CHAPTER 5

ADIPOSE TISSUE AND CERAMIDE BIOSYNTHESIS IN THE PATHOGENESIS OF OBESITY

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

INTRODUCTION

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

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

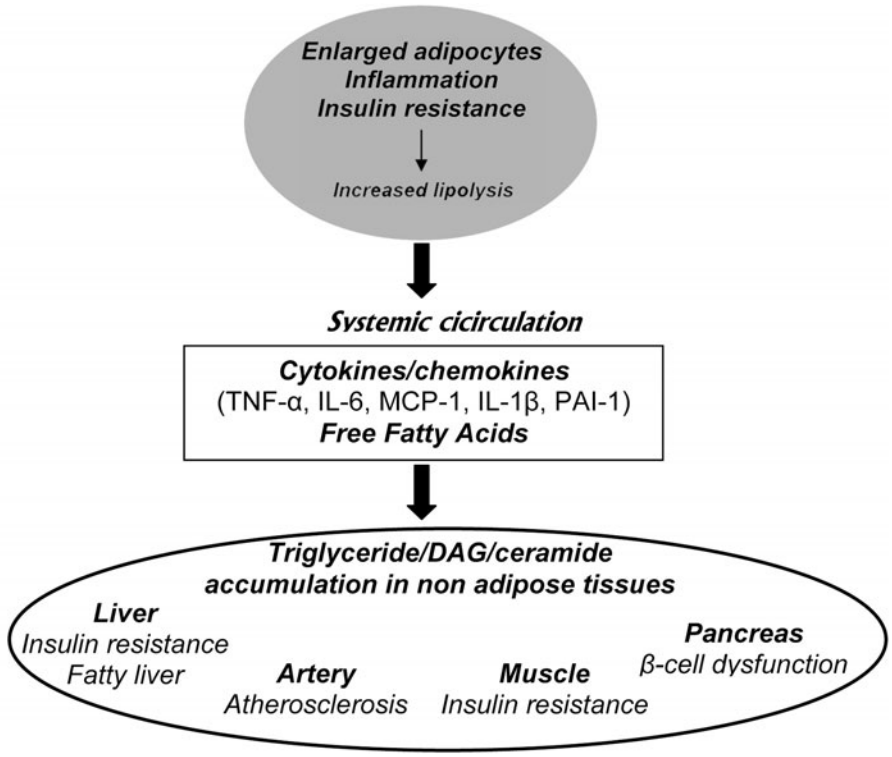

Figure 1. Proposed scheme of how increased adipose tissue mass/enlarged adipocytes contribute to metabolic and cardiovascular risk in obesity.

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

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

REMODELING OF ADIPOSE AND PLASMA CERAMIDE IN OBESITY

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

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

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

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

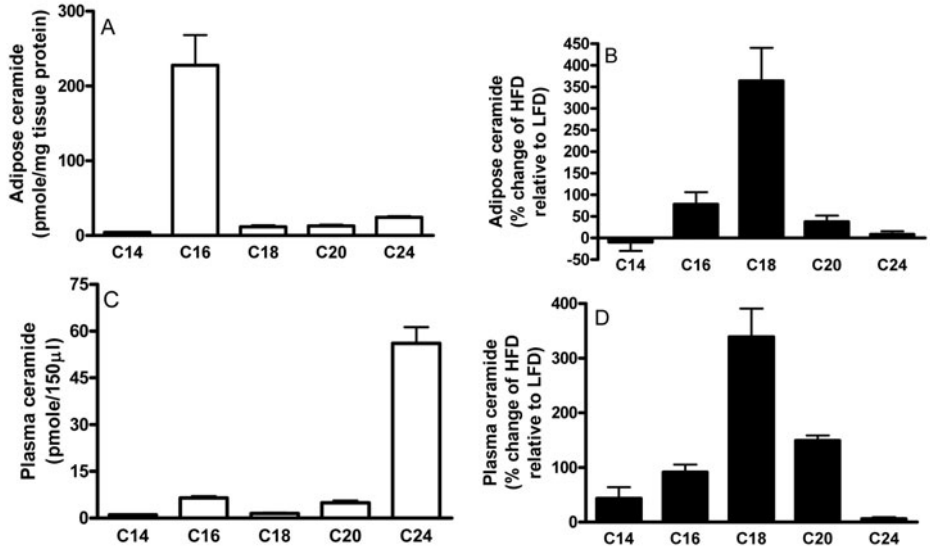

Figure 2. Ceramide expression in lean and diet induced obese (DIO) mice. Panels A and C: Ceramide levels in adipose tissues and plasma of lean mice respectively. Panels B and D: Ceramide expression in adipose tissues and plasma of DIO mice respectively. Obese mice were generated by placing male C57BL/6J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

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

REGULATION OF CERAMIDE METABOLISM IN OBESITY

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

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

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

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

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

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

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

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

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO BODY WEIGHT REGULATION AND ENERGY METABOLISM

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

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

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

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

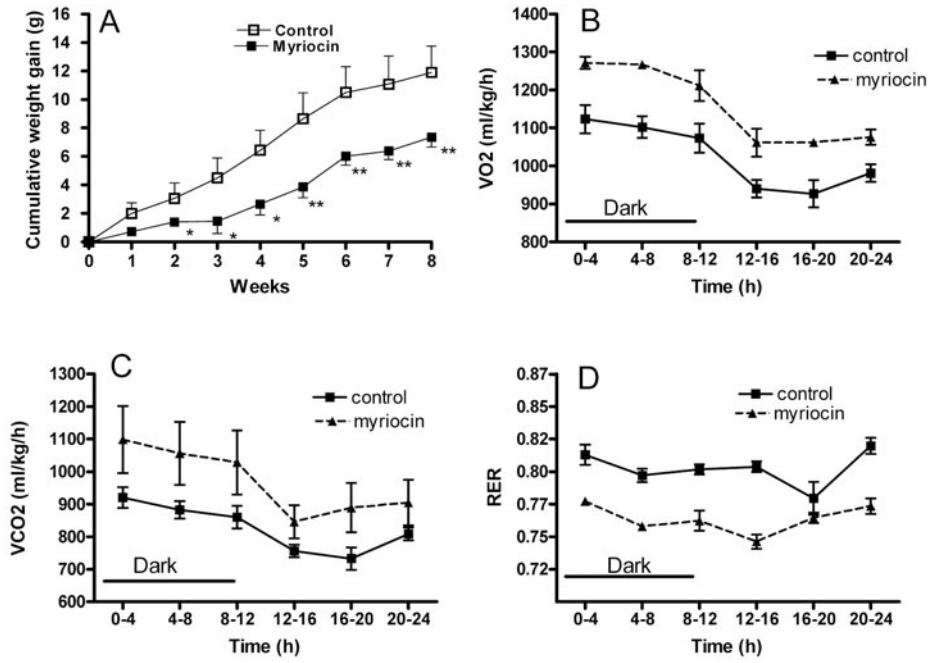

Figure 3. Metabolic parameters in DIO mice after inhibition of de novo ceramide biosynthesis. Panel A: Body weights. Panel B: whole body oxygen consumption. Panel C: Carbon dioxide release. Panel D: Respiratory Exchange Ratio. Adapted from reference 69.

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

SPHINGOLIPIDS AS MEDIATORS OF ADIPOSE INFLAMMATION IN OBESITY

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

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

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO OBESITY MEDIATED INSULIN RESISTANCE

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

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

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

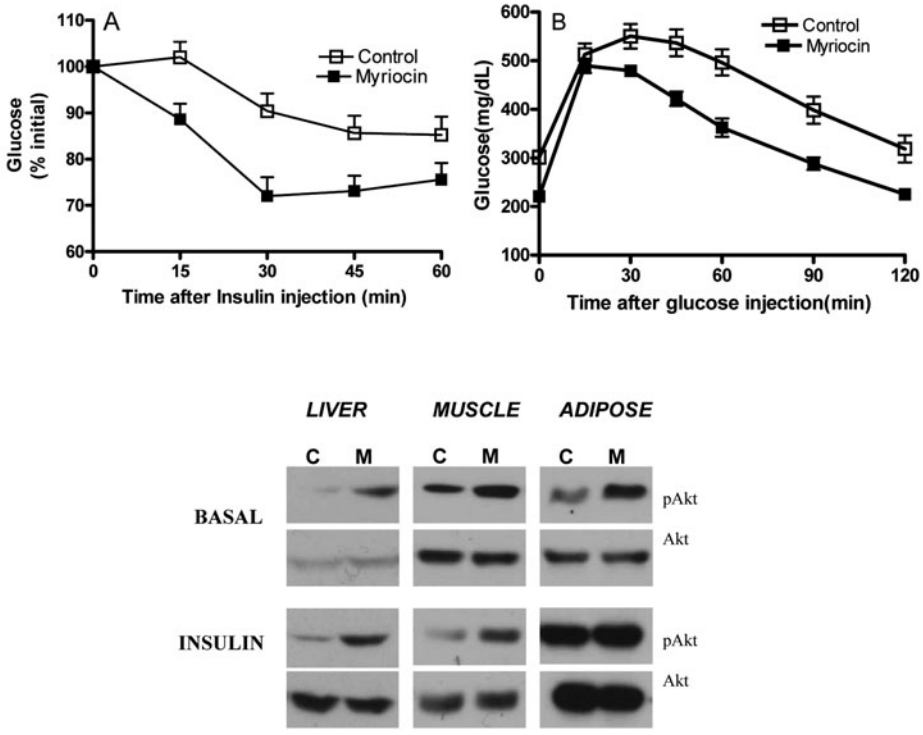

Figure 4. Glucose hemostasis in DIO mice in response to inhibition of de novo ceramide biosynthesis. Panel A: Insulin tolerance test. Panel B: Glucose tolerance test. Bottom panel: Western blot: Basal and Insulin-mediated Akt phosphorylation in Liver, Muscle and Adipose tissues of vehicle-treated controls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

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

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

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

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

CONTRIBUTION OF CERAMIDE BIOSYNTHESIS TO HEPATIC STEATOSIS

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

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

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

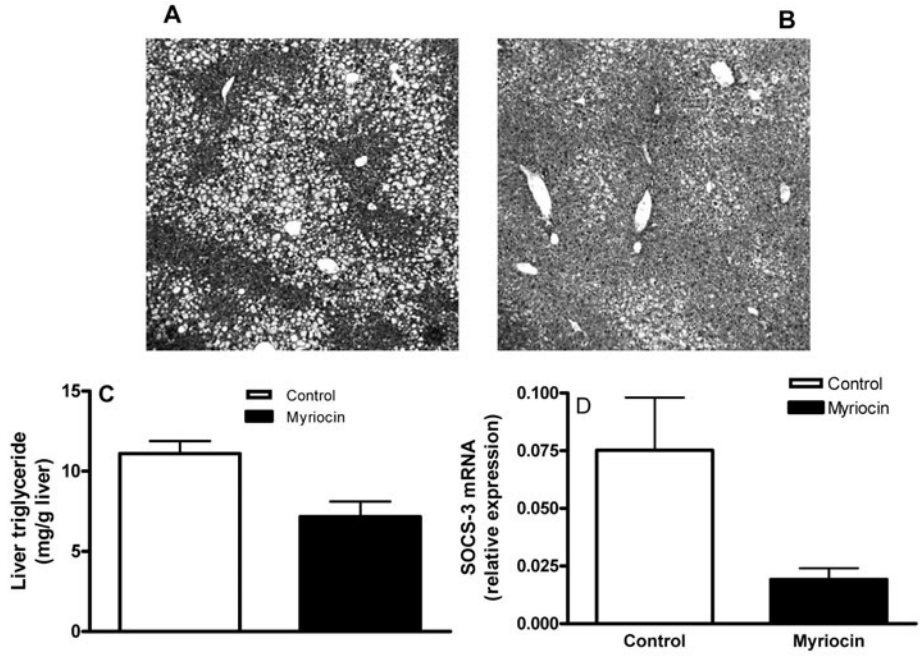

Figure 5. Amelioration of hepatic steatosis and suppressor of cytokine signaling-3 (SOCS-3) in myriocin treated DIO mice. Panels A and B: Liver histology of hematoxylin and eosin stained sections from control and myriocin-treated DIO mice respectively. Panel C: Liver triglyceride content. Panel D: SOCS-3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

CERAMIDE AND CARDIOVASCULAR DISEASE

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

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

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

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

CONCLUSION

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

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