplets or fatty acids (FAs) on myofibrillar function[4] (2) reactive oxygen species (ROS) generated as a toxic by-product of lipid oxidation[4] (3) FA-induced apoptosis[3] (4) diacylglycerol induced activation of signaling pathways such as those mediated by protein kinase Cs.[5] When the balance between oxidation and FA uptake is altered, excess FA must enter pathways for nonoxidative metabolism. This anomalous lipid metabolism is thought to lead to dysfunction of nonadipose tissues. One alternative route to utilize the FA surplus is via the sphingolipid biosynthetic pathway.

Dysregulation of the sphingolipid biosynthetic pathways associated with excess FA uptake and accumulation may be a central metabolic derangement in lipotoxicity and atherosclerosis. This could occur due to changes in plasma levels of circulating lipids or inappropriate lipid uptake by cells/tissues. Correction of the induced sphingolipid biosynthesis excess could become a treatment for diseases such as atherosclerosis and lipotoxicities. Sphingomyelin (SM) is one of the major lipid components in plasma and of cell membranes. Plasma SM levels are an independent risk factor for coronary artery disease, i.e.,independent of cholesterol.[6] In mice, reduction of plasma and liver SM leads to a concomitant reduction of atherosclerosis; this was achieved by pharmacological inhibition of serine palmitoyltransferase (SPT).[7,8] Macrophage deficiency of sphingomyelin synthase 2 (SMS2), the last enzyme for SM biosynthesis, decreases plasma membrane SM levels and decreases atherosclerosis in a mouse model.[9] Cardiac dysfunction caused by lipid accumulation in heart[10] and insulin resistance associated with dietary or infused saturated FAs[11] is ameliorated by inhibition of ceramide biosynthesis. These observations emphasize the need for a better understanding of sphingolipid metabolism in cardiovascular diseases. In this chapter, we will focus on the relationship between de novo synthesis of sphingolipids and lipid metabolism, atherosclerosis and cardiomyopathy.

SPHINGOLIPID BIOSYNTHESIS

The biochemical synthesis of SM occurs through a series of reactions involving the enzymes serine palmitoyltransferase (SPT), 3-ketosphinganine reductase, ceramide synthase, dihydroceramide desaturase and sphingomyelin synthase (Fig. 1).

Located in the endoplasmic reticulum (ER) membrane, SPT is the rate-limiting enzyme in the pathway.[12] Mammalian SPT contains two subunits, Sptlc1 and Sptlc2, encoding 53- and 63-kDa proteins, respectively.[13,14] The subunits are homologous (about 20% identity)[13,14] and this is probably relevant to their formation of a heterodimer. Both Sptlc1 and Sptlc2 have a single, highly hydrophobic N-terminal transmembrane domain.[13,14] Neither appears to be glycosylated.[13] Indirect immunocytochemical analysis with epitope-tagged Sptlc1 indicates that the N- and C-termini of both subunits are oriented to the lumen and cytosol, respectively.[15] A third possible subunit, Sptlc3[16] has 68% homology with Sptlc2 and 20% homology with Sptlc1. Over-expression of Sptlc3 in Hek293 cells led to a 2-3 fold increase in cellular SPT activity.

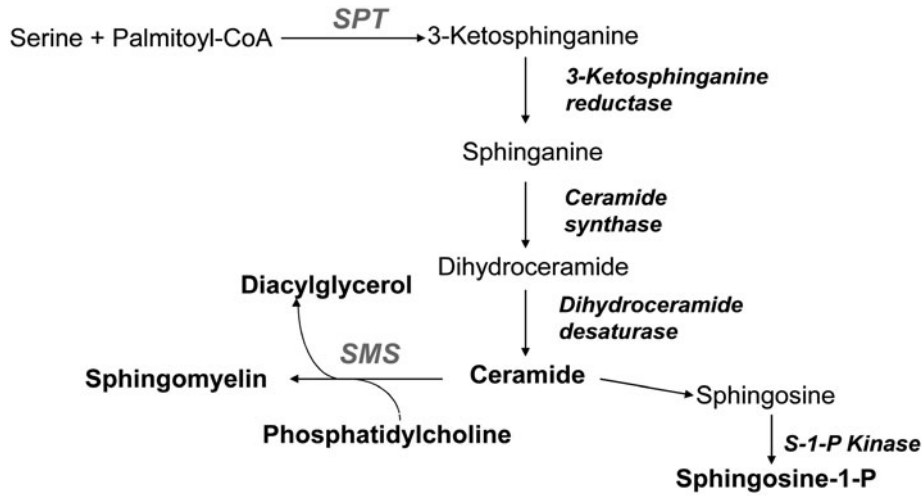

Figure 1. Sphingolipid biosynthesis in mammals. SPT, serine palmitoyltransferase; SMS, sphingomyelin synthase.

Silencing of Sptlc3 expression in HepG2 cells resulted in a significant reduction of cellular SPT activity, but not a complete loss.[16] The authors speculated that Sptlc3 was an isoform of Sptlc2, each binding Sptlc1 independently and that varying the amounts of Sptlc2 and -3 might be a cellular mechanism to adjust SPT activity to meet tissue specific requirements for sphingolipids.

In yeast SPT is composed of a heterodimer of 2 highly-related subunits, Lcb1p and Lcb2p, and a third subunit, Tsc3p, which increases enzyme activity markedly and is required for growth at elevated temperatures.[17] Recently, 2 proteins, ssSPTa and ssSPTb, which despite sharing no homology with Tsc3p, substantially enhances the activity of mammalian SPT expressed in either yeast or mammalian cells, were identified. These proteins are evidence for an evolutionarily conserved family of low molecular weight proteins that confer full SPT enzyme activity.[18] The small subunits of mammalian SPT confer distinct acyl-CoA substrate specificities.[18] SMS is located mainly in the *cis*-, medial-Golgi[19-21] and plasma membranes.[22-24]

There may also be a form of SMS in the *trans*-Golgi network[25] and the nucleus.[26] SMS activity has been found in chromatin and chromatin-associated SMS modifies the SM content.[27] Two SMS genes, SMS1 and SMS2, have been cloned and characterized for their cellular localization.[28,29] SMS1 is found in the *trans*-Golgi apparatus, while SMS2 is predominantly found in the plasma membranes[28,30] and also Golgi apparatus.[28,30] SMS1 and -2 expression positively correlates with levels of cellular SM and SM in lipid rafts.[31-33]

The sphingolipid biosynthesis pathway impacts cellular production of at least three bioactive lipids: ceramide, diglyceride, and sphingosine-1-phosphate; and two structure-related lipids: sphingomyelin and phosphatidylcholine. In this chapter, we focus on sphingomyelin and ceramide.

ROLE OF SPHINGOMYELIN (SM) IN LIPOPROTEIN METABOLISM

Sphingomyelin (SM) is a major component of cell membranes and both atherogenic and anti-atherogenic lipoproteins. Chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) are atherogenic lipoproteins, while high density lipoproteins (HDL) are antiatherogenic ones. Chylomicrons, VLDL and LDL are enriched with SM.[1]

SM and Chylomicron/VLDL Metabolism

The primary metabolic role of VLDL and chylomicrons is to transport endogenous and dietary triglyceride used for energy, structural lipids and storage. Triglyceride located on these particles is hydrolyzed into free FAs by lipoprotein lipase (LpL) associated with the luminal surface of capillaries.[34,35] In vitro studies indicate that SM in these lipoproteins inhibits the action of LpL (Fig. 2). Kuksis et al[36] compared LpL hydrolysis of TG using three emulsions as substrates for the enzyme: phosphatidylcholine (PC)/TG, PC/SM/TG, and PC/phosphatidylethanoloamine (PE)/TG. They found that SM-containing emulsion had the lowest TG clearance rate in vitro. Saito et al[37] and Arimoto et al[38] found that SM, but not cholesterol, significantly inhibited TG lipolysis by LpL in a SM concentration-dependent manner. Lobo and Wilton[39] and Cantin et al[40] showed that cholesterol inclusion in the PC surface coat of a TG emulsion stimulated lipolysis by LpL, but that this stimulation was eliminated by adding SM.

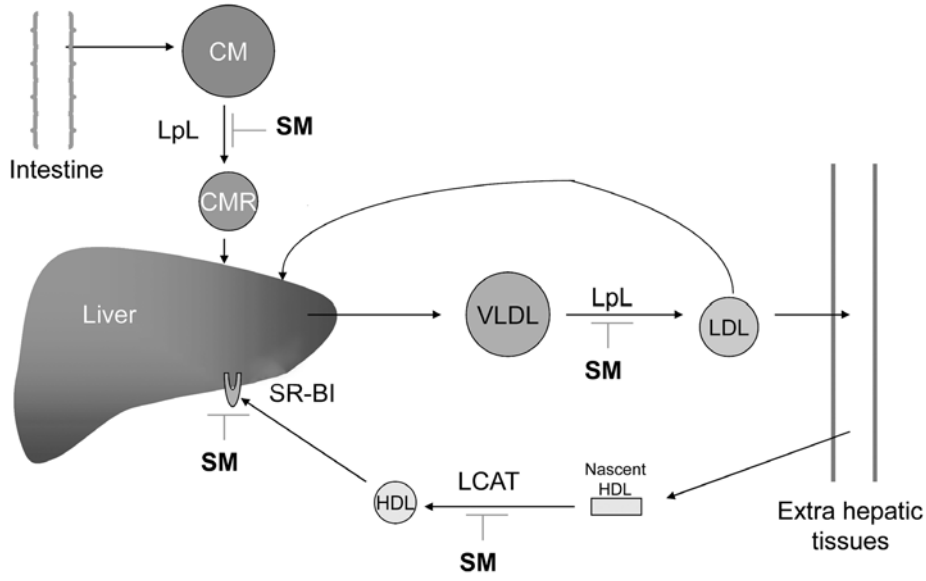

Figure 2. Role of SM in regulation of lipoprotein metabolism. Increased plasma SM inhibits lipoprotein lipase (LpL) and hydrolysis of triglyceride in chylomicrons and VLDL. This latter action reduces the production of LDL. Maturation of nascent HDL is inhibited by SM. SM inhibits LCAT activity and formation of cholesterol ester in HDL is reduced. SM also inhibits SR-BI, a HOL receptor.

SM and Apolipoprotein E-Containing Lipoprotein Metabolism

Apolipoprotein E (ApoE) is a protein which has anti-atherogenic property. Bound to plasma lipoproteins, it is a ligand for cell uptake by the LDL receptor (LDLR), the LDL receptor-related protein (LRP) and heparan sulfate proteoglycans.[41,42] ApoE also influences the assembly and secretion of lipoproteins[43,44] and cholesterol efflux to HDL.[45] Arimoto et al reported that SM in lipoproteins delays remnant clearance by decreasing the binding of apoE to cell membrane receptors.[46] Sphingomyelinase causes particle aggregation in artificial lipid emulsions of TG/PC/SM by degradation of SM to ceramide. ApoE prevents this aggregation,[47] implying an interaction between SM and apoE. To clarify the role of SM in lipoprotein uptake, Morita et al[48] prepared lipid emulsions containing triolein, PC and SM as model particles of reconstituted lipoproteins. Incorporation of SM into the emulsion surface reduced the binding capacity of apoE without changing its affinity for the receptors on the surface of hepatocytes. Surface SM reduced apoE-mediated uptake of emulsions by HepG2 cells because of the decreased amount of binding apoE. The stimulatory effect of LpL on emulsion uptake was decreased by replacing surface PC with SM. These results suggest that SM-induced changes in the binding properties of apoE and LpL correlate with decreased hepatic uptake of lipid particles. Lucic et al found that apoE expression also increased SM secretion from macrophages and this SM was colocalized with apoE in secreted lipoprotein particles.[49] Although the in vivo significance of these findings to apoE-containing lipoprotein metabolism is not yet established, they may be highly relevant during the increase in the SM content of plasma lipoproteins that occurs following a large lipid load[50] or apoE deficiency.[51]

SM and HDL Metabolism

HDLs are well-known anti-atherogenic lipoproteins. HDL particles are involved in the process of reverse cholesterol transport which removes cholesterol from peripheral tissues and cells. The cholesterol on HDL becomes cholesterol ester (CE) through the activity of lecithin:cholesterol acyltransferase (LCAT). The CE of HDL may then be delivered to liver or steroidogenic tissues by the scavenger receptor B1 (SR-B1)[52,53] (primary fate) or transferred, via plasma cholesteryl ester transfer protein (CETP) to LDL and TG-rich lipoproteins in exchange for TG.[54]

These processes may be affected by the presence of SM in HDL. SM inhibits LCAT by decreasing its binding to HDL[55] (Fig. 2). A negative correlation between the SM content of HDL and LCAT activity was observed in studies with proteoliposomes or reconstituted HDL.[56]

Rye, Hime and Barter[57] found that SM influences the structure of discoidal and spherical HDL and confirmed that SM inhibits the LCAT reaction.

Macrophage cholesterol efflux plays an important role in reverse cholesterol transport, an anti-atherogenic process.[58] However the capacity of mouse plasma to mediate cholesterol efflux from mouse macrophages supplemented with different concentrations of SM was not altered. This result indicated that plasma SM levels might not have a direct effect on cholesterol efflux (Li and Jiang unpublished observation).

SR-BI is the first molecularly defined receptor for HDL and can mediate the selective uptake of CE into cells. Subbaiah et al[59] investigated the effect of SM in lipoproteins on the selective uptake in three different cell lines: SR-BI-transfected CHO cells, hepatocytes

(HepG2) and adrenocortical cells (Y1BS1). They found that SM in the lipoproteins regulates the SR-BImediated selective uptake of CE, possibly by interacting with the sterol ring or with SR-BI itself.

A key function of HDL SM may be to regenerate HDL during normal lipid metabolism such as after liver SR-B1 extracts CE from spherical HDL and releases some lipid-free apoA1 protein. Addition of SM-enriched phospholipids and cholesterol to apoA1 produces a new lipoprotein species optimized to accept cholesterol from cells, thus restarting HDL growth.[60]

SPHINGOMYELIN AND ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disease characterized by the production of a wide range of chemokines and cytokines. Atherogenesis is initiated by the interaction of cholesterol-rich lipoproteins with the arterial wall.[61] Many processes have been implicated in early atherogenesis, including lipoprotein oxidation,[62,63] lipoprotein retention and aggregation,[64-67] endothelial alteration,[61] monocyte recruitment, macrophage chemotaxis and foam cell formation[61] and smooth muscle cell migration and alteration.[61]

Two sets of evidence indicate that both the amount of SM in the aortic wall and the SM levels in plasma are closely related to the development of atherosclerosis: (1) SM accumulates in atheromas formed in human and animal models.[68-73] LDL extracted from human atherosclerotic lesions is much richer in SM than LDL from plasma.[74-77] A substantial amount of the SM found in arteries and atherosclerotic lesions appears to arise from synthesis in the arterial tissues.[78,79] SM concentration is also significantly increased in macrophages treated with acetyl-LDL plus an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor.[80] (2) Even in atherosclerotic lesions, the rate of SM formation is relatively slow compared with the rate of total choline-containing phospholipid synthesis,[81] suggesting that additional factors contribute to intimal SM accumulation. The ratio of SM to PC is increased by 5-fold in VLDL from hypercholesterolemic rabbits.[82] ApoE knockout (KO) mice are a well known atherogenic model. Plasma SM levels in these mice are 4-fold higher than in wild type mice[51] and this may contribute to the increased atherosclerosis.[83,84] We also found that human plasma SM levels and SM/PC ratio are independent risk factors for coronary heart disease.[6,85] Moreover, SM-rich (1%) diet significantly increases plasma SM levels, LDL aggregation and atherosclerotic lesions in LDL receptor KO mice.[86] All these data suggest that plasma SM plays a critical role in the development of atherosclerosis. SM on LDL retained in atherosclerotic lesions is hydrolyzed by an arterial wall sphingomyelinase, which promotes aggregation by converting SM to ceramide[51,76] (Fig. 3).

There are two ways of preventing this atherogenic event, the first being to reduce sphingomyelinase levels. Indeed, it has recently been reported that apoE KO mice lacking sphingomyelinase have decreased development of early atherosclerotic lesions and, more important, decreased retention of atherogenic lipoproteins compared with apoE KO matched for similar lipoprotein levels.[87] The second way of preventing atherogenicity is by reducing SM levels in the atherogenic lipoproteins through inhibition of the SM biosynthesis pathway in the lipoprotein-producing tissues such as the liver and small intestine.

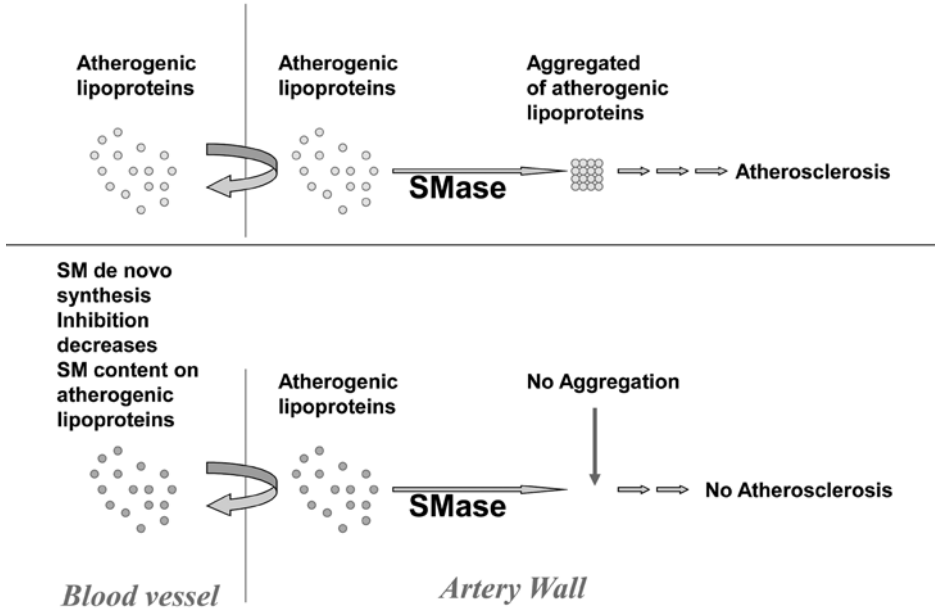

Figure 3. Role of sphingomyelin in aggregation of atherogenic lipoprotein. Generation of ceramide by sphingomyelinase aggregates intimal atherogenic lipoproteins and leads to early atherogenesis. Inhibition of SM de novo synthesis decreases sphingomyelinase substrate SM, thus decreasing aggregation of intimal lipoproteins.

SM and Cholesterol Metabolism

Given that all pathways of cholesterol efflux require cholesterol transport to the plasma membranes, the identification of molecules mediating or regulating this transport process is an important goal. Leventhal et al,[88] reported that lysosomal sphingomyelinase is involved in cholesterol transport from lysosomes to the plasma membranes. Sphingomyelinase hydrolyzes SM in late endosomes and lysosomes.[89] Because SM avidly binds cholesterol,[90,91] SM hydrolysis by sphingomyelinase enables cholesterol transport through preventing cholesterol sequestration by SM. Interestingly, humans with sphingomyelinase deficiency (Types A and B Niemann-Pick disease) have low plasma HDL cholesterol levels,[92] which could result from defective cholesterol efflux.[93]

In macrophages, ABCA1 exports cholesterol and phospholipid to lipid-free apolipoproteins, while ABCG1 and SR-BI export cholesterol to phospholipid-containing acceptors.[94] ABCA1-dependent cholesterol export involves an initial interaction of apoA-I with lipid raft membrane domains.[95,96] ABCA1 expression causes a change in overall lipid packing of the plasma membrane, probably through its ATPase-related functions. Such reorganization by ABCA1 effectively expands the nonraft membrane fractions and consequently preconditions cells for cholesterol efflux.[97] ABCG1 exports cholesterol to

HDL and other phospholipid-containing acceptors. These include particles generated during lipidation of apoA-I by ABCA1, suggesting that the two transporters cooperate in cholesterol export.[98] ABCG1 is mainly found intracellularly in the basal state, with little cell surface presentation. But on stimulation, for example by liver X receptor (LXR) agonist treatment, ABCG1 redistributes to the plasma membranes and increases cholesterol mass efflux to HDL.[99] SR-BI facilitates cholesterol efflux from macrophages.[100] Inactivation of macrophage SR-BI promotes atherosclerotic lesion development in apoE KO mice.[101] In the liver, ABCA1 makes a major contribution to the HDL in the circulation. Liver-specific ABCA1 KO mice decrease plasma HDL by about 80% compared with controls.[102,103] SR-BI is a multifunctional receptor capable of binding a wide array of native and modified lipoproteins. SRBI is abundantly expressed in liver and steroidogenic tissues, where it mediates the selective uptake of cholesteryl esters from HDL.[104,105] A definitive role for SR-BI in HDL metabolism and reverse cholesterol transport in vivo has been demonstrated using different transgenic and knockout mouse models. ABCG1 is also expressed in the liver,[106] but its function in the liver is still not well characterized.

The apical membranes of intestinal cells are enriched in SM and cholesterol,[107] indicative of the presence of lipid rafts.[108,109] Lipid rafts on the enterocyte apical membrane play an important role in cholesterol absorption and trafficking.[110,111]

ABCA1, ABCG1, and SR-BI are located in the plasma membrane rafts (SR-BI),[112,113] or associated with membrane lipid rafts (ABCA1 and ABCG1).[94-97,114] It is therefore conceivable that fundamental changes in SM levels of the plasma membranes influence the functions of these proteins and alter cholesterol homeostasis. In peripheral tissues, SM regulation of reverse cholesterol transport proteins could affect atherosclerosis. Enhanced apoAI-dependent cholesterol efflux by ABCA1 from SM-deficient Chinese hamster ovary (CHO) cells has been reported.[115]

Serine Palmitoyltransferase (SPT) and Atherosclerosis

Plasma SM and other sphingolipid intermediates play important roles in the development of coronary artery disease. It is conceivable that regulation of SM biosynthesis can alter SM levels in the plasma and on the membrane, thus influencing the process of atherosclerosis. How is SM synthesis regulated in vivo and which steps are critical?

SPT Inhibitors

Myriocin, sphingofungins and lipoxamycin are potent and highly selective naturally occurring inhibitors of SPT, inhibiting fungal and mammalian SPT in cell-free preparations with IC_{50} values in the nanomolar range[116-118] (Fig. 4). Structurally they resemble the transient intermediate postulated to form in the condensation of L-serine and palmitoyl CoA. Consistent with this, the inhibitory activity of sphingofungin B is highly dependent on its stereochemistry.[119] Myriocin-linked resins bind the Sptlc1/Sptlc2 complex tightly.[120] All the inhibitors significantly inhibit SM accumulation in both cultured cells and in vivo.[116-118]

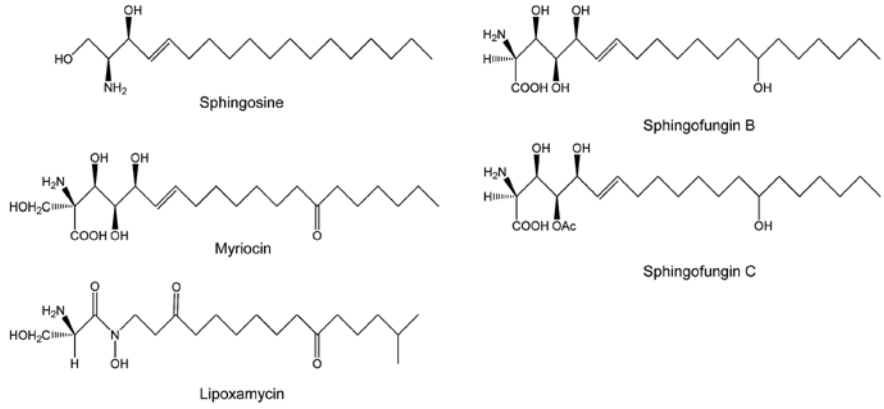

Figure 4. Structures of sphingosine and inhibitors of serine palmitoyltransferase.

The Effect of SPT Inhibition in Mouse Atherosclerosis

Park et al[7] and Hojjatti et al[8] have reported that myriocin treatment (oral administration and intraperitoneal injection, respectively) decreases plasma SM levels and atherosclerosis in apoE KO mice. Myriocin treatment also can induce lower lipid levels in apoE KO mice.[121] However, both administration methods led to reduced atherosclerosis, whereas only oral administration of myriocin lowered plasma cholesterol levels.[7] Oral administration may reduce cholesterol absorption in small intestine. When wild type and apoE KO animals were treated with myriocin, the mice absorbed significantly less cholesterol than controls with no observable pathological changes in the small intestine. Myriocin treatment also increased insulin sensitivity.[11] Thus, myriocin has direct anti-atherosclerosis vascular effects and also has the potential to function as a plasma lipid-lowering agent.

Homozygous Sptlc1 and Sptlc2 KO mice are embryonic lethal, whereas heterozygous versions of both animals, Sptlc1$^{+/-}$ and Sptlc2$^{+/-}$, are healthy. Compared with wild type mice, Sptlc1$^{+/-}$ and Sptlc2$^{+/-}$ mice had: (1) decreased liver Sptlc1 and Sptlc2 mRNA by 44% and 57%, respectively and (2) decreased liver SPT activity by 45% and 60%, respectively. However, these animals had no change in plasma SM, triglyceride, total cholesterol, phospholipids and liver SM levels.[123]

Heterozygous Sptlc1 (a subunit of SPT) knockout mice also absorbed significantly less cholesterol than controls. To understand the mechanism, the protein levels of Niemann-Pick C1-like 1 (NPC1L1), ABCG5 and ABCA1, three key factors involved in intestinal cholesterol absorption were measured. NPC1L1 and ABCA1 were decreased, whereas ABCG5 was increased in the SPT deficient small intestine. SM levels on the apical membrane were also measured and they were significantly decreased in SPT deficient mice, compared with controls.[122] These results suggested that SPT deficiency might reduce intestinal cholesterol absorption by altering NPC1L1 and ABCG5 protein levels in the apical membranes of enterocytes through lowering apical membrane SM levels. Thus, manipulation of SPT activity could provide a novel alternative treatment for dyslipidemia.

Regulation of SPT

A. Transcriptional and posttranscriptional regulation: Although the mechanism underlying the regulation of SPT is largely unknown, studies described below have begun to provide insight into transcriptional and posttranscriptional regulation. Sptlc1 and Sptlc2 mRNA and SPT enzyme activity levels increase in response to several types of inflammatory and stress stimuli. Intraperitoneal administration of endotoxin to Syrian hamsters stimulates SPT activity 2 to 3-fold in the liver, spleen and kidney, with a concomitant increase in the levels of Sptlc2 mRNA and SM.[124,125] Similar changes are observed upon administration of interleukin 1β (IL-1), an inflammatory cytokine.[124] Irradiation of epidermal cells with ultraviolet light also affects Sptlc1 and Sptlc2 mRNA levels.[126] The expression of Sptlc1 mRNA is also up-regulated in the pancreatic islets of leptin receptor-deficient obese *fa*/*fa* rats. This is suggested to be a response to an increase in intracellular fatty acid.[127]
B. Activity regulation: Consistent with the ubiquitous presence of SM in mammalian cells, SPT enzyme activity has been detected in many tissues and cell preparations.[128] The levels of SPT activity varies with development. For example, in rat lung, SPT activity increases progressively from the fetal to neonatal period and reaches a plateau at the adult stage.[129] The levels of SPT activity in several animal tissues are affected by diet.[130,131] A major mode of regulation of SPT activity occurs through substrate supply. It has been reported that the greatest activity was obtained with palmitoyl-CoA supply.[132] Pentadecanoyl-and heptadecanoyl-CoAs are also effective. In mammalian cells, palmitoyl CoA is one of the most abundant acyl-CoA types. Therefore, palmitoyl CoA is the predominant acyl-CoA substrate of SPT in vivo.[133]

Sphingomyelin Synthase (SMS) and Atherosclerosis

SMS is the last enzyme for SM biosynthesis. Its activity directly influences SM, PC and ceramide, as well as diacylglycerol (DAG) levels.[12] Manipulating SMS activity also can regulate plasma and membrane SM levels.

The Effect of SMS2 Overexpression and Deficiency on Mouse Lipoprotein Metabalism

To evaluate the in vivo role of SMS2 in SM metabolism, SMS2 KO and SMS2 liver-specific transgenic (LTg) mice were created and their plasma SM and lipoprotein metabolism were characterized.[134] On a chow diet, SMS2 KO mice had reduced plasma SM levels, but no significant changes in total cholesterol, total phospholipids, or triglyceride, compared with wild type littermates. On a high-fat diet, SMS2 KO mice showed a significant decrease in plasma SM levels, whereas SMS2LTg mice showed a significant increase in those levels, but no significant changes in other lipids. Atherogenic lipoproteins from SMS2LTg mice displayed a significantly stronger tendency toward aggregation after mammalian sphingomyelinase treatment, compared with controls. Moreover, SMS2 deficiency significantly increased plasma apoE levels

(2.0-fold), whereas liver-specific SMS2 overexpression significantly decreased those levels (1.8-fold). Finally, SMS2 KO mouse plasma promoted cholesterol efflux from macrophages, whereas SMS2LTg mouse plasma prevented it.[134] These results indicated that SMS2 is one of the determinants for plasma and liver SM levels in mice.

SMS and Apoptosis

It is believed that disordered apoptosis may occur in atherogenesis, leading to death of lipid-rich foam cells, promoting lipid core formation.[135] In two previous papers, investigators reported controversial results regarding the relationship between SMS activity and cell apoptosis. Van der Luit et al[33] reported that SMS1 gene knockdown alters the plasma membrane raft structure and reduces the internalization of alkyl-lysophospholipid, thereby reducing cell apoptosis rates. Separovic et al[136] reported that SMS1 overexpression suppresses cell apoptosis mediated by photo damage; however, they did not show the effect of SMS1 overexpression on membrane lipid rafts. Based on previous results,[137] it is believed that SMS1 and SMS2 overexpression increases SM levels in the lipid rafts on plasma membranes and promotes a more external appearance for TNFα receptor 1 (a well-known receptor in lipid rafts)[138] following TNFα stimulation, thereby promoting CHO cell apoptosis. SMS1 and SMS2 knockdown by siRNA reduces SM levels in lipid rafts on the plasma membrane and reduces the number of TLR4 (which are also well known for their presence in lipid rafts)[139] that are presented on the plasma membrane after LPS stimulation, thereby reducing macrophage apoptosis. Indeed, after LPS stimulation, SMS1/SMS2 knockdown macrophages contained significantly less TLR4 on the cell surface than control macrophages.[137]

Manipulation of SMS activity alters cellular DAG levels and, thus, may also contribute to apoptosis. Cerbon et al demonstrated that pharmacological inhibition of SMS reduces cellular DAG levels and PKC activity.[140] SMS1 or SMS2 overexpression significantly increases DAG levels in CHO cells, while SMS1 or SMS2 gene knockdown significantly reduces DAG levels in THP-1-derived macrophages.[137] DAG can regulate both conventional and novel PKCs,[141] a family of serine/threonine kinases that regulate a diverse set of cellular processes, including proapoptotic and pro-survival processes. PKCδ is generally considered a growth inhibitory or proapoptotic PKC,[142,143] while PKCε is considered a pro-survival factor.[142,144] It is possible that in both CHO cells and THP-1–derived macrophages, regulation of SMS1 or SMS2 activity by either the overexpression of their genes or gene knockdown could modulate DAG-mediated PKC activity, thereby influencing cell apoptosis.[137]

Manipulation of SMS activity also alters cellular ceramide levels and this may also contribute to apoptosis. Overexpression of both SMS1 and SMS2 is accompanied by increased levels of ceramide, as well as SM.[137] Separovic et al reported the same phenomenon when they overexpressed SMS1 in Jurkat cells.[136] This may be due the complexity of this enzyme which can catalyze bidirectional reaction.[28] The ceramide:SM ratio increases in cells that overexpress SMS compared with controls.[137] This may represent another mechanism for the increased apoptotic potential of these cells, given that ceramide is a bioactive lipid that is well known for promoting cell apoptosis.[145,146] However, ceramide levels did not change in THPderived macrophages after they were exposed to SMS siRNA.[137] This indicates that the ceramide level might not be important in SMS-knockdown–induced macrophage apoptosis.

SMS and NFκB and MAP Kinase Activation

The accumulation of macrophage-derived foam cells in the vessel wall is always accompanied by the production of a wide range of chemokines, cytokines and growth factors.[147] These factors regulate the turnover and differentiation of immigrating and resident cells, eventually influencing plaque development. One of the key regulators of inflammation is NFκB,[148] which has long been regarded as an atherogenic factor, mainly because of its regulation of many of the inflammatory genes linked to atherosclerosis.[149,150]

Depletion of cholesterol from rafts causes a redistribution of TNFα receptor 1 to nonraft plasma membrane, preventing NFκB activation[151] or ligand-induced RhoA activation[152] and such treatment also inhibits inflammatory signals mediated by TLRs.[139] Studies also suggest that NFκB activation is triggered by SM-derived ceramide.[153] On the contrary, it has been also shown that ceramide is not necessary or even inhibits NFκB activation.[154] Luberto et al[155] found that D609, a nonspecific SMS inhibitor, blocks TNFα-and phorbol ester-mediated NFκB activation that was concomitant with decreased levels of SM and DAG. This did not affect the generation of ceramide, suggesting SM and DAG derived from SM synthesis are involved in NFκB activation. Hailemariam et al found that NFκB activation and its target gene expression are attenuated in macrophages from SMS2 KO mice in response to LPS stimulation and in SMS2 siRNA-treated HEK 293 cells after TNFα simulation.[156] In line with attenuated NFκB activation, it was found that SMS2 deficiency substantially diminished the abundance of toll like receptor 4 (TLR4)-MD2 complex levels on the surface of macrophages after LPS stimulation and SMS2 siRNA treatment reduced TNFα-stimulated lipid raft recruitment of TNFα receptor-1 in HEK293 cells.[156] Thus, SMS2 is a modulator of NFκB activation and could play an important role in NFκB-mediated atherogenic process (Fig. 5).

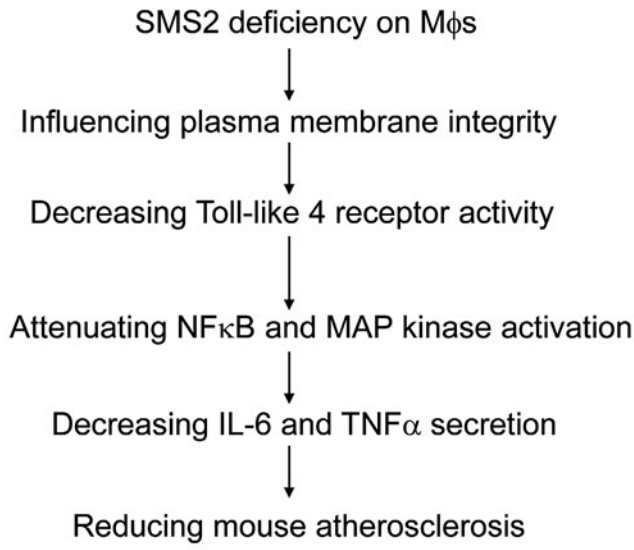

Figure 5. Mechanism of reduced atherosclerosis by deficiency of sphingomyelin synthase 2.

SMS2 deficiency may also influence signal transduction pathways other than NFκB activation. The activation of MAP kinases was attenuated in SMS2 KO macrophages.[156]

Macrophage SMS2 Deficiency and Atherosclerosis

In order to evaluate the relationship between macrophage SMS2 deficiency and atherosclerosis, we transplanted SMS2 KO mouse bone marrow into LDL receptor KO (*Ldlr*$^{-/-}$) mice (SMS2$^{-/-}$ macrophages *Ldlr*$^{-/-}$), creating a mouse model of SMS2 deficiency in the macrophages. After 3 months on a Western diet, SMS2 deficiency decreased atherosclerotic lesions in the aortic arch, root (57%, $P < 0.001$) and the entire aorta (42%, $P < 0.01$), compared with wild type macrophages transplanted into *Ldlr*$^{-/-}$ mice. Analysis of plaque morphology revealed that SMS2 macrophage deficiency produced less necrotic core area (71%, $P < 0.001$) and more collagen content (35%, $P < 0.05$) in atherosclerotic lesions and less free cholesterol and cholesteryl ester levels in the brachiocephalic artery. Therefore, SMS2 deficiency in the macrophages reduces atherosclerosis in mice. Macrophage SMS2 is thus a potential therapeutic target for treatment of this disease[9] (Fig. 5).

CERAMIDE IN LIPOTOXIC CARDIOMYOPATHY

Cardiomyopathy, an outcome of many chronic cardiovascular diseases that is often found in patients with diabetes, is sometimes associated with increased heart content of lipids.

Diabetic cardiomyopathy accounts for increased morbidity and mortality after myocardial infarction in diabetic compared with nondiabetic patients.[157]

The heart utilizes FFA as an important major fuel source. Over 70% of the energy needs for cardiac function comes from oxidation of FA, with the balance provided by carbohydrates and lactate.[158,159] FFAs derived from the hydrolysis of TG stored in adipose tissue bind to circulating albumin and are delivered to heart. Alternatively, TG-rich lipoproteins are internalized by receptor-mediated processes and are hydrolyzed to liberate FFA inside the cells.[160] When cardiac FA uptake is elevated due to fasting, diabetes or obesity,[161,162] excess fatty acyl CoAs and unesterified FA not used for oxidation can be stored in TG. In animal models of obesity and diabetes, TG accumulation in heart is associated with impaired contractile function; this suggests that excess TG or its metabolites are toxic.[3,163] These potentially toxic metabolites include ceramide and DAG. Ceramide is also increased in hearts of obese Zucker with impaired myocardial contractility.[3] Cardiac overexpression of PPARα and long chain acyl-CoA synthetase1 traps greater levels of FFAs in the hearts and the synthesis of ceramide are activated due to increased substrate availability for ceramide synthesis.[164]

Mice with cardiac overexpression of glycosylphosphatidylinositol membrane-anchored LpL mice (LpL_{GPI}) also have increased increased cardiac ceramide and apoptosis markers including cytosolic cytochrome c, caspase 3 expression and activity.[165] Park et al demonstrated that inhibition of ceramide biosynthesis by myriocin or heterozygous deletion of Sptlc1 leads to decreased expression of some apoptotic genes and improved cardiac contraction in LpL_{GPI}.[10] In this study, blockage of ceramide biosynthesis appears to modulate mitochondrial substrate oxidation (Fig. 6). LpL_{GPI} hearts have increased uptake of FFA and rely on FA oxidation for cardiac energy production. A potential

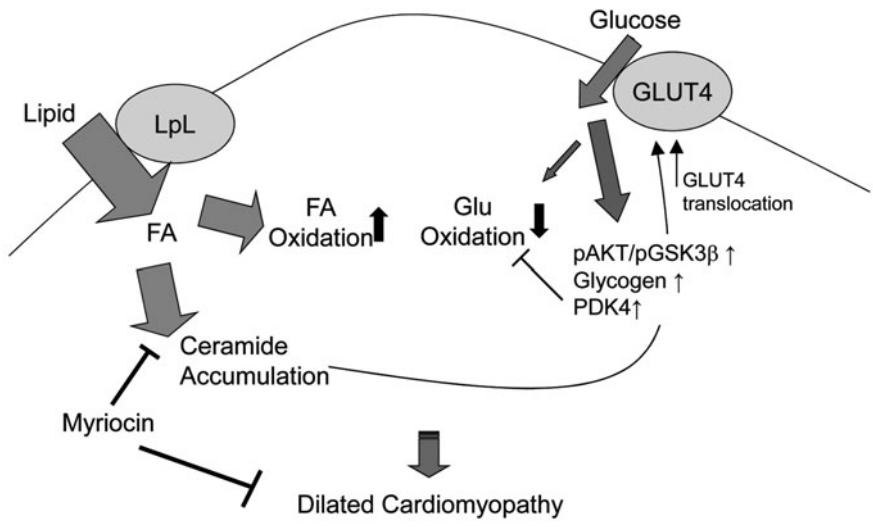

Figure 6. Role of ceramide in modulation of cardiac energetics. Increased uptake of lipids into the hearts alters the balance of glucose and fatty acid metabolism and causes dilated cardiomyopathy. Inhibition of SPT ameliorates cardiac failure by restoring cardiac function by balancing the substrate oxidation of glucose and fatty acids.

mechanism is that pharmacological and genetic inhibition of SPT upregulated pyruvate dehydrogenase kinase-4 and decreased the rate of glucose oxidation.

However, glucose uptake is increased in LpL_{GPI} hearts. This paradoxical fate of glucose is explained by accumulation of glucose in a form of glycogen with increased phosphorylated glycogen synthase 3β.[10] In isolated perfused LpL_{GPI} hearts, myriocin restored cardiac efficiency, enhancing myocardial energetics by maintaining cardiac performance at a lower oxygen cost. Even with improved cardiac function and balanced substrate utilization by myriocin treatment, a longterm treatment of LpL_{GPI} mice with myriocin only partially rescued the survival rate. A potential reason is due to involvement of other lipid metabolites in cardiac dysfunction. Altered PKC signalling pathway by DAG and toxicity of FFA are the probable candidates for cardiac failure. More studies should be followed to distinguish the role of ceramide from other lipid metabolites.

CONCLUSION

Coronary heart disease and heart failure are major causes of mortality in developed countries.

Although presently known risk factors have some predictive value for the disease, a major part of the variability in this process remains unexplained. In addition, therapy aimed at lowering LDL cholesterol reduces only a small fraction (roughly 30%) of the burden of atherosclerotic disease. It is extremely important to find new approaches for better understanding of the disease and for treating it. Exploration of the SM biosynthesis pathway is one such approach. SM is implicated as a biochemical modulator of lipoprotein metabolism and atherosclerosis and ceramide is suggested as a metabolic switch

determining substrate preference for cardiac energetics. One highly important aspect of the reported work is the discovery that inhibiting sphingolipid biosynthesis may have great therapeutic value for the treatment of atherosclerosis and cardiac failure. To confirm this, a prospective clinical study in human populations should be followed to evaluate whether plasma SM is a real risk factor for atherosclerosis. In addition, sphingolipid biosynthesis is tightly linked to development of insulin resistance, obesity and atherosclerosis. The mechanisms of sphingolipid biosynthesis involved in these chronic diseases need to be further elucidated in detail. Thus, sphingolipid modulation in atherosclerosis and ardiomyopathy will provide a therapeutic rationale to cure the patients inflicting with these chronic diseases.

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CHAPTER 3

HEART SPHINGOLIPIDS IN HEALTH AND DISEASE

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Abstract: In recent years, the role of sphingolipids in physiology and pathophysiology of the heart attracted much attention. Ceramide was found to be involved in the pathogenesis of cardiac dysfunction in animal models of ischemia/reperfusion injury, Type 2 diabetes and lipotoxic cardiomyopathy. On the other hand, another member of this lipid family, namely sphingosine-1-phosphate, has been shown to possess potent cardioprotective properties. This chapter provides a review of the role of ceramide and other bioactive sphingolipids in physiology and pathophysiology of the heart. We describe the role of PPARs and exercise in regulation of myocardial sphingolipid metabolism. We also summarize the present state of knowledge on the involvement of ceramide and sphingosine-1-phosphate in the development and prevention of ischemia/reperfusion injury of the heart. In the last section of this chapter we discuss the evidence for a role of ceramide in myocardial lipotoxicity.

INTRODUCTION

Sphingolipids were discovered over 120 years ago and for many decades were considered to serve only as structural components of biological membranes. Nowadays, many of them are known to be highly bioactive compounds that play a significant role in signal transduction and regulation of a host of cellular processes. Ceramide, the central molecule in sphingolipid structure and metabolism, is the best studied member of this family. It was first recognized as a second messenger in 1990 by Okazaki et al[1] who showed that ceramide mediates the effect of 1α,25-dihydroxyvitamin D_3 on HL-60 cell differentiation. Ceramide is also a precursor for other bioactive sphingolipids including

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

ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate (S1P) (Fig. 1). Intensive studies conducted over the next two decades revealed that sphingolipids regulate numerous cellular processes such as cell proliferation, differentiation and apoptosis as well as responses to cytokines and stress.[2] Ceramide has also emerged as a putative mediator of muscle insulin resistance and lipotoxicity in certain cell types, including cardiomyocytes.[3] In addition, the role of sphingolipids in the pathogenesis of cardiac dysfunction in ischemia/reperfusion injury, Type 2 diabetes and obesity has recently attracted much attention.

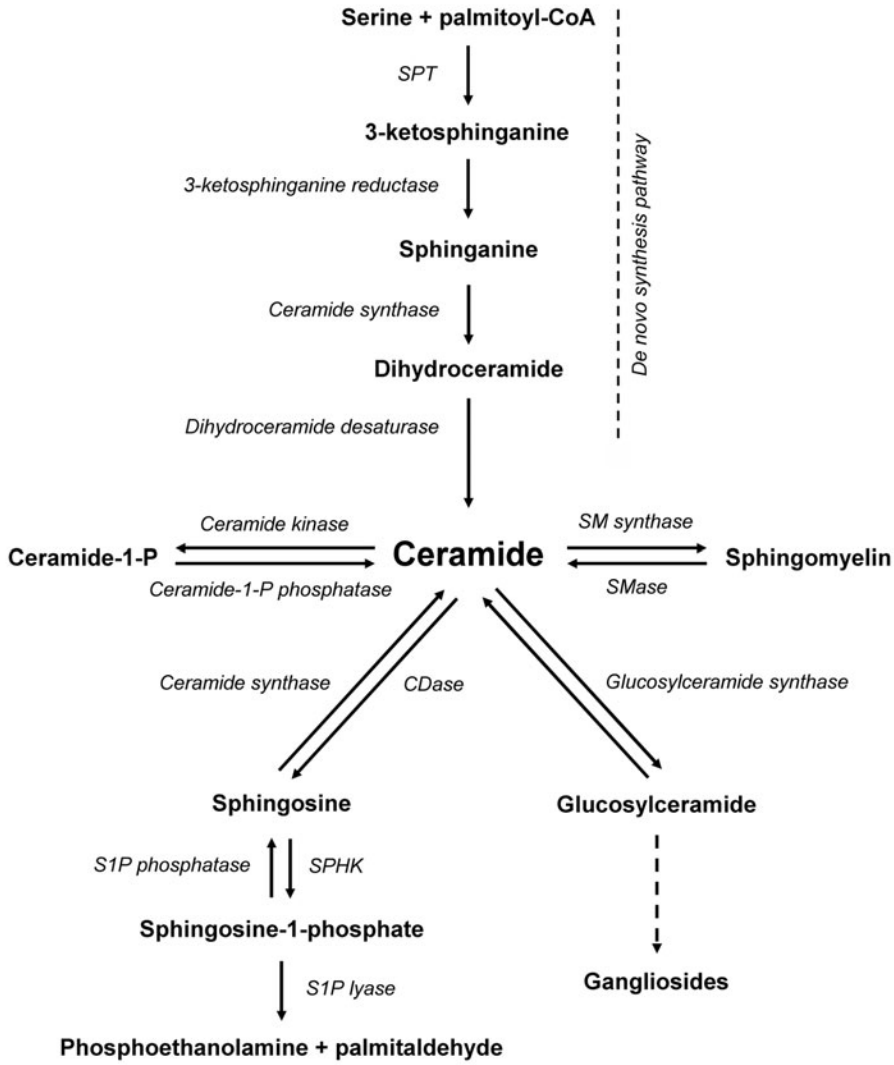

Figure 1. Schematic representation of ceramide metabolism. SPT-serine palmitoyltransferase, SM-sphingomyelin, SMase-sphingomyelinase, CDase-ceramidase, SPHK-sphingosine kinase, S1P-sphingosine-1-phosphate.

CHARACTERIZATION OF MYOCARDIAL SPHINGOLIPID METABOLISM

Both cardiac and skeletal muscle expresses different enzymes and receptors involved in sphingolipid metabolism and can, therefore, synthesize and respond to different bioactive sphingolipids. There are, however, considerable differences between myocardial and skeletal muscle sphingolipid metabolism. As shown on Table 1, the rat heart is characterized by approximately 2-fold higher content of sphingosine, S1P and sphingomyelin as compared to the soleus muscle, whereas the level of ceramide is virtually the same. The activity of most of the enzymes involved in sphingolipid metabolism is also much higher in the myocardium. We observed similar differences in the content of sphingoid bases also between human cardiac and skeletal muscle (see Table 1).

In addition, considerable species differences are found in myocardial sphingolipid metabolism. As compared to rat myocardium, human heart is characterized by similar level of ceramide, however, the content of sphingosine, sphinganine and S1P is substantially lower (Table 1). Another difference is relative contribution of individual ceramidase isoforms to total ceramidase activity. We showed that in the rat heart acid ceramidase (a-CDase) activity is much lower compared with activity of alkaline or neutral isoform of the enzyme.[4] However, in the human myocardium expression of a-CDase is markedly higher than alkaline/neutral-CDase, suggesting the dominance of the former isoform.[5] These results are in line with other reports showing high level of a-CDase mRNA in the

Table 1. Sphingolipid content and activity of enzymes related to ceramide metabolism in heart vs skeletal muscle

	Rat		Human	
	Soleus	Heart	Vastus Lateralis	Heart
Content of sphingolipids (pmol/mg)				
Sphingosine	1.0	2.2	0.33	1.3
Sphinganine	0.38	0.55	0.06	0.14
Sphingosine-1P	0.25	0.49	0.06	0.14
Ceramide	25	23	16	17
Sphingomyelin	316	645		
Enzyme activity (nmol of product/h/mg of protein)				
SPT	0.59	0.94		
al-CDase	1.5	6.0		
n-CDase	1.2	8.0		
a-CDase	not detected	2.7		
n-SMase	16.5	6.6		
a-SMase	23	125		

SPT-serine palmitoyltransferase, al-CDase-alkaline ceramidase, n-CDase-neutral ceramidase, a-CDase-acid ceramidase, n-SMase-neutral sphingomyelinase, a-SMase-acid sphingomyelinase. Created from data in M. Baranowski et al 2007 J Physiol Pharmacol, M. Baranowski et al 2010 J Lipid Res, Błachnio-Zabielska et al 2008 J Cell Biochem and from unpublished results of M. Baranowski.

human but not murine heart.[6,7] We found similar species differences also for expression of sphingosine kinase isoforms.[5] Our data indicate that sphingosine kinase (SPHK) 1 is the dominant subtype in the human myocardium, whereas the rodent heart expresses predominantly SPHK2.[8]

Moreover, we have recently shown significant regional differences in sphingolipid metabolism in the human heart.[5] In patients undergoing coronary bypass graft surgery, papillary muscle of the mitral apparatus was characterized by lower level of ceramide, sphinganine and S1P as compared to the right atrial appendage. On the other hand, expression of enzymes involved in both ceramide synthesis (serine palmitoyltransferase—SPT) and degradation (ceramidases, sphingosine kinases) was markedly higher in the papillary muscle which suggests higher ceramide turnover in the ventricular tissue. However, physiological and pathophysiological consequences of species as well as regional differences in myocardial sphingolipid metabolism remain obscure.

PPARs AS REGULATORS OF SPHINGOLIPID METABOLISM IN THE HEART

Although the role of sphingolipids in the heart has recently attracted much attention, regulation of myocardial sphingolipid metabolism is only poorly investigated. There is, however, some evidence indicating that peroxisome proliferator-activated receptors (PPARs) play a significant role in this process. PPARs are ligand-activated transcription factors of the nuclear hormone receptor superfamily. Three distinct PPAR isoforms termed α, δ and γ have been described. The former two are highly expressed in the heart and are considered to be key transcriptional regulators of fatty acid metabolism in cardiomyocytes.[9] Finck et al[10] demonstrated that high-fat feeding of mice with cardiac-specific overexpression of PPARα leads to accumulation of ceramide in the myocardium. Such effect was not observed in wild type animals which suggests that PPARα may be involved in regulation of myocardial ceramide metabolism. To investigate the mechanism of this phenomenon we examined effects of WY-14643 (a selective PPARα agonist) on sphingolipid metabolism in the rat heart.[11] Activation of PPARα in high-fat fed rats resulted in accumulation of myocardial ceramide and sphingomyelin. This effect was related to stimulation of the de novo sphingolipid synthesis as evidenced by elevated activity of SPT and increased availability of intramyocardial palmitate. Enzymes involved in other pathways i.e., sphingomyelinases and ceramidases were not affected. The mechanism of WY-14643 action on SPT activity is unclear. PPARα stimulation was shown to upregulate expression of SPT in reconstructed human epidermis[12] which suggests involvement of a transcriptional regulation. However, it remains obscure whether PPARα affects SPT expression directly or via changes in cellular lipid metabolism. The latter hypothesis is supported by the fact that in standard chow-fed rats, which did not show accumulation of myocardial free palmitate upon PPARα activation, WY-14643 did not increase either ceramide content or SPT activity.[11] We also observed a modest increase in ceramide content in the heart of rats treated with a selective PPARδ agonist (GW0742) (M. Baranowski, unpublished observation). This increase was accompanied by elevation in the level of sphinganine and long chain fatty acyl-CoA which suggests that the rate of de novo ceramide synthesis pathway was augmented upon PPARδ activation.

Expression of PPARγ in cardiomyocytes is very low and it is the least investigated of all myocardial PPAR isoforms.[9] Several studies demonstrated that thiazolidinediones,

which are selective PPARγ activators, induce expression of glucose transporter 1 and 4 and increase basal and insulin-stimulated glucose uptake in cultured rat cardiomyocytes as well as in the heart of diabetic and insulin-resistant rodents.[13-17] Zhou et al[18] found that administration of troglitazone to Zucker diabetic fatty (ZDF) rats reduced accumulation of myocardial ceramide. This prompted us to examine effect of PPARγ activation on myocardial sphingolipid metabolism. To our surprise, pioglitazone administration increased ceramide level in the heart of rats fed either standard chow or a high-fat diet. However, it should be noted, that this effect was more pronounced in the latter group. A plausible explanation of the discrepancy between our results and those of Zhou et al is that thiazolidinediones lower cardiac ceramide level only in chronically obese and diabetic animals which show excessive lipid deposition in the heart. Accumulation of myocardial ceramide observed in our study was likely a result of its augmented synthesis de novo. This is supported by the fact that administration of PPARγ agonist did not produce evident changes in sphingomyalinase or ceramidase activity, whereas the activity of SPT and the availability of intracellular palmitate was markedly increased in rats fed on either diet. Pioglitazone also induced a modest elevation in the content of SPT protein. However, it did not match the increase in enzyme activity, thus suggesting that the effect of PPARγ agonist was predominantly a result of postranslational modification of SPT protein. It was shown that activity of this enzyme can be modulated independently of changes in the level of its mRNA or protein.[19] However, our data do not exclude a possibility that pioglitazone affects the expression of this enzyme at the transcriptional level. It is widely accepted that increased availability of palmitate induces accumulation of ceramide in cells due to its augmented synthesis de novo.[3] However, in vitro studies on rat astrocytes and pancreatic islets showed that incubation with palmitate also increases activity and expression of SPT.[20,21] These data indicate that palmitate-induced accumulation of ceramide is not solely a result of increased availability of substrate for its synthesis de novo but is also a consequence of activation of the rate-limiting enzyme in this pathway. Therefore, it is likely that the stimulatory effect of pioglitazone on SPT observed in our study was related to accumulation of free palmitate evoked by PPARγ agonist. Collectively, the results of our experiments indicate that PPARs regulate myocardial sphingolipid metabolism predominantly at the level of de novo synthesis (see Table 2). Interestingly, all three PPAR isoforms seem to activate this pathway in the heart, at least in our animal models.

EXERCISE MODULATES MYOCARDIAL CERAMIDE METABOLISM

We found that exercise exerts profound effects on the myocardial content of sphingolipids and affects many pathways of their metabolism.[4] Interestingly, this effect to a large extent depended on exercise duration. We found that 30-minute burst of exercise decreased myocardial content of ceramide. However, its level returned to the baseline after 90 minutes of running and increased further at the point of exhaustion, significantly exceeding the content found in the control animals (Fig. 2). Interestingly, it was recently reported that endurance training consisting of 30-minute exercise bouts reduced ceramide level in the murine heart to a similar extent as observed in our study.[22] Analyses of the activities of enzymes involved in ceramide metabolism revealed that the initial reduction in its content observed in our experiment was likely a result of augmented ceramide degradation, since a concomitant elevation in the activity of acid ceramidase and the level of sphingosine was observed. The subsequent

Table 2. Summary of effects induced by activation of distinct PPAR isoforms on myocardial sphingolipid metabolism

	PPARα		PPARδ		PPARγ	
	Standard Chow	High-Fat Diet	Standard Chow	High-Fat Diet	Standard Chow	High-Fat Diet
Content of sphingolipids						
Sphingosine	↓	↔	↔		↓	↔
Sphinganine	↓	↔	↑		↓	↔
Sphingosine-1P	↔	↔	↑		↔	↔
Ceramide	↔	↑↑	↑		↑	↑↑
Sphingomyelin	↔	↑↑			↔	↑
Enzyme activity						
SPT	↔	↑			↑↑	↑↑
al-CDase	↔	↔			↔	↔
n-CDase	↔	↔			↔	↔
a-CDase	↔	↔			↑	↔
n-SMase	↓↓	↔			↑	↔
a-SMase	↑	↔			↔	↔

PPAR, peroxisome proliferator-activated receptor; SPT, serine palmitoyltransferase; al-CDase, alkaline ceramidase; n-CDase, neutral ceramidase; a-CDase, acid ceramidase; n-SMase, neutral sphingomyelinase; a-SMase, acid sphingomyelinase; ↔, no significant change; ↑, increase; ↓, decrease. Created from data in M. Baranowski et al 2007 J Physiol Pharmacol, M. Baranowski et al 2007 Prostaglandins Other Lipid Mediat and from unpublished results of M. Baranowski.

transition from initial decrease in ceramide content to its accumulation at the point of exhaustion was a consequence of gradual reduction in the activity of acid ceramidase and simultaneous increase in the rate of de novo ceramide synthesis, as evidenced by progressive activation of SPT and accumulation of sphinganine (Fig. 2). This effect could be a result of increased plasma nonesterified fatty acid (NEFA) concentration which occurred during prolonged exercise. As already mentioned in the previous section, in vitro studies showed that incubation of the cells with palmitate increases activity and expression of SPT. Interestingly, in our study the changes in the myocardial activity of SPT reflected those of plasma NEFA concentration.

It should be mentioned that exercise until exhaustion increased not only SPT activity but also content of SPT2 protein (Fig. 2). However, the magnitude of increase in the enzyme activity was over 3-fold higher than that of enzyme protein content. Moreover, SPT2 mRNA level was not affected by treadmill running. Taking together, it indicates that exercise-induced activation of SPT was not a result of its increased expression, but rather a consequence of posttranslational modification of serine palmitoyltransferase protein.

Accumulation of sphingosine is thought to be an important factor contributing to the development of muscle fatigue.[23] We showed previously that exercise until exhaustion induced accumulation of this sphingoid base in rat skeletal muscles.[24] We observed a similar, although much less pronounced, effect also in the heart.[4] There are many reports showing that sphingosine and to a lesser extent also sphinganine, reduce contractility

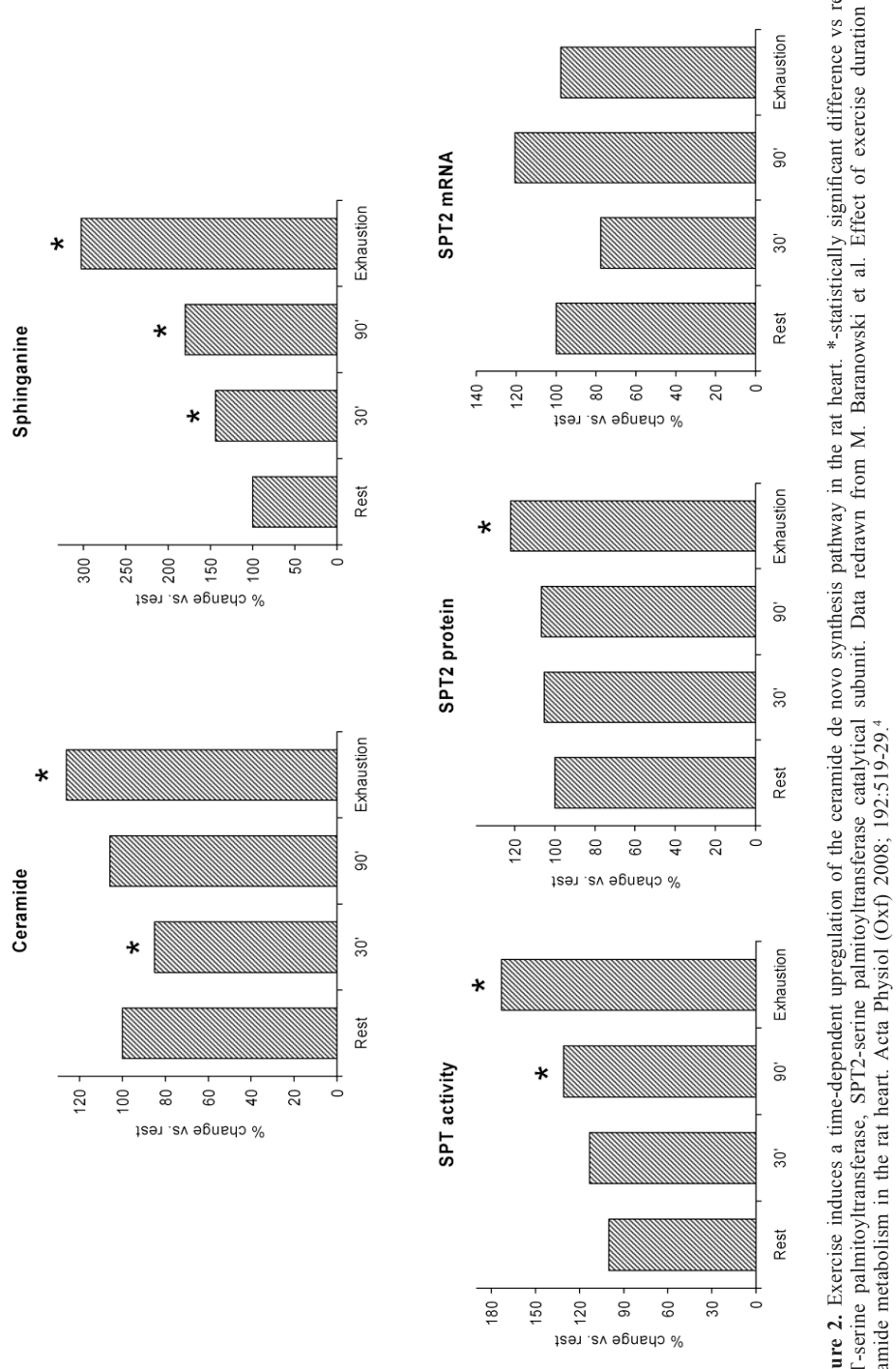

Figure 2. Exercise induces a time-dependent upregulation of the ceramide de novo synthesis pathway in the rat heart. *-statistically significant difference vs rest, SPT-serine palmitoyltransferase, SPT2-serine palmitoyltransferase catalytical subunit. Data redrawn from M. Baranowski et al. Effect of exercise duration on ceramide metabolism in the rat heart. Acta Physiol (Oxf) 2008; 192:519-29.[4]

of cardiomyocytes by blocking calcium release through the sarcoplasmatic reticulum ryanodine receptor.[25,26] It was found that prolonged exercise may induce a reduction in left ventricular systolic function, a phenomenon often called exercise-induced cardiac fatigue.[27] In view of the above data, it is tempting to speculate that accumulation of sphingosine and sphinganine can contribute to the development of fatigue not only in skeletal muscle but also in the heart. However, further studies are required to determine the physiological relevance of this effect.

SPHINGOLIPIDS IN ISCHEMIA/REPERFUSION INJURY OF THE HEART

Krown et al[28] were the first to demonstrate that cell permeable C_2-ceramide induces apoptotic cell death in cardiomyocytes. Shortly after this discovery Bielawska et al[29] using the rat coronary artery occlusion model found that ischemic myocardium was characterized by increased ceramide level that was further elevated upon subsequent reperfusion. This study provided the first evidence that ceramide may be involved in induction of cardiomyocyte apoptosis by ischemia/reperfusion injury. This observation was later confirmed by our and other groups in perfused rat heart and in the rabbit myocardium in vivo.[30-33] However, the mechanism underlying ischemia/reperfusion-induced ceramide accumulation remains unclear. There is some data indicating that increased ceramide production from sphingomyelin is responsible for this effect. Argaud et al[32] found that pretreatment with sphingomyelinase inhibitor—D609 completely prevented ischemia-induced accumulation of myocardial ceramide and reduced cardiomyocyte apoptosis in rabbits. Moreover, depletion of sphingomyelin pool was reported in ischemic/reperfused rat heart.[31] In line with the above observations, hypoxia/reoxygenation was found to rapidly activate neutral (but not acid) sphingomyelinase in neonatal rat cardiomyocytes.[34] Interestingly, pretreatment of the cells with antioxidant prevented both sphingomyelinase activation and ceramide accumulation indicating that increased oxidative stress plays a key role in the effect of reoxygenation on ceramide metabolism in cardiomyocytes. In contrast to the above data, Zhang et al[30] reported inhibition of both neutral and acid sphingomyelinase in the ischemic/reperfused rat heart. Accumulation of ceramide observed in their study was attributed to decreased activity of ceramidase.

We found that ischemic preconditioning (IPC) may afford myocardial protection, at least in part, via modulation of ceramide metabolism.[33] In our experiment, IPC resulted in marked reduction in the ischemia/reperfusion-induced accumulation of ceramide in the perfused rat heart. These results were conformed by Argaud et al[32] who showed that IPC prevented elevation in ceramide content in ischemic rabbit myocardium.

S1P has recently attracted much attention as an important factor protecting the heart against ischemia-reperfusion injury. S1P is not only an intracellular messenger, in fact most of its effects are exerted by binding to a family of plasma membrane G protein-coupled receptors (S1PRs). S1P is normally found in high nanomolar concentrations in human and rodent plasma.[35] Interestingly, this mediator acts also in an autocrine and/or paracrine fashion, as endogenous S1P can be transported to the extracellular space, most likely with the help of cassette transporters.[36] Five subtypes of the S1P receptor have been identified ($S1PR_{1\text{-}5}$). Cardiomyocytes express $S1PR_1$, $S1PR_2$ and $S1PR_3$, however, the first subtype was found to be predominant in these cells.[37]

Karliner et al[38] provided the first evidence for cardioprotective effect of S1P. They showed that preincubation of rat neonatal cardiomyocytes with S1P or the ganglioside GM-1, which stimulates endogenous S1P production via activation of SPHK, prevents hypoxia-induced cell death. Cardioprotective action of extracellular S1P and GM-1 was subsequently confirmed in rodent models of ischemia/reperfusion injury.[39,40] The early study by Jin et al[40] suggested that exogenous and endogenous S1P exerts its effects through distinct pathways. They found that cardioprotection afforded by intracellular S1P (generated in response to GM-1) required PKCε, whereas the action of exogenous S1P did not. However, it was recently reported that pertussis toxin (preventing G proteins from interacting with G protein-coupled receptors) as well as the $S1PR_{1/3}$ antagonist abolished GM-1 mediated cardioprotection.[41] These data indicate that intracellulary produced S1P is exported from cardiomyocytes and exerts its protective action via cell surface S1PRs. Therefore, PKCε seems to mediate GM-1 induced SPHK activation rather than action of intracellular S1P. Interestingly, in the perfused rat heart ischemia markedly inhibited SPHK activity and reduced myocardial S1P content, and this effect was maintained over the period of subsequent reperfusion.[42] Consistently, deletion of the SPHK1 gene increased susceptibility of the heart to ischemia/reperfusion injury[43], whereas adenovirus-mediated SPHK1 gene transfer was found to induce protective effect and attenuate postischemic heart failure.[44] It was also suggested that cardioprotective properties of high-density lipoprotein (HDL) involve S1P, as most of plasma S1P is contained within HDL.[45] Interestingly, sphingosine infused at physiological concentrations (high concentrations are cardiotoxic) also exerts protective effect in the perfused rat heart. However, the mechanism of its action is independent of S1PRs and involves cyclic nucleotide-dependent pathways.[46]

There is a controversy as to which S1PR subtype mediates the cardioprotective action of S1P. Experiments made by Karliner's group suggested predominant role of $S1PR_1$. They found that antibody which functions as a $S1PR_1$-specific agonist as well as a synthetic $S1PR_1$ activator protected adult mouse cardiomyocytes from hypoxia to the same extent as exogenous S1P.[47] In addition, the $S1PR_1$ antagonist VPC23019 blocked protection afforded by S1P or its synthetic analog FTY720. The effect of S1P was mediated by a phosphatidylinositol 3-kinase and likely involved activation of Akt/PKB and inhibition of glycogen synthase kinase-3β. However, reports by other groups indicate that $S1PR_2$ and/or $S1PR_3$ rather than $S1PR_1$ mediate the cardioprotective action of S1P. Means et al[48] found that $S1PR_{2/3}$ double knockout mice are characterized by markedly increased myocardial infarct size following ischemia/reperfusion that is accompanied by impaired activation of Akt/PKB. Interestingly, neither $S1PR_2$ nor $S1PR_3$ deletion alone augmented ischemia/reperfusion injury demonstrating some redundancy in S1P receptor function. In addition, Theilmeier et al[49] reported that S1P-mediated cardioprotection was completely absent in $S1PR_3$-deficient mice. It was also abolished by pharmacological nitric oxide synthase inhibition, implicating a crucial role of nitric oxide in this pathway. Recently, Levkau's group demonstrated that mice with cardiac-specific $S1PR_1$ deficiency are susceptible to ischemia/reperfusion injury to the same extent as wild type animals which strongly argues against the involvement of this receptor in S1P-induced cardioprotection.[37] The discrepancy between the results by Karliner's group and those by other investigators may result from differences in experimental models, since protective role of $S1PR_1$ was demonstrated on isolated cardiomyocytes, whereas $S1PR_{2/3}$-mediated cardioprotection was reported in the intact heart. This notion is supported by a recent study by Hofmann

et al[50] who demonstrated that selective $S1PR_1$ agonist exerts protective effect in neonatal rat cardiomyocytes but not in perfused rat heart. In fact, one report indicated that $S1PR_1$ stimulation exaggerates myocardial ischemia/reperfusion injury.[51]

S1P was also found to mediate IPC in the heart. SPHK1 is activated and translocated to the plasma membrane in response to IPC[52] which to a large extent prevents decrease in the enzyme activity and myocardial S1P level upon ischemia/reperfusion.[42] Consistently, pharmacological or genetic inhibition of SPHK1 abolished IPC-induced cardioprotection in the murine heart.[43,52] A recent report by Vessey et al[53] suggests that the mechanism of IPC involves export of produced S1P from cardiomyocytes to the extracellular space and stimulation of $S1PR_1$ and/or $S1PR_3$. Interestingly, it was found that ischemic postconditioning, similarly to IPC, protected isolated mouse hearts against ischemia/reperfusion injury via SPHK1 activation.[54]

We recently found that plasma concentration of S1P in patients with acute myocardial infarction upon admission to intensive heart care unit was markedly lower as compared to healthy controls.[55] Moreover, further reduction in S1P level was observed in these patients within the next five days. Therefore, our data suggest a sustained reduction of the protective effect of plasma S1P after the infarction. However, the mechanism underlying this decrease remains unclear. The main sources of plasma S1P are platelets and erythrocytes.[35] It is, therefore, likely that S1P release from these cells is reduced upon myocardial infarction. However, factors affecting S1P release from blood cells are only poorly recognized. Each patient was subjected to standard antiplatelet treatment, it is then tempting to speculate that this treatment reduced liberation of S1P from thrombocytes. It would be so far unknown and undesired effect of these drugs, especially in view of the recent report showing that exogenous S1P is cardioprotective also in human myocardial tissue.[50]

CERAMIDE AS A MEDIATOR OF LIPOTOXICITY IN THE HEART

Lipotoxicity is the process through which lipid overload leads to cellular dysfunction, cell death and eventually impaired organ function.[56] Accumulation of neutral lipids within cardiomyocytes is a hallmark of the myocardium of humans and rodents with nonischemic heart failure.[56,57] In recent years a hypothesis suggesting that lipotoxicity may contribute to the development of cardiac dysfunction has emerged.[58,59] Direct evidence supporting this notion comes from experiments on mice with cardiac-restricted overexpression of long-chain acyl coenzyme A synthetase 1 (MHC-ACS1),[60] fatty acid transport protein 1[61] and the cell membrane anchored form of lipoprotein lipase (LpL(GPI)).[62] All these transgenic animal models are characterized by increased myocardial fatty acid uptake, lipid deposition in the heart and cardiomyopathy that develops in the absence of disturbances in systemic metabolism or cardiac fatty acid oxidation.

It is still unclear how lipid overload leads to cardiomyopathy, however, there is increasing evidence indicating that accumulation of ceramide plays a key role in this phenomenon. When the balance between fatty acid uptake and oxidation is altered, excess fatty acids is directed towards synthesis of complex lipids, some of which, like ceramide, are toxic. It was found that increase in myocardial ceramide content accompanies cardiac dysfunction in several genetic models of lipotoxic cardiomyopathy (see Table 3). These models include mice with cardiac-specific overexpression of PPARγ, ACS1 and

Table 3. Relationship between myocardial ceramide, cardiomyocyte apoptosis and heart function in various animal models of obesity, diabetes and lipotoxic cardiomyopathy

Experimental Model	Effect on Ceramide Level	Effect on Apoptosis	Effect on Heart Function	Reference
n6-PUFA rich high-fat diet, rats	↔			Baranowski et al. J Physiol Pharmacol 2007
High saturated-fat diet, rat infarct model of heart failure	+ 75%		↔	Rennison et al. Am J Physiol Heart Circ Physiol 2007
High saturated-fat diet, rat infarct model of heart failure	↔		↔	Morgan et al. Am J Physiol Heart Circ Physiol 2005
High saturated-fat diet, rats	↔	↔	↔	Okere et al. Am J Physiol Heart Circ Physiol 2006
n6-PUFA rich high-fat diet, rats	– 39%	↔	↔	
High saturated-fat diet, wild type mice	↔		↔	Finck et al. Proc Natl Acad Sci USA 2003
High saturated-fat diet, MHC-PPARα mice	+ 31%		↓	
High-saturated fat diet, rats	+ 65%	↔		Torre-Villalvazo et al. J Nutr 2009
ob/ob mice	+ 166%	↔		
Streptozotocin-diabetes, rats	+ 14%			M. Baranowski, unpublished observation
Streptozotocin-diabetes, rats	↔		↓	Hayashi et al. Life Sci 2001
Akita Ins2(WT/C96Y) mice (genetic model of Type 1 diabetes)	+ 69%		↓	Basu et al. Am J Physiol Heart Circ Physiol 2009
Zucker diabetic fatty rats	+ 164%	↑	↓	Zhou et al. Proc Natl Acad Sci USA 2000
MHC-PPARγ mice	+ 40%	↑	↓	Son et al. J Clin Invest 2007
MHC-ACS1 mice	+ 230%	↑	↓	Chiu et al. J Clin Invest 2001
LpL(GPI) mice	+ 45%	↔	↓	Park et al. J Lipid Res 2008

PUFA, polyunsaturated fatty acids; MHC-PPARα, cardiac-specific overexpression of peroxisome proliferator-activated receptor α; MHC-PPARγ, cardiac-specific overexpression of peroxisome proliferator-activated receptor γ; MHC-ACS1, cardiac-specific overexpression of long-chain acyl CoA synthetase 1; LpL(GPI), cardiac-specific overexpression of the cell membrane anchored form of lipoprotein lipase; ↔, no significant change; ↑, activation of cardiac apoptosis; ↓, impairment of heart function.

LpL(GPI).[60,63,64] In addition, Zhou et al[18] reported that heart of ZDF rats is characterized by progressive accumulation of ceramide that precedes development of cardiomyopathy. Increased myocardial ceramide content associated with diastolic dysfunction was recently observed also in Akita Ins2(WT/C96Y) mice (a genetic model of nonobese Type 1 diabetes).[65] Interestingly, improvement in cardiac function observed in ZDF rats, Akita Ins2(WT/C96Y) mice and in MHC-ACS1 mice after pharmacological or genetic intervention (administration of troglitazone, insulin and overexpression of diacylglycerol acyl transferase 1 in ZDF rats, Akita Ins2(WT/C96Y) mice and MHC-ACS1 mice, respectively) was associated with decreased myocardial ceramide level.[18,22,65] However, definitive evidence for the key role of ceramide in lipotoxic cardiomyopathy was provided by Park et al[64] They found that inhibition of SPT with myriocin prevented myocardial accumulation of ceramide (but not of other lipids), reduced mortality rate, improved heart function and reduced expression of cardiac failure markers in LpL(GPI) mice. Similar effect was induced by heterozygous deletion of LCB1 gene (encoding one of SPT subunits) which excludes involvement of nonspecific pharmacological effects of myriocin.

Cardiomyocyte apoptosis is one of the mechanisms underlying development of diabetic cardiomyopathy and heart failure.[66,67] Therefore, considering the proapoptotic action of ceramide, its cardiotoxic effects are commonly considered to be mediated primarily by activation of programmed cell death. Accumulation of ceramide was found to be associated with myocardial apoptosis in ZDF rats as well as in mice with cardiac specific overexpression of PPARγ or ACS1[18,60,63] (see Table 3). In addition, interventions leading to a decrease in heart ceramide simultaneously reduced cardiomyocyte apoptosis in ZDF rats and in MHC-ACS1 mice.[18,22] Moreover, in vitro studies on isolated rat cardiomyocytes revealed that incubation of the cells with palmitate induced ceramide accumulation and activation of apoptosis that was attenuated by inhibition of ceramide synthesis.[68,69]

It should be noted, however, that a recent report by Park et al[64] indicates that cardiotoxicity of ceramide may result from its effect on myocardial glucose and fatty acid metabolism rather than induction of apoptotic loss of cardiomyocytes. They used LpL(GPI) mice which were characterized by impaired systolic function, increased rate of fatty acid oxidation and reduced glucose oxidation rate in the heart. Accumulation of myocardial ceramide was observed as well, however, it was not accompanied by cardiomyocyte apoptosis. Interestingly, pharmacological (with myriocin) or genetic (heterozygous deletion of LCB1 gene) inhibition of de novo ceramide synthesis normalized fatty acid and glucose oxidation rate in the heart as well as systolic function. These interventions also corrected the mismatch between myocardial glucose uptake and oxidation, which likely contributed to the development of cardiomyopathy in this model. Park et al also reported that incubation of human cardiomyocyte AC16 cells with C6-ceramide induced downregulation of glucose transporter 4 and upregulation of pyruvate dehydrogenase kinase 4, atrial natriuretic peptide and brain natriuretic peptide gene expression. These changes were consistent with those observed in LpL(GPI) mice that show elevated myocardial ceramide level. The above data strongly suggest that ceramide is able to modulate cardiomyocyte energetic substrate metabolism via transcriptional regulation of the relevant proteins.

In addition, it was recently reported that myocardium of ob/ob mice and rats chronically fed high saturated-fat diet did not show evidence of cardiomyocyte apoptosis

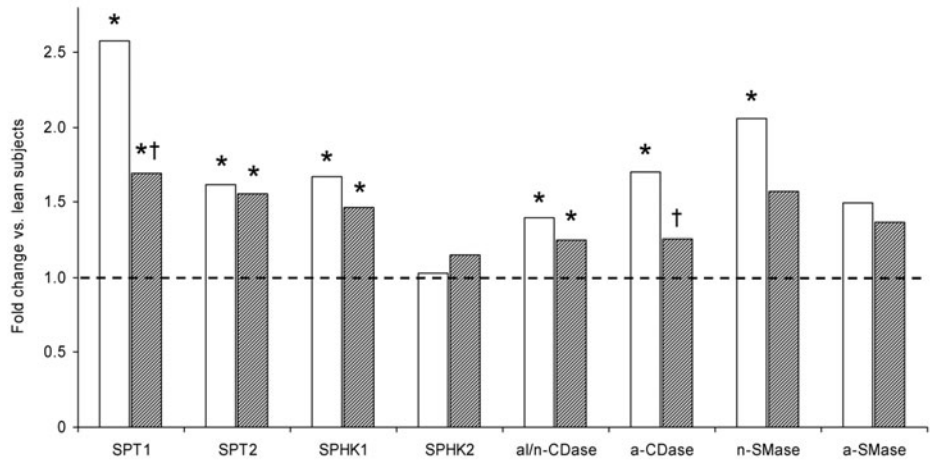

Figure 3. Effect of obesity (open bars) and obesity combined with Type 2 diabetes (hatched bars) on mRNA level of enzymes related to ceramide metabolism in the human heart. *-statistically significant change vs lean subjects, †-statistically significant change vs obese nondiabetic patients, SPT-serine palmitoyltransferase, SPHK-sphingosine kinase, al/n-CDase-alkaline/neutral ceramidase, a-CDase-acid ceramidase, n-SMase-neutral sphingomyelinase, a-SMase-acid sphingomyelinase. Data redrawn from M. Baranowski et al. Myocardium of Type 2 diabetic and obese patients is characterized by alterations in sphingolipid metabolic enzymes but not by accumulation of ceramide. J Lipid Res 2010; 51:74-80.[5]

despite accumulation of ceramide.[70] These data further indicate that increased myocardial ceramide levels do not necessarily lead to activation of apoptosis. Unfortunately, heart function was not assessed in these experiments.

Although data from animal experiments strongly suggest that ceramide may be involved in pathogenesis of heart dysfunction associated with obesity and diabetes, it remains unknown whether similar relationship is present also in the human heart. We attempted to answer this question in our recent study using samples of the right atrial appendage obtained from patients undergoing coronary bypass graft surgery.[5] We found that, compared with lean subjects, myocardium of overweight patients was characterized by marked upregulation of sphingolipid metabolic enzymes expression (Fig. 3). These enzymes included neutral sphingomyelinase, SPT subunits, ceramidases and SPHK1. Interestingly, mRNA level of some genes upregulated by overweight was reduced if concomitant diabetes was present, however, their expression was still higher than in lean subjects (Fig. 3). In addition, we observed elevated DNA fragmentation level (a marker of apoptosis) in the heart of overweight nondiabetic patients that was increased further in overweight Type 2 diabetic subjects. However, to our surprise, there was no accumulation of myocardial ceramide in either group. This was likely due to the fact that cardiac expression of enzymes involved in synthesis and degradation of ceramide was regulated in concert. Our results suggest, that in contrast to rodents, obesity and Type 2 diabetes do not induce ceramide accumulation in the human heart, or at least in the atrium. In addition, ceramide does not seem to be a major factor in cardiomyocyte apoptosis observed in patients suffering from these diseases.

CONCLUSION AND FUTURE PROSPECTS

As reviewed here, evidence from animal models strongly suggest that ceramide accumulation plays a casual role in the pathogenesis of heart dysfunction. However, to date, it remains an open question whether a similar relationship is present also in the human myocardium. Moreover, further research is required to fully elucidate the mechanisms underlying cardiotoxic effects of ceramide. Such information might allow development of new approaches to prevention and treatment of myocardial disease in patients with diabetes and obesity. In contrast to ceramide, S1P plays a critical role in maintaining cardiac cell survival and function. S1P itself as well as agonists of its cognate receptors were found to be potent cardioprotective agents against ischemia/reperfusion injury of the heart in experimental animals. The fact that these compounds are effective also when applied during reperfusion makes them potential candidates for pharmacological postconditioning therapy after myocardial ischemia. Undoubtedly, many new exciting discoveries in the field of myocardial sphingolipid signaling are to be expected in the near future.

ACKNOWLEDGEMENTS

This work was supported by the Polish Ministry of Science and Higher Education (grant no. N N402 470937).

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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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CHAPTER 5

ADIPOSE TISSUE AND CERAMIDE BIOSYNTHESIS IN THE PATHOGENESIS OF OBESITY

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Abstract: Although obesity is a complex metabolic disorder often associated with insulin resistance, hyperinsulinemia and Type 2 diabetes, as well as with accelerated atherosclerosis, the molecular changes in obesity that promote these disorders are not completely understood. Several mechanisms have been proposed to explain how increased adipose tissue mass affects whole body insulin resistance and cardiovascular risk. One theory is that increased adipose derived inflammatory cytokines induces a chronic inflammatory state that not only increases cardiovascular risk, but also antagonizes insulin signaling and mitochondrial function and thereby impair glucose hemostasis. Another suggests that lipid accumulation in nonadipose tissues not suited for fat storage leads to the buildup of bioactive lipids that inhibit insulin signaling and metabolism. Recent evidence demonstrates that sphingolipid metabolism is dysregulated in obesity and specific sphingolipids may provide a common pathway that link excess nutrients and inflammation to increased metabolic and cardiovascular risk. This chapter will focus primarily on the expression and regulation of adipose and plasma ceramide biosynthesis in obesity and, its potential contribution to the pathogenesis of obesity and the metabolic syndrome.

INTRODUCTION

Obesity has reached epidemic proportions in western societies with 65% of the adult US population being either overweight or obese.[1] Obesity contributes significantly to morbidity and mortality as it is a major risk factor in the etiology of heart disease, diabetes and cancer. Within the last decade, the adipose tissue has become a central focus in the pathogenesis of obesity-associated metabolic and cardiovascular complications.

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

The adipose tissue is now recognized not only as a lipid storing organ, but represents an active endocrine organ producing a variety of factors, that include pro inflammatory cytokines, chemokines, hormones, coagulation and fibrinolytic proteins etc (collectively termed "adipokines"), that affect multiple cellular processes including energy hemostasis, insulin sensitivity and cardiovascular risk.[2,3] Obesity mediated adipose tissue inflammation is characterized by infiltration of immune cells including macrophages and T cells and, elevated expression of a variety of pro-inflammatory mediators including tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and keratinocyte-derived chemokine (KC:functional homolog of human IL8); and the expression and secretion of these cytokines and chemokines are elevated in the adipose tissues of obese rodents and humans.[2,4-10,11] Plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of plasminogen activation in vivo and an established risk factor for cardiovascular disease,[12] is also dramatically elevated in adipose tissues in obesity and adipose PAI-1 is considered to be an important contributor to elevated plasma PAI-1 associated with obesity.[13-17] Evidence suggests that PAI-1 may also contribute directly to the complications of obesity including insulin resistance, Type 2 diabetes and atherothrombosis.[18-20] Thus, the dysregulated secretion of adipokines from the adipose tissues in obesity, acts in an autocrine, paracrine and endocrine manner to promote insulin resistance in peripheral tissues (muscle, liver) and increase cardiovascular risk (Fig. 1).

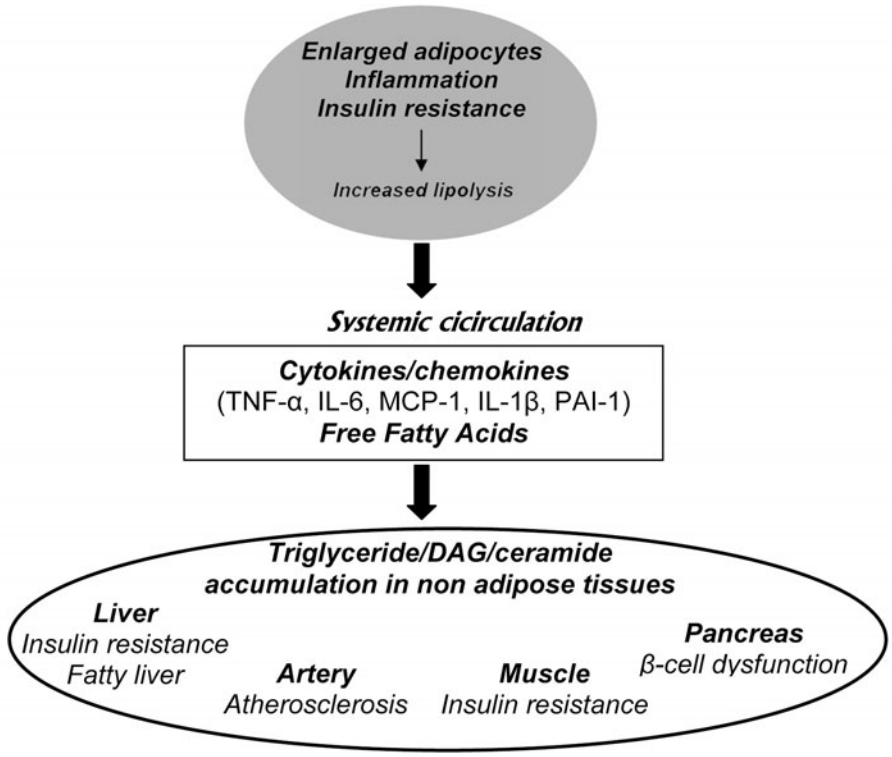

Figure 1. Proposed scheme of how increased adipose tissue mass/enlarged adipocytes contribute to metabolic and cardiovascular risk in obesity.

Another mechanism by which obesity leads to insulin resistance in the liver and muscle is via increased intracellular lipid accumulation in these tissues.[21-25] Release of free fatty acids (FFAs) from adipose tissue stores via lipolysis coupled with the inability of the adipose tissue to store excess energy results in ectopic lipid accumulation in tissues unsuited for triglyceride storage such as the liver, muscle and pancreas. Inflammatory cytokines and FFAs activate stress signaling pathways including c-Jun N terminal kinase and NF-KB that interfere with insulin signaling and contribute to insulin resistance in adipose, muscle and liver (Fig. 1).

Studies demonstrate that sphingolipid metabolism is altered in adipose tissues in obesity and bioactive sphingolipids such as ceramide and/or its metabolites, sphingosine and sphingosine 1 phosphate (S1P) may provide a common pathway that link both elevated FFAs resulting from excess nutrients and, adipose inflammation to increased metabolic and cardiovascular risk.

REMODELING OF ADIPOSE AND PLASMA CERAMIDE IN OBESITY

Sphingolipid metabolism is controlled by a complex network of highly regulated interconnected pathways leading to the production of bioactive molecules including ceramide, sphingosine, S1P and ceramide 1 phosphate (C1P). Ceramide is the central molecule in sphingolipid metabolism and the common precursor in the generation of complex sphingolipids. The production of ceramide, is mediated by de novo synthesis via serine palmitoyl transferase (SPT) and ceramide synthase (CerS) or the hydrolysis of membrane sphingomyelin by acid or neutral sphingomyelinase (ASMase or NSMase).[26-28] A number of isoforms of CerS have been identified and these enzymes regulate the fatty acid composition of ceramide leading to the generation of multiple ceramide species.[29] Ceramide is subsequently deacylated to produce sphingosine through the action of ceramidases (alkaline or acid ceramidase); and sphingosine can be then phosphorylated to S1P via sphingosine kinase.[27,28] Ceramide can be phosphorylated by ceramide kinase to produce C1P and converted to the complex sphingolipid glucosyl ceramide, by the addition of glucose molecules in a reaction catalyzed by glucosylceramide synthase.[30,31] Accumulating evidence suggest that these bioactive sphingolipids (e.g., ceramide, sphingosine, S1P, C1P) serve as signaling molecules involved in multiple signaling pathways regulating a variety of physiological and pathological biological events including cell growth and survival, differentiation, apoptosis and inflammation.[27,28]

It is now well established that sphingolipid metabolism can be activated by a variety of conditions such as pro inflammatory cytokines (e.g., TNF-α), growth factors, oxidative stress and increased availability of FFAs. All of these conditions characterize the local milieu of the obese adipose tissue, suggesting that sphingolipid metabolism may be altered in adipose tissues in obesity. Detailed changes in sphingolipid metabolites produced in the adipose tissue and plasma of genetically obese (ob/ob) was reported using a lipidomics approach.[32] In the leptin deficient ob/ob mice, total sphingomyelin and ceramide were reduced, whereas sphingosine was increased when compared to their lean counterparts. In contrast with sphingolipid levels in adipose tissue, total sphingomyelin, ceramide, sphingosine and S1P levels were all elevated in the plasma of ob/ob mice. Significant decreases were observed in adipose tissues for C18:1, C24 and C24:1 ceramide, whereas a general increase was observed in plasma for all detectable

species of ceramide. However, the largest increase of almost 90% was observed for C18 ceramide. The observed changes in adipose ceramide levels in the ob/ob mice paralleled increases in gene expression of enzymes involved in ceramide generation (SPT, NSMase and ASMase) and ceramide hydrolysis (Ceramidase). The decrease in ceramide observed in the adipose tissue and the corresponding increase in plasma may reflect secretion from adipose tissue into the circulation. In this respect, it was shown that in Sprague-Dawley rats, dexamathasone treatment induced a dramatic increase in ceramide within the portal vein, suggesting that elevated circulating ceramide may originate from the generation and secretion of ceramide from adipose tissue stores.[33] Ceramide secretion was also observed in cultured adipocytes (Samad et al, unpublished observations).

Since the ob/ob mice lack the satiety hormone leptin and human obesity is characterized by leptin resistance and increased levels of circulating leptin, the relevance of the ob/ob model to human obesity may be questionable. These studies were therefore also extended to a high fat diet (HFD) induced obese mouse model, which better mimics human obesity. Here, C57BL/6J mice were placed on a high (60% kcals from fat) or low fat (10% kcals from fat) diet for 16 weeks. In this model the HFD lead to a significant increase in ceramide levels in both the plasma and adipose tissue via a mechanism that involves the induction of enzymes that increase ceramide synthesis (SPT, acid-SMase and neutral-SMase).[34] Lipidomics analysis revealed that the predominant ceramide species in adipose tissues of normal lean mice was C16 (Fig. 2A), whereas C24 ceramide was the dominant species in the plasma (Fig. 2C). HFD-induced obesity resulted in more than 300% increase in C18 ceramide in both

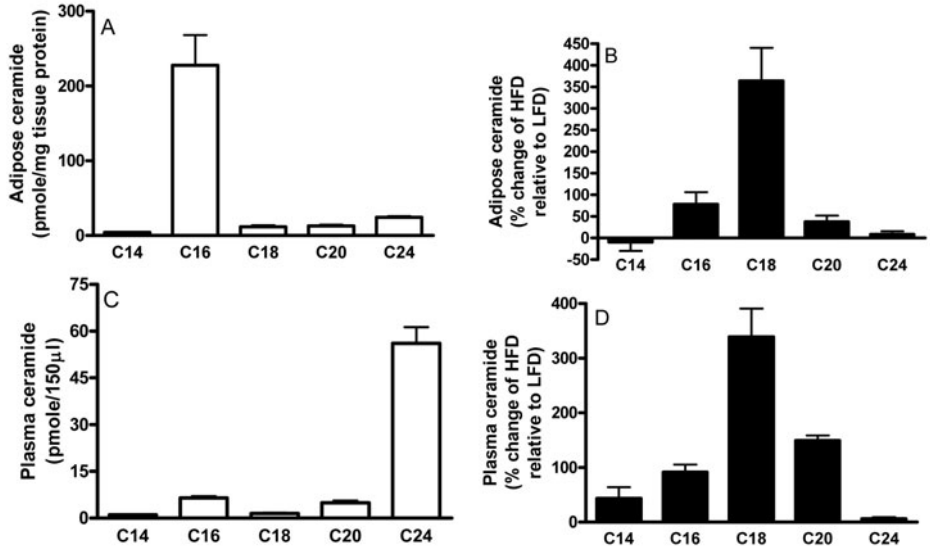

Figure 2. Ceramide expression in lean and diet induced obese (DIO) mice. Panels A and C: Ceramide levels in adipose tissues and plasma of lean mice respectively. Panels B and D: Ceramide expression in adipose tissues and plasma of DIO mice respectively. Obese mice were generated by placing male C57BL/6J mice on a high fat diet (60% of total calories derived from fat) for 16 weeks, whereas lean mice were fed a low fat diet (10% total calories from fat). Adapted from reference 34.

adipose and plasma, whereas C16 ceramide was induced by approximately 75% (Fig. 2B,D). Interestingly, C18 ceramide which was maximally stimulated in response to the HFD, constituted a relatively minor species in the plasma and adipose tissue of normal lean mice. The dramatic increase in adipose C18 ceramide correlated with elevated levels of ceramide synthase 1 (CerS1) which preferentially leads to the production of C18 ceramide. These studies suggest that increases in specific ceramide species such as C18 or C16, rather that total ceramide may be significant in the pathogenesis of obesity and the metabolic syndrome. A recent study showed that Type 2 diabetic subjects had higher concentrations of plasma total and C18, C20 and C24:1 ceramide which correlated with severity of insulin resistance.[35] Increased levels of the ceramide metabolites, sphingosine and S1P have also been reported in Type 2 diabetic patients indicating the activation of sphingolipid metabolism leading to ceramide generation and degradation in this population.[36] Adipose ceramide was also elevated in subjects with increased liver fat that was independent of obesity.[37] Aging increases the risk for insulin resistance and Type 2 diabetes and, adipocytes from old mice expressed higher levels of ceramide compared to those from young mice.[38] Thus, aberrant adipose tissue ceramide accumulation appears to be associated with obesity and may play a role in the pathogenesis of the metabolic syndrome.

REGULATION OF CERAMIDE METABOLISM IN OBESITY

TNF-α expression is elevated in adipose tissues in obesity[39] and this cytokine has been shown to activate genes involved in ceramide generation via hydrolysis of sphingomyelin (ASMase and NSMase) and the de novo pathway of ceramide (SPT) generation in other cell systems.[28] Intraperitoneal injection of TNF-α into C57BL/6J lean mice significantly increased adipose tissue ASMase, NSMase and SPT mRNA expression.[32] The contribution of TNF-α to obesity mediated increase in plasma ceramide was also directly investigated in ob/ob mice that lack both the p55 and p75 TNF-α receptors. Compared with wild type ob/ob mice, plasma ceramide levels were significantly decreased in ob/ob mice that lack both the p55 and p75 TNF receptors (Samad, et al, unpublished observations). These results suggest that TNF-α is upstream of the pathway leading to ceramide generation in obesity. Obesity is associated with insulin resistance and hyperinsulinemia and, insulin dramatically induced ASMase, NSMase and SPT mRNA gene expression in adipose tissues of lean and insulin-resistant ob/ob mice.[32] The magnitude of induction of these genes was significantly higher in insulin-treated ob/ob mice compared with insulin-treated lean mice, suggesting that the hyperinsulinemia that frequently accompanies obesity and insulin resistance may promote the abnormal expression of genes involved in the activation of the ceramide pathway in the obese adipose tissue.

Obesity is additionally characterized by elevated plasma FFA and oxidative stress. Increased adiposity and associated insulin resistance, particularly in abdominal adipose tissue leads to increased lipolysis and release of FFA to the systemic circulation. This increased availability of FFAs drives the de novo generation of ceramide synthesis in tissues such as the skeletal muscle, liver and pancreas thus causing metabolic derangements in these tissues. However, palmitate and oleate both failed to induce ceramide synthesis in cultured 3T3-L1 adipocytes.[40] Whether FFAs directly contributes to the increase in adipose ceramide levels in obesity has not been conclusively demonstrated.

Glucocorticoids are known to contribute to adipose dysfunction and the metabolic syndrome. Levels of the glucocorticoid activating enzyme, 11-beta-hydroxysteroid dehydrogenase Type 1 (11beta-HSD1), are increased in adipose tissues in obesity.[41] Mice lacking 11beta-HSD1 are protected from obesity induced diabetes, whereas its overexpression leads to the manifestation of features of the metabolic syndrome including obesity, insulin resistance and hypertension.[42-44] The expression of 11beta-HSD1 in 3T3-L1 pre adipocytes were significantly induced in response to cell permeable ceramide analogue C2 ceramide, bacterial sphingomyelinase and S1P suggesting a direct role for ceramide in the regulation of adipose glucocorticoids.[45] Glucocorticoids in turn induce sphingolipid biosynthesis (ceramide, sphingosine and sphingomyelin) in a variety of cell types.[46-48] In vivo, dexamathasone treatment of rats dramatically induced ceramide levels in the portal circulation, suggesting increased induction and secretion from adipose tissues stores.[33]

Obesity induces a condition of systemic oxidative stress and increased oxidative stress in tissues such as the adipose tissue, may at last in part contribute to the dysregulated expression of adipocytokines and the development of the metabolic syndrome.[49-52] While oxidative stress and mitochondrial dysfunction per se could promote ceramide accumulation, this has not been directly demonstrated for adipose ceramide in obesity. A number of studies also demonstrate that ceramide directly increases mitochondrial dysfunction/oxidative stress. For example, ceramide triggers ROS production[53] and regulates mitochondrial membrane permeability.[54,55] Recent studies suggest a role for mitochondrial ceramide in the recruitment and activation of Bax,[56,57] a pro-apoptotic member of the Bcl2 family that regulates mitochondrial permeability.[58] While potentially important mechanistic links are present between obesity, ceramide and oxidative stress, it still remains unclear however, whether aberrant ceramide accumulation is a cause or consequence of oxidative stress associated with obesity.

NOVEL LINKS BETWEEN SPHINGOLIPID METABOLISM AND PLASMINOGEN ACTIVATOR INHIBITOR 1 (PAI-1)

PAI-1 is the primary physiological inhibitor of plasminogen activation in vivo and increased PAI-1 compromises normal fibrin clearance mechanisms and promotes thrombosis.[12] PAI-1 levels are consistently increased in the adipose tissues and plasma in obesity and correlate strongly with parameters of the metabolic syndrome, including body mass index, insulin resistance and hyperinsulinemia.[18] Increasing evidence suggests that PAI-1 may contribute directly to the complications of obesity, including insulin resistance, Type 2 diabetes and atherothrombosis[18] and thus central to increased adiposity and its metabolic consequences.

Interestingly, the increase in adipose and plasma ceramide observed in diet induced obsess (DIO) wild type C57BL/6J mice was attenuated in mice lacking PAI-1.[34] HFD fed PAI-1 deficient mice were protected from the diet-induced increase in SPT, ASMase and NSMase mRNA, providing a mechanistic link for decreased ceramide in PAI-$1^{-/-}$ mice.[34] The improvements in the ceramide profile in mice lacking PAI-1 may, at least in part, be also mediated by the decreased levels of plasma FFAs and adipose TNF-α observed in these mice. This study suggests that PAI-1 may interact in previously unrecognized ways with pathways involved in sphingolipid metabolism in obesity. It also suggests that the improvements in the metabolic phenotype (improved insulin signaling, reduced weight) observed in HFD fed PAI-$1^{-/-}$ mice may at least in part, be mediated by the significant

decrease in ceramide, an intermediary molecule linking excess nutrients, inflammatory cytokines and insulin resistance.

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO BODY WEIGHT REGULATION AND ENERGY METABOLISM

Regulation of body weight is governed by multiple pathways, one of which is leptin signaling. Leptin, the hormone secreted primarily by adipocytes regulates central and peripheral signaling pathways that ultimately lead to decreased food intake and/or increased metabolism/energy expenditure.[59-61] However, most obese humans are resistant to the effects of leptin on body weight regulation.[62-64] DIO in mice is a physiologically relevant model of human obesity, since obesity in these mice is associated with the hallmarks of leptin resistance: hyperleptinemia, increased food intake and decreased metabolism.[63,65] Recent studies provide convincing evidence that the development of leptin resistance is a pre requisite for HFD-induced increase in fat storage in adipocytes and thereby to weight gain/obesity.[66]

We determined whether accumulation of ceramide in response to a HFD contributes to weight gain and leptin resistance. De novo ceramide synthesis was inhibited by treating mice with myriocin, which inhibits SPT, the rate limiting enzyme in de novo ceramide synthesis. C57BL/6J mice (8 week old males) were fed a HFD (60% kcal from fat) and treated with vehicle or myriocin as previously described[67,68] (IP, O.3mg/kg body weight) every other day for 8 weeks. Lipidomics profiling showed that myriocin treatment of mice on the HFD results in significant decreases in a number of ceramide species, the largest decrease was observed for C16 and C18 ceramide.[69] While the body weights of vehicle- treated mice increased rapidly, myriocin treated mice gained significantly less weight (Fig. 3A). Inhibiting de novo ceramide synthesis also resulted in increased oxygen consumption and CO_2 output (Fig. 3B,C) indicative of increased metabolism and energy expenditure. Myriocin treatment decreased the respiratory exchange ratio (RER), a ratio of carbohydrate oxidation to lipid oxidation, indicating a shift towards fat oxidation in these mice (Fig. 3D). The weight reduction, decreased fat pad weight/adipocyte size, increased metabolism and fat oxidation in myriocin treated mice, are all hallmarks of a leptin sensitive state. These studies hence implicate that aberrant accumulation of ceramide via increased in vivo ceramide biosynthesis may potentially contribute to the development of leptin resistance, a prerequisite to weight gain and associated metabolic and cardiovascular disorders.

High fat feeding also leads to a large increase in the suppressor of cytokine signaling 3 (SOCS3), a post receptor inhibitor of leptin signaling, important in the development of leptin resistance.[66,70,71-74] Inhibition of de novo ceramide synthesis dramatically reduced the adipose expression of SOCS3 in HFD fed mice.[69] Moreover, direct treatment of 3T3-L1 adipocytes with the short chain ceramide analogue C6 increased SOCS3 mRNA.[69] Thus SOCS3, may mechanistically link aberrant ceramide accumulation to peripheral leptin resistance in adipocytes.

Leptin, regulates a number of target genes and signaling pathways involved in energy homeostasis including the mitochondrial uncoupling proteins (UCP), whose expression is decreased in leptin resistant states.[74-78] UCPs are mitochondrial inner membrane proteins that promote mitochondrial energy expenditure via fatty acid oxidation and thereby play important roles in whole body energy expenditure.[79] Enzymes

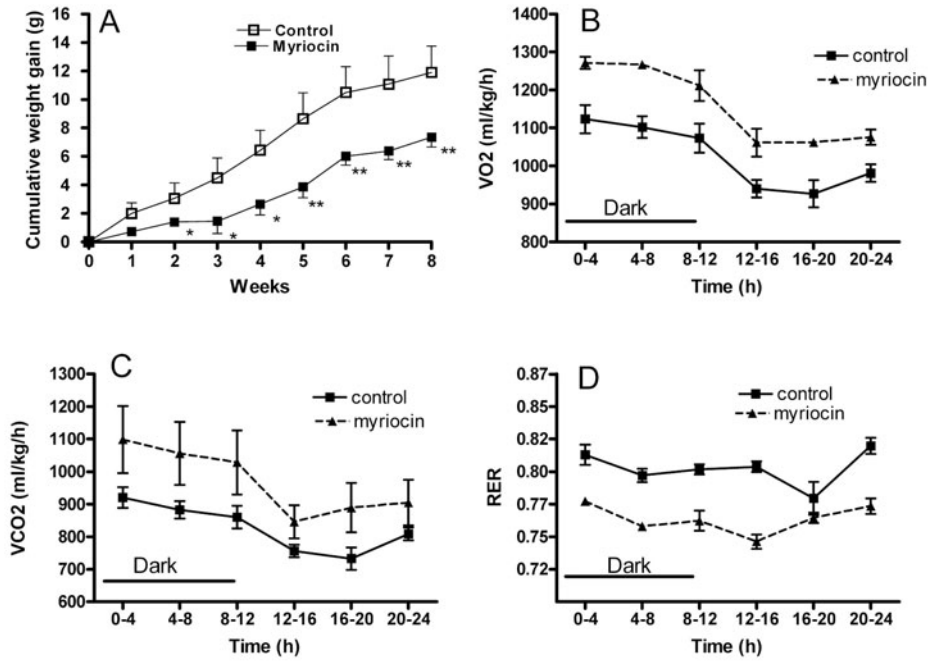

Figure 3. Metabolic parameters in DIO mice after inhibition of de novo ceramide biosynthesis. Panel A: Body weights. Panel B: whole body oxygen consumption. Panel C: Carbon dioxide release. Panel D: Respiratory Exchange Ratio. Adapted from reference 69.

involved in ceramide biosynthesis are expressed in mitochondria and mitochondria are capable of ceramide generation.[54,80-82] Mitochondrial ceramide was shown to be induced by TNF-α, a cytokine whose expression is elevated in obesity.[83] In parallel with the decrease in SOCS-3 expression, inhibition of de novo ceramide synthesis significantly increased the expression of the downstream leptin target gene UCP-3 in adipose tissues of mice on the HFD.[69] Furthermore, UCP-3 mRNA expression was also significantly reduced in 3T3-L1 adipocytes treated with the ceramide analogue C6.[69] While the proposition that ceramide accumulation may be involved in the pathogenesis of obesity mediated leptin resistance is intriguing, further studies are needed to definitively prove this hypothesis.

SPHINGOLIPIDS AS MEDIATORS OF ADIPOSE INFLAMMATION IN OBESITY

Obesity is associated with changes in adipose tissue expression of chemokines, cytokines, hormones and other adipokines that is thought to underlie the cardiovascular and metabolic risk associated with obesity. These adipokines secreted by adipocytes and other cell types such as the macrophages that accumulate in the adipose tissue during weight gain have local and systemic effects on insulin signaling pathways in muscle and liver and contributes to chronic systemic inflammation that increases cardiovascular

risk. Treatment of 3T3-L1 adipocytes with short chain ceramide, sphingosine, or S1P induced the expression of PAI-1 and the pro inflammatory molecules, TNF-α, IL-6, MCP-1 and KC to various extents.[32] Ceramide also increased PAI-1 expression in cultured endothelial cells[84] and astrocytes.[85] Inhibition of de novo ceramide generation in DIO mice, reduced adipose tissue PAI-1 and MCP-1 expression, providing evidence that ceramide accumulation contributes to adipose tissue PAI-1 and MCP-1 expression in vivo.[69] Ceramide however can be readily converted to sphingosine, which in turn can be phosphorylated to S1P and all of these reactions are reversible. Approaches using specific inhibitors or small interfering RNA of enzymes involved in these conversions are needed to specifically identify the roles of individual sphingolipids in the regulation of these pro inflammatory adipokines in adipose tissues in obesity.

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO OBESITY MEDIATED INSULIN RESISTANCE

Obesity is associated with an increased risk for insulin resistance, characterized by an impaired responsiveness of the primary insulin sensitive tissue, the adipose, muscle and liver to the anabolic responses of a normal physiological dose of insulin. Insulin maintains glucose hemostasis by promoting glucose uptake by the muscle and adipose tissues and inhibiting glucose efflux from the liver. Additionally, insulin induces fatty acid uptake and storage as triglycerides in adipose tissues and, insulin resistance is associated with increased adipose tissue lipolysis resulting in secretion of FFA leading to impairment of insulin signaling in the muscle and liver and to pancreatic dysfunction. Thus insulin resistance in the adipose tissue may be the primary event that precipitates whole body insulin resistance and the subsequent development of Type 2 diabetes.

Both in vitro and in vivo studies unequivocally demonstrate a role for sphingolipids, specifically ceramide in the development of insulin resistance (reviewed in refs. 25,86) and ceramide appears to be a putative intermediate linking both excess nutrients such as saturated FFA and inflammatory cytokines such as TNF-α to the induction of insulin resistance. Ceramide and sphingosine inhibits insulin action and signaling in various cell culture systems.[25,87-91] In vivo studies by Holland et al demonstrated that inhibition of ceramide synthesis by preventing de novo ceramide synthesis using the specific SPT inhibitor myriocin ameliorated glucocorticoid, saturated fat and obesity induced insulin resistance.[33] Heterozygous dihydroceramide desaturase mice exhibited enhanced insulin sensitivity and protection against dexamathasone-induced insulin resistance.[33]

Inhibition of ceramide biosynthesis using myriocin, also improved glucose hemostasis in high fat diet induced obese mice, as indicated by the significant improvement in both the glucose and insulin tolerance tests (Fig. 4A,B).[69] While the above studies clearly demonstrate that ceramide may play a role in the development of insulin resistance, the molecular mechanisms by which it does so remain controversial and may depend on the model system used for study. A number of reports demonstrate that ceramide inhibits insulin-stimulated glucose uptake, GLUT4 translocation and/or glycogen synthesis.[87-93] Ceramide has been shown to inhibit several distinct intermediates in the insulin signaling pathway including insulin receptor substrate (IRS)-1, PI3-kinase and Akt/PKB.[94,95] However, these results have been controversial. While some studies have shown that in cultured cells, ceramide inhibited insulin mediated tyrosine

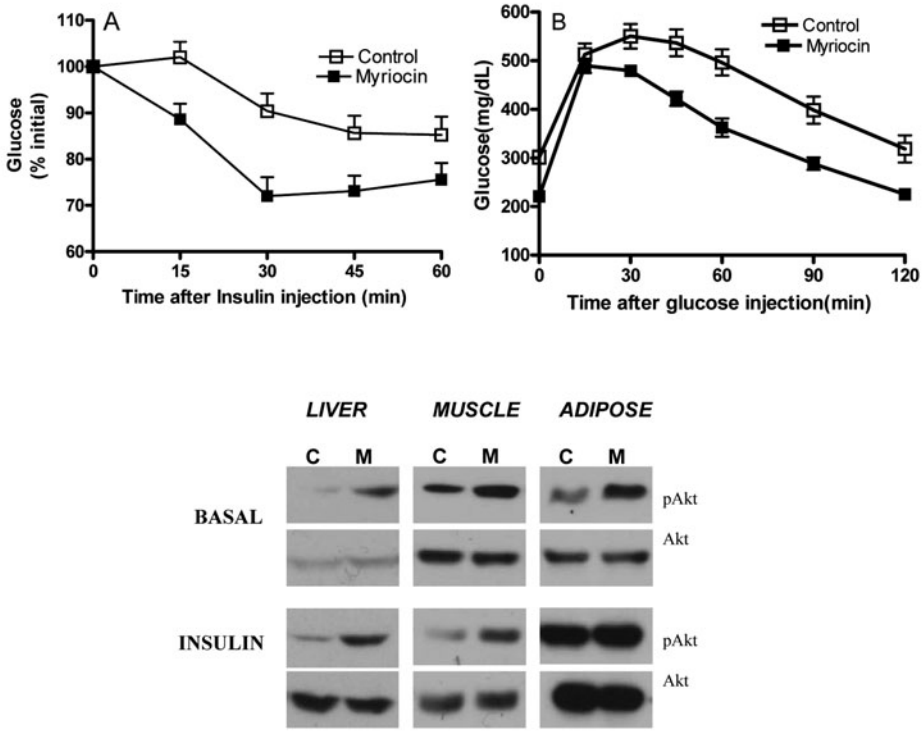

Figure 4. Glucose hemostasis in DIO mice in response to inhibition of de novo ceramide biosynthesis. Panel A: Insulin tolerance test. Panel B: Glucose tolerance test. Bottom panel: Western blot: Basal and Insulin-mediated Akt phosphorylation in Liver, Muscle and Adipose tissues of vehicle-treated controls (C) myriocin treated (M) DIO mice. Adapted from reference 69.

phosphorylation of IRS-1 and subsequent activation of PI3-kinase, others have failed to observe this effect.[87,89,96-103] All studies thus far, however, have consistently demonstrated that ceramide inhibits phosphorylation and activation of Akt/PKB and, this effect of ceramide could potentially occur independently of IRS-1.[87,98-100,103] Ceramide directly activates protein phosphatases 2A (PP2A),[104] the primary phosphatases responsible for dephosphorylating Akt/PKB.[105] Ceramide also activates PKCζ, an enzyme that inhibits Akt/PKB translocation to the membrane.[106,107] Inhibition of de novo ceramide biosynthesis significantly induced basal Akt phosphorylation in adipose tissue, whereas both basal and insulin-mediated Akt phosphorylation was induced in the liver and muscle (Fig. 4). Thus the amelioration of whole body insulin resistance in response to decreased ceramide biosynthesis in obese mice can be attributed to the combined restoration of Akt activity and insulin sensitivity in all three insulin sensitive tissues.

More recently potentially important roles for c-Jun N terminal kinase (JNK) and inhibitor of KB-kinase β (IKKβ) in insulin signaling have been recognized.[108,109] JNK activity is increased in obesity and obese mice lacking JNK showed improved glucose homeostasis.[109] Similarly, pharmacological inhibition of IKKβ or mice deficient (heterozygous or tissue specific) for IKKβ were protected from insulin resistance.[108] Ceramide activates both JNK and IKKβ , mechanisms that may additionally contribute

to ceramide mediated insulin resistance.[110] Thus, therapeutic strategies that lower in vivo ceramide generation are proving to be useful to combat obesity mediated insulin resistance and cardiovascular risk.

Although there is compelling evidence in the literature pointing to a direct or indirect role for ceramide in the inhibition of insulin signaling, a role for glycosphingolipid metabolites of ceramide in the development of insulin resistance is also increasingly being recognized.[111-113] Addition of GM3 to cultured adipocytes suppressed phosphorylation of the insulin receptor and IRS-1 and decreased glucose transport.[111] Pharmacological depletion of GM3 in adipocytes using a glucosylceramide synthase inhibitor prevented the TNF-induced inhibition of IRS-1 signaling.[111] Mutant mice that lack GM3 demonstrate increased sensitivity to insulin and were protected from high fat diet induced insulin resistance.[114] Treatment of ob/ob mice with a highly specific small molecule inhibitor of glucosylceramide synthase normalized their elevated tissue glucosylceramide levels and significantly improved glucose homeostasis.[113] Similar improved insulin resistance was observed in high fat fed mice and in ZDF rats in response to inhibiting glucosylceramide synthase.[113] Thus, glycosphingolipid metabolites of ceramide that are also elevated in obese rodents may contribute to the development of insulin resistance in obesity.

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO HEPATIC STEATOSIS

Despite the high prevalence of Nonalcoholic fatty liver disease (NAFLD), a component of the metabolic syndrome, its pathogenesis is not completely understood. The primary event of NAFLD is the accumulation of triacylglycerols (TAGs) in hepatocytes. While fatty acids required for TAG synthesis are available from both plasma FFA pool and the pool of de novo synthesized fatty acids by the liver, the plasma FFA pool accounts for approximately 60% of the TAG content in the livers of NAFLD patients. Importantly, the adipose tissue contributes approximately 80% of fatty acid content to the plasma FFA pool.[115] Thus the overproduction of fatty acids in adipose tissue (due to insulin resistance in adipose tissue leading to enhanced lipolysis) that flow to the liver via the circulating FFA pool contributes significantly to the excess TAG accumulation in NAFLD. In insulin-resistant states, insulin does not fully suppress the activity of hormone-sensitive lipase, which catalyses the release of fatty acids from TAGs and results in enhanced lipolysis and flux of fatty acids into the plasma. In addition, reduced glucose uptake due to insulin resistance reduces glycerol 3 phosphate levels, thereby reducing the reutilization of fatty acids for TAG synthesis. Thus insulin resistance in the adipose tissue appears to be important in the pathogenesis of NAFLD. In this context, adipose tissue ceramides were increased in subjects with fatty liver compared to equally obese subjects with normal liver fat content.[37] Gene array studies of human liver samples revealed that genes involved in ceramide signaling and metabolism were positively correlated with liver fat.[116] Bioinformatics analysis demonstrated strong associations between hepatic ceramide content and the extent of steatosis in the genetically obese ob/ob mice.[117] A recent study however demonstrated that diacylglycerols but not ceramides are increased in nonalcoholic human fatty liver.[118] Mice deficient in both acid sphingomyelinase and low density lipoprotein receptor were protected from high fat diet induced increase in hepatic triglyceride accumulation, body weight, hyperglycemia and insulin resistance in

spite of elevated sphingomyelin and other sphingolipids.[119] These results implicate that hepatic sphingolipids may not directly contribute to fatty liver. An alternate possibility is that improved adipose insulin resistance resulting in reduced total plasma FFA may contribute to protection from hepatic steatosis.

The contribution of ceramide biosynthesis to the pathogenesis of hepatic steatosis was directly addressed in HFD induced obese mice, where de novo ceramide biosynthesis was inhibited using myriocin.[69] Hematoxylin and eosin staining showed pronounced steatosis with macrovesicular fat accumulation in DIO mice, which was significantly reduced after myriocin treatment (Fig. 5A,B). Hepatic triglycerides, one of the major storage forms of lipids in the liver, were also reduced in myriocin treated obese mice (Fig. 5C). The reduced hepatic steatosis observed in myriocin treated obese mice was accompanied by a significant reduction in SOCS-3 gene expression (Fig. 5D), a molecule that plays a central role in the pathology of hepatic steatosis.[120] Thus the mechanism by which ceramide contributes to hepatic steatosis may at least in part be related to its effects on SOCS-3 expression. While these studies suggest that aberrant ceramide accumulation does contribute to hepatic steatosis, whether hepatic ceramide directly contributes to fatty liver and/or whether ceramide mediated insulin resistance in the fat leading to increased plasma FFAs is the main contributing factor is currently under investigation.

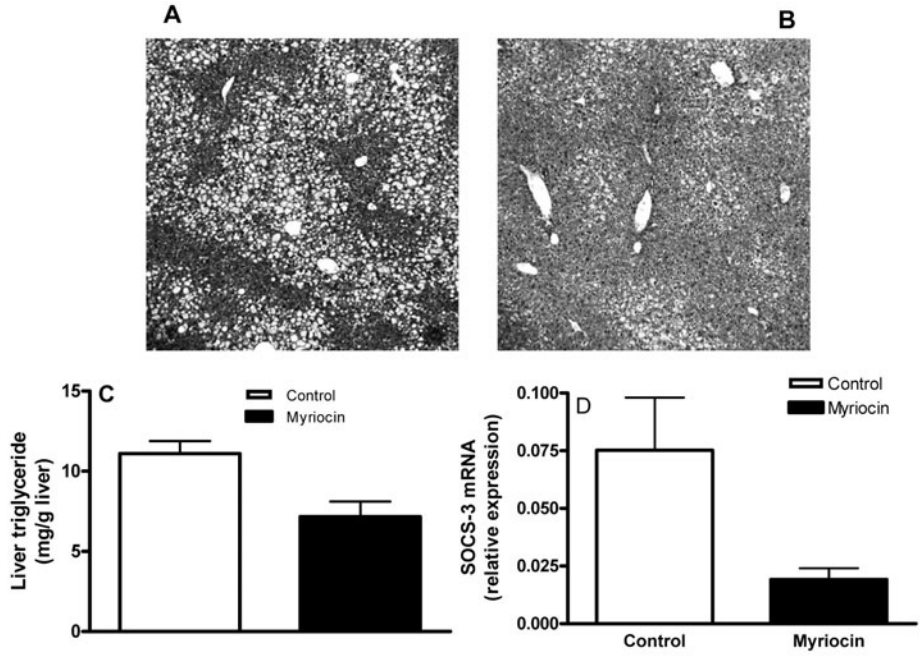

Figure 5. Amelioration of hepatic steatosis and suppressor of cytokine signaling-3 (SOCS-3) in myriocin treated DIO mice. Panels A and B: Liver histology of hematoxylin and eosin stained sections from control and myriocin-treated DIO mice respectively. Panel C: Liver triglyceride content. Panel D: SOCS-3 gene expression in liver from vehicle control and myriocin-treated mice. Adapted from reference 69.

CERAMIDE AND CARDIOVASCULAR DISEASE

The etiology of obesity associated increase in cardiovascular disease is obviously complex and may involve a combination of hyperlipidemia, insulin resistance, inflammation and an increased prothrombotic state.[121-123] A classical perspective of cardiovascular risk however, does not adequately account for all of the cardiovascular events associated with obesity and exciting new studies have demonstrated that ceramide and/or other sphingolipids may play a critical role in the pathogenesis of cardiovascular disease.[67,124-128] The increase in cardiovascular risk in obesity is associated with a systemic prothrombotic and pro inflammatory state, primarily via increased synthesis and secretion of these molecules from the adipose tissue. Increased expression of genes encoding prothrombotic proteins such as PAI-1 and inflammatory proteins (e.g., TNF-α, IL-6, MCP-1 and KC) has been consistently demonstrated in adipose tissues from obese animals and humans. Importantly, ceramide has been shown to induce the expression of PAI-1 from endothelial cells[84] and adipocytes[32] and increase pro-inflammatory cytokine (TNF-α, IL-6) and chemokine (MCP-1, KC) production from adipocytes.[32]

Animal and human studies have shown that plasma sphingomyelin and ceramide levels are closely related to the development of atherosclerosis.[67,68,129,130] For example, in LDL receptor deficient mice (a murine model of atherosclerosis), atherosclerotic lesion formation was significantly increased in response to sphingolipid rich diet.[129] Similarly, inhibition of de novo ceramide synthesis significantly reduced atheroroslorotic lesions in these mice thereby identifying a definitive role for ceramide biosynthesis in the pathogenesis of atherosclerosis.[67,68] Ceramide may also play a role in cardiomyocyte apoptosis and cardiac failure. Treatment of cardiomyocytes with FFAs that stimulate ceramide synthesis (e.g., palmitate and stearate) induced apoptosis in these cells.[131-133] In rat left ventricular myocytes, ceramide contributed to leptin-mediated cardiac contractile dysfunction.[134]

Ceramide may increase atherosclerosis via several mechanisms. Sphingomyelin carried into the arterial wall on atherogenic lipoproteins may be locally hydrolyzed to ceramide by sphingomyelinase, promoting lipoprotein aggregation and macrophage foam cell formation.[135] Ceramide levels of aggregated LDL is almost 10-15 fold higher than that of plasma LDL and exposing LDL to bacterial sphingomyelinase promotes its aggregation.[136] Moreover, ceramide may contribute to the instability and rupture of atherosclerotic plaques because of its pro-apoptotic potential on macrophages and smooth muscle cells.[137,138] The role of the ceramide metabolite S1P in the pathogenesis of atherosclerosis is less clear and appears to be somewhat controversial.[139,140] Platelets store and release S1P[141] and 60% of the S1P in serum is bound to HDL.[142-144] While some functions of S1P may point to a pro-atherogenic effect, others suggest an arthero-protective effect. This outcome with respect to a chronic disease such as arthrosclerosis in all probability depends on the expression pattern of the specific S1P receptors ($S1P_{1-5}$) on specific cells of the vessel wall at any given time during the progression of the atherosclerotic lesion. S1P stimulates the proliferation of endothelial and smooth muscle cells, suggesting a role for S1P in lesion formation and plaque stabilization.[145] S1P has also been shown to induce the expression of adhesion molecules including E-selectin, ICAM-1 and VCAM-1 on endothelial cells, leading to enhanced adhesion to monocytic cells, a process expected to enhance arthrosclerosis.[146,147] Other studies reveal anti-atherogenic functions for S1P.[148] In endothelial cells, S1P stimulates several functions such as survival, migration and nitric oxide synthesis in a manner

that is arthero-protective.[144,148] S1P added to cultured neonatal rat ventricular myocytes was protective against hypoxia induced cell death and S1P administered via an aortic cannula before ischemic/reperfusion injury improved hemodynamics, reduced creatine kinase release and diminished infarct size in both mice and rats.[149-151] Studies show that S1P carried in HDL accounts at least in part for the potent anti-inflammatory potential of HDL.[152-155] These include inhibition of endothelial apoptosis and cell migration, inhibition of adhesion molecule (VCAM-1, I-CAM-1) expression and, stimulation of nitric oxide generation, all of which are anti-atherogenic events.[148,152-154] Two in vivo studies show reduced arthrosclerosis in apolipoprotein $E^{-/-}$ or LDL $receptor^{-/-}$ mice treated with a synthetic S1P analogue, FTY720 which acts as a high affinity agonist for receptors, $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$.[155,156] While this compound shows few toxic effects in either animal or human studies,[157,158] it lowers peripheral blood lymphocytes counts by redirecting them from the circulation to the lymph nodes.[155,158] S1P can also induce cyclooxygenase (COX)-2 expression.[159] COX-2 contributes to the pathogenesis of arthrosclerosis by the induction of lipid accumulation in smooth muscle cells and macrophages, by neovessel formation and plaque stability via its antiproliferative and antimigratory effects on vascular smooth muscle cells.[148,159-161]

CONCLUSION

Research over the past decade has placed the adipose tissue at the center of obesity associated pathologies and, increased adiposity contributes to both insulin resistance and cardiovascular risk. It does so by increased secretion of pro inflammatory/pro thrombotic adipokines and FFAs which not only antagonize insulin signaling in adipose and other tissues such as the skeletal muscle and liver, but also promotes pro-atherogenic events including vascular inflammation/dysfunction and thrombosis. Increasing evidence suggests that sphingolipids, such as ceramide may provide mechanistic links between inflammation, elevated FFAs and increased metabolic/cardiovascular risk. Pharmacological strategies that modulate sphingolipids, such as the use of myriocin to inhibit de novo ceramide synthesis have beneficial effects on the complications associated with obesity. Since myriocin inhibits the initial rate limiting step in de novo ceramide synthesis the mechanism(s) by which myriocin exerts its protective effects are not clear. This remains a major challenge. Given the interconnectedness of the sphingolipid metabolic pathways, it is possible that myriocin may exert its effects via its downstream ceramide metabolites such as glycosphingolipids, C1P and S1P. Future studies need to focus on mechanism of action of specific sphingolipids and identifying therapeutic targets in the sphingolipid metabolic pathway that can be more selectively regulated.

ACKNOWLEDGEMENTS

F Samad is supported by grants from the National Institute of Health (R01HL071146), The Department of Defense (BC045748), American Heart Association (AHA no. 0230054N) and the Diabetes National Research Group (DNRG0709SAMAF).

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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

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

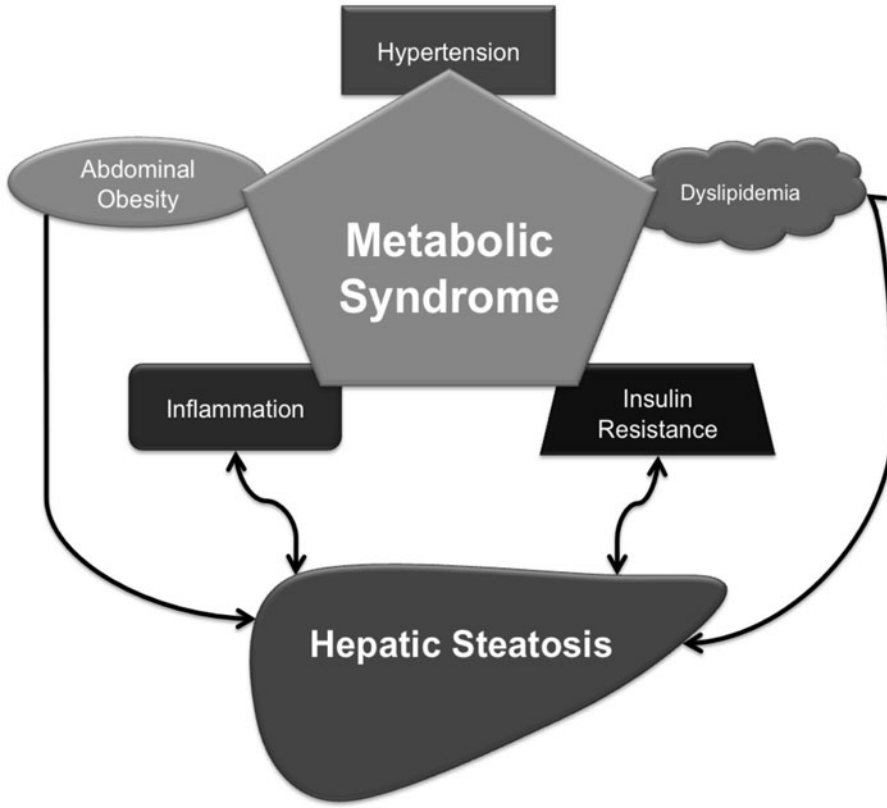

Figure 1. Schematic depicting various features of metabolic diseases.

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

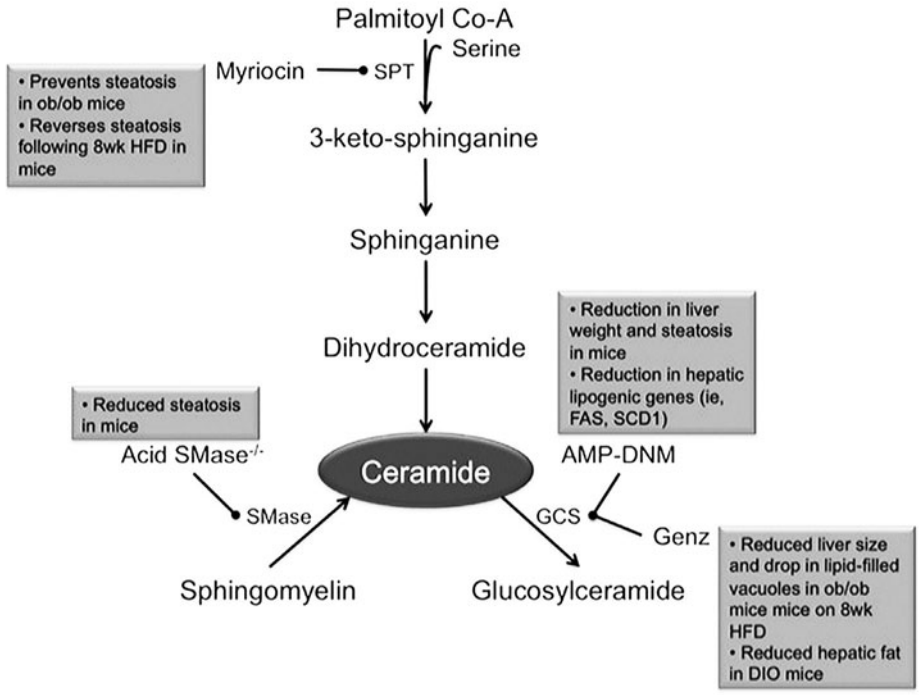

Figure 2. Shematic summarizing interventional studies showing how the modulation of sphingolipid synthesis impacts steatosis.

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).

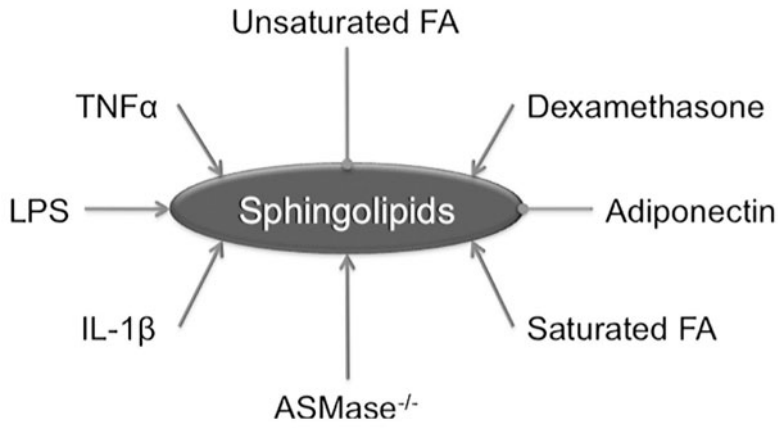

Figure 3. Schematic depicting factors discussed herein which impact hepatic sphingolipid levels.

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.

ACKNOWLEDGEMENTS

The author is grateful to the members of his laboratory for helpful discussions and feedback. Much of the work that was discussed herein is based on research findings obtained using support from the National Institutes of Health (DK081456) and the Agency for Science, Technology and Research (A*STAR) of Singapore.

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CHAPTER 7

GLYCOSPHINGOLIPIDS AND INSULIN RESISTANCE

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Abstract: Glycosphingolipids are structural membrane components, residing largely in the plasma membrane with their sugar-moieties exposed at the cell's surface. In recent times a crucial role for glycosphingolipids in insulin resistance has been proposed. A chronic state of insulin resistance is a rapidly increasing disease condition in Western and developing countries. It is considered to be the major underlying cause of the metabolic syndrome, a combination of metabolic abnormalities that increases the risk for an individual to develop Type 2 diabetes, obesity, cardiovascular disease, polycystic ovary syndrome and nonalcoholic fatty liver disease. As discussed in this chapter, the evidence for a direct regulatory interaction of glycosphingolipids with insulin signaling is still largely indirect. However, the recent finding in animal models that pharmacological reduction of glycosphingolipid biosynthesis ameliorates insulin resistance and prevents some manifestations of metabolic syndrome, supports the view that somehow glycosphingolipids act as critical regulators, Importantly, since reductions in glycosphingolipid biosynthesis have been found to be well tolerated, such approaches may have a therapeutic potential.

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

INTRODUCTION

Serendipitously, Herman Boerhaave may have linked as early as the end of the 17th century agents influencing glycosphingolipid metabolism with the body's responsiveness to insulin. Boerhaave (1668, Voorhout—1738, Leiden) was one of the most influential biomedical researchers who made major contributions to the fields of botany, chemistry and medicine. Boerhaave, called *communis Europae praeceptor*—the teacher of Europe, was already during his life internationally renowned: of the 2000 students who were enrolled in his courses at the university of Leiden in the 18th century, 690 came from English-speaking countries, 600 from German speaking countries and several from the Near Orient and America. Boerhaave's views on medicine and science are even today still remarkably modern. He strongly advocated the use of objective measurements in the analysis of clinical complications and remedies, an approach nowadays coined evidence-based medicine. In his inaugural address entitled *De usu ratiocinii mechanici in medicina,* Boerhaave called 'the chemical art the best and fittest means of improving natural knowledge'. He propagated the use of chemical analyses in clinical management, i.e., molecular medicine avant la lettre. Boerhaave also was the scientific founder of the world's first company producing a medicament at industrial scale, the so-called *Haarlem oil* (1796) that was for centuries internationally widely used for various ailments including Type 2 diabetes. Boerhaave exploited for the development of *Haarlem oil* his broad knowledge about the medicinal potential of local and exotic plants. One of the most important constituents of the original medicament was an extract from *Morus alba*, the white mulberry. Interestingly, leaves of *Morus alba* are rich in deoxynojirimycin-type iminosugars. These compounds are now known to be potent inhibitors of steps in glycosphingolipid synthesis and degradation. The value of deoxynojirimycin and more in particular that of recently developed superior hydrophobic N-alkylated analogues, to ameliorate insulin resistance in various rodent models offers an intriguing indication for the involvement of glycosphingolipids in this condition.

INSULIN RESISTANCE

Insulin and Glucose Homeostasis

The hormone insulin fulfils a vital role in the regulation of glucose homeostasis. Increased concentrations of circulating glucose following a meal or drink are counteracted by release of insulin by pancreatic beta-cells. Binding of insulin to the insulin receptor (IR) results in various cell types in increased glucose uptake by the GLUT4 transporter. In addition, insulin signaling in hepatocytes results in reduced gluconeogenesis and increased formation of glycogen. All these insulin-driven responses contribute to the desired swift normalization of the plasma glucose concentration.[1]

The insulin receptor (IR) is a heterodimer consisting of two alpha- and two beta-subunits.[1] Binding of insulin to the extracellular alpha-subunits induces a conformational change, resulting in autophosphorylation of particular tyrosine residues in the intracellular beta-subunits. Recruitment of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) to the phosphorylated IR results in their phosphorylation, which allows binding and activation of class I phosphoinositide-3-kinase (PI3 kinase). Activated PI3 kinase produces phosphatidylinositol (3,4,5)-triphosphate (PIP3), serving as a binding site

for proteins containing pleckstrin homology (PH) domains. The PH domain containing serine/threonine kinases PKB and phosphatidylinositol-3-phosphate dependent kinase 1 (PDK1) are brought into close proximity with each other by their interactions with PIP3. Additionally, PIP3 helps to activate PKB, by inducing conformational changes that expose two regulatory phosphorylation sites. First, the mammalian target of rapamycin (mTOR)-RICTOR protein complex phosphorylates the exposed regulatory serine (S473) in the C-terminus of PKB. Next, PDK1 phosphorylates the regulatory threonine residue (T307) of PKB that is requisite for enzyme activity.[2-4] All these early signaling steps take place at the plasma membrane. Activation of PKB allows its relocalization to the cytosol. Part of the activated PKB enters the nucleus were it affects transcription of several target genes. The precise pathway from PI3 kinase/PKB to GLUT4 translocation is still not known.[5] PKB substrates are glycogen synthase kinase 3 or GSK3,[6] the transcription factor FoxO,[7,8] the Rheb GAP TSC2[9] the phosphodiesterase PDE3b[10] and the RabGAP TBC1D4/AS160.[11] Only TBC1D4 is shown to play an important role in insulin-stimulated GLUT4 translocation. It has recently been argued by Hoehn and coworkers that defects in upstream elements of the insulin cascade, like IR and IRS proteins, are actually an unlikely cause for insulin resistance.[5] This statement sharply contrasts with the existing dogma. The revolutionary view by Hoehn and colleagues is based on their finding in various models of insulin resistance of discordance between upstream insulin signaling and GLUT4 translocation, most strikingly in palmitate treated L6 myotubes and high fat fed mice.[5] They conclude from these findings that while defects in IRS/PI3 kinase/PKB may occur in insulin resistance it is unlikely that such defects contribute to its early development. They further raise the possibility that upstream signaling defects are either corollary or a consequence rather than a cause of insulin resistance possibly exacerbating the insulin resistance state once it has become established and promoting progression to metabolic disease. The apparent present lack of insight in the precise role of the PI3 kinase/PKB pathway in GLUT4 translocation warrants further investigation. Of note, an additional, PI3 kinase independent, pathway for insulin-stimulated GLUT-4 translocation involving the APS–CAP–Cbl protein complex has been postulated.[12] In this pathway, the APS–CAP–Cbl protein complex activates the small GTPase TC10, which in turn signals the cytoskeleton to promote GLUT-4 cell surface recruitment.[12]

It should also be noted that in addition to the activation of the PI3-kinase/PKB and GTPase TC10 pathways, binding of insulin to IR induces a distinct signaling pathway via Growth factor binding protein 2 (Grb2)/Son of sevenless (Sos) and Ras, leading to activation of the mitogen activated protein kinase (MAPK) isoforms extracellular signal-regulated kinases (ERK)1 and ERK2. The MAPK cascade is not involved in insulin-stimulated glucose transport or glycogen synthesis but may relate to regulation of cell survival and proliferation.[13] Insulin, together with other stimuli, is also involved in (PI3 kinase dependent) regulation of autophagy.[14,15]

Insulin Resistance and Obesity: A Remaining Riddle

The incidence of obesity is rapidly increasing in Western and developing countries due to increased consumption of energy-rich food and diminishing physical activity, [4 SS]. A staggering 1.6 billion adults are overweight, of which 400 million are obese and 180 million suffer from Type 2 diabetes mellitus.[16] Obesity is not without negative health consequences. It is strongly associated with an increased risk for chronic diminished

responsiveness to insulin, so-called insulin resistance. In insulin-resistant individuals, normal levels of insulin fail to illicit the adequate responses in peripheral tissues required for swift normalization of plasma glucose concentration.[1,17] To compensate for this, the pancreas is stimulated to release more insulin. This leads to high circulating levels of insulin, a condition called compensatory hyperinsulinemia. The impaired response to glucose administration in insulin-resistant individuals is designated as glucose intolerance. Temporary insulin resistance is most likely not pathological, but rather a useful physiological response, for example to excessive supply of energy or to specific body demands. A temporary state of insulin resistance may be induced by a various factors, for example inflammation. However, in obese individuals insulin-resistance becomes gradually chronic and more prominent, constituting a pathological trait. In obese, insulin-resistant individuals the compensatory (over)production of insulin ultimately becomes irreversibly impaired due to damage of pancreatic beta-cells. Subsequently, chronic hyperglycemia (Type 2 diabetes mellitus) develops which is usually accompanied by dyslipidemia.[18]

It is still an enigma why obese individuals are so prone to develop chronic insulin-resistance. Obese individuals also typically show low-grade inflammation,[19] mediated by cytokines secreted by macrophages in their adipose tissue, which further promotes insulin resistance.[20-24] It is attractive to speculate that in this manner a vicious self-reinforcing cycle of obesity, insulin resistance and inflammation is formed. Importantly, the effect of insulin on lipid metabolism may further add to this. In the liver insulin stimulates lipogenesis by activating SREBP-1c, enhancing its transcription and increasing the amount of nuclear SREBP-1c.[25] SREBO-1c promotes transcription of genes required for fatty acid and triglyceride biosynthesis, such as acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS).[26] Intriguingly, insulin resistance is selective. In obese insulin-resistant individuals, despite clear resistance for the inhibitory effect of insulin on hepatic glucose output, the sensitivity for the stimulatory effect of insulin on lipogenesis is maintained.[27] This selective insulin resistance explains the paradoxical combination of hyperinsulinemia and hyperlipidemia in Type II diabetes.[18] Partial postreceptor hepatic insulin resistance seems a key element in the development of dyslipidemia and hepatic steatosis. A very recent paper by Sajan et al sheds some new light on the puzzling phenomenon.[28] Evidence is presented indicating that in the liver of insulin-resistant obese rats and mice, PKB activation is impaired but concomitantly atypical protein kinase C (aPKC) activation by insulin is conserved and that the latter causes excessive expression and activation of SREBP-1c, as well as activation of IKKβ/NFκB. Sajan et al propose that in diabetic rodent liver, diminished PKB activation may largely reflect impaired IRS-1/PI3K activation, while conserved aPKC activation reflects retained IRS-2/PI3K activity, causing excessive SREPB-1c and NFκB activities.[28]

In hyperinsulinemic individuals, the increased hepatic lipogenesis and the correspondingly increased supply of peripheral tissues with lipid from the liver, is likely to add to the vicious self-reinforcing cycle of obesity- insulin resistance-inflammation. Indeed, there is growing evidence pointing to a key role for excessive lipids in the etiology of obesity-induced insulin resistance.[29] Triglycerides are normally predominantly stored in adipose tissue. In obesity, this storage capacity is often exceeded, hepatic lipogenesis is increased and triglycerides accumulate in nonadipose tissues such as liver and muscle. Lipid excess in these tissues is associated with impaired insulin signaling and action.[30,31] A particular strong inverse correlation exists between the intramyocellular lipid content

and whole body insulin-stimulated glucose uptake.[32] It is generally thought that in obese subjects the surplus of lipid delivered to tissues like muscle is pathogenic, i.e., lipotoxicity driving forward the pathology of insulin-resistance. However, the precise nature of the lipotoxic factors involved, as well as the sequence of pathological events, are still very poorly understood. In a very recent, elegant review Summers and colleagues discuss the present insight in the molecular mechanisms through which glycerolipids contribute to selective insulin resistance and lipotoxicity.[33] They conclude that triglyceride (TAG) is likely to be just an inert bystander. In contrast, diacylglycerol (DAG) is considered to be a strong candidate as lipotoxic factor. DAG is known to be able to activate PKC isoforms, resulting in phosphorylation of insulin receptor substrates and the inhibition of phosphatidylinositol 3-kinase.[34,35] Phosphatidic acid, an intermediate in the synthesis of DAG has also been proposed as antagonist of insulin action.[36]

A completely different class of lipids, the so-called sphingolipids, has recently also been implicated in the etiology of insulin resistance. Sphingolipids have also been considered as primary lipotoxic agents, as will be discussed in detail in the sections below.

Next to specific lipotoxic lipid species, many investigators have proposed that excessive lipid supply to tissues in obese individuals in general forces the mitochondria to produce ever-increasing amounts of ROS, impairing mitochondrial function and inducing a concomitant development of insulin resistance. Houstis et al[37] observed that chronic treatment with an antioxidant agent (MnTBAP) improved insulin sensitivity and glucose homeostasis in insulin-resistant ob/ob mice. More recently, Hoehn and colleagues[38,39] found that ROS scavengers prevented insulin resistance in animal models as well as L6-myotubes exposed to a number of factors which induce insulin resistance, including various exogenous fatty acids.

GLYCOSPHINGOLIPIDS

Sphingolipids (SLs) and glycosphingolipids (GSLs) are structural components of mammalian cell membranes and largely reside at the cell surface. Sphingolipids are composed of a ceramide moiety with an N-acylated sphingosine group.[40-42] Either glucose or galactose is linked to the primary hydroxy group of the sphingosine moiety through a beta-glycosidic bond, thereby giving rise to the simplest glycosphingolipids: glucosylceramide and galactosylceramide. Linkage of a phosphorylcholine moiety to glucosylceramide results in sphingomyelin, a very abundant membrane lipid. Further additions of oligosaccharides and sulfate groups to glycosphingolipids give rise to a broad range of complex glycosphingolipids.[43,44] Those with a capping N-acetylneuraminic acid are known as gangliosides.

The discovery of the glycosphingolipids is generally attributed to Johan L.W. Thudichum, who in 1884 published on the chemical composition of the brain. Thudicum isolated several compounds from ethanolic brain extracts which he coined cerebrosides. He subjected one of these, phrenosin (now known as galactosylceramide) to acid hydrolysis and this produced three distinct components. One he identified as a fatty acid and another proved to be an isomer of D-glucose, which is now known as D-galactose. The third component, with an "alkaloidal nature", presented "many enigmas" to Thudichum and therefore he named it sphingosine, after the mythological riddle of the Sphinx.

Glycosphingolipid Biosynthesis

The biosynthesis of sphingolipids[45-49] starts at the cytosolic leaflet of membranes of the endoplasmic reticulum (ER), where ceramide is synthesized by a sequence of four enzyme-catalyzed reactions from L-serine and two molecules of coenzyme A (CoA) activated fatty acid: (i) formation of 3-ketosphinganine by serine palmitoyl-CoA transferase (SPT), (ii) formation of sphinganine by 3-ketosphinganine reductase, (iii) formation of hydroxyceramide by dihydroceramide synthase (*CerS*) isoenzymes and (iv) formation of ceramide dihydroceramide desaturase (DES). It has recently become clear that ceramide can also be generated for biosynthetic purposes by acylation of sphingosine stemming from lysosomal degradation of sphingolipids. This pathway is generally referred to as the salvage pathway.[50] Ceramide is the key precursor in the synthesis of various sphingolipids and glycosphingolipids. Ceramide is transported from the ER by the transport protein CERT to the cytosolic leaflet of the trans-Golgi apparatus membrane.[51-53] Here it equilibrates between the cytosolic and luminal side of the trans-Golgi membrane. On the luminal inside, sphingomyelin synthase 1 (SMS1) converts ceramide into sphingomyelin (SM) by transfer of a phosphorylcholine head group from phosphoglycerolipids. A second enzyme, SMS2, is located at the plasma membrane and converts ceramide there into sphingomyelin. In an alternative pathway, ceramide is phosphorylated at the plasma membrane by ceramide kinase (CERK). Ceramide, after equilibration to the luminal side of the ER-membrane, is transformed into galactosylceramide by ceramide galactosyltransferase (CGalT) in the lumen of the ER of some cell types.[54] This lipid is further metabolized either by sulfation or glycosylation at its 3-O-position with Neu5Ac or by further extension to oligosaccharides at its 4-O-position. Ceramide is also transported from the ER to the cytosolic side of the cis-Golgi apparatus membrane by a not yet understood CERT-independent mechanism.[53,55] Here the membrane-bound glycosyl transferase, glucosylceramide synthase (GCS), catalyzes the glycosylation of the primary hydroxy group in ceramide using UDP-glucose as a donor glycoside. It has been reported that a region of the ER that is closely associated with mitochondria also shows enzymatic activity that is capable of generating glucosylceramide.[56] Glucosylceramide synthase is an inverting transferase (family 21; GT-A fold).[57,58] It possesses an N-terminal hydrophobic transmembrane stretch that anchors the enzyme to the cytosolic face of the Golgi membrane together with a hydrophobic loop near the C-terminal region.[59]

Glucosylceramide occupies a key position in the biosynthesis of many glycosphingolipids (Fig. 1). The fact that both GCS and glucosylceramide face the cytosolic side of the cellular membrane is an intriguing aspect: further synthesis of complex glycosphingolipids takes place exclusively at the luminal inside the Golgi apparatus. When glucosylceramide is introduced to the outer leaflet of a model membrane it only slowly equilibrates to the luminal side when unassisted ($t_{1/2}$ = 5 h at 20°C). However, glucosylceramide undergoes rapid transbilayer movement in the Golgi-apparatus membrane ($t_{1/2}$ = 3 min at 20°C). An ATP-independent Golgi localized flippase seems responsible for this.[60] The ATP-dependent multidrug transporter P-glycoprotein located throughout the cell acts as a rapid flippase for artificial, fluorescently labeled (NBD) glucosylceramide, galactosylceramide and sphingomyelin, but possibly not for natural glucosylceramide.[61] De Matteis and coworkers recently showed that FAPP2 is required for the synthesis of complex GSLs because it mediates the nonvesicular transport of glucosylceramide to

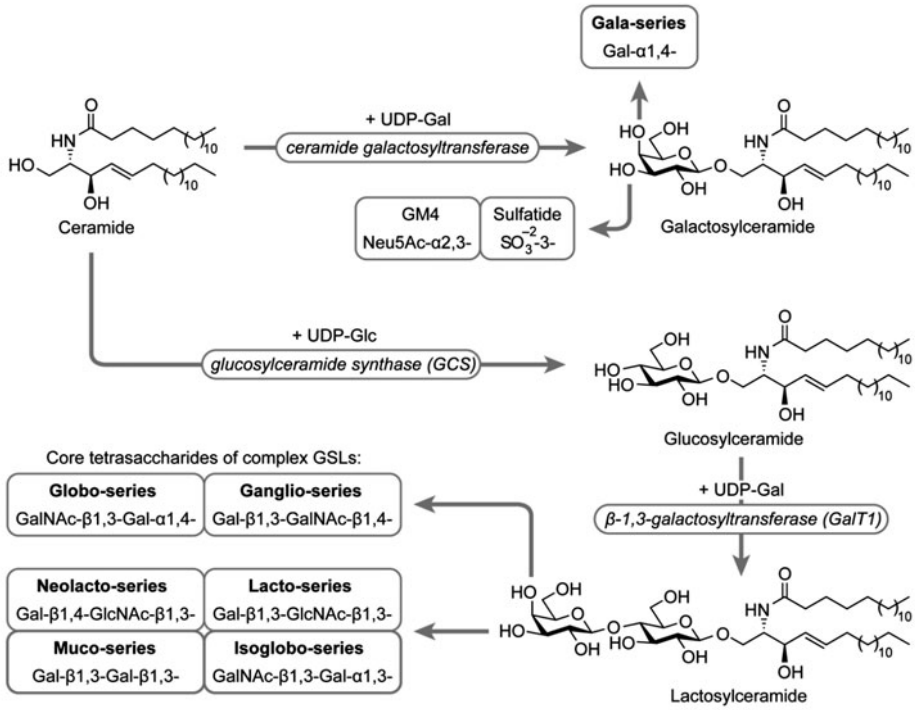

Figure 1. Schematic overview of glycosphingolipid biosynthesis.

distal Golgi compartments and proposed that FAPP2 is also responsible for the relocation of glucosylceramide to the luminal leaflet of trans-Golgi membranes where further GSL synthesis takes place.[62,63] Van Meer and coworkers reported that FAPP2 may also transport glucosylceramide to the ER. In addition to this, the closely related GLTP transport protein is capable of transporting glucosylceramide to the cytosolic leaflet of the plasma membrane.[64,65]

Having arrived at the luminal leaflet of trans-Golgi membranes, the biosynthesis of GSLs continues with the synthesis of lactosylceramide by GalT1. Lactosylceramide is extended sequentially at either the 3-O-positon or the 4-O-position in a stepwise fashion. Most of these glycosphingolipids consist of alternating and branched combinations of alpha- or beta-linked glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine. At their nonreducing end, many of these complex GSLs are terminated with either L-fucose or acidic Neu5Ac. Of particular interest are the gangliosides, that is, lactosylceramide-derived sphingolipids containing capping *N*-acetylneuraminic acid (sialic acid) units. A bewildering number of gangliosides exists and a convenient shorthand nomenclature has been developed by Svennerholm, a pioneer in ganglioside research.[66] In this nomenclature G stands for ganglioside, A for asialo-, M for monosialo-, D for disialo- and T for trisialoganglioside. Specific sialyl transferases convert lactosylceramide stepwise into GM3, GD3 and GT3. Lactosylceramide and each of its sialylated derivatives serve as precursors for complex gangliosides of the 0, a, b and c series. These different series are characterized by the

presence of no (0 series), one (a series), two (b series), or three sialic acid residues (c series) linked to the 3-position of the inner galactose moiety.[67] Gangliosides from the 0 and c series are only found in trace amounts in adult human tissues.

Glycosphingolipid Degradation

Catabolism of complex glycosphingolipids is a stepwise process that predominantly takes place in endosomes and lysosomes. Glycosphingolipids reach the endosomal-lysosomal compartment in various ways. Receptor-mediated endocytosis of low-density lipoprotein (LDL) delivers glycosphingolipids to the lumen of lysosomes. Phagocytosis of larger structures, such as senescent cells containing glycosphingolipids, occurs by specialized phagocytes, such as macrophages. Another major pathway in most cells involves endocytosis of the plasma membrane.[68] Glycosphingolipid-rich membrane parts are internalized and fuse with early endosomes. Here, glycosphingolipids destined for degradation are sorted through formation of intraluminal vesicles (multivesicular bodies) which reach the lysosome.[69] The endolysosomal catabolism of glycosphingolipids takes place at the surface of either the internal membrane vesicles or endocytosed lipoproteins. The lysosomal membrane itself is protected from degradation by a glycocalix, which consists of heavily glycosidated membrane proteins.[68] Carbohydrate residues from the nonreducing end of the glycosphingolipids are sequentially released by the action of exoglycosidases. In contrast to the biosynthetic enzymes, none of the catabolic glycosidases are bound to the membrane. However, their GSL substrates are embedded in intralysosomal membranes. Therefore, GSLs with less than four carbohydrate residues require the presence of specific (glyco)sphingolipid activator proteins (SAPs), which assist the glycosidases in their interaction with their target substrate. Five such proteins are currently known: saposin-A, -B, -C, -D and the GM2-activator protein.[69]

Glucosylceramide is degraded into ceramide and glucose by the enzyme glucocerebrosidase (GBA1; glucosylceramide-beta-glucosidase).[69] GBA1 is a retaining glycosidase (family 30) and the activator protein saposin C is essential for its function in vivo.[69] In 1994, Withers and coworkers identified the catalytic nucleophile at the active site as the side-chain carboxylate group of glutamic acid 340.[70] In 2003, Futerman and coworkers published the first X-ray crystal structure of GBA1,[71] Recently, Rossmann and coworkers published the X-ray crystal structure of the GBA1 activator saposin C.[72] Ceramide is cleaved into sphingosine and fatty acid by acid ceramidase. Ceramide degradation can also take place in other parts of the cell by neutral ceramidases. Sphingosine can be either reacylated to ceramide or used as a substrate for sphingosine-1-phosphate (S1P) synthesis.[73]

Metabolism of endocytosed glycosphingolipids is not restricted to lysosomes. A limited amount of glucosylceramide derived from the degradation of complex glycosphingolipids may escape further lysosomal degradation and re-enter the glycosphingolipid biosynthesis pathway.[74] In addition, direct metabolic remodeling of glycosphingolipids at the plasma membrane may result in local formation of simpler glycosphingolipids from complex ones.[75] The occurrence of nonlysosomal glucosylceramidase activity has long been known and was recently identified as beta-glucosidase 2 (GBA2).[76-78] GBA2, a 105 kDa protein with a transmembrane region, has not yet been assigned to a specific family of glycosidases. In contrast to GBA1, it is not sensitive to inhibition by conduritol B

epoxide. The enzyme has a pH optimum in the neutral region, as opposed to the acidic optimum of GBA1. GBA2 is not located in the lysosomes, but probably close to the cell surface. The function of GBA2 is currently not known, but its inhibition in mice is associated with impaired spermatogenesis, a result that is confirmed in studies with a GBA2 knock-out mouse model.[78-80]

The importance of endolysosomal catabolism of sphingolipids is best illustrated by the existence of a group of inherited disorders in humans caused by deficiency in lysosomal catabolic pathways, the sphingolipidoses.[68] Inherited deficiencies in a specific lysosomal enzyme or activator protein result in accumulation of the corresponding (glyco)sphingolipids. The most common of the sphingolipidoses is Gaucher disease,[81] an autosomal recessive disorder caused by deficient glucocerebrosidase activity.[82,83] The manifestation of Gaucher disease is remarkably heterogeneous: its onset can occur from birth up to an almost asymptomatic course at old age. The underlying mutations in the *GBA1* gene show some correlation with the severity of disease manifestation and, in particular, the development of neurological symptoms. A low residual enzyme activity in leukocytes or fibroblasts is associated with a more severe progression of the disease.[84,85] The most common mutation in the *GBA1* gene, which encodes the amino acid substation N370S, is usually associated with a relatively benign course of the disease, with no neuropathology involved. N370S-GBA1 is normally synthesized and delivered to lysosomes, but shows catalytic abnormalities.[86,87] In sharp contrast, the other common L444P mutation results in a polypeptide that folds poorly in the ER.[86] Homozygotes for L444P-GBA1 develop a severe, neuropathic course of the disease. In contrast to other lysosomal glycosidases, GBA1 does not acquire mannose-6-phosphate moieties, but is sorted and transported to lysosomes by interaction with the integral membrane-protein LIMP-2.[88-90] Deficiency in LIMP-2 may, therefore, also result in reduced cellular GBA1 activity.[91] Since GBA1 requires the activator protein saposin C for efficient intralysosomal degradation of glucosylceramide, deficiency in this accessory protein also results in the accumulation of glucosylceramide in cells.[92] The majority of Gaucher patients have one N370S-GBA1 allele and develop a nonneuropathic, so-called Type 1, disease. In these patients, accumulation of the substrate glucosylceramide is restricted to tissue macrophages. These heavily lipid-laden macrophages, named Gaucher cells, have a characteristic appearance. Gaucher cells are viable and secrete characteristic proteins such as chitotriosidase and CCL-18.[93,94] Elevated levels of these proteins are found in Gaucher patients and their measurement is currently used to monitor disease progression as well as efficacy of therapeutic interventions[95] The presence of large numbers of Gaucher cells in various tissues results in characteristic clinical signs such as hepatosplenomegaly, pancytopenia and skeletal deterioration. The constant release of hydrolases and cytokines by Gaucher cells and surrounding phagocytes is thought to underlie the pathological features of the disorder.[96]

(GLYCO)SPHINGOLIPIDS AND INSULIN RESISTANCE

A Role for Ceramide

Ceramide functions as a mediator in signaling cascades that regulate apoptosis, differentiation and cell cycle arrest.[97] The seminal work by Unger identified the sphingolipid ceramide as candidate lipotoxic agent in obesity-induced insulin resistance.[98] The role

of ceramide in insulin resistance will only be dealt with briefly, for detailed reviews the reader is referred to Summers and coworkers.[99-101] Briefly, many of the circulating factors associated with obesity (e.g., inflammatory cytokines, saturated fatty acids, glucocorticoids, etc.) are known to stimulate sphingolipid formation.[33] Ceramide has been shown in cellular models to inhibit insulin signaling. Exposing cultured myotubes to high doses of the free fatty acid (FFA) palmitate increases de novo ceramide synthesis, followed by inhibition of PKB phosphorylation,[102-104] glucose uptake[105] and glycogen synthesis.[106] Overexpression of acid ceramidase in these cells reverses FFA-induced ceramide accumulation and improves insulin signaling.[107] Addition of short-chain ceramide analogues to cultured 3T3-adipocytes was found to inhibit insulin signaling and action.[108-111] Ceramide does not interfere at the level of IR or IRS-1 phosphorylation, but impairs insulin signaling by inhibition of PKB activation.[112] The inhibition of PKB by ceramide is thought to be accomplished by two mechanisms. Ceramide blocks the translocation of PKB to the plasma membrane and activates PP2A, which impairs PKB activity by removing activating phosphates.[111] Several studies have investigated the concentrations of ceramide in plasma and tissues of animal models of obesity-induced insulin resistance and Type II diabetes.[100,113,114] Inconsistent observations were reported (for a review see ref. 115). For example, Lee and coworkers reported that male rats on a diet rich in saturated fatty acids developed insulin resistance with an increase in muscle diacylglycerol concentration, but without significant changes in muscle ceramide content.[116] In another recent study no significant abnormalities in skeletal muscle ceramide content of insulin-resistant Zucker Diabetic Fatty (ZDF) rats were noted.[117] Recently, studies were conducted in humans on the possible role of ceramide in obesity and FFA induced insulin resistance. Two investigations reported an association between insulin resistance and elevated levels of ceramide in skeletal muscle.[118,119] A more recent study by Skovbro et al showed no increase in muscle ceramide content in insulin-resistant and Type II diabetic individuals compared to insulin sensitive individuals.[120] Infusion of lipid emulsion (Intralipid, an emulsion of soy bean oil and egg phospholipids), known to decrease peripheral insulin sensitivity, was found to increase muscle ceramide content in one study,[121] but not in another investigation.[122] Of note, we noted ourselves that some batches of Intralipid contain very large amounts of ceramide, probably as breakdown product of sphingomyelin. It should be noted that the literature data on ceramide concentrations are difficult to interpret: (i), a variety of analytic methods has been used and (ii) information on ceramide levels of specific cellular membranes are presently lacking. It may be conceived that relevant differences in ceramide concentrations have been overlooked, or vice versa that noted differences in the lipid reflect irrelevant pools.

Consistent with a role of ceramide in insulin resistance are findings made in cell and animal models. For example, inhibition of ceramide formation by myriocin has been found to be improve insulin sensitivity in both muscle and the liver of obese rodents as assessed with hyperinsulinemic–euglycemic clamps.[114] Mouse models with an impairment in biosynthetic enzymes render a similar picture.[33] Mice that are haploinsufficient for DES1 are refractory to dexamethasone-induced insulin resistance. Moreover, muscles isolated from these mice remain insulin sensitive, even in a profoundly hyperlipidemic environment. Consistently, the small molecule fenretinide, a potent inhibitor of DES1, improves insulin sensitivity in mice fed a high fat diet. Haploinsufficiency for a key subunit of SPT has also been reported to prevent features of metabolic disease.[123]

Whilst these findings clearly point to some important role of sphingolipids in insulin resistance, they do not pinpoint one specific lipid species. It should be kept in mind that disruption of ceramide metabolism also impacts metabolites thereof such as sphingomyelin, ceramide-1-phosphate and glycosphingolipids.

Lipid Microdomains, Glycosphingolipids and the Insulin Receptor

The insulin receptor is localized at the cell surface in glycosphingolipid-containing lipid microdomains. The interaction of gangliosides with the insulin receptor was originally described by Nojiri et al,[124] who demonstrated the ganglioside-mediated inhibition of the insulin-dependent cell growth of leukemic cell lines. Tagami et al were the first to demonstrate that the addition of GM3 ganglioside to cultured adipocytes suppresses phosphorylation of the insulin receptor and its downstream substrate IRS-1, thereby resulting in reduced glucose uptake.[125] Inokuchi and coworkers reported that exposure of cultured adipocytes to TNF-alpha increases GM3 and inhibits IR and IRS-1 phosphorylation. This was found to be counteracted by PDMP, an inhibitor of glycosphingolipid biosynthesis.[126] Mutant mice lacking GM3 have been reported to show an enhanced phosphorylation of the insulin receptor of skeletal muscle after ligand binding and to be protected from high fat diet induced insulin resistance.[127] Consistent with this is the recent report on increased insulin sensitivity and glucose tolerance in mice with increased expression of the GM3-degrading sialidase Neu3.[128] In contrast, GM3 levels are elevated in the muscle of certain obese, insulin-resistant mouse and rat models.[117] Altered sphingolipid metabolism, as reflected by increased glycosphingolipid levels, has recently also been documented in relation to neuronal pathology in diabetic retinopathy.[129] More recently, Kabayami et al provided evidence that the interaction of GM3 with the insulin receptor is mediated by a specific lysine residue located just above the transmembrane domain of the receptor and that excess levels of GM3 promote dissociation of the insulin receptor from caveolae, a location which is essential for transduction of the insulin signal (Fig. 2).[130]

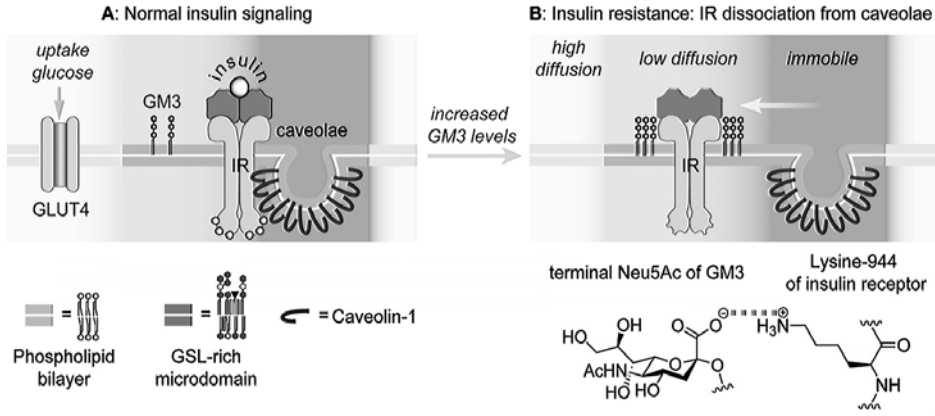

Figure 2. Schematic overview of tentative role of GM3 in modulation of insulin signaling based on work by Inokuchi and coworkers.[130]

It should be noted that the recent data on interaction of glycosphingolipids like GM3 with upstream elements of the signaling pathway can be poorly reconciled with the view of some researchers that defects in upstream elements of the insulin cascade, like IR and IRS proteins, are an unlikely cause for insulin resistance (see section *Insulin and Glucose Homeostasis*).

Gaucher Disease and Insulin Resistance

Our laboratory was for the first time confronted with a possible link between glycosphingolipids and insulin resistance during investigations on Gaucher disease. It was firstly noted that Gaucher patients show a markedly increased hepatic glucose production in combination with elevated insulin levels in the fasted state.[131,132] Next, we encountered reduced adiponectin levels in Gaucher patients, again a feature usually associated with insulin resistance.[133] Finally, an euglycemic clamp revealed that insulin-mediated whole body glucose uptake in Gaucher patients is reduced compared to healthy control subjects.[134] The apparent insulin resistance in Gaucher patients may be the result of their increased production of gangliosides like GM3.[135] Probably, as compensation for the inability to degrade glucosylceramide sufficiently by the action of glucocerebrosidase, glucosylceramide is metabolized more than normal to gangliosides. Increased levels of GM3 can indeed be detected in plasma and tissues of Gaucher patients. One could therefore hypothesize that in Gaucher patients altered glycosphingolipid levels in muscle and/or fat tissue result in their apparent insulin resistance. Of interest, Gaucher patients, despite insulin resistance, rarely show overt hyperglycemia or develop frank diabetes. It is conceivable that the massive presence of macrophages in their tissues, thriving on glycolysis, requires a major change in glucose homeostasis such as reduced insulin responsiveness. This would help to prevent attacks of hypoglycemia. It has been noted that following enzyme replacement therapy some Gaucher patients gain considerable body weight and develop diabetes.[136,137] Apparently, the patient's body metabolism can not adapt appropriately to the rapid loss of kilograms of glucose consuming storage macrophages.

PHARMACOLOGICAL MODULATION OF GLYCOSPHINGOLIPIDS AND INSULIN RESISTANCE

Inhibitors of Glucosylceramide Synthesis

Next to the enzyme replacement therapy, based on chronic intravenous administration of human glucocerebrosidase, so-called substrate deprivation therapy (SRT) has been developed for the treatment of Gaucher disease (see ref. 138 for recent review). This approach implies a chronic inhibition of the biosynthesis of glucosylceramide. Platt and Butters were the first to recognize that N-butyl-1-deoxynojirimycin is an inhibitor of glucosylceramide synthase (GCS).[139] Treatment of mild to moderately affected Type 1 Gaucher patients has been found to be effective and led to the registration of Zavesca (Miglustat; *N*-butyl-1-deoxynoijirimycin) at a dose of 3 × 100 mg/day.[140,141] SRT with Zavesca is generally well tolerated, although intestinal side-effects are encountered at higher doses. During the last decade, we developed more hydrophobic

N-alkylated 1-deoxynojirimycins of which some proved to be very potent inhibitors of GCS.[142-145] One of the most appealing compounds is the iminosugar derivative *N*-(5-adamantane-1-yl-methoxy)- pentyl-1-deoxynojirimycin (AMP-DNM) that shows attractive pharmacokinetics and is hardly metabolized. Oral administration of AMP-DNM at high concentrations is well tolerated in rodents and dogs. The IC_{50} value of AMP-DNM for GCS is around 150 nM.[143] Such a steady-state plasma concentration of the compound can be obtained by administration of 50 mg/kg bw/day in mice and 20 mg/kg bw/day in rats. Incubation of cultured cells with AMP-DNM results in dose-dependent reductions of glucosylceramide and gangliosides without a concomitant increase in ceramide levels. The same is observed in drug-treated mice and rodents.

The impact of AMP-DNM on insulin resistance was investigated. Treatment with AMP-DNM of obese, insulin resistant ob/ob mice corrected their elevated tissue glucosylceramide levels, markedly lowered circulating glucose levels, improved oral glucose tolerance, reduced HbA1C and improved insulin sensitivity in muscle and liver as measured by euglycemic clamps.[143] Similarly beneficial metabolic effects were seen in high fat–fed (DIO) mice and ZDF rats.[143] In cultured 3T3-L1 adipocytes, AMP-DNM counteracted tumor necrosis factor-alfa–induced abnormalities in glycosphingolipid concentrations and concomitantly reversed abnormalities in insulin signal transduction. Thus, AMP-DNM rendered improvements in upstream elements of the insulin signaling pathway like IR and IRS-1 protein, both in the 3T3-L1 cell model as in various tissues of treated animals. Very similar findings were made by Zhao and coworkers with a chemically unrelated inhibitor of GCS, named GENZ-123346. (1R,2R)-nonanoic acid[2-(2′,3′-dihydro-benzo [1,4] dioxin-6′-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide.[114,146] Inhibition of glycosphingolipid formation in ZDF rats and diet-induced insulin-resistant mice with Genz-123346 had comparable beneficial effects on glucose homeostasis: correction of hyperinsulinemia, reduction of nonfasted blood glucose levels, lowering of glycated hemoglobin and improvement of glucose tolerance. Treatment with Genz-123346, like AMP-DNM, promoted insulin-stimulated phosphorylation of the insulin receptor in muscle.[114] Of interest, both AMP-DNM and GENZ-123346 treatment were found to offer protection against beta-cell damage in the pancreas of ZDF-rats.

Mode of Action of Inhibitors

GENZ-123346, being a very specific inhibitor of GCS, has a clear target: glucosylceramide synthase.[146] In sharp contrast, AMP-DNM not only inhibits GCS (IC_{50} ~ 150 nM), but also GBA2 (IC_{50} ~ 1 nM) and GBA, the lysosomal glucocerebrosidase (IC_{50} 220 nM).[143] Inhibition of GBA2 seems to have limited consequences as suggested by the normal phenotype of GBA2 deficient mice. We only noted a spermatogenesis impairment in some mouse strains, an effect also observed with Zavesca and low dose AMP-DNM (see section *Glycosphingolipid degradation*). In rabbits and dogs this effect does not occur. Partial inhibition of GBA1 at the plasma concentrations of AMP-DNM reached in conducted animal studies (20-200 nM) can not be excluded. However, even at a steady-state plasma concentrations of 200 nM AMP-DNM, there should be sufficient residual GBA1 enzyme capacity to prevent lysosomal accumulation of glucosylceramide. Indeed, no elevations in the lipid were noted in muscle or liver of AMP-DNM treated animals. Another potential target of AMP-DNM are intestinal glycosidases. Particularly

sucrase-isomaltase is sensitive to inhibition by 1-deoxynojirimycin-type compounds. Buffering of carbohydrate assimilation by inhibition of intestinal glycosidases is an existing therapeutic approach for diabetes.[144] To dissect whether AMP-DNM treatment exerts its beneficial effect on glucose homeostasis partly via buffering of carbohydrate assimilation, we designed a related compound, L-*ido*-AMP-DNM that inhibits GCS and GBA2 comparable to a AMP-DNM, but is a much poorer inhibitor of sucrase-isomaltase and GBA1.[147] The pharmacokinetics of L-*ido*-AMP-DNM and AMP-DNMin in ZDF rats were equivalent. L-*ido*-AMP-DNM lowered visceral glycosphingolipids in *ob*/*ob* mice and ZDF rats on a par with AMP-DNM and improvements in oral glucose tolerance and insulin signalling in the liver were almost comparable. L-*ido*-AMP-DNM was less potent in lowering blood glucose and reducing HbA1C in these animals.[147] Apparently, combined reduction of glycosphingolipids in tissue and buffering of carbohydrate assimilation produces an optimal correction of glucose homeostasis (Fig. 3). Therefore AMP-DNM seems intrinsically more suited for controlling Type 2 diabetes associated hyperglycemia, whilst L-*ido*-AMP-DNM appears to be more attractive for the treatment of those diseases where the exclusive reduction of glycosphingolipids is required such as the hereditary lysosomal glycosphingolipidoses.

AMP-DNM treatment has a number of additional effects in animal models. Oral administration of AMP-DNM to two distinct mouse models of inflammatory bowel disease was found to result in beneficial effects.[148] The anti-inflammatory action of AMP-DNM is also apparent in ZDF rats and ob/ob mice: drug-treatment results in a reduced expression of genes encoding proteins involved in inflammation in liver as well as adipose tissue.[149,150] Markedly reduced numbers of macrophages were also observed in adipose tissue of AMP-DNM treated ob/ob mice.[149] AMP-DNM treatment of ob/ob mice restored insulin signalling in adipose tissue and in isolated ex vivo insulin-stimulated adipocytes. Drug treatment led to improved adipogenesis as the number of larger adipocytes was reduced and expression of genes like peroxisome proliferator-activated receptor (PPAR) c, insulin responsive glucose transporter (GLUT)-4 and adipsin increased.[149] In addition, adiponectin gene expression and protein were found to be increased by AMP-DNM treatment.[149] Thus, treatment of ob/ob mice improved adipocyte function and reduced inflammation in the adipose tissue.

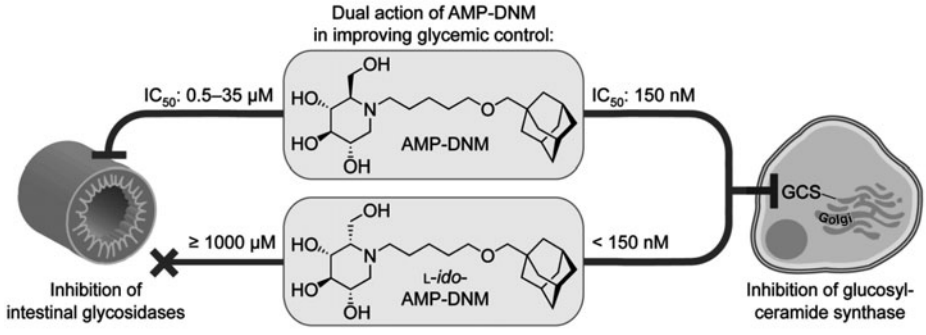

Figure 3. Dual action of AMP-DNM: buffering of carbohydrate assimilation and improvement of insulin sensitivity. L-*ido*-AMP-DNM is a poor inhibitor of intestinal glycosidases.

AMP-DNM has also been found to beneficially affect lipid homeostasis and, in particular, the reverse cholesterol transport pathway.[151] Treatment of C57BL/6J mice with AMP-DNM for 5 weeks decreased plasma levels of triglycerides and cholesterol by 35%, whereas neutral sterol excretion increased twofold. Secretion of biliary lipid also increased twofold, which resulted in a similar rise in bile flow.[151] Treatment of ob/ob mice with AMP-DNM, while restoring insulin signaling in the liver, correcting blood glucose and insulin levels, also reduced the expression of SREBP-1c target genes involved in fatty acid synthesis. AMP-DNM treatment significantly reduced liver to body weight ratio and reversed hepatic steatosis, comprising fat as well as inflammatory markers.[150] Treatment of obese ob/ob mice with GENZ 123346 was also found to result in correction of hepatic steatosis.[152]

The pleiotropic beneficial effects of AMP-DNM remain puzzling. It is far from clear that all effects can be ascribed to drug-induced lowering of glycosphingolipids. A comparison of AMP-DNM with the much more specific GCS inhibitor GENZ-123346 is informative. Both compounds reduce glycosphingolipids and concomitantly correct insulin resistance and hepatosteatosis. The superior efficacy of AMP-DNM in this respect may be ascribed to its concomitant buffering of carbohydrate assimilation. Since the broad anti-inflammatory effect of AMP-DNM and its ability to influence reverse cholesterol transport are not mimicked by GENZ 123346, it is very questionable that these actions require inhibition of glucosylceramide formation.

CONCLUSION

At present it still remains open whether glycosphingolipids directly interact with the insulin signaling pathway. On the one hand, Inokuchi and coworkers have demonstrated in a series of elegant studies that the insulin receptor (IR) reside in lipid domains enriched in gangliosides and they presented data suggesting that gangliosides like GM3 directly interact with a specific lysine residue located just above the transmembrane domain of the receptor, (see Fig. 2). On the other hand its has been postulated by Hoehn and colleagues that upstream elements of the insulin signaling pathway are not the underlying cause of insulin resistance. A reconciliation of the views may be possible by assuming that the physiological chronic insulin resistance in obese subjects requires the sustained impairment of upstream elements of the signaling pathway by excessive gangliosides.

Agents such as AMP-DNM and GENZ 123346 that lower glycosphingolipid levels by inhibition of glucosylceramide formation correct insulin resistance in cell and animal models. These findings are consistent with the hypothesis of Inokuchi and coworkers, but obviously do not proof that glycosphingolipid levels at the cell surface are the culprit during insulin resistance. The observed concomitant correction of hepatosteatosis and insulin resistance observed with AMP-DNM and GENZ 123346 is not entirely surprising. Correction of hyperinsulinemia itself is likely to result in reduced lipogenesis and inflammation in the liver.

Questions remain about the mode of action of AMP-DNM, or more simple deoxynoijirmycins as present in the leaves of Morus albus. It seems at present that the beneficial pleiotropic effects of AMP-DNM can not be explained by reduction of glycosphingolipid synthesis alone. Future investigations with conditional tissue-specific knock outs of GCS may help to shed light on the contribution of glycosphingolipid

lowering to the observed corrections of insulin resitance by AMP-DNM and GENZ 123346. Since compounds like AMP-DNM are very well tolerated, they seem to offer an attractive approach for the treatment of insulin resistance and some other aspects of the metabolic syndrome. A better understanding of the mode(s) of action of the iminosugars is required to expedite such applications.

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CHAPTER 8

GLYCOSPHINGOLIPIDS AND KIDNEY DISEASE

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Abstract: Glycosphingolipids, derived from the addition of sugar-moieties to the sphingolipid ceramide, are highly abundant in the kidney. Glycosphingolipids are known to play an important role in organ function at least in part from inherited lipid storage diseases such as Anderson-Fabry disease (Fabry's disease; FD) that results from a mutation in alpha-galactosidase A (α-GLA or α-Gal A), the enzyme responsible for catalyzing the removal of terminal galactose residues from glycosphingolipids. The inactivation in α-GLA in FD results in the accumulation of glycosphingolipids, including globosides and lactosylceramides, which manifests as several common pathologies including end-stage kidney disease. More recently, glycosphingolipids and other sphingolipids have become increasingly recognized for their roles in a variety of other kidney diseases including polycystic kidney disease, acute kidney injury, glomerulonephritis, diabetic nephropathy and kidney cancer. This chapter reviews evidence supporting a mechanistic role for glycosphingolipids in kidney disease and discusses data implicating a role for these lipids in kidney disease resulting from metabolic syndrome. Importantly, inhibitors of glycosphingolipid synthesis are well tolerated in animal models as well as in humans. Thus, an increased understanding of the mechanisms by which altered renal glycosphingolipid metabolism leads to kidney disease has great therapeutic potential.

INTRODUCTION

Glycosphingolipids are a very heterogeneous class of lipids, varying dramatically in both the hydrophilic and hydrophobic region of the molecule. The hydrophilic portion of glycosphingolipids consists of a sugar headgroup and can contain neutral or charged groups. The hydrophobic portion can vary in the length of the *N*-linked fatty acyl chain and sphingoid base as well as the degree of unsaturation, hydroxylation and branching. Glycosphingolipids have numerous roles in regulating cellular processes,

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

including cell proliferation, apoptosis, inflammation and cellular signaling. Dysregulation of glycosphingolipid metabolism leads to the accumulation of particular species of glycosphingolipids and induces several different pathologies and developmental abnormalities as evidenced from knockout studies in mice. Thus, it is not surprising that glycosphingolipids have been implicated in numerous diseases and that mutations in several enzymes involved in glycosphingolipid metabolism occur in a wide variety of human diseases. Glycosphingolipids are particularly abundant in the kidney and are thought to play an important role in kidney function. Indeed, altered glycosphingolipid metabolism occurs in a variety of kidney diseases. This chapter reviews current literature on the role of glycosphingolipids in kidney disease and discusses implications for their potential role in kidney pathologies associated with metabolic disease.

METABOLISM OF SPHINGOLIPIDS

Glycosphingolipids belong to the sphingolipid family, which all share a common sphingoid base backbone. Sphingolipid metabolism is a complex process involving many different sphingolipids and enzymes each with important and distinct cellular functions.[1] At the heart of sphingolipid metabolism is ceramide, which is comprised of a *N*-acylated (14-26 carbons) sphingosine (16 or 18 carbons). Ceramide can be generated by multiple pathways in cells, namely sphingomyelin hydrolysis, de novo synthesis, the salvage pathway, or breakdown of more complex sphingolipids (see Fig. 1). Sphingomyelinases (SMases) catalyze the hydrolysis of sphingomyelin (SM) to form ceramide and phosphorylcholine and are classified by their pH optima.[2,3] Of these, the acid and Mg^{+2}-dependent neutral sphingomyelinases have been implicated in stress-induced ceramide generation.[2,3]

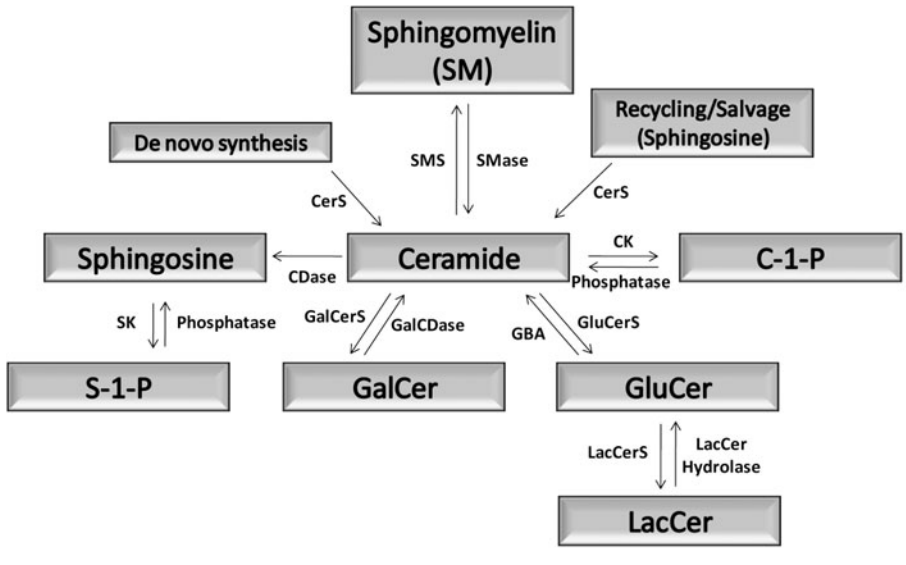

Figure 1. Skematic overview of sphingolipid metabolism.

De novo ceramide synthesis[4,5] occurs in the ER.[6] The enzyme serine palmitoyl transferase (SPT) catalyzes the first and rate limiting step in de novo synthesis, namely the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine. The enzyme 3-ketosphinganine reductase catalyzes the reduction of 3-ketosphinganine to form sphinganine. Ceramide synthases catalyze the *N*-acylation of sphinganine to form dihydroceramide (DH). A desaturase catalyzes the conversion of dihydroceramide to ceramide through the insertion of a trans double bond at the 4-5 position of the sphingoid base backbone.

Once generated, ceramide's *N*-linked fatty acyl chain can be removed to generate sphingosine as catalyzed by ceramidases (CDase). Several CDases have been identified in mammals and are classified by their pH optima.[7] The sphingosine generated by the action of CDases can be used by ceramide synthases to regenerate ceramide in the recycling/salvage pathway. Thus, ceramide synthases are thought to occupy a central position in the sphingolipid metabolic pathway as they catalyze the formation of ceramide in two distinct pathways: de novo synthesis and the recycling/salvage pathway (see Fig. 1).

In mammals, there are six ceramide synthase isoforms (CerS1-6) that have preferences for fatty acyl CoAs of particular chain lengths[8-10] to form the corresponding ceramides. It is becoming increasingly recognized that specific CerS and their resulting ceramide products contribute differently to cellular processes.[11-23] Once generated, ceramides can serve as a metabolic precursor to complex sphingolipids, such as SM and glycosphingolipids. Of note, synthesis of complex sphingolipids can influence other lipid metabolic pathways; for example sphingomyelin synthase can catalyze the transfer of a phosphocholine headgroup from phosphatidylcholine to ceramide to generate SM and diacylglycerol in the Golgi apparatus. Ceramide can also be phosphorylated by ceramide kinase to generate ceramide-1-phosphate (C1P).

Ceramide is broken down when its *N*-linked fatty acyl chain is removed to liberate sphingosine (catalyzed by CDase), which in turn is phosphorylated to generate sphingosine-1-phosphate (S1P) as catalyzed by sphingosine kinases (SK). Two isoforms of SK have been cloned and characterized in mammals, SK1 and SK2. These enzymes are emerging as important and regulated enzymes that not only modulate the levels of S1P, but also those of sphingosine and ceramide.[24,25] S1P is broken down to yield hexadecenal and ethanolamine phosphate as catalyzed by sphingosine lyase. S1P is a pro-inflammatory, pro-proliferative and anti-apoptotic sphingolipid.[25] The dynamic balance between the levels of different sphingolipids is tightly regulated by the activities of the enzymes that catalyze their formation and breakdown. The relative cellular levels of different sphingolipids are proposed to influence cellular fate because of their opposing effects and their ability to be interconverted.

METABOLISM OF GLYCOSPHINGOLIPIDS

In general, glycosphingolipids are initially divided into one of two main classes based on the first sugar moiety linked to the ceramide backbone, namely galactose or glucose of galactosylceramide (GalCer) and glucosylceramide (GluCer), respectively. The GalCer is formed in the ER via the enzyme UDP-Gal:ceramide galactosyltransferase or GalCer synthase (CGalT) via the transfer of galactose from UDP-galactose to ceramide (see Fig. 1). GalCer is then transported to the Golgi where sulfatide or galactose groups can be added, processes which mainly occur in the kidney.

Glucosylceramides are generated from ceramides synthesized in the ER that are transported to the Golgi where UDP-Glc:ceramide glucosyltransferase (glucosylceramide synthase) catalyzes the addition of glucose onto ceramide from an activated nucleotide precursor (UDP-glucose). This reaction occurs on the cytosolic face of the Golgi membranes. In mammals, a galactose group is then added from UDP-galactose to generate lactosylceramide, a reaction catalyzed by lactosylceramide synthases on the luminal surface of the Golgi. In fact, all other stepwise additions to lactosylceramide occur in the Golgi lumen. The flipping of the GlcCer from the cytosolic to the luminal leaflet of the membrane for its metabolism is a highly energetically unfavorable event and is thought to be catalyzed by a flippase.[26] Additionally, it has been proposed that the protein glycosphingolipid transport protein FAPP2 may also be involved in this transbilayer movement of GlcCer.[27]

Several different sequential modifications to lactosylceramides can occur in the lumen of the Golgi to give rise to the more complex gangliosides, lactosylsulfatides, and globotriosylceramides.[28] Thus, LacCer is essential to the formation of complex glycosphingolipids. These complex glycosphingolipids arise from additions to either the 3-O- or the 4-O-position of lactosylceramide and involve combinations of alpha- or beta-linked groups, including glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid (sialic acid) (see Fig. 2). Gangliosides are a class of complex glycosphingolipids that arise from the addition of sialic acid residues to lactosylceramide. Their names are abbreviations assigned to them according to the number of sialic acid residues and the order in which they migrate in chromatography. (For a review of glycosphingolipid metabolism, for description of the root names, or glycosphingolipid structures see refs. 29-33). Gangliosides are highly abundant in the kidney and are thought to play an important role in numerous kidney functions as described below.

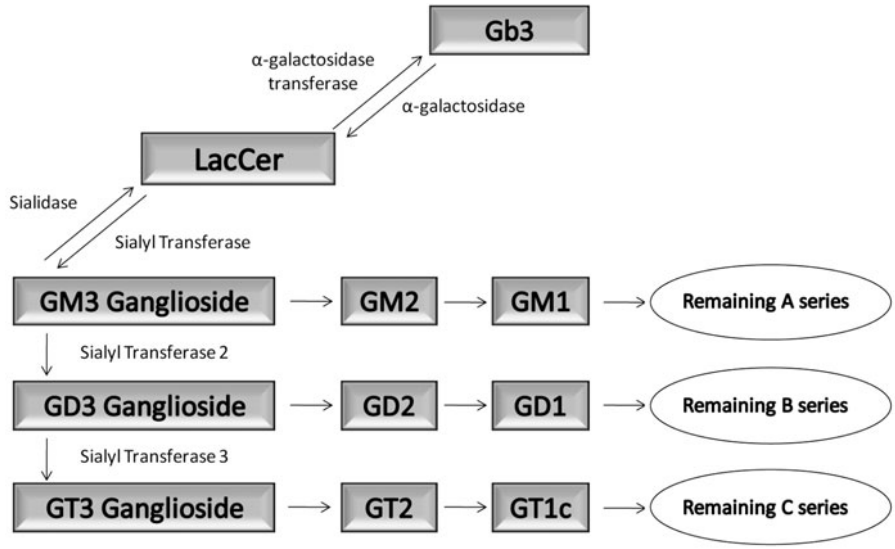

Figure 2. Skematic overview depicting of the major pathways of glycosphingolipid metabolism.

Glycosphingolipids can also be formed by degradation of more complex glycosphingolipids, which occurs mainly in the lysosome, but is known to occur at the plasma membrane and mitochondrion as well.[33-41] Indeed, several lysosomal storage diseases result from mutations in enzymes responsible for the degradation of glycosphingolipids.[32,42-49] Defective degradation of glycosphingolipids can result in serious pathologies as is described below for Fabry's kidney disease.

GLYCOSPHINGOLIPID SUBCELLULAR LOCALIZATION AND TRANSPORT

Glycosphingolipids are found in several cellular locations. On the outside of the plasma membrane, glycosphingolipids are a minor component except for the apical plasma membrane of epithelial cells in the urinary and intestinal tracts where they are highly enriched in the apical plasma membrane, representing 30-40 mole percent of the total lipids.[50-53] The myelin is enriched in galactosylceramide derived glycosphingolipids and the neuronal plasma membranes have particularly high levels of gangliosides. Intracellularly, glycosphingolipids are also found in the exocytic and endocytic pathways. Mitochondria and mitochondrial associated ER derived membranes (MAMs) have been reported to contain particular glycosphingolipids, namely GD1 and GD3 gangliosides and particular enzymes of glycosphingolipid metabolism have been localized to mitochondria and/or MAMs, including neuraminidase isoform 4 which is a sialidase and can act on both gangliosides and glycoproteins.[40,41] Enzymes that breakdown glycosphingolipids are also localized to lysosomes with inactivating mutations leading to the accumulation of particular species of glycosphingolipids as is the case with Fabry's disease and numerous others. In addition, there are also a few enzymes responsible for breakdown of glycosphingolipids that localize to the cytosol, mitochondria and the plasma membrane as is the case for sialidase isoforms 2, 3 and 4.[36,38-41,54-56] As the majority of glycosphingolipids are synthesized in the Golgi, but are localized in other subcellular locations, a system for their transport must exist.

The main system for transport of glycosphingolipids is vesicular along the exocytic and endocytic recycling pathways, which is consistent with their synthesis on the luminal side of the Golgi and enrichment in luminal surface of the endocytic vesicles and the outer surface of the plasma membrane as described in detail elsewhere.[33,57-59] It is interesting that despite the fact that vesicular transport is bidirectional, glycosphingolipids are preferentially maintained in certain subcellular compartments over others. Data in the literature suggest that it is a highly regulated process and is cell-type specific.[33,57-59] Lipid rafts and caveolin are thought to play an important role in the preferential sorting of glycosphingolipids.[58,60-62] In addition to vesicular transport, there is evidence to suggest that glucosylceramide transport to the plasma membrane occurs independently of the Golgi pathway and a glycosphingolipid transport protein has been identified.[63,64]

FUNCTIONS OF GLYCOSPHINGOLIPIDS

Glycosphingolipids regulate several different cellular processes, including cell proliferation, cell signaling, apoptosis and cell recognition. (Only an overview of the function of glycosphingolipids is given below and is not the purpose of this chapter.

For additional information the reader is referred to[28,65-74] and additional references in the section to follow). The study of roles of glycosphingolipids in cell proliferation has been facilitated through studies in mouse models.[32] Glycosphingolipids, including lactosylceramide and galactosylceramide are elevated in several types of tumors.[28,66,75-91] It has been proposed that their positive regulation of cell proliferation promotes tumor formation and growth.[28,69,92-99] Increased production of certain glycosphingolipids inhibits apoptotic mechanisms of the cell and potentially causes cancer.[100] Indeed, increased conversion of the pro-apoptotic sphingolipid ceramide to glucosylceramide occurs in a variety of cancer cells and is thought to play a role in resistance of cancer cells to radiation and chemotherapy.[85,101,102] Alternatively, other glycosphingolipids such as GD3 ganglioside have been shown to play a role in apoptosis at least in part through induction of cytochrome c release from mitochondria.[100,103-117] It is currently unclear how GD3 ganglioside is transported to mitochondria to exert its pro-apoptotic role. However, a mitochondrial role for GD3 ganglioside is supported at least in part by the fact that the sialidase isoform NEU4 localizes to mitochondrial outer membranes and that GD3 ganglioside is one of its known substrates.[41,106]

Glycosphingolipids, especially GM3, are especially important in signal transduction pathways through their carbohydrate interactions.[118] Lipid rafts in the plasma membranes of cells are lipid microdomains thought to be enriched in glycosphingolipids, sphingomyelin and cholesterol and have been proposed to play an important role in cell signaling and cell recognition.[65] Glycosphingolipid containing lipid rafts at the plasma membrane are thought to be important in organizing membrane receptors, helping to transmit various aspects of signal transduction pathways[65] and in cellular surface recognition.[119-127] At the plasma membrane, gangliosides faciliate cell-cell adhesion and are important in recognition of growth factors and immune responses.[120-127]

Gangliosides activate macrophages, inducing the production of proinflammatory cytokines.[32,128] The role of glycosphingolipids in the inflammatory response has been well studied in neuroinflammatory disease and glycosphingolipids like LacCer are throught to play an important role.[128] Glycosphingolipid accumulation in lysosomal storage diseases activates an inflammatory response at least in part due to dysfunctional trafficking.[129] The use of anti-inflammatory agents in mutant mouse models of glycosphingolipid storage diseases reduces disease progression, suggesting that glycospingolipids act in conjunction with other pro-inflammatory factors to mediate inflammation.[32] TNF-α induced ceramide has been implicated in the activation of transcription factors that encode various cytokines and chemokines.[130] Indeed, TNF-α has also been implicated in LacCer metabolism through its activation of nSMase and production of ceramide.[128,130] β-glucosidase 1 (GBA) downregulation increases production of the cytokine IL-6, which plays an important role in inflammation.[131] Pharmacological inhibition of glycosphingolipid synthesis in leptin-deficient Ob mice reduced levels of inflammatory markers in adipose tissue and improved glycemic control, further implicating the pro-inflammatory function of glycosphingolipids as the mechanism by which they mediate disease.[132]

Malfunctions in the concentrations and arrangement of glycosphingolipids can have devastating effects on many organ systems.[32] Glycosphingolipids are found in many types of cells, but are particularly abundant in the myelin sheath and epithelial cells of the renal tubules.[33] In the kidney, defects in glycosphingolipid metabolism that either result in their accumulation or enhanced degradation occurs in a variety of kidney diseases as described in the sections to follow.

FABRY'S DISEASE

Fabry's disease is an example of a lipid storage disease that in the last forty years has progressed from beginning to understand its cause to the development of effective treatments; much of this progress has been driven by breakthroughs in basic scientific research.[133] Fabry's disease is a rare X-linked lysosomal storage disease that results from mutations in the gene that encodes the lysosomal enzyme alpha-galactosidase A (α-GLA or α-Gal A). Over 150 different mutations in the gene that encodes for α-GLA have been documented to lead to the defective or reduced enzyme activity in Fabry's disease and these mutations have been described in all 7 exons of the gene.[134] The disease is estimated to occur in 1 out of every 117,000 male live births and the estimated survival in males is approximately 50 years without the use of recently available treatments.[135] Hemizygous males frequently suffer from severe renal disease and middle age onset of end stage renal failure (ESRF). Heterozygous females normally do not present with proteinuria or ESRF.[129,136,137]

α-GLA is responsible for catalyzing the hydrolysis of terminal galactosyl species from globotriaosylceramide (galactosyl-α-galactosyl-β-glucosylceramide; Gb3) as well as other glycolipids and glycoproteins (see Fig. 2). The glycosphingolipid Gb3 is one of the main glycolipid species that accumulates within the lysosomes of cells in Fabry's disease patients, resulting in the characteristic lamellar lysosomal membrane structures in cells; the most profound accumulations of Gb3 in Fabry's disease occur in the heart and kidney as well as endothelial cells throughout the vasculature.[129] α-GLA expression has been documented in all types of renal tubular and interstitial cells[138] in normal kidneys. In Fabry's disease Gb3 buildup occurs in several areas within the kidney, including glomerular cells (podocytes, mesangial and endothelial cells) and epithelial cells of the Loop of Henle and in the distal tubule, impairing the ability of the kidneys to form concentrated urine.[134] Normally, Fabry's disease patients present with proteinuria, along with tubular malfunctions such as vasopressin-resistant nephrogenic diabetes insipidus and distal tubule acidosis.[136] Advanced Fabry's disease normally leads to ESRF, but onset of ESRF depends on the type of mutation and degree of α-GLA activity present. Management of symptoms associated with kidney malfunction is required along with dialysis and renal transplantation in patients with ESRF.[134] Treatment for Fabry's through enzyme replacement with α-GLA has shown an increase in α-GLA activity, but the damage already sustained to the kidneys is often irreversible.[129] Thus, early diagnosis and intervention is key to the most effective treatment.

Although no definite mechanism has been proposed, there are several hypotheses explaining the progression of Fabry's disease to complete renal failure. Many environmental factors could contribute, but it is thought that ESRF is brought on by vascular changes inside the kidney that eventually lead to ischaemic nephropathy.[136] Other hypotheses include the accumulation of neutral glycosphingolipids that can have been shown to alter charge selective filtration as well as elevated inflammation within the kidney.[32,129,139]

POLYCYSTIC KIDNEY DISEASE

Polycystic kidney disease (PKD) represents a wide variety of renal disorders that are characterized by cystic growth in the kidneys, eventually leading to kidney failure. PKD is a common genetic disorder that affects over 500,000 Americans, with 7,000 new cases every year.[99] The major form of PKD is the result of an autosomal dominant mutation in

polycystin-1 and -2 and has been mapped to human chromosome 6.[99] Recessive forms of the disease result in childhood onset and early end-stage renal failure (ESRF).[140] Patients normally present with enlarged kidneys due to the presence of cysts in the collecting duct of the nephron. Cysts grow in size and number with increasing age. Although there are many forms of PKD, most are caused by uncontrolled tubular epithelial cell proliferation, altered regulation of apoptosis and the activation of growth factors.[141] Glycosphingolipids such as glucosylceramide (GluCer), lactosylceramide (LacCer) and GM3 have shown to regulate these processes.

The role of glycosphingolipid metabolism in PKD has been studied in several mouse models of PKD. The renal levels of GM3 and GluCer were found to be higher in PKD mouse models than in the corresponding control mice, suggesting that these glycosphingolipids might play a role in PKD. Renal glycosphingolipids are elevated in both the recessive and dominant forms of PKD have accumulations of renal glycosphingolipids. The PKD mouse models jck, Pkd1 cKO and pcy all show elevated levels of GM3, GluCer, with slightly increased, but not statistically significant, increases in ceramide. These sphingolipid profile changes are consistent with those present in human PKD kidneys.[141]

The inhibition of glycosphingolipid synthesis and the subsequent reduction in their concentrations has shown promise for translating to new treatments for PKD. The GluCer inhibitor Genz-123346 inhibits the glucosylceramide synthase and depletes GluCer without inducing accumulations in ceramide.[141] Studies have shown that administration of Genz-123346 to mouse models of PKD decreased renal levels of GluCer and GM3.[141] Importantly, Genz-123346 not only reduced cyst volume and fibrosis, but also delayed the progression/onset of PKD in mouse models.[141]

The role of glycosphingolipids in the regulation of cell cycle as well as activation of growth factors is thought to be mechanisms by which they mediate PKD development. The improvement in PKD with Genz-123346 is thought to be due to decreased cell proliferation through regulation of cell cycle progression as its use on kidney cells grown in culture delayed progression of the cell cycle at G2/M.[141] The activation of growth factors, particularly epidermal growth factors (EGF), through the phosphorylation of mitogen-activated protein kinases (MAPK) has been linked to the formation of cysts along with increased inflammation due to the upregulated activity of cyclic-AMP.[99] Importantly, in vitro lactosylceramide addition to human kidney proximal tubule cells induces phosphorylation of MAPK.[99] The recent development of novel inhibitors of glucosylceramide synthase that are well tolerated in mice as well as in humans shows much promise for treating PKD in humans.

KIDNEY CANCER

Several types of glycosphingolipids are elevated in a variety of tumors. In renal cancer, glycosphingolipids reported to be elevated include GM1, GM2, GD2, GD3 and NeuGc-GM3.[142-145] Other glycosphingolipids have been found at much lower levels in kidney cancers. For example, the glycosphingolipid disialylgalactosylgloboside and the sialyltransferase ST6GalNAc VI responsible for its synthesis were found to be downregulated in renal cancer cell lines and cancer tissues.[146] Indeed, human renal cell carcinomas (RCC) are characterized by significant changes in ganglioside composition, which is supported by documented changes in expression of enzymes involved in their metabolism in RCC.[87,147,148] For example, the sialidase NEU3 specifically degrades

gangliosides and is markedly upregulated in many renal carcinomas.[87] In addition, the glycosphingolipid GalNAc disialosyl lactotetraosylceramide is abundant in metastatic renal cell carcinomas in humans.[149] Thus, altered glycosphingolipid metabolism may be key to the growth and metastasis of renal tumors.

Various glycosphingolipids have been implicated in cellular proliferation as discussed above and this may be one mechanism by which they play a role in renal cancers. In addition to regulating cell proliferation, certain gangliosides have shown to inhibit the immune response mounted by the body against tumor cells. Gangliosides such as GM2, GD2 and GD3 accumulate in renal tumors and are shed from these tumors where they are taken up by activated T cells, inducing their apoptosis and causing immune dysfunction.[142,144,150] Understanding how glycosphingolipid metabolism is altered in renal cancers and the mechanism by which glycosphingolipids contribute to the formation and metastasis of renal tumors will allow this pathway to be targeted for the development of novel therapeutics. Indeed, inhibitors of glycosphingolipid synthesis are well tolerated in rodents as well as humans and could potentially be useful in the treatment of renal cell carcinoma.

GLOMERULONEPHRITIS

Glomerulonephritis encompasses several disorders grouped according to varying symptoms such as proteinuria, renal hypertrophy and inflammation with some variations culminating in end-stage renal disease.[151,152] Although no single mechanism has been accepted, recent studies indicate that the loss of a negative charge on proteins, glycolipids and carbohydrates in the podocytes is at least in part responsible for the decreased filtration of the glomerular capillaries.[153-155] Sialic acid is located on the outside of the podocyte and it is responsible for the filtration due to the charge repulsion of adjacent negatively charged sialic acids. This repulsion is at least in part responsible for the creation of filtration gaps.[151] The loss of sialic acid on the surface of the podocytes has been linked to proteinuria, a major indicator of human glomerular disease.[151] Sialic acid residues are cleaved by sialidases (neuraminidases) that are present in the plasma membrane, cytosol, mitochondria and lysosomes.[155] Indeed, removal of negatively charged sialic acid residues from the apical plasma membrane of podocytes via in vivo treatment with bacterial sialidase is sufficient to induce foot process effacement and proteinuria.[156-159] Importantly, the kidneys of mice carrying a mutated form of a key enzyme involved in the production of sialic acid have podocyte effacement and splitting of the glomerular basement membrane resulting in severe proteinuria that is partially rescued with dietary supplementation,[160,161] further illustrating the importance of sialic acid residues in podocyte function.

Sialidases do indeed desialylate the gangliosides GM3, GM2 and GM1.[155] The plasma membrane sialidase NEU3, acts exclusively on gangliosides and in renal cancers has been shown to be upregulated.[162] This upregulation leads to an accumulation of lactosylceramide with a concomitant decrease in GM3, influencing the ratio between negatively charged and neutral glycosphingolipids which alters the balance between cell proliferation and cell death.[162] Likewise, in puromycin induced mouse models of minimal change nephropathy, a disappearance of GD3 was noted at day 10.[163] Around day 30, the gangliosides were measured again and found to be close to those of the control mice with disease symptoms subsiding.[163] These data suggest that the loss of renal gangliosides plays a major role in minimal change nephropathy.[163]

Sialidases have been implicated in other glomerular diseases and patterns of glomerular injury, including lupus nephritis, primary and secondary focal segmental glomerulosclerosis (FSGS) and membranous glomerulopathy. Lupus nephritis is an autoimmune systemic disease which has a variable histology including membranous injury and/or extracapillary, mesangiocapillary, or mesangial proliferation and characterized by decreased renal filtration, proteinuria and inflammation. The decreased renal filtration is due to the destruction of the foot processes of the podocytes,[164] which has been proposed to be at least in part caused by defective removal of sialic acid residues from glycoproteins and glycolipids via increased activity of sialidases, resulting in the closure of filtration slits.[151] Our own unpublished data indicates that the sialidase Neu1 is upregulated in a mouse model of lupus nephritis and that the substrate for Neu1 is not a glycoprotein, but rather the ganglioside GM3 as there are concomitant accumulations in the neutral glycosphingolipid products lactosylceramide and glucosylceramide. It is possible that the immune cells within the kidney are the source of changes in glycosphingolipid metabolism as GM1 gangliosides levels are altered in the peripheral CD4+ T cells of lupus patients.[165] As lupus nephritis is an autoimmune disorder, it is not surprising we detect altered glycosphingolipid metabolism as gangliosides have long been known to play a role in a variety of autoimmune diseases.[166-172] In glomerular diseases, studies have focused on identifying changes in the patterns of the glycol-epitopes on glycoproteins in the glomerular basement membrane with little success. The decrease in negative charges was once thought to be due to loss of heparan sulfate, but this has been challenged by studies indicating that its removal from the glomerular basement membrane was insufficient to induce proteinuria.[173,174] In addition, studies examining changes in the patterns of sialoproteins have not identified a particular protein target with decreased sialysation despite evidence of increased sialic acid in the urine.[153,175-177] Even though the patient numbers were very low, one study showed an elevated level of the sialidase Neu1 of membranous glomerulopathy with elevated urinary sialic acid levels, but not changes in the glycoprotein dystroglycan.[153] Thus, it is possible that the negatively charged sialic acid residues are lost not from glycoproteins such as dystroglycan, but rather from gangliosides.

DIABETIC NEPHROPATHY AND METABOLIC SYNDROME

In STZ-induced diabetic rats, the levels of kidney glucosylceramides are significantly elevated without changes in the activity of glucosylceramide synthase.[178] However, kidney levels of glucose and UDP-glucose (a substrate for the formation of glucosylceramides) are also significantly elevated in the kidneys of STZ-induced diabetic rats.[178] Importantly, Zador et al 1993 found that the level of UDP-glucose present in the kidney under normal conditions was below the Km for glucosylceramide synthase, indicating that it is a rate limiting substrate in the synthesis of glycosphingolipids.[178] Glycosphingolipid metabolic enzymes have been shown to be regulated in vivo by elevated glucose utilization.[179] When liver glucose utilization was elevated via the overexpression of glucokinase (hexokinase IV) in the livers of STZ-induced Type I diabetic rats, there was a 6-fold upregulation in the levels of lactosylceramide synthase mRNA.[179] Alternatively, glycosphingolipids may regulate directly or indirectly glucose utilization through regulation of insulin signaling.[180,181] GM3 is thought to play a role in insulin resistance as mutant mice deficient in this ganglioside have increased insulin sensitivity.[182] In addition, pharmacological inhibition of glucosylceramide synthase in ob/ob mice that are insulin resistant was sufficient to improve glucose tolerance.[180] Likewise, inhibition of glucosylceramide synthase with two different glucosylceramide

synthase inhibitors improved glucose tolerance and insulin sensitivity in diet-induced as well as STZ-induced diabetic mice.[180,181] Reduced glycosphingolipid levels in cells induces GLUT4 translocation to the plasma membrane via enhanced formation of GLUT4 storage vesicles.[183] This recent data from the Pagano laboratory provides mechanistic insight for in vivo studies utilizing glucosylceramide synthase inhibitors to improve glycemic control and insulin sensitivity in rodent models of diabetes.[183]

Chronic hyperglycemia and insulin resistance dramatically alter kidney function and lead to diabetic nephropathy. Indeed, complex glycosphingolipids have been implicated in diabetic nephropathy where changes in glomerular sialic acids and/or sialidase activity correlate with onset of proteinuria.[184-186] In addition, studies indicate a role for ceramide in renal injury resulting from high fat diet induced nephropathy.[187] No measurements of glycosphingolipids were reported in these studies, but given that ceramides are required for the formation of glycosphingolipids, it is possible that they also are also elevated and play a role.[187] A link between glucose metabolism, insulin resistance and glycosphingolipid metabolism is clear from numerous reports in the literature,[180,181,183,188-190] suggesting a role for glycosphingolipids in diabetic nephropathy and kidney disease resulting from metabolic syndrome.

CONCLUSION

Data in the literature clearly indicate a role for glycosphingolipids in a variety of kidney diseases, including Fabry's kidney disease, polycystic disease, kidney cancers, several types of glomerulonephritis and diabetic nephropathy. Data strongly suggest a role for glycosphingolipids in kidney disease resulting from metabolic syndrome and future studies are aimed at uncovering these specific roles. Data with novel inhibitors of glucosylceramide synthase that deplete kidney glycosphingolipids without elevating ceramides show promise, not only for the translation to treatment of polycystic kidney disease in humans, but also the other kidney diseases mentioned above.

Kidney glycosphingolipids encompass a complex and highly diverse family of lipids with diverse cellular roles. Thus, the identification of the specific glycosphingolipid species involved in specific kidney pathologies is essential. The recent development of novel mass spectrometry techniques that allow for the quantification of complex glycosphingolipids will help with this challenging and essential endeavor. Inhibitors of glucosylceramide synthase deplete a plethora of different glycosphingolipids and development of additional inhibitors that target specific points in the glycosphingolipid metabolic pathway is essential. In addition, deciphering the mechanism by which the accumulation of specific glycosphingolipid species leads to specific kidney pathologies will allow for the identification of novel therapeutic targets for the development of drugs aimed at interfering with their actions in addition to their metabolism.

ACKNOWLEDGEMENTS

L.J.S. gratefully acknowledges research support from a VA CDA-2 Award, pilot project funding from the VA REAP, a ACS IRG #IRG-97-219-11 subaward and pilot project funding from the NIH/NCRR P20 RR17677 COBRE in Lipidomics and Pathobiology.

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CHAPTER 9

SPHINGOLIPID SYNTHETIC PATHWAYS ARE MAJOR REGULATORS OF LIPID HOMEOSTASIS

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Abstract: This chapter focuses on the role of sphingolipids in the regulation of sterol-regulatory element binding protein (SREBP) dependent lipid synthesis and ATP-binding cassette protein ABCA1 and ABCG1 mediated lipid efflux, key regulators of cellular lipid homeostasis. Sphingolipid synthesis activates SREBPs independently of whether sphingolipid synthesis occurs through recycling or de novo pathways. SREBPs are major transcription factors of lipid metabolism that regulate more than 30 genes of cholesterol, fatty acid and phospholipid synthetic enzymes and they required NADPH cofactors. SREBPs are downstream of sphingolipid synthesis and do not regulate activity of sphingolipid synthetic enzymes. Cells that cannot synthesize sphingolipids fail to increase SREBP in response to lipid depletion. Similar mechanisms are found in *D. melanogaster* in which SREBP activity depends on expression of a ceramide synthase analog. SREBP is inhibited by its end products cholesterol and unsaturated fatty acids. Ceramide decreases SREBP by inhibiting sphingolipid synthesis. Molecular mechanisms of regulation are related to the effect of sphingolipids on intracellular trafficking but are overall not clear. Several groups have investigated the effect of sphingolipids in the regulation of cholesterol efflux receptors ABCA1 and ABCG1, major regulators of plasma high-density lipoprotein (HDL) concentration, an important anti-atherogenic lipoprotein. Data indicate an inverse relationship between sphingolipid de novo synthesis and cholesterol efflux. Inhibition of sphingolipid de-novo synthesis increases ABCA1 mediated cholesterol efflux independent of sphingomyelin. Potential mechanisms include the physical interaction of subunit 1 of serine-palmitoyl transferase (SPT), the rate limiting enzyme of de-novo sphingolipid synthesis, with ABCA1. ABCG1 mediated efflux, in contrast, is dependent on sphingomyelin mass. Animal studies support the findings made in cultured cells. Inhibition of sphingolipid de novo synthesis increases anti-atherogenic lipoproteins and decreases atherosclerosis in mouse models. Together, manipulation of sphingolipid synthetic pathways is a potentially promising therapeutic target for treatment of low-HDL dyslipidemia and atherosclerosis.

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

INTRODUCTION

Sphingolipids are key components of cellular and subcellular eukaryotic membranes and regulate essential cell functions that include growth, apoptosis and immune function.[1,2] This chapter focuses on the role of sphingolipids in the regulation of sterol-regulatory element binding protein (SREBP) dependent lipid synthesis and ATP-binding cassette transporters ABCA1 and ABCG1-mediated cholesterol efflux, important contributors of cellular lipid homeostasis.

SREBPs ARE KEY TRANSCRIPTION FACTORS OF PROTEIN THAT SYNTHESIZE CHOLESTEROL, FATTY ACIDS AND PHOSPHOLIPIDS

The SREBPs are transcription factors that were discovered in 1993.[3,4] SREBPs directly activate the expression of more than thirty genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglyceride and phospholipids, as well as the NADPH cofactor required to synthesize these molecules.[5] Three SREBP isoforms are found in mammalian cells, one SREBP isoform is found in drosophila.[5-7] Regulation of SREBPs occurs transcriptionally and posttranscriptionally. Insulin mediated increase of the liver X receptor (LXR) factor is the major transcriptional activator of SREBP.[8] The posttranscriptional activation of SREBP is more complex and can occur at different levels (Fig. 1). The inactive precursor form of SREBP (pSREBP) is located in the endoplasmic reticulum where it is bound to a sterol-sensing protein, SCAP. Cholesterol promotes the association of the Insig protein to the SREBP-SCAP complex. This association anchors SREBP in the endoplasmic reticulum and inhibits SRE-mediated gene transcription. When cells are lipid depleted, SREBP and SCAP are trafficked by vesicular transport to the Golgi apparatus. SCAP is recycled to the endoplasmic reticulum. In the Golgi, pSREBP undergoes proteolysis by two distinct enzymes. These steps generate mature SREBP (mSREBP), basic helix-loop-helix-leucine zipper (bHLH) transcription factors.[9] mSREBP-1 activated genes include the rate limiting enzymes of fatty acid synthesis as well as Δ6-desaturase that generates long chain unsaturated fatty acids from essential fatty acids and fatty acid CoA ligase, an enzyme that enables the incorporation of fatty acids into triglycerides, phospholipids and ceramides. mSREBP-2 activates the LDL receptor gene and essentially all genes required for cholesterol synthesis.[6] Sphingolipid synthetic enzymes are not regulated by SREBPs.

SPHINGOLIPIDS SYNTHESIS IS REQUIRED TO INCREASE SREBP IN LIPID DEPLETION

SREBPs are activated by lipid depletion. This observation had led to the discovery of this class of transcription factors.[3,4] Consecutive studies in mammalian cells and drosophila demonstrated that sphingolipid synthesis is required for the activation of SREBP.[7,10-12] Synthesis of sphingolipids occurs through recycling and de-novo pathways. The de-novo pathway is increased when the recycling pathway cannot supply enough sphingoid bases. Long chain sphingoid bases are derived from sphingomyelin or glucosylceramides. Sphingolipid de-novo synthesis is increased in rapidly dividing cells and in the epidermis. The initial step in de-novo sphingolipid synthesis is the condensation

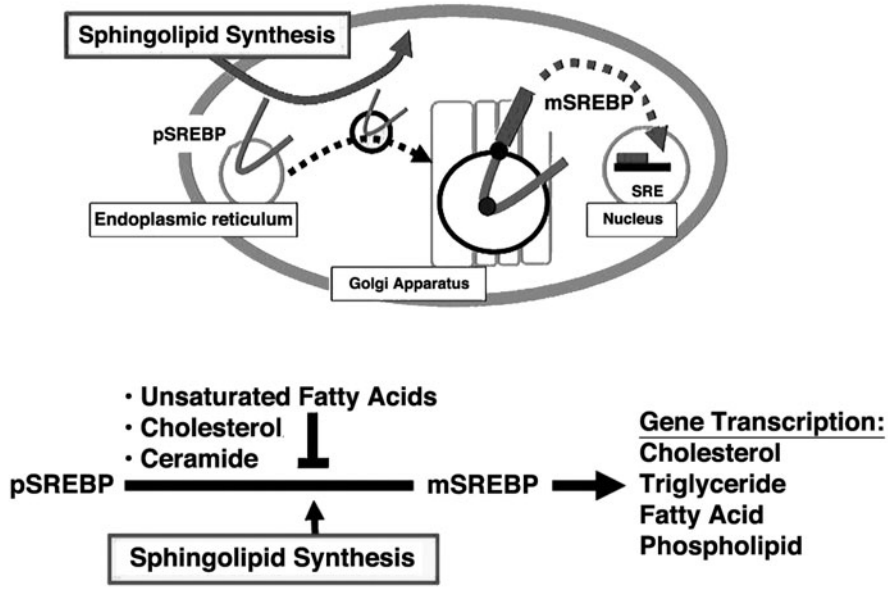

Figure 1. Sphingolipid synthesis activates SREBP transcription factors. Sterol-regulatory-element binding proteins (SREBPs) are membrane proteins of the endoplasmic reticulum. The inactive precursor SREBP (pSREBP) resides in the endoplasmic reticulum. When cells are lipid depleted pSREBP translocates to the Golgi apparatus. In the Golgi, two distinct proteases cleave pSREBP and release the transcriptional active mature SREBP (mSREBP). SREBPs regulate a total of thirty-three genes of cholesterol, triglyceride, fatty acid and phospholipid metabolism. Sphingolipid synthesis is necessary to activate SREBP. Cells that are defective in sphingolipid synthesis fail to increase SREBP in lipid depletion. Unsaturated fatty acids, cholesterol and ceramide inhibit SREBP. Ceramide inhibits SREBP by inhibiting sphingolipid synthesis.

of L-serine and palmitoyl-CoA. This reaction takes place on the cytosolic surface of the endoplasmic reticulum and is catalyzed by serine-palmitoyl transferase (SPT). SPT is a heterodimer in which the SPTLC1subunit associates with one of the other known subunits, SPTLC2 or SPTLC3.[13,14] The different SPT subunits confer distinct acyl-CoA substrate specificities.[15] The SPTLC3 unit generates short chain sphingoid bases.[16] The SPT reaction generates 3-ketodihydrosphingosine. 3-Ketodihydrosphingosine is reduced to sphinganine.[17] Sphinganine is acetylated with saturated fatty acyl-CoAs to generate ceramides or dihydroceramide. This reaction is mediated by ceramide synthases. There are six different ceramide synthases in humans and one ceramide synthase, schlank, in drosophila melanogaster.[11,18,19] In humans, ceramide synthases display overlapping fatty acid preferences and tissue expression patterns. Ceramide is the central product of sphingolipid synthesis. Accumulation of ceramide leads to apoptosis. Most of the ceramide is further metabolized to lipids including sphingosine, ceramide-1-phosphate, glucosylceramides and sphingomyelin. For sphingomyelin synthesis, ceramide is transported from the ER membrane to the Golgi. This reaction is mediated by the CERT protein and requires active oxysterol binding protein proteins.[20-22] Sphingomyelin is synthesized by two sphingomyelin synthases, they are site specific and located on the luminal side of the Golgi apparatus or at the plasma membrane.[23,24]

We showed that sphingolipid synthesis is necessary to activate SREBP.[10] These studies were carried out in Chinese-hamster ovary (CHO) cells and in LY-B cells. LY-B cells are CHO cells in which the SPTLC1 subunit is mutated. LY-B cells are unable to produce sphingolipids de-novo and depend on exogenous sphingolipids for survival. When LY-B cells are incubated in lipid depleted medium, SREBP is not increased and cells die. Cells increase SREBP and SRE-mediated gene expression when sphingosine or sphinganine, substrates of sphingolipid synthesis, are supplied in the incubation medium. Sphingolipid mediated regulation of SREBP occurs independently of cellular cholesterol concentration. This mechanism of regulation is also observed, albeit to a smaller degree in wild type CHO cells. Sphingosine or sphinganine increase SREBP and SRE-mediated gene transcription in CHO cells. Similar mechanisms that demonstrated a sphingolipid synthesis dependency of SREBP were recently found in *D. melanogaster*.[11] One SREBP is found in drosophila. Drosophila do not produce sterols, but in this model SREBP controls fatty acid metabolism and is regulated by phosphatidylethanolamine, its major phospholipid.[7,25,26] In drosophila, activation of SREBP depends on functional 'schlank', a ceramide synthase analogue. Mutations in 'schlank' or siRNA mediated downregulation of schlank decreases (dihydro)ceramides and SREBP. Decreased SREBP decreases triacylglycerol synthesis and mass of storage fat.[11] A compensatory increase of sphingolipid synthesis could also underlie the observation that glycosphingolipid deficiency caused inhibition of ceramide glucosyltransferase, increases SREBP.[12] Animal models have confirmed the relevance of sphingolipid synthesis as a regulator of lipid metabolism. Two independent groups demonstrated decreased atherosclerotic lesions in apolipoprotein E knock-out mice treated with the SPT inhibitor myriocin.[27-31] One group related the beneficial effect of sphingolipid inhibition to a reduction in SREBP activity and showed that lowering of sphingolipids and atherogenic plasma lipids led to regression of pre-existing atherosclerotic lesions.[29,31] Further support for the role of sphingolipids as regulators of lipid metabolism comes from studies that evaluated targets of sphingosine-1-phosphate, an endproduct of sphingolipid synthesis and important lipid messenger. It was shown that sphingosine-1-phosphate increases Cyp-17, an important steroidogenic gene of the adrenal cortex is mediated by activating SREBP. Mechanisms by which sphingosine-1-phosphate increases SREBP are currently not known.[32]

CHOLESTEROL, UNSATURATED FATTY ACIDS AND CERAMIDES INHIBIT SREBP

SREBP is inhibited by cholesterol and polyunsaturated fatty acids, end-products of SREBP-dependent lipid synthesis and ceramide.[3,4,33] Mechanisms by which cholesterol inhibits SREBP are well known. Cholesterol promotes residence of SREBP in the endoplasmic reticulum by promoting the interaction of SCAP with Insig. This interaction prevents translocation of pSREBP to the Golgi. Mechanisms how polyunsaturated fatty acids inhibit SREBP are less clear. Characteristic for fatty acid mediated inhibition of SREBP is that inhibition increases with chain length and degree of unsaturation. Notably, unsaturated fatty acid mediated inhibition of SREBP is cholesterol independent as it persists in sterol depletion.[33] One mechanism by which fatty acids decrease SREBP is linked to their ability to affect the biophysical interaction between cholesterol and sphingomyelin in membranes. Unsaturated fatty acids mobilize transfer of cholesterol and sphingomyelin between model membranes.[34,35] Inhibition of SREBP could be a result of fatty acid mediated

displacement of cholesterol to intracellular pools. Another mechanism is linked to the ability of unsaturated fatty acids to increase sphingomyelinase activity. Sphingomyelinase decreases sphingomyelin mass and forces the associated cholesterol to translocate to extracellular acceptors or to translocate to intracellular sites. Increased intracellular cholesterol, in turn, inhibits SREBP.[34,36,37] Another, cholesterol-independent mechanism of regulation occurs though the generation of ceramide, a product of sphingomyelinase. We showed that ceramides inhibit SREBP, independent whether ceramides are generated endogenously or exogenously. The effect of ceramides is additive to polyunsaturated fatty acids and occurs also in lipid depletion mediated by incubation in lipid free medium or cationic amphiphiles that sequester cholesterol. Ceramides increase levels of pSREBP and decrease levels of mSREBP. This finding indicates that ceramides inhibit translocation of pSREBP from the endoplasmic reticulum to the Golgi.[10] More recent studies indicate that the ceramide mediated inhibition of SREBP is more significant than cholesterol translocation mediated pathways. Experiments in cultured cells compared the effect of sphingomyelinase C and sphingomyelinase D on cholesterol synthesis. Both treatments decrease sphingomyelin mass, the difference is that sphingomyelinase C generates ceramides and phosphocholine while sphingomyelinase D generates ceramide phosphate. Results demonstrated that sphingomyelinase C decreased HMG-CoA reductase by 90% in comparison to sphingomyelinase D, which decreased HMG-CoA reductase by 10%.[38] Extrapolation of these results indicates that sphingomyelinase mediated inhibition of SREBP is mainly mediated by generation of ceramides. Our studies demonstrated that ceramide decreased SREBP by inhibiting sphingolipid synthesis.[10] Together, the data are consistent with a model in which sphingolipid synthesis and sphingolipids coordinate the transcriptional control of lipids that maintain membrane composition.

ABCA1 AND ABCG1 ARE KEY LIPID EFFLUX RECEPTORS THAT DETERMINE ANTI-ATHEROGENIC HIGH-DENSITY LIPOPROTEIN (HDL) LEVELS

Extensive epidemiological data have shown an inverse relationship between high density lipoprotein (HDL) levels and cardiovascular disease.[39,40] Notably, plasma HDL levels remain an independent predictor for suffering cardiovascular events even in patients with low low-density lipoprotein (LDL) levels.[41] Plasma HDL levels are determined by cholesterol efflux. Studies from several laboratories have demonstrated a major role for the ATP-binding cassette receptors A1 (ABCA1) and ABCG1 as regulators of cholesterol efflux and plasma HDL levels. ABCA1, the mutant molecule in Tangier disease, promotes net cholesterol efflux to lipid-poor Apo A1 while ABCG1 facilitates net cholesterol efflux to HDL particles.[42-45]

SPHINGOLIPIDS AFFECT ABCA1 AND ABCG1 BY DIFFERENT MECHANISMS

Several groups have investigated the effect of sphingolipids in the regulation of cholesterol efflux receptors ABCA1 and ABCG1 (Fig. 2). Sphingolipids affect cholesterol efflux through mechanisms related to sphingolipid de-novo synthesis and related to sphingomyelin mass in the plasma membrane. ABCA1mediated cholesterol efflux

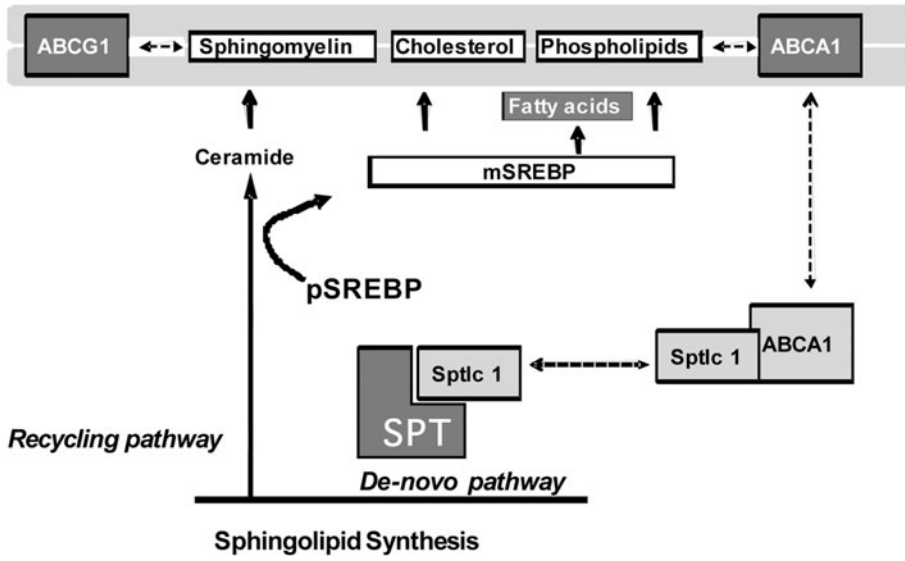

Figure 2. Sphingolipid synthesis through recycling and de-novo pathways regulates a feedback loop that determines cholesterol synthesis and efflux—A model. Sphingolipid homeostasis is maintained by synthesis through recycling and de-novo pathways. The de-novo pathway is activated when cells are sphingolipid depleted. Serine-palmitoyl transferase (SPT) is the rate limiting enzyme of sphingolipid de-novo synthesis. SPT is a heterodimer. Subunit SPTL1 dimerizes with subunit SPTLC2 or subunit SPTLC3. Both pathways function to maintain membrane integrity. Both pathways produce sphingomyelin, a key component of the plasma membrane activate that associates with cholesterol, a product of SREBP within the phospholipid bilayer. ABCG1 and ABCA1 are important cholesterol efflux receptors. ABCG1 promotes cholesterol and sphingomyelin efflux. ABCG1 mediated cholesterol efflux to HDL depends on sphingomyelin. Decreased sphingomyelin inhibits ABCG1 mediated cholesterol efflux. ABCA1 promotes cholesterol and phosphatidylcholine efflux. ABCA1 mediated cholesterol efflux to apoA1 is independent of sphingomyelin and inversely related to sphingolipid de-novo synthesis. Potential mechanisms include the physical interaction of ABCA1 with SPTLC1 that decreases ABCA1 expression at the plasma membrane. In the above model, all conditions that increase the de-novo pathway reduce cholesterol efflux. Cholesterol efflux is restored when endogenous sphingolipid synthesis reaches a threshold that inhibits de-novo pathway. Under these conditions, characterized by presence of sphingomyelin and absence of LCB1, cholesterol efflux by ABCG1 and ABCA1 is restored.

has been shown to be increased by ceramide and glucosylceramide synthase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP).[46,47] Mechanisms for this effect are not entirely known. It has been proposed that ceramide shape and the amide bond are critical for the effect on ABCA1 mediated cholesterol efflux.[48] Interestingly neither group found an effect of sphingomyelin on transcription or expression of ABCA1. Based on a recent study, one possibility is that the effects are mediated by inhibition of sphingolipid de-novo synthesis. Notably, ceramide is a negative feedback inhibitor of sphingolipid de-novo synthesis and PDMP is known to increase ceramide.[10,49] It was recently demonstrated that the SPT subunit SPTLC1 interacts physically with ABCA1.[50] This interaction blocks ABCA1 in the endoplasmic reticulum, leads to decreased expression of ABCA1 at the plasma membrane and results in decreased cholesterol efflux. Myriocin, a competitive inhibitor of SPT, abolishes the interaction of SPTLC1 with ABCA1. This results in increased expression of ABCA1 at the plasma membrane

and increases cholesterol efflux. According to this model, ceramide and PDMP mediated increase in cholesterol efflux would be caused by their inhibitory effect on sphingolipid de-novo synthesis. In contrast to ABCA1 mediated cholesterol efflux, ABCG1 mediated cholesterol efflux depends on sphingomyelin. Pivotal studies were carried out in LY-A. LY-A cells are low in sphingomyelin due to mutations in the CERT protein. Expression of ABCG1 in LY-A cells fails to promote cholesterol efflux. Overexpression of CERT and reconstitution of membrane sphingomyelin restores the ability of ABCG1 to promote cholesterol efflux to HDL. These studies demonstrated that ABCG1 mediated efflux depends on sphingomyelin mass.[51] Overall, studies that evaluated the effect of sphingolipids on cholesterol efflux are consistent with a model in which sphingolipid de-novo synthesis regulates cholesterol efflux by affecting ABCA1 mediated cholesterol efflux directly and affecting ABCG1mediated cholesterol efflux indirectly. Conditions that increase the de-novo pathway reduce cholesterol efflux and increase SREBP mediated cholesterol synthesis. Cholesterol efflux is restored when the de-novo pathway is inhibited by endogenous feedback inhibition. This is achieved when sufficient sphingolipids are available to supply long chain bases for the recycling pathway. Under these conditions, characterized by presence of sphingomyelin and absence of LCB1, cholesterol efflux by ABCG1 and ABCA1 is restored.

SPHINGOMYELIN INDUCES LIPOPROTEIN PATTERNS THAT CORRELATE WITH INCREASED CARDIOVASCULAR RISK

Epidemiological studies demonstrated that sphingomyelin plasma levels are an independent risk factor for coronary heart disease.[52,53] Consistent with this observation is that over-expression of sphingomyelin synthase shifts plasma cholesterol and sphingomyelin concentration to the pro-atherogenic nonHDL lipoproteins.[54] Potential mechanisms for the undesired effect of sphingomyelin are the effect of sphingomyelin to enhance lipoprotein aggregation and the effect of sphingomyelin on lipoprotein kinetics.[55,56] Increased sphingomyelin content in triglyceride rich particles decreases both affinity for and catalytic activity of lipoprotein lipase resulting in decreased lipolysis.[57] Increased sphingomyelin in HDL particles decreases the activity of lecithin cholesterol acyl transferase (LCAT). This results in decreased generation of mature HDL and thus decreased HDL mediated reverse cholesterol flux.[58,59] Together, increased triglyceride and decreased plasma HDL concentration constitute a lipoprotein profile associated with increased cardiovascular risk, formation of atherosclerotic lesions and macrophage accumulation. Data obtained from the sphingolipid storage disease Niemann Pick Type A and B are consistent with this concept. Niemann Pick disease Type A and B is caused by mutations in the gene for acid sphingomyelinase. Sphingomyelin and cholesterol are severely increased in Niemann Pick disease Type A and B. Sphingomyelin constitutes up to 70 % of phospholipids compared to normal concentrations of 5-20%, cholesterol mass is increased up to ten fold.[60] The lipid abnormalities are clinically significant. A cross sectional study of 10 children and adolescents with Niemann Pick disease Type A and 30 patients with Niemann Pick disease Type B demonstrated significant atherogenic lipoprotein profiles specifically characterized by low plasma HDL-cholesterol.[61] Coronary artery calcium scores, an indicator for the development of atherosclerosis, were positive in 10 of 18 Type B patients studied.

CONCLUSION

Sphingolipids regulate lipid homeostasis by affecting SREBP dependent lipid synthesis and ABCA1 and ABCG1 dependent cholesterol efflux, major regulators of plasma high-density lipoprotein (HDL) concentration, an important anti-atherogenic lipoprotein. Sphingolipid synthesis is an obligate activator of SREBPs, key transcription factors of cholesterol, fatty acid and phospholipid synthesis. Inhibition of sphingolipid synthesis decreases SREBP on the post transcriptional level. The precursor form of SREBP is not processed to the transcriptional active mSREBP. Sphingolipid de-novo synthesis is inversely related to cholesterol efflux by ABCA1 and ABCG1. Mechanisms for decreased ABCA1 mediated cholesterol efflux include the physical interaction of the SPT subunit SPTLC1 with ABCA1. ABCG1 mediated cholesterol efflux depends on sphingomyelin. Cholesterol efflux by ABCG1 and ABCA1 is restored in conditions that inhibit sphingolipid de-novo synthesis. Another aspect of the effect of sphingolipid synthesis on cardiovascular disease is that sphingomyelin increases pro-atherogenic lipoprotein patterns and susceptibility to particle aggregation. Animal studies support the findings made in cultured cells. Inhibition of sphingolipid de-novo synthesis increases anti-atherogenic lipoproteins and decreases atherosclerosis in mouse models. Together, manipulation of sphingolipid synthetic pathways is a potentially promising therapeutic target for treatment of low-HDL dyslipidemia and atherosclerosis.

ACKNOWLEDGEMENT

T.S. Worgall is supported by NIH Grant NIA KO8 AG025833 and Cystic Fibrosis Pilot and Feasibility Award 07010.

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INDEX

E

F

G

H

I

K

L

M

N

O

P

S

T

U

V