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Sphingolipid regulators of cellular dysfunction in Type 2 diabetes mellitus: a systems overview

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Abstract

Climbing obesity rates have contributed to worldwide increases in obesity-associated diseases, including the metabolic syndrome and Type 2 diabetes mellitus (T2DM). Sphingolipids, an important class of structural and signaling lipids, have emerged as key players in the development and pathogenesis of insulin resistance and T2DM. More specifically, sphingolipids have been demonstrated to play integral roles in lipotoxicity and other aspects of pathogenesis in T2DM, although the cellular mechanisms by which this occurs and by which sphingolipid metabolism is dysregulated in T2DM remain under investigation. This review summarizes current knowledge of sphingolipid metabolism and signaling in key organs and tissues affected by T2DM, including the pancreas, adipose tissue, skeletal muscle, cardiovascular system and liver, and highlights areas that ripe for future investigation.

Keywords

free fatty acids; lipotoxicity; obesity; sphingolipid; Type 2 diabetes mellitus

Recent decades have witnessed an increase in the incidence of obesity and obesity-associated diseases [1-6] with 30% of American adults classified as obese [7-10]. Obesity and obesity-associated diseases are the fifth leading risk for death and account for at least 2.8 million adult deaths annually [7]. In addition to a heavy mortality burden, obesity and obesity-associated diseases also account for a large financial burden, resulting in an estimated US\$147 billion in medical costs in the USA [7,11]. With approximately 34% of Americans obese, and 68% maintaining a weight above a healthy range [8,9], these mortality rates and costs are expected to continue to rise.

It is not necessarily obesity *per se* that presents these challenges to human health, but rather, the deleterious outcomes of obesity, which include diseases, such as coronary heart disease, stroke, hypertension, high cholesterol and triglycerides, liver dysfunction and Type 2

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diabetes mellitus (T2DM) [11]. Obesity also contributes to the development of the metabolic syndrome (MetS) [12]. MetS is a cluster of symptoms defined by six major components: abdominal obesity, atherogenic dyslipidemia, hypertension, a prothrombotic state, a proinflammatory state and insulin resistance [12]. Researchers are building a firm foundation to assist in understanding the pathophysiology underlying these deleterious health outcomes of obesity at the cellular and molecular level, with the hope that furthering knowledge may lead to potential treatments. T2DM is mediated by a multitude of factors, including the consumption of a diet high in saturated fatty acids [13-15]. Therefore, one area that has been of particular interest is the oversupply of fatty acids, or, more broadly, the perturbation of lipid metabolism that occurs in T2DM, that may contribute to deleterious outcomes. This process has been generally termed 'lipotoxicity' (Figure 1).

Lipotoxicity & its mechanisms

Excess lipid supply to tissues and organs has arisen as a potential mechanism by which obesity and/or diabetes causes disease. In cellular homeostasis, there lies a delicate balance between processes that generate lipids and those that result in their transport, oxidation and utilization. Due to alterations in these processes, plasma-free fatty acids (FFA) in obese individuals are elevated and may be as much as double those that occur in their lean counterparts [16]. Moreover, increases in levels of plasma lipoproteins that carry triacylglycerols (TAG) also provide excess lipid to organs that express endothelial lipoprotein lipase, which releases FFAs for immediate uptake by these tissues. These organs include skeletal muscle, heart and liver, three major sites of obesity-induced disease (reviewed in [17,18]). These lipids can disrupt cell signaling, promote mitochondrial dysfunction and/or lead to aberrant cell processes including maladaptive autophagy and/or cell death, and this process has been termed 'lipotoxicity' [19,20] (Figure 1).

FFAs are taken up by cells through both protein-dependent and independent mechanisms [21] and can accumulate in nonadipose tissue, which may in part mediate pathological outcomes through several mechanisms. One of the most appreciated mechanisms by which excess fatty acids mediate lipotoxic cellular dysfunction is through perturbation of normal mitochondrial function. Mitochondrial dysfunction in T2DM was first described by Kelley *et al.* [22]. This study demonstrated that in T2DM patients, mitochondria exhibited loss of integrity, as evidenced by a decrease in size and an elevation in the number of damaged mitochondria. Moreover, in cells, fatty acid oversupply increased flux of lipid through the mitochondria, resulting in incomplete fatty acid oxidation, oxidative stress and impaired glucose utilization [23,24]. Studies have also demonstrated that in animal models of diet-induced obesity or treatment of cells with palmitate resulted in oxidative stress and mitochondrial damage [25,26]. It remains unclear if mitochondrial dysfunction is a direct cause or occurs as a result of insulin resistance [27,28] or if mitochondrial damage plays a significant role in insulin resistance at all [29-31]. However, overload of mitochondria with substrate for β -oxidation can increase cell oxidative stress, potentially leading to many deleterious outcomes.

Perturbation of the biosynthesis of signaling lipids is another mechanism for lipotoxicity. Lipid oversupply has been demonstrated to increase cell levels of potentially harmful lipid

mediators such as diacylglycerols (DAG) and bioactive sphingolipids (Figure 2). Not only do these lipids perturb cell signaling and function through mechanisms described below, but there may also be cross-talk between this pathway and mitochondria-dependent pathways of lipotoxicity. Indeed, oxidative stress has been directly shown to trigger ceramide accumulation [32]. Furthermore, ceramides found in the mitochondria were demonstrated to play a role in high-fat diet-induced mitochondrial dysfunction, and inhibition of *de novo* sphingolipid synthesis depleted ceramides and enhanced mitochondrial function in high-fat-fed mice [32,33].

Aberrant sphingolipid biosynthesis perturbs the functions of multiple organelles. For example, elevated sphingolipid synthesis has been shown to contribute to cell death through endoplasmic reticulum (ER) stress. The ER is important in cellular function and has been shown to contribute to the insulin resistance that leads to the development of T2DM [34]. Studies have implicated ceramide as both a causative agent and a product of ER stress [35,36]. Roles for specific sphingolipid species and isoforms of the enzymes of sphingolipid metabolism are presently emerging in diabetes and other contexts, and have been thoroughly reviewed elsewhere [37,38]. In interpreting the results of many of these and other studies, one should keep in mind that sphingolipid metabolism is a highly connected web of related pathways, and that most steps in sphingolipid metabolism employ enzymes that catalyze the opposite reaction (reviewed in [39]). Thus, many sphingolipids are not merely products of one enzyme, but can be generated by more than one metabolic route (i.e., anabolic and catabolic pathways). Moreover, most sphingolipids serve as substrates for biosynthesis of other lipids which may also have biological actions. Therefore, direct treatment of cells with sphingolipids, use of inhibitors for specific enzymes and/or genetic deletion of enzymes may not reveal the specific bioactive lipid in an experimental situation.

Sphingolipids & obesity-associated disease: a systems overview

Pancreatic β -cells

β -cell dysfunction is a characteristic disease outcome of T2DM and underlies much of its resultant pathology [40]. In obesity, oversupply of FFA induces insulin resistance in peripheral tissues, as discussed elsewhere in this review, and pancreatic β -cells initially maintain homeostatic glucose levels by increasing insulin secretion [40,41]. However, β -cells gradually lose their ability to compensate for peripheral insulin resistance. Plasma hyperlipidemia ultimately causes insulin resistance in the pancreas itself, resulting in a gradual decline in insulin production and consequent increases in blood glucose. This results in hyperglycemia, which combines with oversupply of FFA to induce cellular dysfunction and death [42]. Importantly, sphingolipids have emerged as key players mediating both glucotoxic and lipotoxic cell death.

The deleterious effects of fatty acids on β -cells have long been appreciated as an important component of the pathology of T2DM and were, in fact, the phenomenon for which the term 'lipotoxicity' was originally coined [41]. Subsequent investigations revealed that DNA laddering, a marker of apoptosis, was elevated in pancreatic islets from obese, diabetic mice and that these cells displayed an increased sensitivity to *in vitro* FFA treatment compared with cells isolated from lean controls [42]. Additionally, ceramide, which is known to

promote apoptosis [43], increased in islets from these obese, diabetic mice and in islets exposed to FFA *in vitro*. Substantiating a mechanistic link between increased ceramide and β -cell apoptosis, protection against apoptosis was conferred by inhibition of serine palmitoyltransferase (SPT), the first enzyme of *de novo* sphingolipid synthesis, or (dihydro) ceramide synthase (CerS), which is responsible for acylation of sphingoid bases to form ceramide or dihydroceramide [44,45]. Furthermore, only treatment with specific FFA that promoted increases in ceramide levels was sufficient to induce lipotoxicity and insulin resistance *in vitro* [46,47], and treatment with a cell-permeable synthetic ceramide, C₂-ceramide, in itself induced β -cell death [42]. These foundational results suggested a role for sphingolipids in lipotoxic β -cell death, which was elaborated by subsequent studies.

Augmenting the deleterious effects of lipotoxicity, glucotoxicity has also been shown to play an important role in sphingolipid-mediated pancreatic damage after decompensation of insulin resistance. Indeed, direct exposure to excess glucose was shown to induce cytotoxicity in β -cells, and pharmacologic inhibition of CerS provided protection from this effect [46]. While this study provided important insights into cross-talk between glucose oversupply and pathways of lipotoxicity, the experimental approach employed did not reveal whether this protection was due to effects on ceramide levels *per se* or rather to alterations in levels of glycosphingolipids, which contain carbohydrate head groups. Further studies demonstrated that oversupply of glucose and FFA combined to potentiate ceramide production to a greater degree than oversupply of either nutrient alone, reflecting a greater role for ceramide in more physiologically relevant conditions [48-50]. Together, these results provided the rationale for later studies that characterized sphingolipid-mediated β -cell death in T2DM, as discussed below.

Mechanistic studies have addressed the roles of sphingolipids in multiple facets of β -cell dysfunction, including altered insulin production and secretion, ER stress and apoptosis. Ceramide was directly implicated in pancreatic insulin resistance and decline in insulin production, both at a molecular level and due to a reduction in the population of β -cells [47]. Specifically, ceramide reduced proinsulin mRNA levels by reducing transcription, possibly *via* a reduction in ERK phosphorylation or upregulation of the transcription factor C/EBP- β [50-53]. Some data have also suggested a role for aberrant sphingolipid levels in insulin secretion. Specifically, it was shown that β -cells of sphingomyelin synthase 1 knockout mice displayed an accumulation of ceramide and decreased insulin secretion [54], and knockdown or inhibition of sphingomyelin synthase 1 and sphingomyelin synthase 2 in INS-1 cells was shown to inhibit insulin secretion *in vitro* [55]. This may occur in part due to the effect of sphingomyelin synthesis on DAG concentrations at the Golgi [55]. Indeed, SMS activity simultaneously produces both sphingomyelin and DAG, which is released when the choline head group of phosphatidylcholine is transferred to ceramide. It has been proposed that a reduction in SMS activity diminishes the pool of DAG available for binding to protein kinase D at the Golgi, thus impairing secretion of insulin through the trans-Golgi network [55].

Similarly, ceramide production was shown to promote ER stress in β -cells through mechanisms that are still being elucidated [56,57]. Recently, altered membrane dynamics have been proposed as a key mechanism by which this occurs. Chronic treatment of MIN6

pancreatic cells with palmitate resulted in enrichment of ceramide and glucosylceramide and depletion of sphingomyelin in the ER, accompanied by decreased vesicle budding and altered membrane properties [58]. This decrease in vesicle budding had the effect of diminishing ER-to-Golgi protein trafficking, resulting in protein accumulation in the ER and subsequent ER stress [59]. Inhibition of sphingomyelinase (SMase), which catabolizes sphingomyelin to generate ceramide and phosphorylcholine, or overexpression of glucosylceramide synthase restored lipid levels and membrane properties, improved ER-to-Golgi trafficking and protected cells against lipotoxic ER stress [58,59].

Finally, sphingolipids have been implicated in the induction of apoptosis in the diabetic pancreas. Ceramide accumulation was shown to stimulate the apoptotic cascade through several other mechanisms, including loss of mitochondrial membrane potential, release of cytochrome C into the cytosol, reduced expression of Bcl-2 and activation of caspases, especially caspases 3/7 [46,56,60,61]. Indeed, one study specifically implicated ceramide production through one specific isoform of CerS, CerS4, in activation of caspases 3/7 in glucolipotoxicity in INS-1 cells [62]. These findings are consistent with other recent studies suggesting a proapoptotic function for CerS4 and its metabolites [63,64]. While the authors suggested C18:0- and C22:0-ceramide as the proapoptotic metabolites in the INS-1 line, regulation and distribution of specific ceramide chain lengths appear to vary markedly across species and cultured β -cell models, perhaps limiting the broader applicability of this finding [49,57]. Other authors have also hypothesized a role for the salvage pathway of ceramide generation in lipotoxic β -cell apoptosis, citing evidence for a role for acid SMase (aSMase) in other mechanisms of β -cell death in T2DM [57]. Glycosphingolipids have also been suggested as proapoptotic lipids in the diabetic pancreas, although this view is controversial. Palmitate treatment of MIN6 insulinoma cells resulted in an increase in glucosylceramide levels, and expression of GM3 ganglioside synthase was shown to increase in rodent models of T2DM with defective leptin signaling [56,65]. Treatment of diabetic rats with inhibitors of glycosphingolipid synthesis improved blood glucose levels, alleviated hyperinsulinemia and protected pancreatic islet architecture, suggesting harmful roles for glycosphingolipids [66,67]. However, the inhibitors used in this study do not diminish production of all branches of glycosphingolipid metabolism, and protection may have, in fact, occurred due to redirection of precursors into sulfatides or other complex sphingolipids. Additionally, *in vitro* experiments showed that glycosphingolipid synthesis actually protected β -cells from apoptosis and ER stress [59,68]. While it has been suggested that the discrepancies lie in the effects of glycosphingolipid synthase inhibitors on whole-body insulin resistance and its subsequent effects on the pancreas, further work is required to reach a more definitive conclusion [57].

While most studies of sphingolipids in the diabetic pancreas have focused on their harmful effects in cell death and insulin resistance, some sphingolipid metabolites also appear to serve important functions in β -cell function and survival. For example, the glycosphingolipid sulfatide (3'-sulfogalactosyl-ceramide), particularly C16:0-sulfatide, has been shown to promote proper insulin folding and secretion, to protect cells from cytokine-mediated apoptosis and to improve insulin response to fasting or glucose (reviewed in [57,69]). Similarly, sphingosine-1-phosphate (S1P) has been proposed to play an important role in determining β -cell fate and in governing insulin resistance [70]. S1P has been shown

to accumulate both in isolated pancreatic islet cells and MIN6 cells in response to exposure to high levels of glucose, putatively through increased sphingosine kinase 2 (SphK2) activity [71]. Accumulation of S1P increased glucose-stimulated insulin secretion, and inhibition or knockdown of SphK2 decreased glucose-stimulated insulin secretion *in vitro* and impaired glucose tolerance and diminished insulin secretion in response to elevated blood glucose *in vivo*. Additionally, S1P is believed to protect against inflammation-mediated β -cell death. Supporting this notion, exposure to IL-1 β and TNF- α increased SphK activity, likely through activation of SphK2, in isolated pancreatic islets and INS-1 cells [72], and treatment with S1P protected isolated pancreatic islets from apoptosis in response to IL-1 β exposure [73]. The specific S1P receptors (S1PRs) and molecular mechanisms underlying this protection remain under investigation.

Adipose tissue

Increases in the incidence of obesity have brought significant attention to the role that adipose tissue plays in the development of insulin resistance and T2DM [74,75]. Excess adipose tissue not only may contribute to the physiologic perturbations associated with obesity, but these perturbations, for example, insulin resistance, may in turn also act on adipose tissue to exacerbate and/or accelerate disease processes. Therefore adipose function may lie at the crux of obesity-associated disease.

An important component of homeostasis is the maintenance of insulin sensitivity in adipose tissue [76]. Insulin signaling in adipose promotes the storage of excess nonesterified FFAs for their release to distal tissues when needed. This storage is accomplished through the promotion of glucose transport and lipogenesis [77] and by the inhibition of lipolysis [76]. Under obesogenic conditions, however, excess FFA, in other words, above what the tissue can handle, can lead to the development of insulin resistance, prompting elevations in tissue lipolysis, which then exacerbates elevation of plasma FFA. Disruption of adipose tissue endocrine function may also prompt the secretion of adipocyte-derived cytokines, or 'adipokines', which, in addition to hormones specific to adipose tissue, including include leptin, resistin and adiponectin, may disrupt whole-body physiology, include leptin, resistin and TNF- α [74,78-80]. In adipose tissue, TNF- α functions to inhibit lipogenesis and increase lipolysis; blocking TNF- α function in obese *fa/fa* rats restored the insulin sensitivity that was diminished in control animals [77,81]. A potential mechanism for this may include that TNF- α can impair insulin signaling through the phosphorylation of IRS-1 and the reduction of glucose transporter 4 (GLUT4) gene expression [81,82]. In general, the production of adipokines often results in fatty acid release from adipose tissue and the deposition of fatty acids in distal tissues and, thus, promotes the development of lipotoxicity. It should be noted that several studies demonstrate a neutral or even beneficial role for palmitoleate, a C16:1 unsaturated fatty acid, in insulin sensitivity [83-85]; palmitoleate does not efficiently undergo metabolism to sphingolipids, which is consistent with the notion that saturated fats drive pathophysiology of obesity to a greater extent than unsaturated fatty acids. This is also consistent with findings that gluteofemoral fat, which is not as highly correlated to insulin resistance as abdominal fat, is higher in palmitoleate [86].

Many of the deleterious outcomes of obesity discussed above may be mediated in part by disruption of normal sphingolipid biosynthesis and metabolism. For example, the adipokine TNF- α has been linked with the activation of enzymes, such as SPT, the rate-limiting step of *de novo* sphingolipid synthesis [87] and other sphingolipid catabolic enzymes including aSMase and neutral sphingomyelinase (nSMase), as well as decreases in adipose tissue levels of sphingomyelin and ceramide. Specifically, injecting wild-type mice with TNF- α increased adipose tissue expression of *aSMase*, *nSMase* and *SPT* [88]. Moreover, genetically obese (*ob/ob*) mice were found to have increased expression levels of *aSMase*, *nSMase* and *SPT*. Feeding of C57BL/6J mice with a high-fat diet for 16 weeks demonstrated that high-fat feeding promoted increased levels of ceramide in both plasma and adipose tissue as well as elevations in the expression and activity of *aSMase* and *nSMase* [89]. Additionally, insulin injections of *ob/ob* mice also demonstrated elevations in the expression of *aSMase*, *nSMase* and *SPT* in adipose tissue when compared with their lean counterparts, indicating that hyperinsulinemia may promote production of ceramide (an insulin-desensitizing lipid) through manipulation of these enzymes [88]. These studies demonstrate that adipose tissue homeostasis and sphingolipid metabolism are disrupted in response to obesity as a result of changes in fatty acid flux. Together with reports that elevated plasma sphingolipid levels are associated with elevated cardiovascular risk in obesity [90,91], these studies support the hypothesis that dysfunctional adipose tissue promotes lipotoxicity through causing deleterious modifications to bioactive sphingolipid metabolism. Thus, adipose dysfunction may lie upstream from lipotoxicity in distal tissues due to its contribution(s) to increased circulating lipids and also its altered endocrine and/or proinflammatory functions.

Skeletal muscle

Skeletal muscle plays an integral role in the removal of glucose from the bloodstream, as it accounts for 40% of total body mass and 80% of whole-body insulin-stimulated glucose disposal [92,93]. Therefore, skeletal muscle is thought to make significant contributions to the systemic glucose intolerance and insulin insensitivity that can occur concomitantly with obesity. Glucose removal in response to insulin stimulation is accomplished through the interaction of insulin with its receptors and the subsequent phosphorylation and activation of insulin receptor substrates [3,94,95]. The stimulation of the insulin receptor results in the recruitment and activation of phosphatidylinositol 3-kinase [94], resulting in the production of the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), a bioactive lipid that mediates the recruitment of Akt/protein kinase B (PKB) to the plasma membrane. The activation of Akt/PKB results in the uptake of glucose by skeletal muscle through the stimulation and translocation of GLUT4, ultimately resulting in the increased uptake of glucose, effectively decreasing the amount of circulating glucose and an increasing generation of energy storage molecules [87]. The response of Akt/PKB to insulin can be perturbed by a host of stimuli including TNF- α , glucocorticoids and long-chain fatty acids such as palmitate [96,97].

Under normal conditions in skeletal muscle, FFAs are sequestered into lipid droplets after their conversion to TAG; this is generally considered to render them benign to the tissue [96,98]. However, this storage mechanism is often overwhelmed in response to exposure to the high amounts of fatty acids seen during obesity, thus allowing excess fatty acids to

remain available for biosynthesis of signaling molecules such as DAG and sphingolipids (i.e., ceramide) [97,99-101]. However, it is not currently understood whether aberrant lipolysis of these intracellular TAGs can also provide substrates for the aberrant synthesis of bioactive lipids.

The involvement of ceramide in the development of insulin resistance was initially demonstrated by Turinsky *et al.* [102]. This study revealed that ceramide accumulated in the skeletal muscle of insulin-resistant Zucker *fa/fa* rats. Subsequent *in vitro* studies utilizing the saturated fatty acid palmitate determined that the central mechanism employed by skeletal muscle derived ceramide to block insulin action is through the inhibition of insulin-stimulated Akt/PKB activation [103]. Further studies employing exogenous ceramide and ceramide analogs (e.g., C2- and C6-ceramide) demonstrated effects similar to those seen in cells treated with palmitate [99,101,103,104], further bolstering the notion that ceramide promotes insulin resistance through interfering with insulin signaling. Later mechanistic studies by the groups of Summers, Biden and Lorenzo demonstrated that ceramide blocked Akt/PKB activation through one of two molecular mechanisms: by blocking the translocation of Akt/PKB to the plasma membrane where it can be activated by phosphorylation [105,106] or by mediating the dephosphorylation of Akt/PKB by protein phosphatase 2A [101,106-108]. In addition to demonstrating that palmitate inhibits Akt/PKB activation through directly incorporating into ceramide and thus increasing its levels, Hu *et al.* [109] demonstrated that the saturated fatty acid increased mRNA and activity of dihydroceramide desaturase 1, the enzyme catalyzing the final step in *de novo* ceramide biosynthesis. Moreover, palmitate-induced inhibition of Akt/PKB activation could be overcome by treatment with the unsaturated fatty acid oleate, which occurred through repressing the increase in dihydroceramide desaturase 1. Intriguingly, although DAG has been proposed as another insulin-desensitizing lipid in skeletal muscle [3,110], oleate drove significant accumulations of DAG despite protecting cells from palmitate-induced insulin resistance. These discrepancies have yet to be resolved; however, insulin desensitization in response to DAG may occur in a manner dependent on specific acyl chain lengths of the molecule, as oleate treatment increased only those DAG species containing oleoyl (i.e., C18:1) acyl chains. Additionally, Hu *et al.* [5], demonstrated that treatment of skeletal muscle cells with palmitate elevated the expression and activity of sphingosine kinase 1 (SphK1), the enzyme responsible for the generation of sphingosine-1-phosphate (S1P), in a manner that was independent of *de novo* sphingolipid synthesis. Further studies revealed that palmitate-mediated *SphK1* expression occurred in PPAR- α -dependent manner [111], demonstrating a role for SphK1 in the context of the MetS.

Cardiovascular system

Cardiovascular disease is one of the most pressing long-term threats facing patients with T2DM. Adults with T2DM are two to four times more likely to die of heart disease than nondiabetics, and 67% of adults with diabetes had hypertension or took antihypertensive drugs [112]. Patients are often subject to a confluence of several conditions, including hypertension, atherosclerosis, ischemia/reperfusion injury and a unique diabetic cardiomyopathy (DbCM), which come together to cause cardiac hypertrophy, fibrosis and

dysfunction [113]. Strikingly, sphingolipids regulate key aspects of disease progression and cardiac pathology in all of these conditions [114,115].

Hypertension is a potentially dangerous complication that is often present in diabetic patients, resulting in cardiac hypertrophy and dilation as well as damage to other organs [116]. Roles of sphingolipids in regulation of vascular tone and blood pressure have been the subject of intense study. Multiple sphingolipid species, including S1P, ceramide, sphingosine and sphingosylphosphorylcholine (*lyso*-sphingomyelin) have been implicated in regulation of vascular tone and blood pressure, and their roles in vasoconstriction and vasodilation are often conflicting and vary among vascular beds (reviewed in [117-119]). The roles of sphingolipids in cardiac remodeling and dysfunction in pressure overload/hypertension are much less well studied [120-122], but ceramide appears to increase in models of pressure overload. Sphingolipids have also been shown to modulate cardiac hypertrophy in other physiological contexts [123-126], supporting the potential for sphingolipid involvement in hypertension as well. Future studies will be needed to provide mechanistic insight and better characterization of the influence of sphingolipids on cardiac hypertrophy in hypertension.

Atherosclerosis is another serious vascular problem in T2DM in which sphingolipids are significantly involved. In brief, atherosclerosis is characterized by dysfunction and structural alterations of the endothelium, subendothelial accumulation and oxidation of LDL, immune infiltration and chronic inflammation (reviewed in [127]). The roles of sphingolipids in atherosclerosis are multifaceted, have been thoroughly reviewed elsewhere and will be touched on only briefly here [128-130]. In particular, S1P and sphingomyelin have been shown to be key players in the pathogenesis of atherosclerosis. Elevated circulating sphingomyelin levels have been shown to constitute an independent risk factor for coronary artery disease [128,131], and sphingomyelin is much more concentrated in LDL found in atherosclerotic plaques than in circulating LDL in humans [132-135]. Further studies have demonstrated the sphingomyelin in these plaques is hydrolyzed by SMase in the arterial wall, generating ceramide that then promotes aggregation of intimal atherogenic lipoproteins [134,136]. Sphingomyelin was also implicated in proinflammatory processes underlying plaque formation, as well as formation of foam cells (reviewed in [130]). The story of S1P and its receptors appears to be a bit more complex. Patients with stable coronary artery disease (i.e., buildup of atherosclerotic plaque in the arteries supplying blood to the myocardium) were shown to have higher levels of non-HDL-bound S1P in the blood plasma [137], and circulating S1P has been shown to promote expression of markers of inflammation [138]. The proatherogenic, proinflammatory effects of S1P in this context appear to be mediated by signaling through S1PR_{2/3} [139-142]. In contrast, treatment with FTY720, which functions as a functional antagonist of S1PR₁ and S1PR₃₋₅, ameliorated plaque formation and recruitment of monocytes and macrophages in mouse models of atherosclerosis [143,144]. However, protective roles for S1P in atherosclerosis have also been demonstrated. Erythrocyte-derived S1P was shown to improve endothelial barrier function, possibly by promoting formation of adherens junctions through S1PR₁ [145]. Together, these results emphasize the need for further clarification of the involvement of sphingolipids in atherosclerotic and atheroprotective processes.

Sphingolipids have also emerged as important players in cardiac ischemia/reperfusion injury [114]. Initial studies indicated that ceramide increased in the ischemic myocardium, and reperfusion further elevated ceramide levels and ceramide-mediated apoptosis [146-149]. Furthermore, ischemic preconditioning attenuated these increases in ceramide levels [149,150]. As in other pathological conditions, ceramide profiles appeared to be governed in a chain-length-specific manner in cardiac ischemia and reperfusion. For example, one study found increases in C16:0, C18:0, C18:1, C18:2 and C20:4-ceramides with ischemia and further increases upon reperfusion, while ischemic preconditioning prevented the additional reperfusion-induced increases in all species, except C18:1-ceramide without restoring ceramides to normal levels [149]. These altered ceramide levels have been proposed to originate from catabolism of sphingomyelin or decreased ceramidase activity [114,147]. Supporting the former view, it was found that sphingomyelin levels decreased in hearts subjected to ischemia/reperfusion, and hypoxia/reoxygenation of cultured cardiomyocytes specifically activated nSMase in a manner that was inhibited by antioxidant treatment [148,151]. Furthermore, pharmacologic inhibition of SMase attenuated apoptosis and prevented accumulation of ceramide in response to ischemia/reperfusion [150].

In contrast to the pro-death effects of ceramide, S1P has been shown to have a protective effect in ischemia/reperfusion. Plasma S1P decreased in both human patients and rat models following ischemia/reperfusion, and direct treatment with S1P was shown to protect cells from apoptosis in response to hypoxia and ischemia/reperfusion injury [152-156]. Furthermore, exposure to the ganglioside GM-1 was shown to activate SphK, producing a similar protective effect. Ischemic preconditioning also appears to activate SphK1 and to promote translocation of the enzyme to the cell membrane [157]. Importantly, endogenously produced S1P appears to act through different pathway than exogenous S1P (e.g., S1P in the blood plasma) [158]. In particular, only endogenously produced S1P required PKC- ϵ for cardioprotection, and only cardioprotection by endogenous S1P was abolished in response to an S1PR_{1/3} antagonist or pertussis toxin, which inhibits $G_{i/o}$ proteins [154,158]. These results suggested that the protective effect of endogenously produced S1P required the S1P to be secreted and to act on S1PRs at the plasma membrane. The roles of particular S1PRs in cardioprotection and ischemic preconditioning have remained controversial, with some studies implicating S1PR₁ and others suggesting roles for S1PR_{2/3}, either alone or in combination [157,159-162]. The proposed cardioprotective mechanisms for S1P action appear to be partially overlapping across receptor subtypes. Activation of S1PR₁ promoted Akt activation in a phosphatidylinositol 3-kinase- and G_i -dependent manner, resulting in inactivation of GSK-3 β and reduced release of cytochrome C from the mitochondria [159]. Similarly, loss of both S1PR₂ and S1PR₃ diminished Akt activation but had no effect on activation of ERK, JNK or p38 MAP kinases [160].

In addition to increased incidence of hypertension, ischemic events and atherosclerosis, T2DM patients also suffer from a unique cardiomyopathy, termed DbCM [163]. This condition is characterized by reduced contractility, systolic and diastolic dysfunction, hypertrophy, fibrosis and a shift away from utilization of glucose as a metabolic substrate in excess of what can be accounted for by other risk factors [164]. Numerous studies have shown that sphingolipids are key players in DbCM and cardiomyocyte lipotoxicity, which is a major mechanism underlying heart disease in T2DM (reviewed in [115]). Indeed, many

enzymes in the sphingolipid metabolic pathway were overexpressed in the myocardium of diabetic humans [165], suggesting increased flux through sphingolipid metabolism, and ceramide levels were shown to be elevated in both diet-induced and genetic models of DbCM and cardiac lipotoxicity [123,166-168]. Inhibition of *de novo* sphingolipid synthesis was sufficient to ameliorate the structural, functional and metabolic DbCM phenotype in these animal models, and other interventions that normalized the cardiac phenotype often also prevented ceramide accumulation and overexpression of enzymes of sphingolipid metabolism.

Mechanisms by which ceramide accumulation induces cardiomyocyte death and dysfunction are still under investigation but may reflect general mechanisms of sphingolipid-dependent lipotoxicity. At a cellular level, accumulation of ceramide was shown to induce expression of the heart failure markers atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), to be required for lipotoxic and glucolipotoxic cardiomyocyte death, to promote cardiomyocyte hypertrophy and to induce β -adrenergic dysfunction [17,18,123,169]. In particular, it has been shown that ceramide induced lipotoxic cell death in a caspase 3- and 8-dependent manner in cultured cardiomyocytes [170], while β -adrenergic dysfunction was shown to result from PKC- α and PKC- δ activation [169]. In a novel finding, lipotoxic cardiomyocyte hypertrophy was shown to require induction of autophagy by CerS5 [123]. Similarly, ceramides have also been shown to mediate the shift in substrate utilization in lipotoxicity. For example, treatment of AC-16 immortalized human ventricular cardiomyocytes with the cell-permeable ceramide analog C₆-ceramide diminished glucose oxidation by decreasing expression of the GLUT-4 and increasing expression of PDK4, which inhibits the pyruvate dehydrogenase complex [18]. The accumulation of ceramide is believed to result from a combination of excess substrate supply, due to the inability of the cell to adequately incorporate excess fatty acids into TAG, and upregulation of the enzymes of sphingolipid synthesis through mechanisms that are only crudely defined [69,126,171,172].

Sphingolipids other than ceramide also serve important functions in DbCM. SphK1 overexpression and direct treatment with S1P were shown to confer protection from lipotoxic cardiomyocyte hypertrophy and death *in vitro* and *in vivo* in adult cardiomyocytes [173,174], although S1P appeared to promote hypertrophy in neonatal cardiomyocytes through S1PR₁ signaling [175]. Likewise, treatment with a ganglioside preparation attenuated atrial dysfunction and protected norepinephrine concentration in a mouse model of obesity and diabetes [176,177]. Based on this intriguing information, roles for sphingolipids other than ceramides in DbCM are in need of further study.

In conclusion, sphingolipids have been shown to play important roles in several components of cardiovascular disease in T2DM. Ceramide and sphingomyelin have been shown, generally, to promote cell death and dysfunction, while S1P, gangliosides and sulfatides generally have protective effects. Further investigations are needed to better define roles for specific sphingolipid species in cardiovascular disease and to identify potential drug targets for future clinical interventions.

Liver

Nonalcoholic fatty liver disease (NAFLD) begins with the accumulation of TAG in the liver and is a very common chronic condition that affects approximately 90% of the morbidly obese and 20–30% of all adults in the USA [178-180]. Clinically, NAFLD is diagnosed when the accumulation of TAG deposits within the liver exceeds 5–10% of the total liver weight [181,182]. In obesity, NAFLD is promoted in part by adipose tissue dysfunction and consequent oversupply of fatty acids to the liver [183,184] and is accompanied by defects in glucose and fatty acid handling. Liver dysfunction is problematic as the liver is responsible for the metabolism and storage of dietary fats, glucose and glycogen [185,186], and thus impaired function has significant effects on whole-body physiology. In response to nutrient uptake, insulin signaling normally causes hepatocytes to increase glucose uptake and glycogen synthesis [186]; however, elevated plasma FFA concentrations inhibit insulin signaling and thus promote gluconeogenesis and glycogenolysis [187,188]. Furthermore, fatty acid oversupply results in the accumulation of TAG-rich lipid droplets [189-191] and, ultimately, the development of fatty liver and lipotoxicity [191].

Studies have demonstrated that treatment of hepatocytes with palmitate induced an increase in TAG and perturbation of sphingolipid synthesis. Data from genetically obese (*ob/ob*) and high-fat-fed C57BL/6J mice demonstrated that myriocin treatment reduced ceramide production, the accumulation of TAG and the development of hyperglycemia, further indicating a role for sphingolipid synthesis in liver dysfunction [192]. Another study demonstrated that TAG accumulation in the liver inhibited the activity of aSMase, the enzyme that hydrolyzes sphingomyelin derived from the recycling/endocytic pathway [191], and these increases in TAG were ablated in response to high-fat feeding in the absence of aSMase [191]. These animals did, however, demonstrate an increase in *de novo* sphingolipid synthesis and, in contrast to the situation in skeletal muscle, were actually protected from diet-induced hyperglycemia and insulin resistance [191], which underscores the complexity of sphingolipid involvement in insulin resistance. Additionally, a recent study by Park *et al.*, revealed that mice lacking the CerS2 enzyme demonstrated diminished glucose sensitivity when compared with their wild-type counter parts [193].

Future perspective

In the past several decades, many advances have been made in understanding the molecular mechanisms underlying the pathology of T2DM. Literature discussed here supports a robust role for sphingolipids in the development and pathogenesis of T2DM. Ceramide was shown to potentiate apoptosis, insulin resistance and cellular dysfunction in multiple disease contexts. S1P, on the other hand, seemed to promote pathological molecular programs in some systems, yet to serve protective roles in others. Complex sphingolipids, including sphingomyelin and glycosphingolipids, also functioned in a context-specific manner. These results indicate the important contributions of sphingolipids to tissue homeostasis and pathogenesis in T2DM. However, this field is relatively young, and most studies have opened doors for much-needed studies to facilitate obtaining fine-grained mechanistic insight. Many key enzymes of sphingolipid metabolism exist as several different isoforms,

each of which has a unique substrate profile, localization within the cell, and/or pattern of regulation [194].

Studies addressing precisely which specific enzymes are involved in cellular dysfunction in T2DM are sparse [115]. Furthermore, sphingolipid metabolism is composed of multiple interconnected pathways, and many strategies of disrupting sphingolipid metabolism at specific points, particularly *de novo* synthesis, have a ripple effect across other metabolite pools [194]. To identify potential targets for future drug discovery efforts and to dissect molecular mechanisms, it is critical to select approaches that can tease apart these nuanced aspects of sphingolipid metabolism and signaling. Due to recent advances in methodology, these advanced approaches are now feasible and will undoubtedly significantly advance this area of research.

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Executive summary

Lipotoxicity & its mechanisms

- Lipotoxicity is a lipid-induced disruption of cell signaling, mitochondrial function and cellular viability.
- Sphingolipids have emerged as key regulators of lipotoxic processes in cells.
- Ceramides found in the mitochondria have been demonstrated to mediate oxidative stress-induced mitochondrial dysfunction.
- Ceramide has been shown to be both a causative agent and a product of endoplasmic reticulum stress.

Pancreatic β -cells

- Glucotoxicity and lipotoxicity both contribute to sphingolipid-mediated β -cell dysfunction and death in diabetes.
- Ceramide, particularly that produced by (dihydro)ceramide synthase 4, has been implicated in insulin resistance, reduced insulin production, endoplasmic reticulum stress and apoptosis.
- Sphingosine-1-phosphate, especially that produced by sphingosine kinase 2, has been shown to protect against inflammation-mediated β -cell death and to preserve insulin secretion in response to elevated blood glucose.
- Roles for glycosphingolipids appear to be variable, with sulfatides serving protective functions and some other glycosphingolipids promoting apoptosis.

Adipose tissue

- The accumulation of excess free fatty acids in adipose tissue promotes the development of insulin resistance, elevations in tissue lipolysis and the production of adipokines.
- The adipokine TNF- α has been demonstrated to activate the enzymes SPT, nSMase and acid aSMase.
- TNF- α has also been shown to increase adipose tissue levels of sphingomyelin and ceramide.
- Injection of insulin into genetically obese mice demonstrated elevated expression levels of aSMase, nSMase and SPT.
- Adipose tissue dysfunction may lie upstream of lipotoxicity in distal organs and tissues.

Skeletal muscle

- Ceramide accumulation in skeletal muscle was observed in genetically obese mice.

- Treatment of cells with palmitate revealed that ceramide blocks insulin action through the inhibition of Akt/protein kinase B (PKB) activation.
- Ceramide inhibits Akt/PKB activation by blocking the translocation of Akt/PKB to the plasma membrane or by mediating the dephosphorylation of Akt/PKB by protein phosphatase 2A.
- Palmitate increases the activity of dihydroceramide desaturase 1 and sphingosine kinase 1.
- Palmitate-induced inhibition of Akt/PKB can be overcome by treatment with the unsaturated fatty acid oleate.
- Palmitate-induced sphingosine kinase 1 expression occurs independently of *de novo* sphingolipid synthesis and is PPAR- α dependent.

Cardiovascular system

- Sphingolipids are very important in many aspects of cardiovascular disease that commonly occur in diabetics.
- Several sphingolipids regulate vascular tone and blood pressure and may play roles in hypertension.
- Ceramide has been implicated in cardiac hypertrophy and ischemia/reperfusion injury.
- Sphingosine-1-phosphate protected cardiomyocytes from lipotoxic hypertrophy and from cell death in lipotoxicity, hypoxia and ischemia/reperfusion; however, it was also shown to be proinflammatory and potentially proatherogenic.
- Sphingomyelin has been proposed to act as a reservoir that releases toxic ceramides in atherosclerotic plaque formation and cardiomyocyte postreperfusion cardiomyocyte cell death.

Liver

- Excess free fatty acids inhibit insulin signaling and promote the accumulation of TAG-rich lipid droplets.
- Studies in genetically obese and high-fat-fed wild-type mice revealed that myriocin treatment reduced ceramide production, the accumulation of TAG and the development of hyperglycemia.
- TAG accumulation in the liver inhibits the activity of aSMase, the enzyme that hydrolyzes sphingomyelin.
- Mice lacking *Cers2* exhibited diminished glucose sensitivity.

Figure 1. Mechanisms of lipotoxicity in the pancreas, adipose tissue, skeletal muscle, and liver
 FA: Fatty acid; FFA: Free fatty acids; NAFLD: Nonalcoholic fatty liver disease; TAG: Triacylglycerol.

Figure 2. Metabolism of sphingolipids