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Sphingolipids in Obesity, Type 2 Diabetes, and Metabolic Disease

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Abstract

Metabolic disease, including obesity and type 2 diabetes, constitutes a major emerging health crisis in Western nations. Although the symptoms and clinical pathology and physiology of these conditions are well understood, the molecular mechanisms underlying the disease process have largely remained obscure. Sphingolipids, a lipid class with both signaling and structural properties, have recently emerged as key players in most major tissues affected by diabetes and are required components in the molecular etiology of this disease. Indeed, sphingolipids have been shown to mediate loss of insulin sensitivity, to promote the characteristic diabetic pro-inflammatory state, and to induce cell death and dysfunction in important organs such as the pancreas and heart. Furthermore, plasma sphingolipid levels are emerging as potential biomarkers for the decompensation of insulin resistance to frank type 2 diabetes. Despite these discoveries, the roles of specific sphingolipid species and sphingolipid metabolic pathways remain obscure, and newly developed experimental approaches must be employed to elucidate the detailed molecular mechanisms necessary for rational drug development and other clinical applications.

Introduction

Type 2 diabetes is an increasingly important healthcare concern in Western nations, with incidence on the rise in both Europe and the United States (Passa 2002; Wei et al. 2011). This disease, which is diagnosed based on blood plasma hyperglycemia, has been linked to lipid overload and abdominal obesity and may synergize with these conditions to promote negative clinical outcomes (Maheux et al. 1994; Nussey and Whitehead 2001; Pontiroli and Camisasca 2002; Grundy et al. 2004; Banegas et al. 2007; Qiao and Nyamdorj 2010). This connection is significant, as the incidence of obesity is also increasing, with greater than 65% of individuals classified as overweight or obese in both the United States and the European Union (Martinez et al. 1999; Baskin et al. 2005; Fry and Finley 2005; Samad et al. 2006; Mueller-Riemenschneider et al. 2008). This chapter summarizes current knowledge of the sphingolipid-dependent manifestations of obesity and diabetes and points to areas for future study and potential clinical utility.

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Pathogenesis of Type 2 Diabetes Mellitus

Diabetes develops not as a single-organ disease but as a syndrome progressively affecting multiple organ systems. Because diabetes is silent early on and often is only discovered after onset of complications, it has been difficult to tease out a complete natural history of this disease. However, careful epidemiological and laboratory studies have revealed a general outline of disease progression.

It is believed that insulin resistance is the primary defect in type 2 diabetes mellitus, preceding frank hyperglycemia (Cavaghan et al. 2000). In many diabetics, the initial cause of insulin resistance may be hyperlipidemia consequent to abdominal obesity (Pouliot et al. 1990). Excess free fatty acids promote insulin resistance in peripheral tissues, including the skeletal muscle and liver; at first, pancreatic β-cells are able to compensate for this by increasing insulin secretion (Bevilacqua et al. 1987; Hirose et al. 1996; Roden et al. 1996). This compensated state, in which hyperinsulinemia effectively controls plasma glucose levels, may persists for years and, in fact, may never progress to overt diabetes mellitus (Leahy 2008). Over time, the ability of the pancreas to produce high levels of insulin may be exhausted due both to aberrant cell signaling and to β-cell loss to apoptosis (Hirose et al. 1996; Pick et al. 1998). This decompensation results in the hyperglycemia observed in type 2 diabetes, which occurs as a result of both continually elevated hepatic glucose output and reduced peripheral glucose uptake and utilization. At this point, the hyperglycemia itself damages pancreatic β-cells, accelerating the course of β-cell insufficiency and disease progression (Maedler et al. 2003).

Type 2 diabetes is not merely a disease of the pancreas. Rather, insulin resistance and dysregulation of plasma glucose and free fatty acid levels can produce severe dysfunction in multiple organs, including the heart, kidneys, liver, and eyes (as discussed in the following sections), as well as the brain and peripheral nervous system. Insidiously, tissue damage can begin before noticeable decompensation of insulin resistance, causing patients to suffer silent tissue damage long prior to diagnosis (Nerpin et al. 2008; Dinh et al. 2010; Dinh et al. 2011; Manchanayake et al. 2011). Through this multisystemic onslaught, diabetes slowly saps patients of both longevity and quality of life. Thus, it is critical to understand the mechanisms underlying this disease and to develop rational treatment regimens to halt this cascade of dysfunction.

Sphingolipid Metabolism and Regulation

Sphingolipids are a class of structural and signaling molecules incorporating a unique dihydrosphingosine backbone, which may also be present in its desaturated form as sphingosine (Gault et al. 2010). These lipids play important roles in determining membrane biophysical properties, topology, and integrity and in numerous cellular signaling processes, particularly apoptosis and proliferation (Zheng et al. 2006; Hannun and Obeid 2008). Sphingolipid-dependent mechanisms of pathogenesis have been demonstrated in many disease contexts, and sphingolipids have emerged as important players in obesity and diabetes (Shimabukuro et al. 1998; Summers and Nelson 2005; Zheng et al. 2006; Hannun and Obeid 2008). Importantly, sphingolipid metabolism is highly interconnected by a well-

characterized network of enzymes, making it possible to study mechanisms of sphingolipid production and signaling with great precision (Gault et al. 2010). This metabolic system, which we recently reviewed, (Brice and Cowart 2011) is described in detail in several other chapters

Given the great complexity of sphingolipid metabolism, it follows that its regulation may also be complex or multifactorial. Indeed, it has been demonstrated that sphingolipid synthesis can be regulated at both the substrate level and through signaling in response to cellular stimuli. In perhaps the most intuitive manner of regulation, which is extremely relevant to the study of type 2 diabetes, fatty acids impact sphingolipid levels through both substrate supply and regulation of the enzymes of sphingolipid metabolism (Shimabukuro et al. 1998; Hu et al. 2009; Hu et al. 2011). Initially, oversupply of saturated fatty acids directly stimulates sphingolipid synthesis *via* substrate supply (Merrill et al. 1985; Shimabukuro et al. 1998). This excess of fatty acids provides additional acyl-CoA moieties for *de novo* production of sphingoid bases by serine palmitoyltransferase (SPT) and *N*-acylation of sphingoid bases by CerS. In a complementary manner, fatty acid supply also regulates expression of the enzymes of sphingolipid metabolism, including SPT, dihydroceramide desaturase 1 (Des1), and sphingosine kinase 1 (SphK1), thus up-regulating sphingolipid production in conditions of steatosis (lipid overload) (Hu et al. 2009; Hu et al. 2011). Strikingly, regulation of these enzymes by fatty acids appears to occur in a chain length- and saturation-dependent manner, with unsaturated fatty acids protecting against up-regulation of SphK1, SphK2, and Des1 (Hu et al. 2009; Hu et al. 2011).

Sphingolipid metabolism is also regulated in a substrate-independent manner by other components of the diabetic physiologic phenotype. Indeed, sphingolipid metabolism can be activated by several aspects of diabetic physiology, including the presence of inflammatory cytokines, growth factors, and oxidative stress (Goldkorn et al. 1998; Memon et al. 1998; Levy et al. 2006; Samad et al. 2006; Sultan et al. 2006; Aerts et al. 2007; Levy et al. 2009). *In vivo*, these conditions were all observed in the adipose tissue of genetically obese, diabetic *ob/ob* mice (Samad et al. 2006). Compared to their lean counterparts, genetically obese mice demonstrated significantly higher expression of enzymes promoting ceramide and sphingosine/sphingosine-1-phosphate accumulation, including acid- and neutral sphingomyelinase, alkaline- and acid ceramidase, and SPT. In contrast, levels of enzymes that would route ceramide to more complex metabolites, including glucosylceramide synthase and *N*-acetylneuraminylgalactosylceramide (GM3) synthase, were downregulated in the diabetic mice. This indicated that in the diabetic state, expression of sphingolipid-metabolizing enzymes is recalibrated toward generation of pro-inflammatory and pro-apoptotic species. Furthermore, direct exposure of HepG2 hepatocellular carcinoma cells to the inflammatory cytokines TNFα and IL-1 directly stimulated SPT expression and activity *in vitro*, providing a direct mechanistic link between these inflammation and sphingolipid synthesis (Memon et al. 1998). Other work has suggested a similar stimulatory effect of inflammatory cytokines on the sphingolipid recycling pathway, in which ceramides are deacylated to form sphingosine and then reacylated (Sultan et al. 2006). Likewise, it has been shown that oxidative stress promotes ceramide accumulation in other disease contexts, particularly through stimulation of neutral sphingomyelinase 2 (NSMase2) and the recycling

pathway (Goldkorn et al. 1998; Levy et al. 2006; Sultan et al. 2006; Levy et al. 2009). Thus, the conditions present in diabetes stimulate sphingolipid production and increased expression of the enzymes of sphingolipid metabolism.

Adipose Tissue in Obesity and Diabetes

Adipose tissue primarily functions to store free fatty acids (FFA) after food intake and to release FFA in the fasting state (Hajer et al. 2008). However, with the rise in obesity, adipose tissue has emerged as an important tissue in the metabolism of lipids and glucose and possibly the induction of insulin resistance and type 2 diabetes (Samad et al. 2006; Hajer et al. 2008; Bonzon-Kulichenko et al. 2009). Adipose has been demonstrated to act in an endocrine fashion by producing a multitude of factors that include a variety of pro-inflammatory cytokines and chemokines collectively known as adipokines (Mohamed-Ali et al. 1997; Christiansen et al. 2005; Hotamisligil 2006; Samad et al. 2006; Samad et al. 2011), and the production of these inflammatory mediators is believed to mediate the initiation of insulin resistance in distal tissues such as skeletal muscle and liver.

A proposed mechanism for obesity-induced insulin resistance in distal tissues is by increased intracellular lipid accumulation. Induction of the expression and production of adipokines results in increased lipolysis in adipocytes and elevated release of FFA. Adipocytes at this point lose their ability to store FFA and as a result promote the accumulation of lipids such as liver, skeletal muscle and pancreas. The inappropriate storage of lipids in these tissues results in the activation of signaling pathways that interfere with insulin signaling and ultimately contribute to insulin resistance in adipose, muscle, and liver. Collectively, these actions have been termed “lipotoxicity”. A key outcome of lipotoxicity is insulin resistance, which precedes Type 2 diabetes (Turpin et al. 2009).

Indeed, significant changes in the sphingolipids produced by the adipose tissue have been reported in genetically obese (ob/ob) mice (Samad et al. 2006). Compared to their lean counterparts, ob/ob mice demonstrated significantly higher levels of expression of acid sphingomyelinases (ASMase), NSMase, and SPT. When the levels of sphingolipids within the adipose tissue were measured, it was found that there were decreased levels of total sphingomyelin and ceramide and increased levels of sphingosine. These data suggest that sphingomyelin may be hydrolyzed to ceramide and further converted to sphingosine. Consistent with this, the expression of both alkaline- and acid-ceramidases were increased in the adipose tissue of ob/ob mice over their lean counterparts. Analysis of plasma sphingolipids from lean and ob/ob mice found that there were increases in sphingomyelin, ceramide, sphingosine and S1P (Samad et al. 2006). There was no detectable S1P in the adipose tissue of these mice. These data support studies that suggest increased sphingolipid levels in the plasma of obese mice are associated with increased cardiovascular risk in obesity (Auge et al. 2004; Hojjati et al. 2005). Not only have studies evaluated sphingolipid metabolism in genetically obese mice, they have evaluated sphingolipid metabolism in diet induced obesity models as well. In this study, C57BL/6J mice were maintained on a high fat (60% calories from fat) or a low fat (10% calories from fat) diet for 16 weeks. In the mice fed the high fat diet (HFD), ceramide levels in the adipose and the plasma were increased as evidenced by increases of the enzymes SPT, ASMase, and NSMase (Shah et al. 2008).

Studies evaluating changes to sphingolipid metabolism in human adipose tissue remain scarce and have demonstrated that ceramide and triacylglycerides are elevated in the adipose tissue of obese individuals (Kolak et al. 2007). Other studies have demonstrated that inflammation in human adipose tissue affects sphingolipid metabolism in other tissues such as skeletal muscle (Lam et al. 2011).

The data demonstrate that sphingolipid synthesis is disrupted in adipose tissue in response to increased fatty acid influx. As mentioned above, the source of excess FFA delivery from adipose to other tissues such as skeletal muscle leads to loss of insulin sensitivity. In fact, additional roles for sphingolipids in these disease processes are also emerging.

Sphingolipids in Skeletal Muscle

The sensitivity of skeletal muscle to insulin is critical for the removal of glucose from the bloodstream as it accounts for approximately 40% of total body mass and 80% of whole body insulin-stimulated glucose disposal (Gorski et al. 2002; Bruni and Donati 2008). It is for these reasons that skeletal muscle is believed to contribute most significantly to the glucose intolerance associated with nutrient oversupply and obesity.

In insulin-sensitive tissues, insulin promotes the uptake of glucose (Summers 2006). Insulin signaling is initiated through the activation of the insulin receptor. Insulin receptor substrates (IRS proteins) recruit and activate the phosphatidylinositol 3-kinase (PI3K) (Keller and Lienhard 1994; Summers 2006), which in turn produces phospholipid phosphatidylinositol (3,4,5)-triphosphate (PIP3), a bioactive lipid that mediates recruitment of the cytosolic enzyme Akt/protein kinase B (PKB) to the plasma membrane (Keller and Lienhard 1994). Phosphorylation of the Thr308 in the activation loop and Ser473 in the hydrophobic C-terminal domain by PIP3 is required for full activation of Akt/PKB (Vanhaesebroeck and Alessi 2000; Summers 2006).

Akt/PKB promotes the uptake of glucose in skeletal muscle by stimulating translocation of Glucose Transporter 4 (GluT4) resulting in an increase in the rate of glucose flux into the tissue, creating a state of decreased circulating glucose and stimulating the its conversion to energy storage molecules such as glycogen (Hajduch et al. 1998; Hajduch et al. 2001; Whiteman et al. 2002). Akt/PKB response to insulin can be perturbed by stimuli such as TNFα, glucocorticoids, and prolonged exposure to long-chain fatty acids such as palmitate, all of which have been implicated in insulin resistance (Chavez et al. 2003; Holland et al. 2007). Intriguingly, a common feature of these stimuli is their ability also to promote the accumulation of sphingolipids (i.e. ceramide) (Schmitz-Peiffer et al. 1999; Teruel et al. 2001; Holland et al. 2007).

Exposure of skeletal muscle to FFA results in the development and accumulation of intramuscular triacylglycerol (IMTG) and other fatty acid derived molecules (i.e. diacylglycerol and ceramide)(Itani et al. 2002; Moro et al. 2008; Lipina and Hundal 2011). The accumulation of IMTG in skeletal muscle has been associated with endurance trained athletes with significant sensitivity to insulin, indicating that the accumulation of IMTG is not the sole mediator of insulin resistance in skeletal muscle (Goodpaster et al. 2001; Bruce et al. 2004); however, it has been demonstrated that exposure of skeletal muscle to

exogenous palmitate, the most abundant fatty acid in plasma, impaired insulin signaling by preventing insulin-induced phosphorylation of Akt/PKB (Hajduch et al. 2001; Moro et al. 2008). Several studies have demonstrated the insulin-desensitizing effects of palmitate can be mimicked through the acute application of ceramide and ceramide analogues (Schmitz-Peiffer et al. 1999; Teruel et al. 2001; Chavez et al. 2003). And, moreover, inhibitors of sphingolipid biosynthesis protect cells from the insulin-desensitizing effects of palmitate (Chavez et al. 2003)..

The accumulation of IMTG does not appear to be pathogenic by itself but reflective of lipid oversupply, inducing an increase in free palmitate; however, whether IMTG serve as a source of substrate for sphingolipid synthesis is unknown, but in general sequestration of cell lipids into TAG is protective (Dimitrios et al. 2010; Amati et al. 2011).

Sphingolipids in the Diabetic Heart

Heart disease is a major clinical concern in type 2 diabetes: patients with diabetes are several times more likely to die of heart disease than non-diabetics (Ho et al. 1993; Kucharska-Newton et al. 2010). Significantly, diabetics develop a distinct cardiomyopathy that is independent of traditional risk factors (e.g. hypertension and ischemia/reperfusion) (Guha et al. 2008). This condition, termed *diabetic cardiomyopathy*, is characterized by systolic and diastolic dysfunction, reduced contractility, fibrosis, hypertrophy, and a profound shift away from utilization of glucose as a substrate (Boudina and Abel 2010). Strikingly, severity of insulin resistance was associated with both the level of myocardial steatosis and the degree of cardiac remodeling (Utz et al. 2011), while the amount of myocardial steatosis was itself an independent predictor of left-ventricular systolic dysfunction in adult humans (Rijzewijk et al. 2008). In fact, induction of myocardial steatosis is used to replicate the phenotype of diabetic cardiomyopathy in mouse models (discussed below). Together, these observations suggested the potential contribution of lipid overload to the development of diabetic cardiomyopathy.

Similarly to what was seen in pancreatic β-cells and other tissues, excess saturated fatty acids were shown to induce both metabolic alterations and apoptosis in cardiomyocytes (de Vries et al. 1997; Hickson-Bick et al. 2000; Sparagna et al. 2000). In isolated neonatal rat cardiac myocytes, it was shown that palmitate induced a dramatic reduction in fatty acid oxidation, as compared to cells treated with oleate (Hickson-Bick et al. 2000). This downregulation of fatty acid oxidation was thought to occur due to reduced AMPK activity and subsequent accumulation of malonyl-Coenzyme A (CoA), which in turn inhibits fatty acid oxidation through CPT-1. The reduction in fatty acid oxidation shunted fatty acyl-CoAs into non-oxidative pathways: triglyceride and ceramide levels both doubled in response to palmitate, but not oleate, treatment (Hickson-Bick et al. 2000; Sparagna et al. 2000). This increase in ceramide was accompanied by a significant increase in DNA laddering as well a more than two-fold increase in caspase 3 activity in palmitate-treated cells (Hickson-Bick et al. 2000). These results were suggestive of a toxic role for ceramides, as had been demonstrated a short time before in pancreatic β-cell lipotoxicity (Shimabukuro et al. 1998) and in TNF-α-mediated cardiomyocyte toxicity (Krown et al. 1996).

Ceramide was first directly implicated in cardiomyocyte apoptosis through the use of a cell-permeable artificial ceramide; this finding was confirmed and extended using a combination of inhibitor studies and ceramide treatments. Initially, it was shown that treatment with C_2-ceramide, a cell-permeable synthetic ceramide, induced neonatal cardiomyocyte apoptosis in a caspase 3- and 8-dependent manner (Wang et al. 2000). A subsequent, conceptually important study confirmed that *de novo* sphingolipid synthesis was required for induction of lipotoxicity in isolated adult rat ventricular cardiomyocytes. In this system, it was shown that inhibition of ceramide synthesis attenuated lipotoxic and glucolipotoxic TUNEL staining and completely ameliorated lipotoxic myofibrillar degeneration (Dyntar et al. 2001). This approach was important for two reasons: first, primary adult cardiomyocytes are post-mitotic and, therefore, a better model of the adult heart than neonatal cells, and, second, the use of an inhibitor provided redundancy to the short-chain ceramide analogue-based approach, which has caveats that will be discussed later in this chapter. Therefore, through the use of complementary approaches, these experiments demonstrated that an increase in ceramide levels is both necessary and sufficient to induce lipotoxic cell death and myofibrillar degeneration in cardiomyocytes.

Further studies extended the known role for ceramide in development of the diabetic cardiomyopathy phenotype, including induction of heart failure markers, down-regulation of glucose oxidation, and impairment of contractility. Numerous aspects of the response of cardiomyocytes to ceramide were worked out in AC-16 cells, which are an immortalized human ventricular cardiomyocyte-derived cell line (Park et al. 2008). For example, it was shown that C_6-ceramide treatment of AC-16 cells induced up-regulation of the heart failure markers ANF and BNP (Park et al. 2008). Suggesting a role for ceramide in lipotoxic down-regulation of glucose oxidation, C_6-ceramide treatment also suppressed expression of the GLUT-4 glucose transporter and potentiated expression of PDK4, which inhibits the pyruvate dehydrogenase complex. Ceramide has also been implicated in the lipotoxic impairment of cardiac contractility. Specifically, it was shown that treatment with either palmitate or C_6-ceramide induced β-adrenergic dysfunction in AC-16 cells (Drosatos et al. 2011). Strikingly, the induction of β-adrenergic dysfunction by palmitate was completely ameliorated by co-treatment with the *de novo* sphingolipid synthesis inhibitor myriocin. This sphingolipid-dependent impairment of β-adrenergic function was thought to occur as a result of activation of PKCα and PKCδ. However, it should be noted that treatment with diacylglycerol was shown to produce the same effects, and it is likely that sphingolipids and diacylglycerol work in concert in this process. Together, these studies showed that, in an immortalized cardiomyocyte cell system, increased cellular ceramide was sufficient to induce expression of heart failure markers, changes in glucose uptake and metabolism, and β-adrenergic dysfunction, and *de novo* sphingolipid synthesis was required for induction of β-adrenergic dysfunction by palmitate. Critically, results obtained in isolated cell systems have been recapitulated and extended *in vivo*. One model that has provided a great deal of information is the LpL^{GPI} mouse, which expresses a heart-specific membrane-bound form of lipoprotein lipase and thus develops profound cardiac steatosis (Yagyu et al. 2003). Cardiomyocytes bearing this transgene accumulated ceramides, sphingomyelin, and diacylglycerol (Park et al. 2008). Importantly, while myriocin treatment prevented the increase in cardiac ceramides and sphingomyelin, diacylglycerol levels remained elevated;

this allowed the authors to tease out ceramide-specific effects from those of diacylglycerol. The results of this study were striking. It was shown that many of the pathological outcomes in the LpL^{GPI} mouse, including gross cardiac hypertrophy, increased left ventricular systolic diameter, reduced cardiac efficiency, and increased mortality, were completely prevented by myriocin treatment, which inhibits the first step of *de novo* sphingolipid synthesis. Glucose oxidation and palmitate oxidation were also restored to normal levels by myriocin treatment, as was expression of molecular markers of heart failure (ANF and BNP), hypertrophy (GSK3β phosphorylation), and insulin signaling (AKT phosphorylation). These sphingolipid-dependent outcomes effects were also attenuated upon crossing with a mouse heterozygous null for the serine palmitoyltransferase subunit SPTLC1, which also displays impaired de novo sphingolipid biosynthesis. Thus, the role of sphingolipids in the development of lipotoxic cardiac hypertrophy and heart failure was demonstrated *in vivo*.

Another transgenic model system provided additional support for the role of sphingolipids in diabetic cardiomyopathy. The *ob/ob* mouse is a very popular leptin-deficient model of obesity and type 2 diabetes; these mice develop marked cardiac hypertrophy, dysfunction, and steatosis (Barouch et al. 2006; Dobrzyn et al. 2010). Paralleling results in the LpL^{GPI} mouse, these animals dramatically accumulate ceramide and overexpress SPT, the initial enzyme of *de novo* sphingolipid synthesis (Dobrzyn et al. 2010). However, it was shown that loss of stearoyl-CoA desaturase 1 (SCD1), which catalyzes synthesis of monounsaturated fatty acids, not only protected *ob/ob* mice from obesity but also reduced SPT expression and ceramide to normal levels and prevented the hypertrophic cardiac phenotype. This study thereby demonstrated that a mutation that indirectly normalized ceramide levels prevented the development of diabetic cardiomyopathy.

While most studies have focused on the role of ceramide in the heart, other work has revealed a protective role for sphingosine-1-phosphate, similar to that demonstrated in other lipotoxic cell models. In isolated primary cardiomyocytes, treatment with sphingosine-1-phosphate protected against palmitate-induced cell death as effectively as myriocin treatment (Holland et al. 2011). Additionally, overexpression of sphingosine kinase 1 protected diabetic mice against the development of cardiomyopathy (Ma et al. 2007). Intriguingly, though, treatment with sphingosine-1-phosphate promoted hypertrophy of neonatal cardiomyocytes, possibly through signaling of the EDG1/S1PR1 receptor (Robert et al. 2001). It remains unknown whether this effect is recapitulated in adult cardiomyocytes. Therefore, although sphingosine-1-phosphate signaling appears to play a protective role in the lipotoxic heart, its function has yet to be completely understood.

The role for sphingolipids other than ceramide and sphingosine-1-phosphate in the diabetic heart remains almost totally uncharacterized. Two studies have indicated that administration of a ganglioside preparation protected atrial function in genetically obese, diabetic mice and prevented reduction in cardiac norepinephrine concentration in diabetes (Prosdocimi et al. 1987; Tessari et al. 1988); potential actions of other sphingolipids in the diabetic heart remain undescribed. Significantly, many studies addressing the roles of sphingolipids in the heart do so by inhibiting *de novo* sphingolipid synthesis. In addition to reducing ceramide, though, these strategies can reduce levels of downstream metabolites of ceramide (Miyake et al. 1995). Thus, because of the known roles for sphingolipid species other than ceramide and

sphingosine-1-phosphate in other diabetic organs, this represents a major gap in knowledge of sphingolipid functions in the diabetic heart and presents a caveat to attribution of cellular outcomes to ceramide *per se*.

Although numerous studies have addressed the roles of ceramide in diabetic cardiomyopathy, few have addressed the mechanisms by which sphingolipid levels are regulated in diabetes. While general mechanisms parallel those described elsewhere in this chapter, a few cardiac-specific observations are worthy of mention. As discussed above, the prevailing theory of lipotoxicity proposes that accumulation of toxic lipid species, including ceramide, results from the inability of cells to sequester free fatty acids adequately as neutral lipid (Cnop et al. 2001). To test this hypothesis, diacylglycerol acyltransferase 1 (DGAT1), which catalyzes the formation of triacylglycerol from diacylglycerol, was overexpressed in a cardiac-specific manner in mice (Liu et al. 2009). This resulted in accumulation of triacylglycerols and a concomitant reduction in ceramide and diacylglycerol levels. Furthermore, when these animals were crossed with a transgenic strain that developed lipotoxic cardiomyopathy, the resulting mice displayed improved heart function and reduced mortality as well as more normal expression of markers of heart failure, oxidative stress, and apoptosis. Strikingly, statistical analyses revealed a significant correlation between cardiac ceramide levels and the level of cardiac function in a mixed cohort of transgenic and wild type mice. This study thus provided support for the hypothesis that lipid overload induces steatosis by overwhelming cells' capacity to sequester fatty acids as neutral lipid.

Other work has addressed a potential role for the transcription factor PPARα in regulation of ceramide levels (Baranowski et al. 2007). In this study, treatment of wild type rats with a PPARα agonist (WY-14643) synergized with high fat diet to increased SPT activity in a more dramatic manner than observed with diet alone. Likewise, ceramide levels also increased markedly upon administration of WY-14643 in high fat diet, and the fatty acyl chain distributions of both ceramide and sphingomyelin were also altered. This latter finding suggests that PPARα could potentially act on other components of the sphingolipid synthetic pathway besides SPT. Strikingly, other studies have implicated PPARα in heart failure (reviewed in (Finck 2004) and (Madrazo and Kelly 2008)), thus raising the question of whether these effects are mediated by regulation of sphingolipid levels by PPARα. Together, these findings suggest that PPARα-mediated signaling may augment the observed substrate-based effects of lipid oversupply to promote *de novo* sphingolipid synthesis.

In summary, these findings demonstrate roles for sphingolipids in the pathogenesis of cardiac lipotoxicity and diabetic cardiomyopathy. Ceramide was implicated not only in the induction of cardiomyocyte apoptosis but also in insulin resistance, altered cardiac substrate utilization, hypertrophy, β-adrenergic dysfunction, and impaired cardiac function. In contrast, there are hints that sphingosine-1-phosphate may protect cardiomyocytes from apoptosis while, perhaps, potentiating hypertrophy. As of this writing, no published studies have addressed potential roles for other sphingolipids, including glycosphingolipids, sulfatide, and ceramide-1-phosphate, in the diabetic heart. Furthermore, the contributions of particular enzymes of sphingolipid metabolism and individual *N*-acyl chain lengths have been utterly neglected. Additionally, it should be noted that, while much of the literature on sphingolipids in the diabetic heart focuses on their roles in diabetic cardiomyopathy, they

also play important roles in injury following ischemia and reperfusion (I/R) (Maulik et al. 1993; Bielawska et al. 1997; Theilmeier et al. 2006) and in defects in electrical conductance (Constable et al. 2003) and autonomic nervous function (Tessari et al. 1988; Davis et al. 2006), all of which are major concerns in the diabetic heart. Finally, the role of sphingolipids in diabetic glucotoxicity in the heart has been abjectly neglected, despite its potential importance. Thus, while sphingolipids have been shown to play multiple roles in the pathogenesis of cardiac lipotoxicity, much remains to be learned about their regulation, specific mechanisms of action, and broader significance in the diabetic heart.

Sphingolipids in the Diabetic Pancreas

Pancreatic dysfunction is the hallmark disease outcome in diabetes. In insulin resistance, excess plasma free fatty acids initially stimulate insulin secretion by the pancreas, both directly and in response to insulin resistance in peripheral tissues, resulting in a compensatory hyperinsulinemic state (Milburn et al. 1995). However, a gradual decline in insulin release occurs, in part, due to decreased insulin sensitivity in the pancreatic β-cells themselves; this effect has been shown to be mediated by excess uptake of free fatty acids from the plasma (Lee et al. 1994). The resulting loss of compensation results in frank hyperglycemia, which synergizes with the hyperlipidemic state to promote molecular pathology and cell death, as described later in this section.

The process of fatty acid-mediated β-cell dysfunction and, ultimately, death, occurs through *lipotoxicity*. The ability of lipid oversupply to increase ceramide levels in the diabetic pancreas has long been appreciated (Shimabukuro et al. 1998). Initially, it was observed that both DNA laddering (an indicator of apoptosis) and ceramides increased in the pancreatic islets from obese, diabetic rats, compared to lean controls. Furthermore, treatment of isolated islets with free fatty acids recapitulated this DNA laddering and ceramide production *in vitro*. Strikingly, it was found that only free fatty acids that promoted ceramide synthesis were able to cause lipotoxicity and insulin resistance *in vitro*, supporting a link between ceramides and islet lipotoxicity (Maedler et al. 2001; Maedler et al. 2003). Substantiating this relationship, treatment with a membrane-permeable synthetic ceramide, C_2-ceramide, directly potentiated DNA laddering (Shimabukuro et al. 1998). Intriguingly, this study also noted that islet cells from obese, diabetic rats were more vulnerable to free fatty-acid mediated lipotoxicity, suggesting that changes in metabolism or protein expression in the diabetic pancreas augmented susceptibility of these cells to lipotoxicity. Together, these findings suggested both that ceramide plays a key role in lipotoxic β-cell apoptosis and that the diabetic pancreas displays an increased vulnerability to toxicity from excess plasma free fatty acids, possibly due to the already-heavy fatty acid loading of these cells.

Glucotoxicity has also been shown to play an important role in pancreatic damage after decompensation of insulin resistance; this process, too, seems to be governed by sphingolipids. Most directly, glucose-stimulated cytotoxicity was shown to be ameliorated by inhibition of ceramide synthase (Maedler et al. 2003). Unfortunately, the effect of this treatment on downstream, complex sphingolipid species was not determined. This point may be significant because, as described later in this section, ceramides may be adorned with

carbohydrate headgroups to form complex sphingolipids, which have distinct signaling and membrane roles from those of simple ceramide. Regardless of this point, though, it appears that oversupplies of glucose and free fatty acids work in concert to promote ceramide synthesis in the diabetic pancreas. In fact, neither excess glucose nor a low dose of palmitate alone was able to induce ceramide production profoundly in INS832/13 or INS-1 cells, but the two conditions in combination strongly potentiated this effect (El-Assaad et al. 2010; Veret et al. 2011). In contrast, excess glucose and palmitate each provoked ceramide production in isolated rat islet cells, while the combination of both stimulated an even stronger increase in ceramide levels (Kelpe et al. 2003). This suggests a synergy between excess glucose and excess fatty acids in the induction of sphingolipid-dependent diabetic β-cell toxicity. These findings bring to light several unanswered questions, such as whether glucose and fatty acid oversupply act on distinct metabolic routes of sphingolipid production and clearance, whether these two routes of toxicity promote production of different sphingolipid metabolites, and whether they act on the same or complementary lipotoxic pathways.

Further studies more fully characterized the sphingolipid-dependent molecular features of pancreatic functional deterioration, including apoptosis, endoplasmic reticulum (ER) stress, and insulin resistance. For example, increased intracellular ceramides stimulated several components of the apoptotic cascade, such as cytochrome C release into the cytosol, a reduction in Bcl-2 expression, and loss of mitochondrial membrane potential (Maedler et al. 2003; Veluthakal et al. 2005). Broadly, it was found that ceramide acted to promote apoptosis, in part, by inducing caspase activation (Lupi et al. 2002); studies in the MIN6 insulinoma cell line and in glucolipotoxicity in INS-1 cells specifically implicated caspases 3/7 in sphingolipid-mediated cell death (Boslem et al. 2011; Veret et al. 2011). Ceramide accumulation also induced lipotoxic ER stress, as indicated by increased expression of CHOP, in MIN6 cells (Boslem et al. 2011). Finally, ceramide was directly implicated in insulin resistance at a molecular level, in addition to its non-specific actions of reducing β-cell proliferation and promoting apoptosis (Maedler et al. 2001). In particular, ceramide accumulation reduced proinsulin mRNA levels *via* reduced transcription in INS-1 and isolated rat pancreatic islet cells (Kelpe et al. 2003; Guo et al. 2010). This may have been mediated by attenuation of ERK phosphorylation (Guo et al. 2010). Thus, elevated sphingolipid loads in the diabetic pancreas promote insulin resistance and pancreatic failure through a suite of specific molecular outcomes. These findings lead to a larger question of how sphingolipid synthesis is up-regulated in the diabetic pancreas, thereby allowing all of these downstream effects to ensue.

As discussed above, lipid oversupply perturbs sphingolipid homeostasis by diverse mechanisms in many organs and cell types, and this is also true in diabetic pancreas. For example, it has been shown that expression of SPT is up-regulated in the diabetic pancreas (Shimabukuro et al. 1998). This up-regulation may be recapitulated *in vitro* by oversupply of the fatty acid palmitate as well as by other factors, including exposure to elevated levels of hepatocyte growth factor (HGF), which is overproduced in diabetes (Shimabukuro et al. 1998; Gonzalez-Pertusa et al. 2010). Other routes and enzymes of sphingolipid synthesis have been shown to be regulated by saturated fatty acids in skeletal muscle, as discussed

above, and may also play important roles in governance of sphingolipid levels in the diabetic pancreas (Hu et al. 2009). Thus, the diabetic pancreas harbors elevated sphingolipid levels due to both excess substrate supply and up-regulation of the enzymes of sphingolipid synthesis. However, while most studies have focused on bulk sphingolipid levels and general routes of metabolism, the roles of individual metabolites and enzymes in this system have remained undetermined. Largely because the distinct functions of individual *N*-acyl chain lengths of ceramide and specific (dihydro)ceramide synthase (CerS) enzymes have only recently been appreciated, only one study thus far has attempted to implicate an individual CerS isoform in β-cell lipotoxicity: Véret et al. proposed that CerS4 is responsible for glucolipotoxicity in INS-1 cells (Veret et al. 2011). Indeed, overexpression of CerS4 induced caspase 3/7 activity, while siRNA-mediated knockdown of CerS4 partially attenuated caspase activity in response to glucose and fatty acid overload. This work clearly demonstrated a role for CerS4 in glucolipotoxicity; however, additional studies will be required to define or to rule out contributions from other CerS isoforms. There are a few reasons for this. First, although the ceramide chain length profiles in the manuscript are suggestive, they are not sufficiently clear-cut to implicate a specific isoform. Second, the effect of overexpressing or knocking down other CerS isoforms was not tested. Third, and finally, cells were presented with only palmitate in the media. Beta-cells encounter a mixed profile of fatty acids *in vivo*, which are utilized differentially for *N*-acylation by individual CerS isoforms, and exposure to this characteristic mixture of substrates may reveal roles for other CerS isoforms in a physiologic context. Thus, although CerS4 has been implicated in β-cell lipotoxicity, roles for other CerS isoforms have not been ruled out. Furthermore and importantly, no studies have identified a role for specific ceramide species in β-cell lipoapoptosis. Thus, significant work will be required to elucidate the mechanisms by which diabetes and lipotoxicity up-regulate sphingolipid levels in the pancreas, as well as the significance of specific metabolic pathways.

Although most studies discussing sphingolipids in pancreatic lipotoxicity focus on ceramide, ceramide itself is not the only sphingolipid that accumulates during fatty acid overload. Indeed, many downstream metabolites of ceramide are modulated by manipulation of ceramide synthesis, and one must be careful before concluding that it is ceramide, *per se*, that is responsible for particular molecular outcomes. For example, glucosylceramide was shown to increase in MIN6 insulinoma cells treated with palmitate, and this accumulation was, in fact, much more robust than that of simple ceramide (Boslem et al. 2011). Furthermore, this may have important functional implications, as inhibition of glucosylceramide synthase, which may presumably increase its metabolic precursor, ceramide, partially preserved pancreatic insulin secretion in Zucker diabetic fatty rats (Aerts et al. 2007). This suggests a potential role for glucosylceramide, in particular, and sphingolipids other than ceramide, in general, in pancreatic insulin resistance. An additional example of this principle involves sulfatide (3'-sulfogalactosyl-ceramide), a glycosphingolipid present in the islets of Langerhans in the pancreas (Buschard et al. 2002). Like its metabolic precursor ceramide, this lipid has also been implicated in diabetes and insulin resistance. Mechanistically, sulfatide is involved in proper folding of proinsulin, preservation of insulin crystals, and monomerization of stored insulin hexamers (Osterbye et al. 2001). Furthermore, C16:0-sulfatide, specifically, was shown to be required for

stabilization of insulin granules (Blomqvist et al. 2003). C16:0 sulfatide also appears to be required for appropriate management of insulin secretion in normal rat pancreatic islets; it was shown to attenuate insulin secretion by reducing sensitivity of the ATP-dependent potassium channel to inhibition by ATP (Buschard et al. 2002; Buschard et al. 2006). Significantly, this particular sulfatide species was deficient in the pancreas of several rodent models of type 2 diabetes (Blomqvist et al. 2003), and administration of sulfatide appeared to ameliorate some aspects of diabetic pathology *in vivo*. For example, administration of C16:0-sulfatide, specifically, to Zucker fatty rats, which express a defective leptin receptor, reduced fasting insulin and improved the first-phase insulin response and glucose-stimulated insulin response (Blomqvist et al. 2005). More generally, sulfatide was shown to protect isolated islet cells from apoptosis, iNOS expression, and nitric oxide secretion induced by cytokines; this protective effect was not specific to C16:0-sulfatide (Roeske-Nielsen et al. 2010). These findings indicate that sulfatide exerts a protective effect in pancreatic islets *via* normalization of insulin secretion and protection from inflammation. It is still unknown whether these protective effects are also relevant to the context of fatty acid oversupply, and the mechanism by which C16:0 sulfatide is reduced in the diabetic pancreas remains unknown.

Finally, sphingosine-1-phosphate, the product of sphingosine kinase, has been suggested as an anti-apoptotic lipid in inflammation-mediated β-cell death. In particular, supplementation with sphingosine-1-phosphate protected isolated pancreatic islets from IL-1β-induced apoptosis (Rutti et al. 2009), and exposure to both IL-1β and TNF-α up-regulated sphingosine kinase activity in INS-1 cells and isolated rat pancreatic islets (Mastrandrea et al. 2005). It was suggested that this increase in activity was primarily mediated by sphingosine kinase 2 (SPHK2), based on *in vitro* activity assays. These results indicate that, in contrast to ceramide, sphingosine-1-phosphate acts to protect β-cells from the pathological inflammatory state present in diabetes and the metabolic syndrome; indeed, the balance of ceramide and sphingosine-1-phosphate levels has been suggested as a clinically important determinant of β-cell fate (Jessup et al. 2011).

In summary, these findings illustrate an important role for sphingolipids in the pathogenesis of the diabetic pancreas. Fatty acid and glucose oversupply both stimulate ceramide production, which promotes apoptosis and insulin resistance, as well as synthesis of downstream metabolites of ceramide. Like ceramide, glucosylceramide may act in a pro-pathogenic manner by potentiating insulin resistance. In contrast, sulfatide normalizes insulin secretion, and both sulfatide and sphingosine-1-phosphate protect cells from the harmful effects of inflammation. These foundational findings lead to numerous further questions about the detailed mechanisms of sphingolipid action in the diabetic pancreas. For example, it remains unknown which ceramide species play active roles in pathology, which pathways (i.e. salvage or *de novo* synthesis) generate the bulk of these pathogenic lipids *in vivo*, and which specific enzyme isoforms are involved. Functions of sphingosine-1-phosphate and other metabolites also remain incompletely characterized. Finally, the roles of sphingolipids in diabetic glucotoxicity are severely under-studied and may be distinct but synergistic with those observed in lipotoxicity. Thus, several sphingolipid species play key,

but contrasting, roles in β-cells during diabetes, and further studies are needed to clarify them and to understand the underlying molecular mechanisms.

Emerging Concepts in the Sphingolipid-Diabetes Axis: Liver, Kidney, and Retina

While many of the changes in sphingolipid synthesis and signaling have been somewhat well defined in tissues such as skeletal muscle, heart, and pancreas, there are several tissues that have yet to be thoroughly examined. One such tissue is the liver. Located between the intestinal tract and the systemic circulation, the liver plays an important role in the metabolism and storage of dietary fats as well as playing a central role in glucose and lipid metabolism (Coleman and Lee 2004; Yosuke et al. 2011). During times of feeding, hepatocytes increase their uptake of glucose and synthesis of glycogen (Yosuke et al. 2011). The liver also acts to synthesize triacylglycerides (TAG) through β-oxidation of fatty acids obtained from over nutrition or adipocyte cell death and/or inflammation(Coleman and Lee 2004; Bijl et al. 2009). In obesity, the combination of increased fatty acid flux from dysfunctional adipose tissue, coupled with induced fatty acid synthesis in the liver, results in the accumulation of TAG-rich lipid droplets in the cytosol (Preiss and Sattar 2008; Bijl et al. 2009; Deevska et al. 2009) and ultimately the development of fatty liver and lipotoxicity (Deevska et al. 2009). The development of these pathologies can lead to loss of insulin sensitivity, which in the liver, prevents the suppression of glucose generation, signaling the pancreas to increase insulin production resulting in hyperinsulinemia (Brown and Goldstein 2008; Bijl et al. 2009).

As a result of TAG accumulation, lipid synthesis and metabolism are disrupted and pertubations of these pathways have been demonstrated to contribute to loss of insulin sensitivity and the development of hyperglycemia (Deevska et al. 2009; Yosuke et al. 2011). For example, treatment of hepatocytes with palmitate promoted an increase in TAG. TAG accumulation was attenuated by the inhibition of the activity of acid sphingomyelinase (aSMase), which generates ceramide by hydrolyzing sphingomyelin derived from the recycling/endocytic pathway (Deevska et al. 2009). This was confirmed in mice lacking functional aSMase as well as functional LDL receptors. These animals demonstrated that when maintained on a high fat diet, there were no increases in TAG content in the cytosol of hepatocytes. They did however, demonstrate increases in *de novo* sphingolipid synthesis metabolites sphinganine, dihydroceramide, and ceramide as well as the activity SPT (Deevska et al. 2009). These animals were also protected against diet-induced hyperglycemia and insulin resistance.

Sphingolipid synthesis in the kidney is also adversely affected by the development of insulin resistance in other distal tissues. Hyperglycemia and insulin resistance induced by fatty acid oversupply to the liver results in dramatic alterations in kidney function, sphingolipid profiles, and the development of diabetic nephropathy. Indeed, complex glycosphingolipids have been indicated in the development of diabetic nephropathy, particularly where changes in glomerular sialic acids and/or sialidase activity correlate with the onset of proteinuria (Cohen-Forterre et al. 1984; Baricos et al. 1986; Cardenas et al. 1991; Mather and LJ 2011). Ceramide has also been implicated in renal injury as its accumulation is a result of

nephropathy induced by the consumption of a high-fat diet (Boini et al. 2010). While this study pointed to ceramide as the causative agent in high-fat diet-induced renal injury and nephropathy, glycosphingolipid levels were not assessed. As ceramide is a necessary for glycosphingolipid synthesis, it is possible that the synthesis of glycosphingolipids is also increased and may play a role (Boini et al. 2010). Indeed, the link between glucose metabolism, insulin resistance, and glycosphingolipid synthesis in the kidney has been established (Zhao et al. 2007), indicating glycosphingolipids in renal injury and the development of nephropathy.

The retina plays a significant role in vision and is adversely affected in type 2 diabetes; however the effects of changes in sphingolipid metabolism have not been thoroughly evaluated. Studies have indicated that the accumulation of ceramide in response to fatty acid oversupply results in increased apoptosis in retinal pericytes and induction of diabetic retinopathy (Cacicedo et al. 2005; Fox et al. 2006). While these studies provide significant evidence, more has to be done to ascertain the mechanism by which sphingolipid synthesis is perturbed in the retina.

Sphingolipids as Biomarkers of Diabetes

In addition to their direct roles in the pathogenesis of diabetes, sphingolipids have also emerged as potential biomarkers of diabetes and the metabolic syndrome. It has long been appreciated that sphingolipid levels are perturbed in the blood plasma of diabetic patients. The first such finding indicated that glycosphingolipids were elevated in the plasma of some groups of diabetic human patients (Kremer et al. 1975). This was later confirmed by a study demonstrating that glucosylceramide was elevated in the plasma of diabetics (Serlie et al. 2007). Similarly, plasma ceramides have emerged as potential biomarkers in diabetes. For example, plasma ceramides were elevated in humans with type 2 diabetes and correlated negatively with the rate of insulin-stimulated glucose disposal, indicating a relationship between plasma ceramide levels and insulin resistance (Haus et al. 2009). Furthermore, the concentration of ceramide in the blood plasma positively correlated with levels of the pro-inflammatory cytokine IL-6, which has been shown to promote insulin resistance (de Mello et al. 2009). Additionally, it was shown in human patients that sphingomyelin levels in the plasma membranes of erythrocytes positively correlated with circulating insulin levels (Zeghari et al. 2000). These results were echoed in an animal model of diabetes: sphingomyelin, ceramide, sphingosine, and sphingosine-1-phosphate were all elevated in the plasma of *ob/ob* mice, which are leptin-deficient, diabetic, and obese (Samad et al. 2006). Thus, multiple studies have suggested that sphingolipid levels in the plasma correlate with specific diagnostic criteria for diabetes and the metabolic syndrome in both mice and humans.

Despite these highly suggestive observations, relatively few studies have directly analyzed the predictive value of sphingolipid levels for diabetes. In one such study, sphingomyelin content of erythrocyte plasma membranes was shown to be an independent predictor of fasting insulin levels, degree of insulin resistance, and level of glucose tolerance (Candiloros et al. 1996). Indeed, higher levels of membrane sphingomyelin correlated with increased

fasting insulin, insulin resistance, and glucose tolerance in both normal and diabetic human patients.

Another recent study analyzed the potential of uncommon sphingosine bases as biomarkers of type 2 diabetes and the metabolic syndrome (Othman et al. 2011). In this study, plasma sphingolipids were deacylated, and the relative contributions of different sphingoid bases to the total pool were quantified in a small, homogenous population. The authors found that the concentrations of the uncommon sphingoid bases deoxysphinganine and deoxysphingosine, which incorporate the amino acid alanine rather than serine, were increased in patients with metabolic syndrome, with or without type 2 diabetes. Additionally, it was found that the levels of these compounds had a significant positive predictive value for the metabolic syndrome. Furthermore, the concentration of C_{16}-sphingosine, which is derived from myristoyl-CoA rather than palmitoyl-CoA, was decreased in patients with both diabetes and the metabolic syndrome, but not in patients with metabolic syndrome alone. It was also determined that levels of C_{16}-sphingosine had significant predictive value in differentiating patients with type 2 diabetes from pre-diabetic and control patients. Thus, it was suggested that levels of alanine-derived sphingoid bases are a potential biomarker for metabolic syndrome, with or without type 2 diabetes, while levels of C_{16}-sphingosine could be used to detect the transition from compensated insulin resistance to type 2 diabetes.

Together, these studies demonstrate the potential value of sphingolipids, and particularly uncommon sphingolipids, as biomarkers of diabetes and the metabolic syndrome. However, although strong correlations exist between sphingolipid levels and parameters of these diseases, few studies have performed the statistical analyses necessary to determine their predictive value, including ROC curves, which determine specificity and sensitivity of a potential biomarker. Additionally, further studies will be required to validate the findings of Othman *et al*. (2011) and Candiloros *et al*. (1996). These studies would need to incorporate larger and more diverse populations and, ideally, follow a prospective design. Furthermore, studies of ceramides and complex sphingolipids could benefit from analysis of individual *N*-acyl chain lengths. Thus, while plasma sphingolipids display much potential as biomarkers of diabetes, substantial work remains to be done before these tests reach the clinic.

Summary and Future Directions

Diabetes presents as a multifactorial disorder affecting multiple organ systems. Although broader disease outcomes (e.g. liver disease, heart failure, kidney failure) are complex and diverse, the underlying cellular and molecular etiologies flow through some common channels. One major theme seems to be sphingolipid-mediated insulin resistance and cell death, consequent to lipotoxicity. Indeed, a great deal of work has been done to link *de novo* sphingolipid synthesis and individual sphingolipid metabolites to particular molecular events. Broadly, ceramide has been implicated as a toxic lipid promoting insulin resistance, metabolic derangement, and cell death in skeletal muscle, heart, and pancreas (Shimabukuro et al. 1998; Schmitz-Peiffer et al. 1999; Park et al. 2008). In contrast, sphingosine-1-phosphate has been tentatively shown to exert protective effects in these same organ systems, despite its promotion of inflammation (Rutti et al. 2009; Holland et al. 2011). However, these general findings merely reveal an entire network of questions about the

specific molecular mechanisms of disease, especially regarding which pathways, enzyme isoforms, and sphingolipid metabolites are pathogenic or protective. Fortunately, new tools have emerged that will allow researchers to uncover precise aspects of these mechanisms.

Previously, the nature of the available experimental tools limited the resolution to which sphingolipid pathways could be teased apart. For many years, ceramide and its metabolites could only be measured by techniques such as TLC, which revealed changes only in bulk levels of these lipids. However, recent developments now allow researchers to examine detailed changes in sphingolipid *N*-acyl chain length profiles using quantitative mass spectrometry-based approaches (Bielawski et al. 2010). Results in other disease contexts, particularly cancer, have demonstrated that alterations in levels and proportions of ceramide *N*-acyl chain lengths are functionally important (Senkal et al. 2010; Senkal et al. 2011), and it may now be determined whether the lipotoxic effects of ceramide occur due to increases in bulk ceramide levels or to shifts toward particular chain length profiles. Furthermore, mass spectrometry-based labeling strategies, such as the use of ^{13}C-palmitate, allowed investigators to trace the specific metabolic routes that are invoked in their experimental systems, distinguishing between *de novo* synthesis and salvage pathways, for instance (Hu et al. 2009). These findings would lead to a better understanding of the underlying molecular mechanisms of ceramide-mediated lipotoxicity.

Critically, determining the nature of any changes in sphingolipid *N*-acyl profiles would help to reveal which CerS isoforms mediate the lipotoxic effects of ceramide and its downstream metabolites, as each CerS isoform produces a unique *N*-acyl chain length distribution (Pewzner-Jung et al. 2006; Laviad et al. 2008). Once again, newly-developed experimental approaches enable investigators to resolve the cellular functions of particular CerS isoforms. While myriocin, which inhibits SPT, or fumonisin B1, which inhibits all isoforms of CerS, can function as powerful tools to screen for sphingolipid-dependence, they reveal very little about involvement of specific sphingolipid metabolic pathways (Wang et al. 1991; Miyake et al. 1995). In contrast, new approaches based on DNA transfection, RNAi, and knockout animals allow precise insight into the roles of specific CerS isoforms in cellular functions and disease, with the caveat that dysregulation of individual isoforms can perturb global CerS expression patterns (Mullen et al. 2011). These approaches may also be applied to other enzymes of SL synthesis that are present in more than one isoform (e.g. ceramidase, sphingosine kinase, sphingomyelinase). Thoughtful employment of these strategies would do much to advance the field beyond a binary sphingolipid-dependent/sphingolipid-independent understanding of diabetic molecular and cellular phenotypes. Successful identification of specific enzymes for therapeutic targeting would also provide rationale for developing inhibitors selective for specific CerS isoforms, a strategy already well underway for sphingosine kinases (ref some Sk1 vs. SK2 inhibitor papers)

Notwithstanding the need for specific inhibitors, due to the highly interconnected nature of sphingolipid metabolism, any approach based on inhibiting or perturbing these pathways should be interpreted with care. In particular, many studies consider sensitivity to myriocin or fumonisin B1 to indicate a dependence on ceramide itself, excluding potential roles for subsequent metabolites. In fact, although such results are suggestive, they can be misleading; inhibition of *de novo* sphingolipid synthesis can reduce not only levels of

ceramide but of its downstream metabolites (Saito et al. 2005; Mullen et al. 2011). Thus, conclusions based on inhibitor studies actually indicate a dependence on either ceramide or its downstream metabolites. Similar caution should be used when employing knockdown or overexpression strategies to manipulate specific enzyme isoforms. Lipid profiles produced by individual isoforms may partition distinctly either at a metabolic level, due to differential acyl chain length preferences of downstream enzymes, or at the organelle level, due to different localization of particular enzymes of sphingolipid metabolism (reviewed in (Brice and Cowart 2011)). Furthermore, knockdown of individual CerS isoforms has been shown to perturb regulation of non-targeted isoforms (Mullen et al. 2011). Thus, results of inhibitor, knockdown, or overexpression experiments should be interpreted cognizant of potential off-target effects on sphingolipid synthesis and metabolism.

Similar caution should be exerted towards experiments using C_2- and C_6-ceramide, which have served as important tools in the determining the roles of sphingolipids in diabetes. This is because these artificial, short-chain ceramides have different biophysical properties than endogenous ceramides and may induce distinct cellular outcomes (Gidwani et al. 2003; Nybond et al. 2005), including lipotoxic outcomes through mechanisms that are not physiologically relevant. For example, treatment with C_2-ceramide can provoke mitochondrial depolarization and cytochrome c release in cardiomyocytes (Sparagna et al. 2000; Di Paola et al. 2004; Parra et al. 2008); these results would obviously suggest a role for ceramide. However, these cellular events actually precede ceramide accumulation in the context of palmitate-induced neonatal rat cardiomyocyte lipotoxicity, suggesting that this portion of the lipotoxic cascade is not ceramide-dependent in this system (Sparagna et al. 2000). Similarly, while pyridinium ceramides can provide important clues to intracellular functions of ceramide, it should be kept in mind that these compounds preferentially target to the mitochondria, which could potentially cause their functions to diverge from those of natural ceramides (Dindo et al. 2006). Furthermore, the activity of downstream enzymes, particularly the ceramidases, could be quite different toward endogenous ceramides than toward either short-chain or pyridinium-containing ceramides, which have modified *N*-acyl chains. Therefore, although treatment with cell-permeable ceramide analogues can activate multiple pathologic pathways and can provide important insights, other modes of experimentation are required to confirm that these mechanisms of pathology are consistent with those induced by endogenous sphingolipids.

In conclusion, it has been shown that sphingolipids play a multiplicity of both harmful and protective roles in the pathogenesis of the metabolic syndrome and diabetes, but these roles are, as yet, only crudely understood. A number of important questions remain unanswered regarding the sphingolipid-diabetes axis, and tools are now available to investigate mechanisms that would have been inscrutable only a decade ago. In particular, it remains to be shown which enzyme isoforms of sphingolipid metabolism are responsible for potentiating specific aspects of the cellular diabetic phenotype. Furthermore, the individual sphingolipid species involved in these processes are almost totally unknown. It is unknown which specific sphingolipid *N*-acyl chain lengths or classes of chain lengths are important in cellular pathology; just as critically, the roles of complex sphingolipids and, in many tissues, the potently bioactive lipid sphingosine-1-phosphate remain neglected. Furthermore, the vast

majority of studies have focused on the roles of sphingolipids in lipotoxicity; this may owe to the intuitive substrate-level connection. However, glucotoxicity is also an important player in the pathogenesis of diabetes, and the literature has provided a few intriguing hints of a synergistic relationship between sphingolipid-dependent glucotoxicity and lipotoxicity that beg for further investigation. Finally, the possibility of using circulating sphingolipids as clinical biomarkers is only now emerging. To develop this area, larger-scale studies with appropriate statistical approaches will need to be conducted. Furthermore, the physiological basis linking levels of particular circulating sphingolipids to diabetes remains to be determined. Thus, while much fundamental research has already implicated sphingolipids in diabetes in a very general way, this field of study blossoms both with questions regarding specific mechanisms of action and with new technologies and strategies to address them.

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