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Sphingolipids, Insulin Resistance, and Metabolic Disease: New Insights from *in Vivo* Manipulation of Sphingolipid Metabolism

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Obesity and dyslipidemia are risk factors for metabolic disorders including diabetes and cardiovascular disease. Sphingolipids such as ceramide and glucosylceramides, while being a relatively minor component of the lipid milieu in most tissues, may be among the most pathogenic lipids in the onset of the sequelae associated with excess adiposity. Circulating factors associated with obesity (*e.g.*, saturated fatty acids, inflammatory cytokines) selectively induce enzymes that promote sphingolipid synthesis, and lipidomic profiling reveals relationships between tissue sphingolipid levels and certain metabolic diseases. Moreover, studies in cultured cells and isolated tissues implicate sphingolipids in certain cellular events associated with diabetes and cardiovascular disease, including insulin resistance, pancreatic β-cell failure, cardiomyopathy, and vascular dysfunction. However, definitive evidence that sphingolipids contribute to insulin resistance, diabetes, and atherosclerosis has come only recently, as researchers have found that pharmacological inhibition or genetic ablation of enzymes controlling sphingolipid synthesis in rodents ameliorates each of these conditions. Herein we will review the role of ceramide and other sphingolipid metabolites in insulin resistance, β-cell failure, cardiomyopathy, and vascular dysfunction, focusing on these *in vivo* studies that identify enzymes controlling sphingolipid metabolism as therapeutic targets for combating metabolic disease. (*Endocrine Reviews* 29: 381–402, 2008)

I. Introduction

OBESITY PLACES INDIVIDUALS at risk for type 2 diabetes, hypertension, coronary heart disease, hypercoaguability, stroke, gallbladder disease, sleep apnea, osteoarthritis, osteoporosis, and certain types of cancer. With almost two thirds of the American population overweight and 30% clinically obese, obesity-related expenditures account for over 40% of health care costs and represent a significant fraction of the gross national product (1, 2). With the predicted increase in both obesity and costs of treating its associated health abnormalities, these expenditures are predicted to double by 2025 (3). Moreover, as a result of the myriad pathogenic consequences of nutrient oversupply, life expectancy, which has risen steadily for two centuries, is predicted to decline (4).

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Abbreviations: apoE, Apolipoprotein E; CerS, ceramide synthase; CoA, coenzyme A; Des1, dihydroceramide desaturase 1; FFA, free fatty acid; GCS, glucosyl ceramide synthase; GLUT4, glucose transporter 4; 11HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD1, 11β-hydroxysterol desaturase 1; IKK, inhibitor of nuclear factor-κB kinase; IRS, insulin receptor substrate; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; PAI-1, plasminogen activator inhibitor 1; PDK1, phosphatidylinositol-3-phosphate-dependent kinase 1; PH, pleckstrin homology; PI3K, 3-phosphoinositide kinase; PIP_3, phosphatidylinositol 3,4,5 trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; S1P, sphingosine 1-phosphate; SPT, serine palmitoyltransferase; TLR, toll-like receptor; TZD, thiazolidinedione; ZDF, Zucker diabetic fatty.

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Despite considerable attention, the mechanism by which obesity impairs the function of peripheral tissues is unclear. A hypothesis gaining credibility is that the delivery of lipids to tissues in excess of their oxidative or storage capacities is an underlying component of many of the pathogenic conditions associated with obesity. Although sphingolipids are a relatively minor component of the lipid milieu in most mammalian cells, their accumulation in tissues such as the liver, muscle, heart, pancreas, and vasculature has long been speculated to play a role in the onset and development of metabolic diseases.

First, unlike other more abundant lipids, sphingolipid levels are selectively up-regulated by circulating factors associated with obesity and metabolic disease. Indeed, ceramides and related sphingolipids have been shown to accumulate in obese humans and rodents (summarized in Table 1).

Second, the sphingoid backbone of sphingolipids relies on the availability of saturated fatty acids (5, 6), which have generally been regarded to be more pathogenic than unsaturated ones (7). Thus, excess intake or impaired oxidation of saturated fat likely contributes to the accrual of sphingolipids in tissues.

Third, bioinformatic strategies for conducting lipidomic analysis have revealed particularly strong associations between hepatic ceramide levels and the extent of steatosis in a rodent model of obesity (8).

Fourth, the addition of exogenous sphingolipids, including ceramides and glucosylceramides, to isolated cells or tissues recapitulates some of the cellular events associated with metabolic disease.

Despite these observations, however, conclusive evidence that aberrant sphingolipid accumulation contributes to metabolic disease has come only recently. Owing to the development of pharmacological inhibitors of enzymes controlling sphingolipid synthesis and metabolism, coupled with the recent cloning of genes encoding the enzymes that regulate ceramide accrual, scientists have recently demonstrated that inhibiting enzymes controlling sphingolipid synthesis has beneficial effects in rodent models of atherosclerosis, insulin resistance, diabetes, and cardiomyopathy. A discussion of this *in vivo* work is the focus of this review.

II. Regulation of Sphingolipid Synthesis and Metabolism: Effect of Obesity

The recent advances in understanding the role of sphingolipids in metabolic disease have involved the manipulation of enzymes controlling rates of ceramide synthesis, degradation, and metabolism. A series of four sequential reactions promote the synthesis of bioactive ceramide from its precursors, free fatty acid (FFA) and serine (Fig. 1).

- *Serine palmitoyltransferase* (SPT) catalyzes the first reaction, which condenses serine with palmitoyl-coenzyme A (CoA) to produce 3-ketosphinganine (reviewed in Ref. 9). Two gene products (Sptlc1 and 2) that physically associate are necessary for enzyme activity. A putative third subunit has recently been identified in both yeast (10) and mammals (11). In all organisms, the enzyme is highly selective for saturated fatty acyl-CoA containing 16 ± 1 carbons. The rate of this reaction is influenced largely by the avail-

TABLE 1. Ceramide levels in liver, muscle, and serum

Animal model	Liver	Muscle	Serum	Ref.
Female Zucker fa/fa rat	↑ 26%	↑ 52%		71
Male ZDF rat	↑ 40%	↑ 51%	↑ 120%	12
Male Zucker fa/fa	↑ 43%	NC*	↑ 111%*	12
ob/ob mice	↑ 987%	NC*	~ ↑ 200%	73, 75
Lard oil-infused rat	↑ 61%	↑ 89%		12
Liposyn-infused rat	NC	NC		12,127
Intralipid-infused rat		↑ 45%		125
High-fat-fed rat (3 wk)		↑ 70–100%		161,162
High-fat-fed rat (4 wk)		↑ 23%		294
Dexamethasone-dosed rat	↑ 140%	↑ 94%	↑ 310%	12
Streptozotocin diabetic rat		↑ 75–250%		295
LPS-treated rats	↑ 150%			60
LPS-treated hamsters	↑ 150%			59
LPS-treated mice			~ ↑ 1000%	58
Safflower oil diet in mice	↓ 9%	↓ 22%		296
Fish oil diet in mice	NC	↓ 32%		296
Muscle LPL mice		~ ↑ 45%		15
Obese humans		↑ 84%		76
Intralipid-infused humans		↑ 48%		128
Liposyn-infused humans		NC		123
LPS-treated humans			↑ 1000%	58

Insulin-resistant rodents and humans often display elevated ceramide concentrations in liver, muscle, or serum as compared to lean or untreated control subjects. The percentage of change and direction of change compared to controls are indicated in the relevant tissue categories. The relevant studies are listed. *Asterisks* denote unpublished observations. Unpublished samples were enzymatically measured as previously described from flash-frozen samples obtained from anesthetized animals. Muscle ceramide content was analyzed from soleus muscles of dexamethasone-treated (400 μg/kg dexamethasone every 12 h for 36 h) male Sprague Dawley rats (250 g). Ceramide was measured in soleus muscles 4 wk after male Sprague Dawley rats were made diabetic by streptozotocin (60 mg/kg). Ceramide was compared from gastrocnemius muscles of marmots obtained in July (lean) or October (obese).

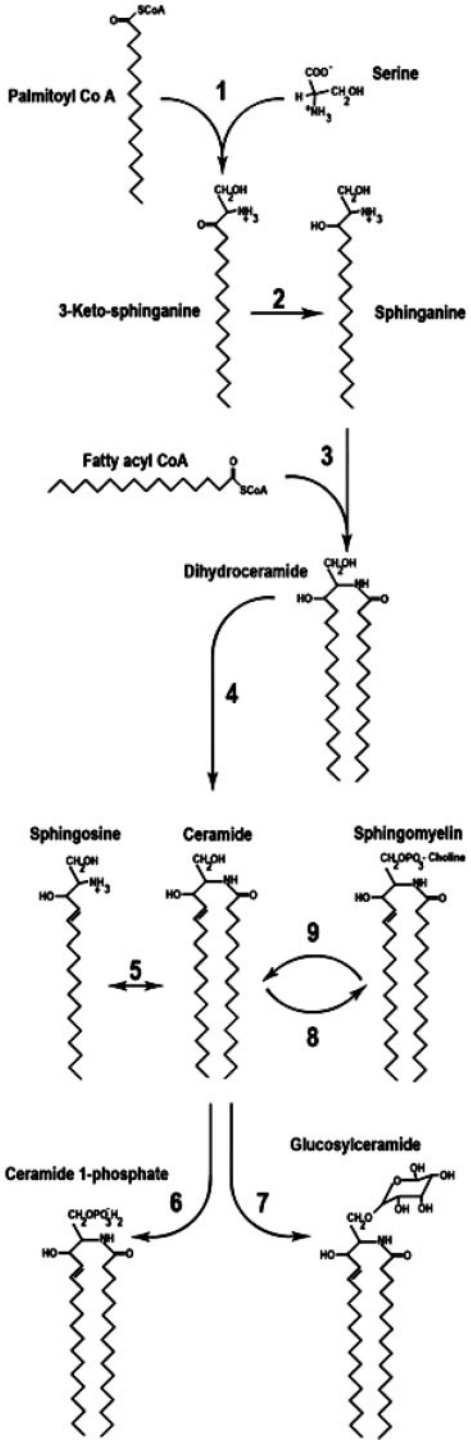

FIG. 1. Schematic diagram illustrating sphingolipid synthesis and metabolism. 1) Serine palmitoyltransferase catalyzes the condensation of serine and palmitoyl CoA. 2) 3-Ketosphinganine reductase catalyzes sphinganine formation. 3) Dihydroceramide synthases add a second acyl chain to sphinganine resulting in dihydroceramide formation. 4) Dihydroceramide desaturase catalyzes formation of bioactive ceramide. 5) Ceramidase deacylates ceramide to form sphingosine and fatty acid. 6) Ceramide kinase phosphorylates ceramide to form ceramide 1-phosphate. 7) Glucosylceramide synthase adds glucose, an initial step in ganglioside formation. 8) Sphingomyelin synthase promotes the addition of phosphocholine to ceramide. 9) Sphingomyelinase regenerates ceramide and choline from the breakdown of sphingomyelin.

ability of the FFA substrate (5), and this explains the mechanism by which saturated fats, but not unsaturated ones, drive the synthesis of sphingolipids (12, 13). Inhibitors of SPT include the sphingofungins lipoxamycin and myriocin and the broad spectrum, and less specific, antibiotic cycloserine (14).

- *Ketosphingaine reductase* reduces 3-ketosphinganine to produce sphinganine through an nicotinamide adenine dinucleotide phosphate-dependent mechanism. The 3-ketosphinganine intermediate is only rarely observed in cells, suggesting that this reaction occurs rapidly (15).
- (*Dihydro)ceramide synthases* (CerS) acylate sphinganine to produce dihydroceramide (reviewed in Ref. 16). Recent studies indicate that a large family of CerS isoforms exists, each demonstrating selectivity for particular fatty acyl-CoA substrates (16). Because of the existence of multiple specific enzymes catalyzing this reaction, it is tempting to speculate that individual ceramide subspecies will have distinct biological functions, but this has not yet been confirmed through experimentation. A number of fungi metabolites have been shown to inhibit this step, with the most widely-used reagent being fumonisin B1 (14).
- *Dihydroceramide desaturases* oxidize inactive dihydroceramide into active ceramide. Two isoforms have been identified. Dihydroceramide desaturase 1 (Des1) inserts this key double bond in most peripheral tissues (17), whereas the Des2 isoform preferentially produces phytosphingolipids and is largely restricted to the gut and kidneys (17). The anticancer and antidiabetic agent fenretinide (18, 19), a cyclopropene-containing sphingolipid (termed GT11), and a rationally designed compound (termed XM642) are inhibitors of this enzyme (14, 20).

Once generated, ceramide is the common precursor of complex sphingolipids, and the molecule can be glucosylated, phosphorylated, or deacylated to produce a wide array of metabolites.

- *Ceramidases* deacylate ceramide to produce sphingosine, which can in turn be phosphorylated by *sphingosine kinase* to produce sphingosine 1-phosphate (S1P). S1P often opposes ceramide action, leading researchers to propose the existence of a ceramide:S1P rheostat that controls cellular responses (21). Ceramidases, which can sometimes catalyze the reverse reaction to convert sphingosine back into ceramide, can be distinguished by their pH optima (22). Collectively, ceramidases are ubiquitously distributed throughout cellular membranes and are also secreted into the extracellular milieu.
- *Ceramide kinase* phosphorylates ceramide to produce ceramide 1-phosphate, which activates intracellular enzymes such as phospholipase A2 and certain phosphatases, and may be important in eicosanoid biosynthesis (23).
- *Glucosylceramide synthase* tethers glucose with ceramide to create glucosylceramide, which is the precursor of compound gangliosides. These complex lipids are particularly abundant in the brain but are less prevalent in other peripheral tissues (24, 25).
- *Sphingomyelin synthase* converts ceramide into sphingomyelin by catalyzing the addition of a phosphocholine

head group (26). Importantly, *sphingomyelinase* performs the reverse reaction to rapidly regenerate ceramide and choline from sphingomyelin (27).

In general, the *de novo* synthesis of ceramide occurs on the outer leaflet of the endoplasmic reticulum. Ceramide is then modified into complex sphingolipids in the golgi complex. However, several lines of evidence suggest that sphingolipid metabolism can also occur in the mitochondria. First, several enzymes involved in ceramide synthesis or metabolism (*e.g.*, ceramide synthase, ceramidase, sphingomyelinase, sphingomyelin synthase) are resident in mitochondrial membranes (28–34). Second, isolated mitochondria were found to be capable of generating ceramide (34). Third, the inflammatory cytokine TNFα was shown to directly stimulate ceramide production in this organelle (35). So, although sphingolipids are ubiquitously present in cell membranes, the intracellular cites of production are of interest because sphingolipids produced in different locales may have distinct functions (34).

A. Sphingolipids in the diet

Sphingolipids are present in all eukaryotes and many prokaryotes and thus are present in the diet. They are particularly prevalent in meat, eggs, and dairy products and may have significant roles within the digestive tract (36). Using radioactive sphingolipid tracers, researchers have observed the appearance of small amounts of label in blood, lymph, and liver (37, 38). Moreover, supplementing the diet with high levels of sphingolipids can increases serum sphingomyelin concentrations and be proatherogenic (39). However, it is unlikely that increased intake of sphingolipids promotes their accumulation during obesity. The vast majority of sphingolipids are degraded in the gut by resident glucoceramidases, sphingomyelinases, and ceramidases (37). About 25% of consumed sphingolipids resist degradation, only to be secreted in the feces, predominantly in the form of ceramide (37).

B. Regulation of sphingolipid synthesis and metabolism during obesity

A number of factors associated with obesity selectively alter rates of ceramide synthesis. Long-chain saturated fats, which are more poorly oxidized than their unsaturated counterparts (40), are required for formation of the sphingoid backbone and are sufficient to drive the formation of ceramide (6). Thus, increased saturated acyl chains within circulating lipoprotein particles likely contribute to the induction of ceramide in peripheral tissues. In addition, obesity is associated with a state of chronic low-level inflammation (41, 42), which likely contributes to the induction of ceramide accumulation.

First, an expanded fat pad secretes a number of inflammatory cytokines, including TNFα, IL-1, IL-6, plasminogen activator inhibitor 1 (PAI-1), and C-reactive protein. These cytokines derive either from enlarged adipocytes or from macrophages that infiltrate the tissue to consume dying adipocytes, and knockout mice lacking PAI-1 or TNFα receptors are protected from many of the metabolic consequences of obesity (43, 44). A number of these factors promote lipolysis, thus increasing delivery of fatty acids to other peripheral tissues. Additionally, some of these pathogenic agents selectively alter metabolic pathways to promote the incorporation of the incoming fat into ceramide. For example, TNFα produces a rapid increase in ceramide by activating acidic and neutral sphingomyelinase isoforms and effects a chronic and sustained elevation in *de novo* ceramide synthesis (45–47). TNFα also stimulates the production of gangliosides (48–50). Similarly, IL-1 is a potent inducer of ceramide (51–55).

Second, Flier and colleagues (56, 57) recently demonstrated that fatty acids could activate toll-like receptors (TLRs), which are involved in innate immune responses. These TLRs produce TNFα, IL-6, and other cytokines capable of producing ceramide. Lipopolysaccharide (LPS), a strongly immunogenic component of Gram-negative bacteria and an activator of TLR4, has been shown to induce ceramide accumulation in serum, liver, kidney, and spleen (58–60). Moreover, MyD88, an essential component of TLR signaling pathways, has been shown to activate sphingomyelinase (51). Supporting the hypothesis that TLRs are essential for promoting ceramide accrual is the observation that the subset of fatty acids that induce ceramide (13) are similar to those that activate TLRs (56, 57).

The mechanism(s) by which these factors influence ceramide synthesis or degradation is incompletely understood because these factors could alter either the activity or the expression of these biosynthetic intermediates.

Another factor associated with obesity-induced metabolic derangements is cortisol, which has long been known to induce adiposity, insulin resistance, hyperlipidemia, and hypertension (Cushing's syndrome). Circulating cortisol levels are not elevated in the obese, but 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), an enzyme that converts inactive cortisone to active cortisol, is increased in sc tissue and correlates with omental fat cell size (61). Transgenic overexpression of 11HSD1 in adipose tissue causes obesity, hypertension, and insulin resistance (62, 63), and knockout mice lacking the enzyme are protected from diabetes (64). Thus, inhibitors of 11HSD1 are being developed as a means of combating metabolic disease (65).

Glucocorticoids have long been known to have a large and specific effect on sphingolipids. In tissue culture systems, dexamethasone was demonstrated to increase membrane sphingomyelin, sphingosine, or ceramide levels in a broad range of cell types (66–70). Epididymal fat cell ghosts isolated from adrenalectomized rats demonstrated decreased sphingomyelin levels, which could be restored by the administration of the synthetic glucocorticoid dexamethasone (68). Moreover, dexamethasone treatment of rats induces ceramide within the portal circulation and the liver, while increasing the hepatic expression of various biosynthetic enzymes including SPT and CerS1 (12).

C. Quantification of sphingolipid levels during obesity

Given the number of factors predicted to induce ceramide during obesity, one would be surprised not to detect selective increases in certain sphingolipids in rodent mod-

els of the condition. Indeed, a growing number of investigators have described elevations in ceramide in muscle and liver of obese rats or mice. For example, Turinsky *et al*. (71) demonstrated that ceramide, as well as the glycerolipid diacylglycerol, accumulates in muscle and liver of female Zucker (fa/fa) rats. Other animals with increased ceramide levels are summarized in Table 1. Advancements in lipidomics technologies have made it possible to quantify a broader range of lipid metabolites in a single sample, as well as to assess differences in fatty acid chain length and degree of saturation. Using such approaches, Samad *et al*. (72) have reported detailed changes in the sphingolipid metabolites produced in adipose tissue and serum of leptin-deficient, diabetic ob/ob mice. Sphingomyelin and ceramide levels were lower in adipose tissue but higher in serum, whereas sphingosine levels were higher from both locales in obese mice. Additionally, they reported increases in sphingosine, SMase, and SPT abundance in adipose tissue of obese mice compared with lean controls. Using a bioinformatics strategy to characterize a broader array of lipid species, Yetukuri *et al*. (8) correlated a variety of lipid metabolites with the induction of hepatic steatosis; C16 ceramide positively correlated with liver triglycerides, whereas a host of other lipid metabolites did not.

Several reports have suggested that glucosylceramides, some of which are implicated in insulin resistance (see *Section IV*) are also elevated in obese rodents: 1) Zucker fa/fa rats and/or ob/ob mice display glucosylceramide in liver (73), and GM3 synthase expression is elevated in adipose tissue (48); 2) streptozotocin-induced diabetic rats have elevated hepatic GM3 levels (74); and 3), Zucker diabetic fatty (ZDF) rats have elevated muscle (quadriceps) GM3 ganglioside levels (75). However, the latter finding is in contrast to that reported by Aerts *et al.* (73), who found that neither glucosylceramide nor GM3 gangliosides were elevated in muscle or liver of ZDF rats.

Studies performed with insulin-resistant human subjects similarly demonstrate aberrant ceramide accumulation. Adams *et al*. (76) demonstrated that obese, insulin-resistant subjects display significantly higher ceramide content in vastus lateralis muscle than lean subjects with no family history of diabetes. By contrast, they found no significant differences in other sphingolipids. Gorska *et al*. (77) demonstrated that serum sphinganine and sphingosine were elevated in type 2 diabetics compared with healthy control subjects, which may suggest elevations in serum ceramide as well. Thus far, these studies have involved analysis of relatively small numbers of people and have not revealed whether ceramide accumulation predicts insulin resistance in lean individuals.

III. Sphingolipids in Atherosclerosis

Atherosclerosis is characterized by the deposition of atheromatous plaques containing cholesterol and other lipids on the innermost layer of arterial walls, and the condition is a leading cause of death in the United States. Aggregation of lipoproteins is a fundamental step in the formation of atherosclerotic lesions.

A. Modulation of sphingolipid levels prevents plaque formation in ApoE-deficient mice

The most abundant lipids within lipoproteins include cholesterol, cholesterol esters, triglycerides, and sphingomyelin. Noting that plasma sphingomyelin levels correlate with coronary artery disease independently of cholesterol levels (78, 79) and that atherosclerotic lesions contained much higher concentrations of ceramide when compared with plasma low-density lipoproteins (LDLs) (80, 81), Park *et al.* (82) investigated whether inhibiting rates of sphingolipid biosynthesis affected plaque formation. They demonstrated that in apolipoprotein E (apoE)-deficient mice, which are a commonly used rodent model of atherosclerosis, SPT activity and plasma sphingomyelin levels increased markedly during high-fat feeding. Treating these animals with the SPT inhibitor myriocin dramatically lowered SPT activity and reduced plasma sphingomyelin levels by 64%, bringing it to the level of standard chow-fed animals. Interestingly, it also caused a reduction in circulating cholesterol, very low-density lipoproteins, and LDLs. Ultimately the treatment strategy led to a 93% reduction in atherosclerotic lesion coverage within the aorta, as well as substantial decrease in plaques in the brachiocephalic artery and aortic valve area.

Shortly after Park *et al.* (82) published their findings, Hojjati *et al.* (83) reported similar results including the lowered SPT activity, decreased plasma sphingomyelin, ceramide, and S1P levels, and decreased atherosclerotic lesion area in fat-fed apoE mice treated with myriocin. Despite the similar conclusions, this group reported substantial differences. First, they used an ip injection approach for administering the drug, claiming that oral administration caused gastrointestinal toxicity. Second, they found that the treatment had no effect on circulating cholesterol and triglyceride levels. Similar conclusions were reached by Glaros *et al*. (84), who found that myriocin additionally decreased serum glycosphongolipid levels.

In a follow-up study, Park *et al.* (85) addressed the issue of gastrointestinal toxicity, noting that their treatment protocol had no deleterious consequences in their subset of animals. In this work, they demonstrated that myriocin prevented the formation of atherosclerotic-like lesions caused by acutely placing a nonocclusive polyethylene cuff on the femoral artery of the apoE knockout mice. After 4 wk on a high-fat diet, the animals developed macrophage-rich atherosclerotic-like lesions, which were again reduced by 98% by myriocin. Moreover, they again saw the decrease in circulating cholesterol, which they attributed to a suppression of sterol regulatory element-binding protein.

Despite the subtle discrepancies between the findings of these groups, their work strongly suggests that one or more sphingolipids contributes to atherosclerotic lesion formation in this animal model. Moreover, these studies were pioneering because they established experimental paradigms that would be repeated in subsequent studies evaluating the toxicity of ceramides in metabolic disease.

B. Mechanism by which sphingolipids promote atherosclerosis and thrombosis

A number of different mechanisms have been proposed to explain how sphingolipids may contribute to lesion formation.

First, aggregation of atherogenic lipoproteins is important for the initiation and progression of atherosclerosis. Studies with purified LDLs suggest that the strong tendency of ceramides to self-aggregate may contribute to the amalgamation of ceramide-enriched LDLs (86, 87). In support of this, bacterial sphingomyelinase promotes LDL aggregation, and the ceramide content of aggregated LDLs is much higher than plasma LDLs (80).

Second, ceramides can induce apoptosis in vascular wall cells, thus contributing to plaque erosion that can induce thrombosis (88, 89). A more complete discussion of ceramides and apoptosis is included in *Section V*.

Third, by blocking access to apoE and lipoprotein lipase, sphingomyelin may block LDL uptake (90). This is consistent with the aforementioned data indicating that myriocin selectively lowers LDL levels.

Fourth, S1P stimulates endothelial and smooth muscle cell proliferation, thus contributing to thickening of the vascular wall and plaque stabilization (91, 92)

And fifth, ceramide may regulate the synthesis of PAI-1, which contributes to atherosclerosis and thrombosis. TNFα has been shown to regulate PAI-1 levels in cultured cells (93–96), rodents (97), and humans (98). However, ceramide may mediate this effect because sphingomyelinase and short chain ceramides stimulate PAI-1 release in human umbilical vein endothelial cells (99, 100) or human astrocytes (101).

IV. Sphingolipids in Insulin Resistance

Canonical insulin target tissues include skeletal muscle, adipose tissue, and the liver. In muscle and fat, insulin promotes glucose uptake by facilitating the translocation of glucose transporter 4 (GLUT4) from intracellular stores to the plasma membrane. In the liver, insulin inhibits glucose efflux by blocking gluconeogenesis. Simultaneously, insulin activates anabolic enzymes and inhibits catabolic ones to promote the storage of the incoming glucose as glycogen. Although insulin has been viewed historically as being primarily involved in glucose uptake, the hormone additionally facilitates the uptake and storage of amino acids and fatty acids, converting them to protein and lipid, respectively (102–105). Recent studies suggest that insulin may have actions on other tissues that enable it to effectively manage postprandial nutrient disposal. In the brain, insulin has been proposed to serve in the regulation of satiety and to initiate central signaling events that modulate anabolic responses in peripheral tissues such as the liver (106–108). In the vasculature, insulin promotes vasodilation, an important component in promoting glucose clearance (109). In the β-cell, insulin inhibits apoptosis and drives survival (110–112). All of these processes are mediated by a common intracellular signaling pathway summarized in *Section IV.B* (Fig. 2).

Insulin resistance occurs when a normal dose of insulin is incapable of eliciting these anabolic responses. The condition, along with central obesity, dyslipidemia, hyperglycemia, glucose intolerance, and hypertension, predicts development of cardiovascular disease and diabetes (113, 114). As proposed initially by Reaven (113, 115), insulin resistance

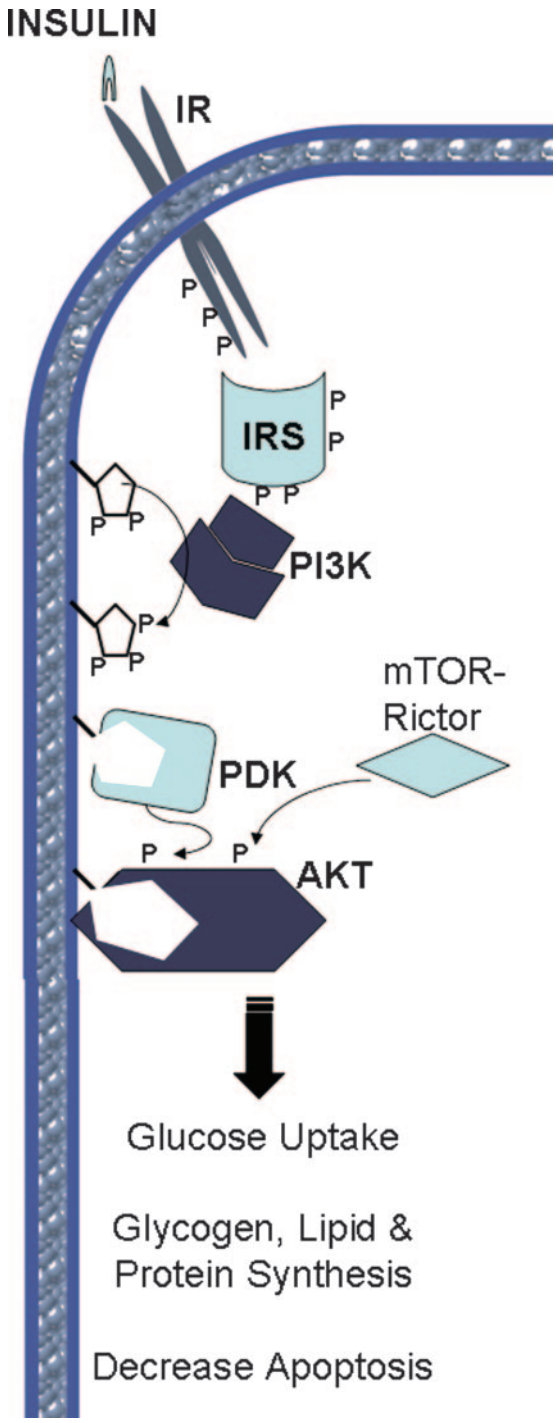

FIG. 2. Schematic diagram illustrating the canonical insulin signaling pathway. The insulin receptor (IR) phosphorylates itself as well as IRS. PI3 kinase (PI3K) phosphorylates 3-phosphoinositides, which produce binding sites for PIP_3 dependent kinase (PDK) and Akt via their PH domains. Akt is phosphorylated by PDK and mTOR-Rictor, which lead to active Akt kinase activity and its pleiotropic effects. P denotes key phosphorylation events.

and its associated compensatory hyperinsulinemia may contribute to the etiology of both of these conditions.

A. Modulating sphingolipid levels impacts insulin sensitivity in vivo

An abundance of studies have evaluated the role of ceramides in the control of insulin sensitivity, using a wide variety of cultured cell and rodent models of the condition. Conclusions from these studies have identified a subset of biosynthetic enzymes as therapeutic targets for improving insulin sensitivity.

1. Lipid-induced insulin resistance. The addition of lipids in excess of a tissue's oxidative or storage capacity is sufficient to induce insulin resistance. Strategies that have been implemented to model this condition include the following: 1) incubating isolated muscle strips with FFAs (12, 116–120); or 2) infusing lipid emulsions into rodents or humans (12, 121–125). In both of these model systems, pharmacological or genetic ablation of enzymes controlling ceramide biosynthesis prevents the induction of insulin resistance.

- Infusing lard oil emulsions (a lipid emulsion composed of diverse triglyceride species, of which 37% are saturated fat) into the bloodstream of Sprague-Dawley rats via jugular catheters promotes ceramide accrual in skeletal muscle and liver while inducing insulin resistance (as assessed by hyperinsulinemic-euglycemic clamps). Coinfusing inhibitors of SPT (*i.e.*, myriocin, cycloserine) prevented the increases in ceramide accumulation and maintained insulin-stimulated glucose disposal (12). The improvement in glucose homeostasis was due to increased glucose disposal into skeletal muscle and a restoration in insulin suppression of hepatic glucose output.
- Administration of saturated fats to isolated rodent muscles has also been shown to induce insulin resistance via a ceramide-dependent mechanism. Administering ceramide or palmitate to isolated muscle strips impairs 2-deoxyglucose uptake. Treating muscles with SPT (12) or CerS inhibitors (our unpublished observation) made them refractory to palmitate inhibition of insulin-stimulated glucose uptake in isolated soleus muscles. Similarly, isolated soleus muscles from mice lacking one allele of dihydroceramide desaturase 1 were impervious to palmitate-induced insulin resistance (12).[1]

An elevated ratio of saturated fats to unsaturated fats is a risk factor for metabolic complications (126). The experiments described above suggest that ceramide derived from saturated fats could be a primary contributor to insulin resistance. However, an interesting observation in these studies was that unsaturated fats induce insulin resistance by a distinct mechanism that is ceramide-independent. Specifically, infusion of soy- or safflower-based lipid emulsions (Liposyn II or Intralipid) that are enriched in the unsaturated fatty acid linoleate promotes insulin resistance but does not reliably induce ceramide (123, 127). [Note: Some researchers have detected significant increases in ceramide content after Intralipid infusion (125, 128), and it is unclear what causes the discrepancy in findings.] Moreover, coinfusing SPT inhibitors fails to prevent their induction of insulin resistance (12). Studies in the isolated muscle system confirmed that linoleate (*i.e.*, the predominant fatty acid side-chain in Intralipid and Liposyn II) antagonized 2-deoxyglucose uptake via a ceramide-independent mechanism (12). Linoleate-induced insulin resistance is likely to involve a glycerolipid intermediate because mice lacking an enzyme that attaches fatty acids to the glycerol backbone (mitochondrial glycerol phosphate acyl-transferase) are protected from Intralipid-induced hepatic insulin resistance (129). Studies conducted by the Shulman laboratory have correlated the production of diacylglycerol with the induction of unsaturated fat-induced insulin resistance, and serine phosphorylation of insulin receptor substrate (IRS)-1 by protein kinase C (PKC) θ and/or inhibitor of nuclear factor-κB kinase (IKK) appears to be involved in these effects (reviewed in Ref. 130). The observation that down-regulation of diacylglycerol kinase elevates diacylglycerol and exacerbates insulin resistance (131) is consistent with this hypothesis. Paradoxically, recent studies in cultured myotubes suggest that di-linoleoyl phosphatidic acid, and not diacylglycerol, may be the primary lipid metabolite that antagonizes insulin action (132).

2. Glucocorticoid-induced insulin resistance. Excess glucocorticoids have long been suspected to produce insulin resistance, and studies over the last few decades have begun to elucidate the importance of these effects. Although it is relatively rare for obese patients to display elevated serum glucocorticoid levels present in classical Cushing's syndrome, numerous studies have suggested that obese and/or diabetic individuals may display an elevated response to circulating glucocorticoids. 11β-Hydroxysterol dehydrogenase 1 (11-HSD1) reactivates glucocorticoid precursors (11 dehydrocorticosterone in rodents or hydrocortisone in humans) to form functionally active glucocorticoids (corticosterone in rodents or cortisol in humans). The expression of the enzyme correlates with obesity and diabetes in rodents (133, 134) and humans (135–137), and manipulating expression of this enzyme *in vivo* has a profound effect on obesity and insulin resistance. Specifically, overexpression of 11β-HSD1 in adipose tissue promotes obesity and insulin-resistant diabetes (62). By contrast, 11β-HSD1 null mice or mice with adipose-specific overexpression of 11β-HSD type 2, which performs the reverse reaction to deactivate active glucocorticoids, are protected from diet-induced obesity and maintain superior glucose homeostasis and insulin sensitivity when challenged with high-fat diets (138, 139). Collectively, these studies have established the potential for heightened glucocorticoid responses to contribute to insulin resistance.

Although glucocorticoids have long been known to promote ceramide biosynthesis, the role of ceramide in their

[1] Unlike the *in vivo* studies described above, which involved the addition of a complex mixture of lipid metabolites, purified fatty acids were added to the isolated muscles. Advantages of this strategy are that it allows researchers to gain insight into the metabolic fates of specific fatty acids and to determine how differences in their utilization may alter disease. However, these results should be viewed with some caution because the experimental model is relatively nonphysiological. Validating the results using lipid infusion or high-fat feeding models is essential for gauging the relative importance in the control of insulin sensitivity *in vivo*.

induction of insulin resistance was not evaluated until recently. Specifically, pretreating rats with the SPT inhibitor myriocin completely prevented glucose intolerance resulting from the administration of the synthetic glucocorticoid dexamethasone (12). This was due to the ability of the compound to maintain suppression of hepatic glucose output and promote whole body 2-deoxyglucose uptake. Mice heterozygous for Des1 were similarly protected from dexamethasone-induced insulin resistance.

Glucocorticoids may also employ ceramide-independent mechanisms in their regulation of hepatic insulin sensitivity. In liver, a glucocorticoid-responsive element in cAMP regulatory element binding protein induces phosphoenolpyruvate carboxykinase, which governs the rate-limiting step in gluconeogenesis (140, 141). Moreover, numerous cell culture studies suggest that dexamethasone can under certain conditions repress expression of other insulin-signaling intermediates (142–145). The Semenkovich laboratory completed a particularly impressive series of studies demonstrating that peroxisome proliferator activated receptor (PPAR) α, a member of the nuclear receptor superfamily that promotes lipid uptake and oxidation, is critical for glucocorticoid-induced insulin resistance. Specifically, they demonstrated that genetic ablation PPARα or disruption of hepatic vagal nerves (which decreases hepatic PPARα expression) prevented dexamethasone-induced glucose intolerance and hepatic glucose output. Curiously, in other tissues, most notably in the heart, PPARα overexpression (146) or activation (147) promotes ceramide accumulation. Thus, the existence of a relationship between PPARα and ceramide signaling remains a formal possibility.

3. Obesity-induced insulin resistance. With animal models, it is difficult (perhaps impossible) to differentiate between effects caused by lipid oversupply, glucocorticoids, and inflammation. However, given the relative role of ceramide as a common molecular intermediate linking many of these metabolic stresses to the induction of insulin resistance, one would predict that inhibition of sphingolipid production would improve insulin sensitivity in obese rodents. Indeed, recent studies suggest that this is in fact the case.

Obese leptin (or leptin receptor) -deficient (*e.g.*, Zucker fa/fa rats, ZDF rats, ob/ob mice, and db/db mice) and high fat-fed animals display evidence of increased inflammation and dyslipidema. Treating ZDF and Zucker fa/fa rats with the SPT inhibitor myriocin prevented aberrant ceramide accumulation in muscle, liver, and serum and improved glucose tolerance and insulin sensitivity (12) (Table 2). Similarly, diet-induced obese mice maintained on oral doses of myriocin displayed vast improvements in insulin sensitivity, as measured by circulating insulin levels during glucose tolerance tests (12). In fact, the improvement in insulin sensitivity was on par with rosiglitazone, one of the most effective insulin-sensitizing drugs currently marketed. Fenretinide, a chemotherapeutic agent that lowers circulating retinol-binding protein levels, improves insulin sensitivity in high fat-fed mice (148). This drug was recently identified as an inhibitor of Des1 (19); thus, some of its insulin-sensitizing actions may result from effects on ceramide synthesis.

Studies with GM3 synthase null mice and inhibitors of glucosylceramide synthase suggest that gangliosides may additionally contribute to obesity-induced insulin resistance. Mice lacking the GM3 synthase gene display lower fasting glucose levels and improved glucose tolerance (149). When challenged with high-fat diets, the GM3 synthase null mice maintained superior glucose tolerance, improved insulin-stimulated glucose uptake, and enhanced suppression of hepatic glucose output measured by hyperinsulinemic-euglycemic clamps.

The enhanced glucose homeostasis of GM3 synthase null mice strongly suggests that targeted pharmacological disruption of glucosylceramide-producing enzymes may provide an effective means of combating insulin resistance and type 2 diabetes. Two recent reports confirm this hypothesis. Using highly specific inhibitors of glucosyl ceramide synthase (GCS), N-(5′-adamantane-1′-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), Aerts *et al*. (73) demonstrated the ability to selectively decrease glucosylceramide content in muscle and liver of ob/ob mice without affecting ceramide content. Administration of the drug decreased fed blood glucose and improved glucose tolerance in ob/ob mice. Moreover, AMP-DNM increased whole body glucose clearance, while decreasing hepatic glucose output, under hyper-

TABLE 2. A summary of the effects of *in vivo* prevention of aberrant sphingolipid accumulation on metabolic diseases

Metabolic condition	Rodent model	Treatment/knockout	Ref.
Atherosclerosis	ApoE-deficient mice	Myriocin	82–85
Insulin resistance	Lipid-infused, dexamethasone-treated, and high fat fed mice; Zucker fa/fa and dexamethasone-treated rats	Myriocin	12
Insulin resistance	Dexamethasone-treated mice	DES1 −/+	12
Insulin resistance	Ob/ob mice	AMP-DNM	73
Insulin resistance	High fat fed mice	Genz-123346 GM3−/−	73, 149
Diabetes (β-cell failure)	ZDF Rats	Myriocin, cycloserine, AMP-DNM Genz-123346	12, 73, 75, 236
Diabetes (β-cell failure)	NOD-Mice	FTY720	243, 297
Diabetes (β-cell failure)	DRBB Rats	FTY720	244
Cardiomyopathy	LPL-GPI Mice	Myriocin, SPT −/+	281

The SPT inhibitors myriocin or cycloserine pharmacologically inhibit *de novo* ceramide biosynthesis. Glucosylceramide synthase is inhibited by the drugs AMP-DNM or Genz-123346, thus decreasing ganglioside synthesis. FTY720 is a phosphorylatable analog of sphingosine, which mimics sphingosine-1-phosphate. Knockout animals lacking SPT, Des1, and GM3 synthase improve various metabolic parameters. GPI, Glycosylphosphatidylinositide.

insulinemic conditions. Similar improvements were detected in diet-induced obese mice because fasting glucose and insulin were decreased in mice treated with the GCS inhibitor. In a separate study, Zhao *et al*. (75) demonstrated that Genz-123346, a GCS inhibitor derived from PDMP that doesn't stimulate ceramide accrual like the parent compound (150), improves glucose homeostasis and insulin sensitivity in ZDF rats and high fat fed mice (75).

4. Antidiabetic interventions decrease sphingolipid accumulation. Insulin-sensitizing drugs are the most commonly prescribed oral hypoglycemic agents. Although these drugs are given for their beneficial effects on glucose homeostasis, their insulin-sensitizing effects may come from lipid-partitioning effects. Metformin, widely prescribed for over 40 yr in Europe, enhances AMP kinase activity via an unknown mechanism that requires the upstream kinase LKB1 (151). Although the exact mechanisms of metformin action remain unclear, the enhanced AMP kinase activity would likely promote lipid oxidation through regulation of acetyl CoA carboxylase, leading to decreased formation of ceramide or other lipid metabolites (152, 153). The antidiabetic agent may additionally decrease lipid uptake, because Smith *et al*. (154) reported that metformin prevents increases in the fatty acid transport protein CD36 as well as aberrant ceramide and diacylglycerol content in skeletal muscle of ZDF rats.

Anorectic agents such as leptin and ciliary neurotrophic factor appear to be protective against aberrant accumulation of sphingolipids. Studies pioneered by the Unger laboratory suggest that a primary function of leptin is to promote proper lipid partitioning during times of nutrient excess (155). Animals that lack leptin or the functional leptin receptor display aberrant triglyceride, diacylglycerol, and ceramide accumulation in nonadipose tissues including muscle, liver, cardiomyocytes, and β-cells. In stark contrast, when leptin is administered to leptin-sensitive animals, the adipokine prevents aberrant accumulation of lipid metabolites in muscle (156), cardiomyocytes (155, 157), and β-cells (158, 159). Thus, leptin appears to oppose lipoapoptosis of β-cells and cardiomyocytes. Ciliary neurotrophic factor, which works by unknown mechanisms, prevents lipid-induced insulin resistance and decreases ceramide accumulation without affecting diacylglycerol (125).

Thiazolidinediones (TZDs) are also a widely prescribed class of insulin-sensitizing agents that stimulate PPARγ, a nuclear receptor that controls fat cell differentiation. A likely mechanism of action of these drugs is that they promote differentiation of preadipocytes, thus increasing the storage capacity of adipose tissue and preventing lipid accumulation in tissues not suited for fat storage (160). As predicted, treatment with rosiglitazone or pioglitazone prevents aberrant ceramide accumulation in muscles from rats or mice fed normal chow or high-fat diets (161–163). However, TZDs fail to protect from insulin resistance induced by acute lipid infusion, consistent with the idea that TZDs act by limiting lipid exposure to nonadipocyte tissues (164, 165).

While TZDs lower sphingolipid levels in skeletal muscle, the effects in cardiac muscle remain unclear. Troglitazone was shown to normalize ceramide content in ZDF rat hearts (166), but pioglitazone may actually increase SPT expression and ceramide synthesis in this tissue (147). These observations are interesting because TZDs have come under recent scrutiny over concerns that they may increase cardiac complications in diabetics (167, 168).

Exercise, which has repeatedly been shown to improve insulin sensitivity and glucose homeostasis, also decreases ceramide accumulation. Acute bouts of exhaustive exercise in rats (169) or routine exercise training increase lipid oxidative capacity and diminish ceramide accumulation in rats and humans (154, 170). Ceramide degradation may also be enhanced during exercise because sphingosine content increased in many of these studies (171, 172).

B. Mechanism by which sphingolipids antagonize insulin action

Insulin binding to its cognate receptor induces autophosphorylation via the receptor's intrinsic tyrosine kinase. The activated receptor phosphorylates a family of IRS proteins that recruit and activate multiple intracellular effector pathways (173). Notably, IRS proteins provide binding sites for the p85 subunit of 3-phosphoinositide kinase (PI3K), which is requisite for most of the hormone's anabolic and antiapoptotic actions. PI3 kinase, which is a dimer consisting of a regulatory subunit (p85) and a catalytic p110 subunit, phosphorylates the membrane lipid phosphatidylinositol 4,5 bisphosphate, producing phosphatidylinositol 3,4,5 trisphosphate (PIP_3). PIP_3 is not a substrate for phospholipases, but rather serves as a binding site for proteins containing pleckstrin homology (PH) domains. Akt/protein kinase B (PKB) and phosphatidylinositol-3-phosphate dependent kinase 1 (PDK1) are serine/threonine kinases that are brought into close proximity with each other by their interactions with PIP_3. Additionally, the membrane lipid helps to activate Akt/PKB, by inducing conformational changes that expose two regulatory phosphorylation sites (174). The mammalian target of rapamycin (mTOR)-Rictor protein complex phosphorylates a regulatory serine (S473) on the C terminus of Akt/PKB (175, 176). Subsequently, PDK1 phosphorylates a regulatory threonine residue (T307) of Akt/PKB that is requisite for enzyme activity (177).

The Akt/PKB kinase includes three family members, each a product of a different gene. Studies involving the introduction of dominant-negative Akt/PKB, small interfering RNA sequences, and/or neutralizing antibodies have confirmed that the kinase, particularly the Akt2/PKBβ isoform, is a central regulator of insulin-stimulated anabolic metabolism, cell survival, and GLUT4 translocation (178, 179). Knockout mice lacking this isoform develop a diabetes-like syndrome consisting of insulin resistance in skeletal muscle and liver (180). A comprehensive analysis of Akt/PKB substrates is beyond the scope of this review, but an abbreviated list includes the following:

- *Akt substrate 160* (AS160), a rab-GTPase activating protein that regulates the subcellular localization of GLUT4 (181–183);
- *Endothelial nitric oxide synthase* (eNOS), which regulates vasodilation (184);
- *Glycogen synthase kinase 3β* (GSK3β), which regulates glycogen synthase (185–187);

- *Tuberous sclerosis complex 2* (TSC2), a component of the tuberous sclerosis heterodimer that deactivates the small GTP-binding protein Rheb. Akt/PKB thus inhibits the Rheb-GTPase-activating function of the TSC1/TSC2 complex, which facilitates Rheb activation of the mammalian target of rapamycin. This is an essential pathway in protein synthesis and the regulation of cell growth (188);
- *Phosphodiesterase-3β* (PDE3β), which hydrolyzes cAMP to block the effects of glucagon on gluconeogenesis, glycogenolysis, and lipolysis (189, 190);
- *BAD*, a Bcl2 family member involved in apoptosis (191–193);
- *Proliferator-activated receptor-coactivator 1a* (PGC-1a), a transcriptional coactivator peroxisome that is a global regulator of hepatic gluconeogenesis and fatty acid oxidation (194, 195);
- and *FOXO*, a transcription factor that regulates gluconeogenesis, the detoxification of reactive oxygen species (ROS), cell cycle, cell survival, and energy homeostasis (196).

The majority of studies evaluating the mechanisms of sphingolipid-induced insulin resistance have employed cultured cell systems. Although a number of sphingolipid entities have been identified as potential inhibitors of insulin signal transduction, ceramide and GM3 gangliosides have received the most attention. Using analogs of these lipids or various approaches to increase endogenous accumulation, researchers have delineated several potential mechanisms by which these lipids impair insulin action (Fig. 3). Despite years of attention, the precise mechanisms governing the effects of these lipids are not fully resolved.

1. Ceramide. When added to cultured myotubes, hepatocytes, or adipocytes, ceramide analogs acutely inhibit glycogen synthesis or glucose uptake (197). The mechanism underlying this effect appears to be the inhibition of Akt/PKB, which is accomplished by one of two mechanisms.

- Ceramide blocks the translocation of Akt/PKB to the plasma membrane (198). Under these conditions, the lipid fails to inhibit insulin signaling through PI3-kinase, the accumulation of 3′-phosphoinositides, or the translocation of PDK1. Studies by Powell *et al.* (199) may have uncovered the mechanism underlying this ceramide action. Specifically, this group demonstrated that ceramide inactivation of Akt/PKB requires the atypical PKC isoform PKCζ. Impressively, they found that PKCζ phosphorylates serine 34 of the Akt/PKB PH domain. Using dot blot assays, they further demonstrated that phosphorylation of the PH domain on this residue blocked its ability to interact with PIP_3, blocking its net translocation. In the L6 myotube cell system used in this assay, ceramide inactivation of Akt/PKB was negated by the administration of PKCζ inhibitors or the expression of dominant-negative PKCζ constructs (200). Moreover, an Akt/PKB isoform with the S34 site converted to an alanine was resistant to ceramide effects. Similar findings were obtained in vascular smooth muscle (201, 202), where it was further demonstrated that ceramide stabilized interactions between Akt/PKB and PKCζ by recruiting the enzymes to detergent-resistant membrane fractions (*e.g.*, membrane rafts or caveolae) (202, 203).
- Among the earliest known ceramide targets was protein phosphatase 2A (PP2A) (204, 205), which was shown by the Olefsky group to dephosphorylate Akt/PKB and alter insulin stimulation of glucose uptake (206). These observations prompted the hypothesis that ceramide would promote dephosphorylation and inactivation of Akt/PKB. Indeed, several groups have demonstrated that ceramide promotes the dephosphorylation of Akt/PKB by protein phosphatase 2A (13, 207–209). In C2C12 myotubes (13), PC12 neurons (207), brown adipocytes (208), or a human glioblastoma cell line (209), the PP2A inhibitor okadaic acid obviates the effects of ceramide on Akt/PKB. Similarly, overexpressing the SV40 small T antigen, which impairs PP2A activity by displacing regulatory subunits that target PP2A to specific substrates, negates ceramide effects on Akt/PKB in certain cell types (13).

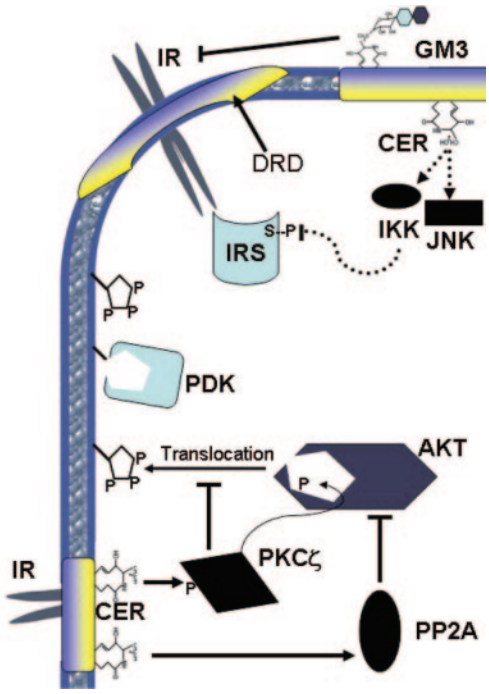

FIG. 3. Schematic diagram depicting the multiple mechanisms by which sphingolipids impair insulin action. *Top*, GM3 gangliosides present in detergent-resistant microdomains (DRD) displace the insulin receptor from these domains and prevent insulin receptor activation. Ceramide (CER) may lead to activation of IKK and c-Jun N-terminal kinase, which inhibit IRS via serine phosphorylation (S-P). *Bottom*, Ceramide activates PKCζ, which via phosphorylation on Akt's PH domain prevents binding to 3-phosphoinositides that aid in Akt activation. Additionally, ceramide activates PP2A, which impairs Akt activity via removal of activating phosphate residues.

In some cell types, such as 3T3-L1 adipocytes, both mechanisms are present (210, 211), whereas in other cultured cell systems (C2C12 myotubes or A7r5 vascular smooth muscle cells) either PP2A or PKCζ appears to play the dominant role (13, 201). It remains unclear which pathway plays the primary role in mammalian insulin resistance because their relative importance has not been assessed *in vivo*.

Although these studies were generally done using cer-

amide analogs, a number of groups subsequently demonstrated that relatively small increases in endogenous ceramide (~50%) were sufficient to activate both of these pathways (13, 199, 200). Schmitz-Peiffer *et al.* (118) first demonstrated that exposing cultured myotubes to palmitate, the most abundant saturate fatty acid in the circulation, increased ceramide accumulation while simultaneously inhibiting Akt/PKB. Blocking ceramide accumulation using myriocin, cycloserine, or fumonisin B1 restores insulin-stimulated Akt and GSK3β phosphorylation, even in the presence of excess palmitate (13, 200). This system was later recapitulated in cultured human myotubes (212, 213). Interestingly, in these studies, coadministering oleate shunted palmitate into triglyceride synthesis pathways, thus preventing aberrant ceramide accumulation and insulin resistance.

As an alternative strategy for manipulating endogenous ceramide, treating cultured cells with inhibitors of ceramide deacylation (NOE) or glucosylation (PDMP) exacerbated palmitate-induced insulin resistance (214), whereas overexpressing acid ceramidase negated these palmitate effects (13, 200). These studies confirmed the requirement for ceramide, rather than another sphingolipid metabolite, in the inhibition of Akt/PKB.

Relatively few studies have addressed the mechanisms by which ceramide impairs insulin action in live animals. We recently demonstrated that treatments (dexamethasone or lipid infusion) that promote ceramide accumulation in muscle and liver of live rats impair insulin signaling to Akt without affecting PI3 kinase (12). These effects on Akt were reversed by SPT inhibition, suggesting that endogenous ceramide impairs insulin action at the level of Akt in *bona fide* muscle and liver.

The concept that PKCζ impairs Akt/PKB, thus inhibiting glucose uptake and anabolic metabolism, is difficult to reconcile with numerous reports identifying the enzyme as an obligate intermediate in insulin effects. Several prior reports revealed that PKCζ and its related isoform PKCλ are inducers of glucose transport. In cultured L6 myotubes, PKCζ or PKCλ inhibition impairs insulin-stimulated glucose uptake, whereas overexpression of either enzyme stimulates glucose uptake (215–217). Moreover, Farese *et al*. (218) demonstrated that muscle-specific PKC-λ knockout mice were glucose intolerant due to muscle insulin resistance. A more intriguing role for ceramide affiliating with PKCζ may be in the liver, where glucose uptake is governed differently. Interestingly, Ron Kahn's group has demonstrated that PKCζ is requisite for insulin induction of lipogenesis in the liver (219). Thus, should ceramide activate this enzyme in the liver, an attractive hypothesis is that ceramide could simultaneously antagonize insulin repression of glucose output (*i.e.*, by inhibiting Akt/PKB) while maintaining the lipogenic pathways that promote hepatic steatosis (*i.e.*, by activating PKCζ). Indeed, bioinformatic strategies for conducting lipidomic analysis have revealed particularly strong associations between hepatic ceramide levels and the extent of steatosis in a rodent model of obesity (8). The potential for aberrant ceramide accumulation to promote fatty liver disease is an intriguing concept, but it hasn't been experimentally validated.

We previously demonstrated that overexpression of a constitutively active isoform of Akt/PKB negated ceramide's inhibitory actions toward glucose uptake (210). These data were consistent with the hypothesis that ceramide-induced insulin resistance is due to its effects on early signaling events. However, JeBailey *et al.* (220) recently found that low doses of ceramide inhibited glucose uptake independently of these effects on Akt/PKB in L6 myotubes. They concluded that ceramide independently blocked actin remodeling by preventing activation of Rac and thus attenuated GLUT4 translocation. Interestingly, Long and Pekala (221) found that ceramide decreased GLUT4 transcription, suggesting another mechanism in 3T3-L1 adipocytes by which the lipid induces insulin resistance.

Another mechanism by which ceramide may impair insulin action is by facilitating signaling pathways initiated by inflammatory cytokines, such as TNFα, that activate serine/threonine kinases (*e.g.*, c-Jun N-terminal kinase, IKK) known to impair insulin signaling. Moreover, TNFα alters the expression of genes that modulate insulin signaling, including suppressor of cytokine signaling-3, an insulin receptor/IRS interacting protein. Lastly, TNFα alters rates of lipid hydrolysis in adipocytes while decreasing lipid oxidation in skeletal muscle, which likely exacerbates rates of formation of deleterious lipid metabolites. TNFα rapidly generates ceramide via the hydrolysis of sphingomyelin and subsequently induces a sustained elevation in ceramide by promoting its *de novo* synthesis (222–224). The acid sphingomyelinase containing death domain of the 55-kDa TNF receptor, which catalyzes this reaction, is required for TNFα's antagonism of insulin action (225). Intriguing work by the Gulbins group indicates that local production of ceramide within membrane microdomains promotes receptor clustering, which is important for signal transmission (225).

2. Glucosylceramide derivatives. Exogenous GM3 gangliosides inhibit insulin receptor tyrosine phosphorylation and IRS-1 tyrosine phosphorylation in cultured 3T3-L1 adipocytes. Mechanistically, gangliosides impair dimerization and activation of tyrosine kinase receptors (226). Similar findings were obtained in cultured cells treated with TNFα, which induces GM3. In these studies, treating with the aforementioned GCS inhibitors negates TNFα effects on IRS-1 (48, 73). The mechanism underlying this effect appears to be that ganglioside production within detergent-resistant raft domains displaces insulin receptors, thus antagonizing insulin receptor signaling to IRS-1 (49, 50).

Studies *in vivo* support the idea that gangliosides impair insulin activation of its receptors. The GM3 synthase knockout mice demonstrate enhanced tyrosine phosphorylation of the receptor when compared with wild-type mice (149). Similarly, the various GCS inhibitors augment insulin-stimulated phosphorylation of the insulin receptor, as well as Akt/PKB and/or the mTOR phosphorylation, in skeletal muscle (75) and liver (73) of obese rodents.

V. Sphingolipids in Pancreatic β-Cell Failure

Diabetes mellitus results from insulin availability that is insufficient to meet tissue insulin needs (227), and recent

studies suggest that both the type 1 and type 2 forms of the disease are associated with decreased β-cell mass resulting from decreased proliferation and increased apoptosis (228). Moreover, the susceptibility of β-cells to both apoptosis and necrosis during isolation or transplantation has hindered attempts to utilize islet transplantation as a treatment for these diseases (229). Numerous findings suggest that β-cell apoptosis, perhaps resulting from increased exposure to glucose, saturated fats, TNFα, or islet-associated amyloid polypeptide could account for this decline in β-cell function (227, 230–232).

Several different sphingolipid metabolites have emerged as potentially important regulators of β-cell survival, proliferation, and function. Ceramide, which can be produced in response to inflammatory cytokines (*e.g.*, TNFα or IL1) or by excessive deposition of saturated fats, inhibits insulin gene expression, blocks β-cell proliferation, and induces β-cell apoptosis (233–237). Gangliosides, which are glycosylated derivatives of ceramide, have been speculated to be antigens implicated in the onset of the autoimmune response (238). In contrast, S1P promotes β-cell growth and survival and augments glucose-stimulated insulin secretion (101, 239–241).

A. Modulating sphingolipid levels impacts the β-cell in rodent models of diabetes

In rodent models of type 2 diabetes, increases in islet ceramide and triglyceride precede β-cell dysfunction and destruction (230, 242). The Unger group, in the first study to evaluate the consequences of ceramide depletion on metabolic disease *in vivo*, reported that treating Zucker diabetic fa/fa (ZDF) rats with cycloserine reduced islet apoptosis (236). In subsequent studies, the SPT (12) or GCS (73, 75) inhibitors, which have more substantial and specific effects on sphingolipid levels, were shown to preserve β-cell function and prevent onset of frank diabetes in this animal model.

FTY720 is a novel immunosuppressant that functions as an S1P receptor agonist. The compound has shown particular efficacy in preventing the demise of β-cells in rodent models of type 1 diabetes (NOD mice and DRBB rats) (243, 244) and during islet transplantation (241, 245–251). Its primary mechanism of action is attributable to its ability to prevent the infiltration of effector lymphocytes into islets. However, although many such immunosuppressive compounds are often toxic to β-cells, S1P actually enhances β-cell function (241). Moreover, studies *in vitro* (discussed in *Section V.B*) suggest that S1P may have insulinotropic capabilities, which render it particularly suited for work in islet transplantation. Moreover, these findings suggest that S1P may in fact be a novel endogenous modulator of β-cell homeostasis.

B. Mechanism of ceramide-mediated β-cell failure

Although inhibition of ceramide production clearly preserves β-cell function in ZDF rats, it is difficult to know whether this was a direct effect of ceramide depletion in β-cells, was due to global alterations in inflammatory responses, or was a consequence of enhanced peripheral insulin sensitivity in liver and muscle (12). Ultimately this will require experiments investigating conditional ablation of ceramide in selected cell types. Nonetheless, numerous *in vitro* studies suggest that ceramide may directly alter β-cell responsiveness.

Noting that palmitate, but not oleate, affected insulin gene transcription in isolated and cultured islets, the Poitout laboratory (233, 252, 253) investigated the hypothesis that ceramide was an intermediary linking the excess lipid to the regulation of the insulin gene. They found that ceramide analogs decreased insulin gene transcription and inhibitors of *de novo* ceramide synthesis prevented the antagonistic effects of palmitate (233, 252, 253). The ceramide effects result from the inhibition of binding of the transcription factors pancreatic/duodenal homeobox-1 and mammalian homolog of avian MafA/L-Maf (MafA) to the insulin promoter. These effects appear to result from the ability of the sphingolipid to inhibit glucose stimulation of the nuclear translocation of pancreatic/duodenal homeobox-1, coupled with its ability to block glucose induction of the MafA transcript, but direct targets of ceramide that account for this action are unknown (233, 252, 253).

Ceramides induce apoptosis in cultured islets or isolated β-cells (234, 236, 237, 254, 255), and inhibitors of *de novo* ceramide synthesis partially prevent palmitate induction of β-cell death *in vitro* (233, 234, 236, 255, 256). Intracellular mechanisms by which ceramide induces apoptosis have been detailed in other cell types (257, 258), but their relevance to the β-cell is only partially elucidated. Briefly, ceramide induces a variety of independent effects, which could ultimately contribute to programmed cell death.

1. Recruitment of Bax to the mitochondria. Bax is a proapoptotic member of the Bcl2 family that functions by promoting cytochrome c release from the mitochondria. As a monomer, Bax is an inactive, largely cytosolic protein. However, after stimulation of cells with apoptotic stimuli, Bax undergoes a conformational change causing it to oligomerize and subsequently induce cytochrome c release from mitochondria. Two recent studies support a role for mitochondrial ceramide in the recruitment and conformational change of Bax. Kashkar *et al.* (259), using small interfering RNA strategies or ASMase (−/−) fibroblasts, found that ASMase was requisite for the induction of a Bax conformational change after UV stimulation. By contrast, they found that treating cells or isolated mitochondria with ceramide, but not dihydroceramide, induced the conformational shift and effected cytochrome c and Smac release. Similarly, Birbes *et al.* (31–33) found that overexpressing a bacterial sphingomyelinase targeted to mitochondria induced Bax translocation in intact cells, or that treating mitochondria with recombinant sphingomyelinase promoted recruitment of Bax to mitochondria in a cell free system. Collectively, these studies suggest that ceramides induce the recruitment of Bax to the mitochondria, thus eliciting a conformational change, oligomerization, and permeabilization of the mitochondria to cytochrome c/Smac.

2. Creation of ROS. Although ROS have generally been regarded as toxic byproducts of aerobic metabolism, scientists now appreciate their roles as signaling intermediates that regulate cell growth or death (260). The major source of ROS in most cells is leakage of electrons from the mitochondrial

respiratory chain to produce O_2^-. Ceramide may directly regulate respiration in isolated mitochondria by inhibiting the mitochondrial ubiquinone pool of complex III (35, 261). Alternatively, ceramides also could generate ROS through the regulation of nicotinamide adenine dinucleotide phosphate oxidase (262, 263).

3. Direct effects on mitochondrial membrane permeability. Siskind *et al.* (40) additionally proposed that ceramide, but not dihydroceramide, has the capacity to form channels in the mitochonrdrial membrane, thus increasing the permeability of organelles to cytochrome c (264–270).

C. Mechanism of glucoslyceramide-mediated β-cell failure

In addition to perhaps serving as an intracellular regulator of β-cell mitogenesis and apoptosis, ceramide is a precursor for galactolipids, which are speculated to serve as autoantigens that target T-lymphocytes to the β-cell in type 1 diabetes. The involvement of gangliosides was prompted by the initial finding of circulating antibodies against ganglioside GT3 in about 30% of newly diagnosed type 1 diabetics (271). Subsequent studies revealed that antibodies toward other gangliosides were also elevated in either prediabetics or diabetics (238). In particular, anti-GM2–1 autoantibodies were expressed in a high percentage (71%) of newly diagnosed type 1 subjects (238).

D. S1P as a regulator of β-cell growth and survival

Little attention has been placed on understanding the mechanism by which S1P regulates insulin secretion (101, 241) and β-cell survival (239). The best known effects of S1P result from its ability to activate a family of G protein-linked receptors (S1P1, -2, -3, -4, -5, formerly EDG1, -3, -5, -6, and -8), which initiate the MAPK and PI3-kinase-Akt/PKB signaling pathways to regulate cell growth and survival (Fig. 4). In addition to serving as an extracellular agonist of S1P receptors, S1P may also function as an intracellular messenger. For example, the overexpression of sphingosine kinase, which produces S1P from sphingosine, stimulates cell proliferation and survival of S1P-receptor null fibroblasts (272). Moreover, dihydro-S1P, which binds to and activates all S1P receptors, does not mimic the effects of S1P on cell survival in some cell types (273). Thus, some S1P actions may be receptor independent.

Four of the five S1P receptors thus far identified are present in mouse pancreatic islets, and three of them are expressed in Ins-1 insulinoma cells (240). Glucose acutely increases expression of the S1PR1 isoform in freshly isolated islets (*e.g.*, after a 2-h treatment), whereas chronic glucose decreased S1PR1 expression (*e.g.*, after 7 d of treatment). These data suggest that physiological regulation of this signaling pathway could underlie nutrient regulation of β-cell proliferation.

VI. Sphingolipids in Cardiomyopathy

Lipid accumulation in the heart is associated with impaired contractile function (166, 274). Transgenic approaches to produce excessive lipid uptake into the heart have allowed for the creation of rodent models of lipotoxic cardiomyopathy (275–280). In some cases this was shown to be associated with increases in ceramide (146, 280).

A. Modulating sphingolipid levels ameliorates cardiac dysfunction in a rodent model of lipotoxic cardiomyopathy

To determine whether ceramide could contribute to the progressive decline in cardiac function associated with a fatty heart, Ira Goldberg's laboratory recently completed a study (281) investigating the functional consequences of ceramide depletion in mice expressing a glycosylphosphatidy-

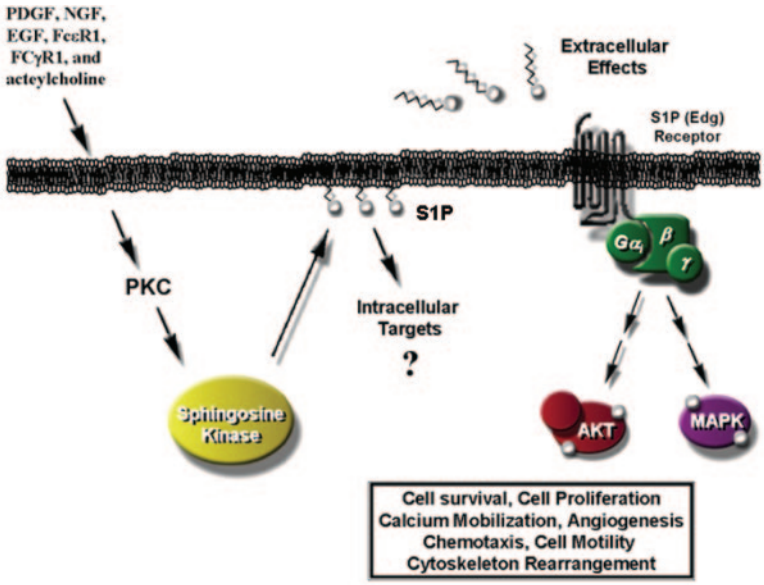

FIG. 4. This schematic depicts the production of S1P by sphingosine kinase and its resulting roles as both an extracellular ligand for S1P receptors and a putative intracellular messenger. Akt/PKB and MAPK are serine/threonine kinases shown previously to stimulate β-cell survival or proliferation, respectively.

linositide-anchored lipoprotein lipase exclusively in the heart. As they described previously (279), these animals exhibit enlarged myocytes with abnormal architecture, which led to cardiac hypertrophy, left ventricular dilatation, and reduced fractional shortening. Treating these animals with myriocin selectively decreased heart ceramide levels without impacting diacylglycerol, triacylglycerol, cholesterol, and FFA levels; restored heart size to normal; and improved fractional shortening. Ultimately, the lipoprotein lipase (LPL) transgenics had decreased survival resulting from the developing cardiomyopathy, which myriocin partially reversed (281).

An important component of the study by the Goldberg laboratory was the demonstration that haploinsufficiency for SPT also rendered mice resistant to lipotoxic cardiomyopathy. Specifically, when the LPL transgenics were crossed onto a background strain lacking a single allele encoding the LCB1 subunit of SPT (282), they demonstrated improved systolic function and fractional shortening when compared with the LPL transgenics. This is a particularly important observation because it demonstrates a genetic complement for the findings with myriocin.

B. Mechanism by which ceramides promote cardiomyocyte dysfunction and apoptosis

Progressive contractile dysfunction and apoptotic cell loss are key features of heart failure (283). Saturated FFAs, not other FFAs, are sufficient to induce cardiomyocyte apoptosis or damage myofibrils (255, 284–287), which C2-ceramide recapitulates and CerS inhibition negates (255). Moreover, altering the ceramide/S1P ratio has been shown to contribute to apoptosis in other models, including that resulting from ischemia or ischemia reperfusion (288–290). In the studies by the Goldberg laboratory, myriocin decreased expression of some apoptotic genes, but there was no evidence of increased 2-deoxyuridine 5-triphosphate nick end labeling staining in the LPL hearts. Ceramide induction of ROS is implicated in cardiomyocyte apoptosis (287, 288, 291) and to induce HERG potassium channel dysfunction, which depresses cardiac repolarization (292). Moreover, ceramides stimulate mitochondrial fission, which is associated with early activation of cardiomyocyte apoptosis (293).

In the aforementioned study by Park *et al.* (281), blocking ceramide synthesis appeared to alter mitochondrial energetics. Specifically, heart-specific LPL overexpression led to a switch in substrate utilization, including an increased reliance on FFAs for energy. Myriocin reversed this by increasing rates of glucose oxidation. A potential mechanism for this was that it prevented LPL-induced increases in pyruvate dehydrogenase kinase-4, which increases phosphorylation of pyruvate dehydrogenase and decreases rates of glucose oxidation. In hearts isolated from the LPL transgenics, myriocin normalized cardiac efficiency, enhancing mitochondrial energetic by maintaining cardiac performance at a lower oxygen cost.

LPL and myriocin had paradoxical and surprising effects on Akt/PKB. Specifically, LPL increased Akt/PKB activity (281), which is consistent with the increase in heart size. By contrast, myriocin prevented this defect. Thus, these results are in opposition to those seen in liver and muscle, where ceramide inhibits Akt/PKB and myriocin enhances activation of the enzyme, in rodent models of obesity.

VII. Conclusions and Considerations

Inhibition of ceramide synthesis has beneficial effects in rodent models of atherosclerosis, insulin resistance, diabetes, and cardiomyopathy. Although myriocin has been a workhorse for these studies, due to its ability to markedly reduce ceramide levels *in vivo*, work involving other pharmacological agents (*e.g.*, cycloserine, fumonisin B1, AMP-DNM, or Genz-123346) and genetic approaches (SPT, Des1, and GM3 knockout mice) have confirmed that the beneficial effects of myriocin likely result from its ability to impact specific sphingolipid levels. Although more work must be done to determine the mechanism of sphingolipid action and to elucidate the regulatory networks controlling rates of sphingolipid synthesis, these studies have identified ceramide and its metabolites as particularly toxic lipids that contribute to obesity-associated metabolic dysfunction.

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