

# Chapter 2

## GLYCOSPHINGOLIPIDS AND INSULIN RESISTANCE

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*Progress in Lipid Research 2009; March*

## **Abstract**

Obesity is associated with an increased risk for insulin resistance, a state characterized by impaired responsiveness of liver, muscle and adipose tissue to insulin. One class of lipids involved in the development of insulin resistance are the (glyco)sphingolipids. Ceramide, the most simple sphingolipid, directly inhibits phosphorylation of the insulin signaling mediator Akt/Protein Kinase B. More complex glycosphingolipids, so-called gangliosides, block phosphorylation of the insulin receptor and down-stream signaling, possibly by exclusion of the insulin receptor from specific membrane domains. Pharmacological inhibition of glycosphingolipid synthesis is found to markedly improve insulin sensitivity in rodent models of insulin resistance. Partial glycosphingolipid reduction is well tolerated and may thus offer an attractive new treatment modality for obesity-induced insulin resistance and type II diabetes.

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# 1 Introduction

The ancient Greek motto  $\mu\epsilon\delta\epsilon\upsilon\ \alpha\gamma\alpha\upsilon$  ('nothing in excess') at the Apollo temple in Delphi, stresses the importance of moderation. In this time of nutrient excess and sedentary life style the motto seems more applicable than ever. Due to increases in the amount and caloric content of food as well as diminishing physical activity, the incidence of obesity is markedly increasing. Obesity is associated with an increased risk for diminished insulin sensitivity. This so-called insulin resistance is characterized by reduced insulin-mediated glucose uptake and impaired suppression of lipolysis as well as hepatic glucose production<sup>1,2</sup>. Another common feature of obese individuals is low-grade inflammation<sup>3</sup>, mediated by cytokines secreted by macrophages in their adipose tissue, which further promotes insulin resistance<sup>4-8</sup>.

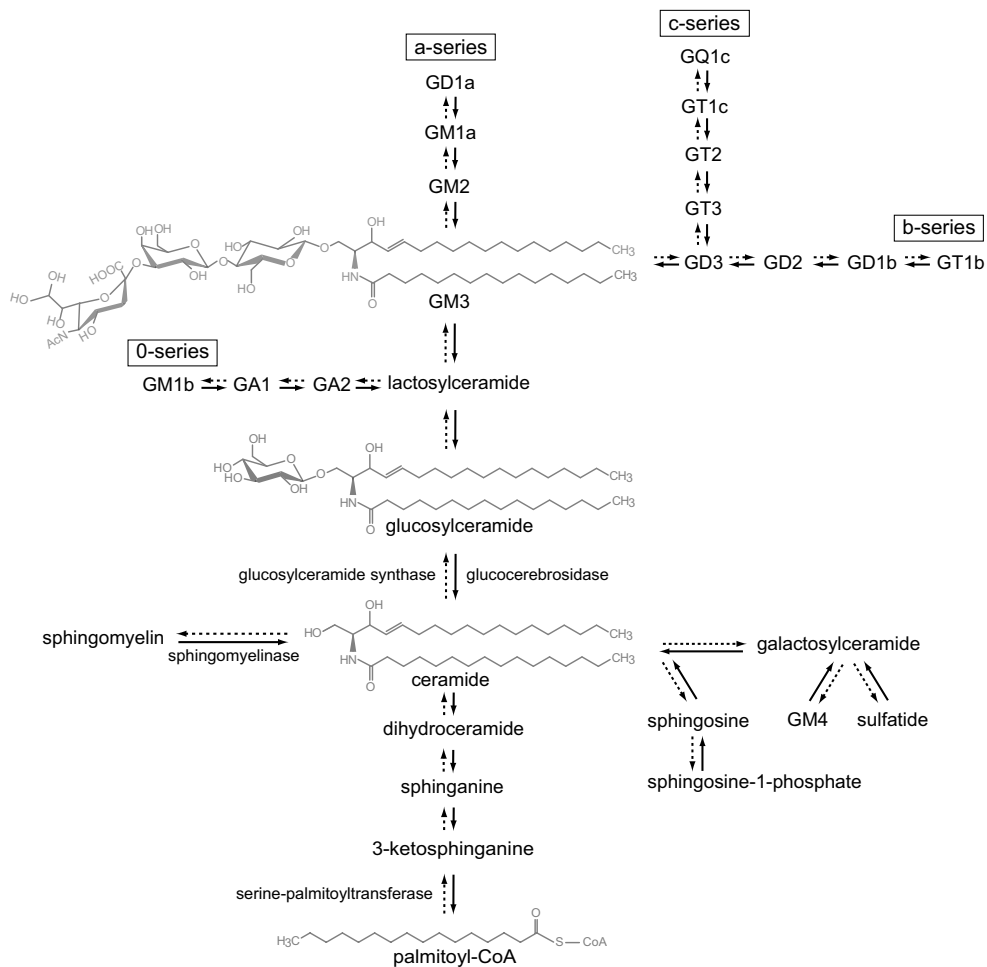
In the last decade evidence has accumulated pointing to a key role for excessive lipids in the etiology of obesity-induced insulin resistance<sup>9</sup>. Triglycerides are normally predominantly stored in adipose tissue. In obesity, this storage capacity is often exceeded and triglycerides accumulate in other tissues such as liver and muscle. Lipid excess in tissues is associated with impaired insulin signaling and action<sup>10,11</sup>. A strong inverse correlation exists between the intramyocellular lipid content and whole body insulin stimulated glucose uptake<sup>12</sup>. It is thought that the surplus of triglycerides and free fatty acids delivered to insulin-responsive tissues results in local high concentrations of other lipids, among which sphingolipids.

Excessive sphingolipids hamper insulin signaling and promote inflammation. For example, increased concentrations of ceramide lead to inhibition of insulin signaling at the level of Akt/Protein Kinase B (PKB) in cell models<sup>13,14</sup>. Since the role of ceramide in insulin resistance has been extensively reviewed<sup>15,16</sup>, this review primarily focuses on the new findings which stress the importance of glycosphingolipids in regulating insulin responsiveness. Recent reports suggest that excessive glycosphingolipids are major contributors to the pathophysiology of obesity-induced insulin resistance. Moreover, pharmacological reduction of glycosphingolipids has been found to exert beneficial effects in animal models of insulin resistance and type II diabetes.

## 2 Glycosphingolipid metabolism

### 2.1 Glycosphingolipid biosynthesis

Ceramide, formed by *de novo* synthesis or by catabolism of glycosphingolipids and sphingomyelin, is the precursor of all complex glycosphingolipids (figure 1). *De novo* synthesis of ceramide takes place at the cytosolic leaflet of the ER. The first step in ceramide synthesis is the formation of 3-ketosphinganine by Serine Palmitoyl Transferase (SPT). The *de novo* synthesis rate of ceramide is regulated by the availability of the precursors palmitoyl-CoA and serine<sup>17</sup>. In addition, extracellular stimuli such as cytokines enhance ceramide formation by increasing expression of SPT<sup>18</sup> (see section 6). Ceramide is the precursor for several types of sphingolipids: sphingomyelin, galactosylceramide and sulfatide, ceramide-1-phosphate, O-acylceramide, and glucosylceramide. This review focuses on glucosylceramide and its anabolites, the more complex glycosphingolipids. Glucosylceramide is formed via glucosylation of ceramide by the enzyme Glucosylceramide Synthase (GCS). GCS transfers the glucose moiety from the donor UDP-glucose to ceramide present at the cytosolic leaflet of the Golgi apparatus<sup>19-21</sup>. Next, glucosylceramide can be either directly transported to the plasma membrane or translocated across the membrane of the Golgi apparatus where it may be transformed to more complex glycosphingolipids<sup>22-24</sup>. Glucosylceramide is first converted to lactosylceramide by (1-4)- $\beta$  linkage of galactose. Next, from lactosylceramide so-called gangliosides can be formed (figure 1). Gangliosides include sphingolipids containing N-acetylneuraminic acid (sialic acid) units. A bewildering amount of gangliosides exists and a convenient shorthand nomenclature has been developed by Svennerholm, a pioneer in ganglioside research<sup>25</sup>. In this nomenclature G stands for ganglioside, A for asialo-, M for monosialo-, D for disialo- and T for trisialo-ganglioside. The principles of the ganglioside-synthesizing machinery and their nomenclature have nicely been reviewed<sup>26,27</sup>. Specific sialyl transferases convert lactosylceramide stepwise to GM3, GD3, and GT3. Lactosylceramide and each of its sialylated derivatives serve as precursors for complex gangliosides of the 0-, a-, b-, and c-series. These different series are characterized by the presence of no (0-series), one (a-series), two (b-series), or three sialic acid residues (c-series) linked to the 3-position of the inner galactose moiety (figure 1). In adult human tissues, gangliosides from the 0- and c-series are only found in trace amounts<sup>26,27</sup>.

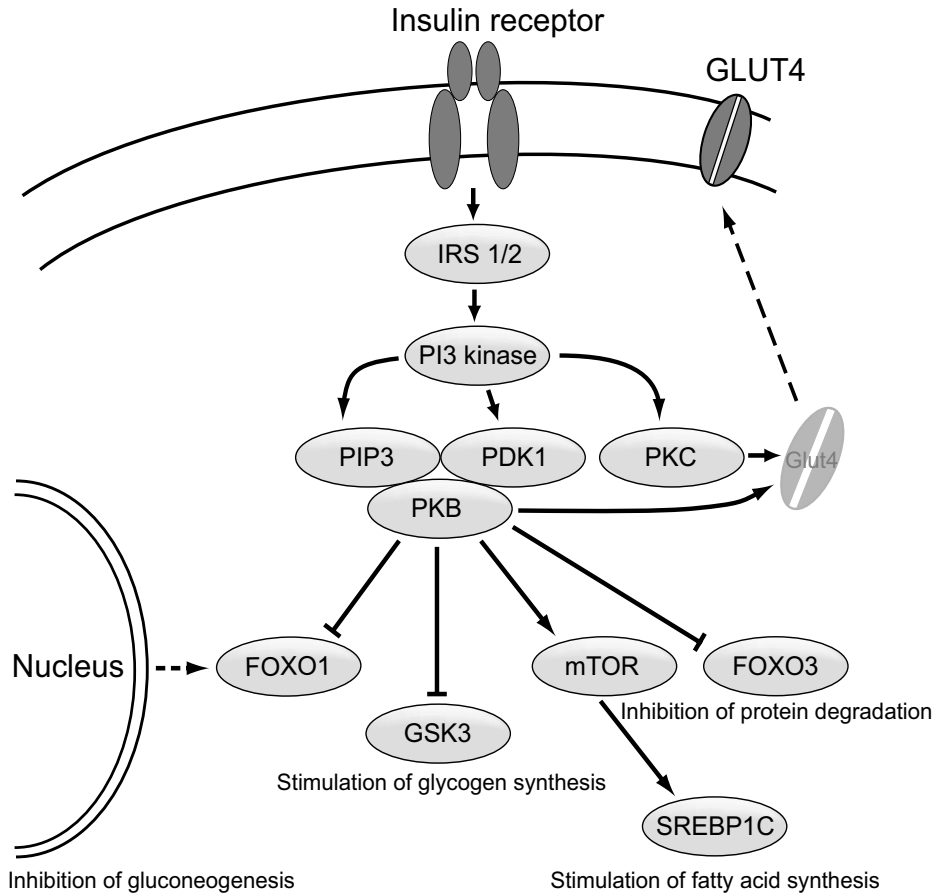


**Figure 1** Schematic overview of glycosphingolipid metabolism. G: ganglioside, A: asialo-, M: monosialo-, D: disialo- and T: trisialo-ganglioside. 0, a, b and c series: see text.

## 2.2 *Glycosphingolipid catabolism*

Catabolism of complex glycosphingolipids is a stepwise process, predominantly taking place in lysosomes<sup>28,29</sup>. Glycosphingolipids can reach the endosomal-lysosomal compartment in various ways<sup>30</sup>. The first route is receptor-mediated endocytosis of LDL, which results in delivery of glycosphingolipids to the lumen of lysosomes. The second way is phagocytosis of larger structures such as senescent cells containing glycosphingolipids by specialized phagocytes like macrophages. Another major pathway in most cells involves endocytosis of plasma membrane. Glycosphingolipid-rich membrane parts are internalized and fuse with early endosomes. Next, glycosphingolipids destined for degradation are sorted out by formation of intraluminal vesicles (multivesicular bodies) which reach the lysosome<sup>31</sup>. In the lysosome, glycosphingolipids are catabolized sequentially by glycosidases. Some steps require the assistance of sphingolipid activator proteins (Sap A, B, C and D and GM2 activator protein)<sup>31</sup>. The product formed by deglycosylation of glycosphingolipids is glucosylceramide, which in turn is degraded into ceramide and glucose by the enzyme glucocerebrosidase<sup>32</sup>. Ceramide can then be cleaved in sphingosine and fatty acid by ceramidase. Of note, ceramide degradation can also take place in other parts of the cell. Sphingosine can be either re-acylated to ceramide or used as a substrate for sphingosine 1-phosphate (S-1-P) synthesis<sup>33,34</sup> (figure 1). It has recently become apparent that metabolism of endocytosed glycosphingolipids is not restricted to lysosomes. Glucosylceramide derived from degradation of complex glycosphingolipids, from both intra- and extracellular sources, may escape lysosomal degradation and re-enter the glycosphingolipid synthesis pathway<sup>35</sup>. In addition, direct metabolic remodeling of glycosphingolipids at the plasma membrane has been proposed. This process may result in local formation of more simple glycosphingolipids from complex substrates<sup>36</sup>. The occurrence of an extra-lysosomal glucosylceramidase, converting glucosylceramide to ceramide, has also been demonstrated<sup>37-39</sup>.

The importance of efficient glycosphingolipid catabolism is illustrated by the existence of inherited deficiencies in many lysosomal glycosidases and activator proteins. This results in accumulation of the corresponding glycosphingolipids and a broad spectrum of pathologies<sup>28,29</sup>. The most common of the so-called sphingolipidoses is Gaucher disease, a disorder caused by deficiency of glucocerebrosidase<sup>32,40</sup>. The diagnosis of Gaucher disease can be conveniently confirmed by demonstration of deficient activity of glucocerebrosidase with artificial substrates such as 4-methylumbelliferyl-beta-glucoside or C6-NBD-glucosylceramide<sup>41,42</sup>.



**Figure 2** Major pathways in insulin receptor signaling. Some of the metabolic effects of insulin may also be mediated via other signaling branches than those depicted. Abbreviations: IRS=Insulin Receptor Substrate, PI3kinase=Phosphoinositide-3-kinase, PIP3=Phosphatidylinositol (3,4,5)-triphosphate, PDK1=Phosphatidylinositol-3-phosphate Dependent Kinase 1, PKC=Protein Kinase C, PKB=Protein Kinase B, FOXO1=Forkhead box O1, GSK3=Glycogen Synthase Kinase 3, mTOR=mammalian Target of Rapamycin, FOXO3=Forkhead box O3, SREBP1C=Sterol Regulatory Element Binding Protein-1C, GLUT4=Glucose Transporter 4.

## 3 Insulin signaling and action

### 3.1 *The insulin signaling cascade*

The insulin receptor (IR) is a heterodimer consisting of two  $\alpha$ - and two  $\beta$ -subunits. Binding of insulin to the extracellular  $\alpha$ -subunits induces a conformational change, resulting in auto-phosphorylation of particular tyrosine residues in the intracellular  $\beta$ -subunits. Recruitment of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) to the phosphorylated IR results in their phosphorylation, which allows binding and activation of class I phospho-inositide-3-kinase (PI 3-kinase). Activated PI 3-kinase produces phosphatidylinositol (3,4,5)-triphosphate (PIP3), serving as a binding site for proteins containing pleckstrin homology (PH) domains. The PH domain containing serine/threonine kinases PKB and phosphatidylinositol-3-phosphate dependent kinase 1 (PDK1) are brought into close proximity with each other by their interactions with PIP3. Additionally, PIP3 helps to activate PKB, by inducing conformational changes that expose two regulatory phosphorylation sites. First, the mammalian target of rapamycin (mTOR)-RICTOR protein complex phosphorylates the exposed regulatory serine (S473) in the C-terminus of PKB. Next, PDK1 phosphorylates the regulatory threonine residue (T307) of PKB that is requisite for enzyme activity<sup>43-45</sup>(figure 2). All these early signaling steps take place at the plasma membrane. Activation of PKB allows its relocalization to the cytosol. Part of the activated PKB enters the nucleus where it affects transcription of several target genes.

In addition to the PI 3-Kinase/PKB pathway, binding of insulin to IR induces another signaling pathway via Growth factor binding protein 2 (Grb2)/Son of sevenless (Sos) and Ras, leading to activation of the mitogen activated protein kinase (MAPK) isoforms extracellular signal-regulated kinases (ERK)1 and ERK2. However, insulin produces most of its metabolic actions through the PI 3-Kinase/PKB pathway. The MAPK cascade is not involved in insulin-stimulated glucose transport or glycogen synthesis but may relate to regulation of cell survival and proliferation<sup>46</sup>. Insulin, together with other stimuli, is also involved in the regulation of autophagy<sup>47,48</sup>. Since studies on the interference of insulin signaling by sphingolipids focus on the PI 3-Kinase/PKB pathway this review will further only discuss this part of the signaling cascade.

### 3.2 Metabolic responses to insulin signaling

An important function of insulin is to rapidly increase glucose uptake by target organs. Following an insulin stimulus, activated PKB, in concert with Protein Kinase C (PKC) activated by PI3-kinase, promotes translocation of glucose transporter 4 (GLUT-4) to the plasma membrane, allowing immediate glucose uptake<sup>49</sup> (figure 2). An additional pathway for insulin-stimulated GLUT-4 translocation involving the APS-CAP-Cbl protein complex has been postulated<sup>50</sup>. In this pathway, the APS-CAP-Cbl protein complex activates the small GTPase TC10, which in turn signals the cytoskeleton to promote GLUT-4 cell surface recruitment<sup>50</sup>.

Insulin enhances the formation of glycogen and inhibits gluconeogenesis. PKB stimulates glycogen synthesis by phosphorylation of glycogen synthase kinase 3 (GSK3), which thus no longer can inhibit the enzyme glycogen synthase<sup>51</sup>. Inhibition of gluconeogenesis, is regulated, at least partially, by the transcription factor FOXO1. Phosphorylation of FOXO1 by PKB excludes it from the nucleus, abolishing FOXO1-mediated expression of the gluconeogenic enzymes glucose-6-phosphatase (G6Pase), fructose-1, 6-biphosphatase and phosphoenolpyruvate carboxykinase (PEPCK)<sup>52</sup> (figure 2). Inhibition of protein degradation by insulin requires activation of the mammalian target of rapamycin (mTOR) and inactivation of the transcriptionfactor FOXO3 by PKB<sup>48</sup>. Insulin stimulates lipogenesis by activating the transcription factor Sterol Regulatory Binding Protein-1c (SREBP-1c) via the PI3K/Akt/mTOR pathway<sup>53</sup>. This enhances transcription of genes required for fatty acid and triglyceride biosynthesis, such as acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS)<sup>54</sup>. Insulin activates SREBP-1c by both enhancing its transcription and increasing the amount of nuclear SREBP-1c, most likely by promoting conversion of the membrane-bound precursor to its cleaved nuclear form<sup>55</sup>. Insulin decreases lipolysis in adipose tissue by activating phosphodiesterase-3B (PI3B)<sup>56</sup> PI3B decreases intracellular cyclic Adenosine Monophosphate (cAMP) concentrations, which in turn leads to dephosphorylation of hormone sensitive lipase (HSL) resulting in inhibition of lipolysis.

Intriguingly, despite clear insulin resistance for the inhibitory of effect of insulin on hepatic glucose output, insulin sensitivity is maintained for lipogenesis in *ob/ob* mice<sup>57</sup>. This selective insulin resistance explains the paradoxical combination of hyperinsulinemia and hyperlipidemia in type II diabetes<sup>58</sup>. Partial postreceptor hepatic insulin resistance thus seems a key element in the development of dyslipidemia and hepatic steatosis.

## 4 Insulin receptor membrane topology

### 4.1 *Insulin receptor localization in caveolae/Detergent Resistant Membranes*

As described above, insulin signaling requires the participation of many components. Proper insulin signaling, in terms of speed and specificity, requires spatial and temporal organization of the key signal components in specific plasma membrane domains<sup>59</sup>. Caveolae are membrane invaginations visible with electron microscopy and characterized by the presence of the structural protein caveolin-1. Caveolae are stable and relatively immobile membrane regions enriched for cholesterol, sphingomyelin and glycosphingolipids. They are most abundant in adipocytes but are also present in other cell types such as fibroblasts and endothelial cells<sup>60,61</sup>. Caveolae are thought to serve as signaling platforms for several receptors including the insulin receptor, though this proposed function is still debated<sup>61</sup>. In tissues with relatively low caveolin-1 expression levels, such as skeletal muscle and liver<sup>62,63</sup>, the IR has been detected in membrane domains enriched in cholesterol and glycosphingolipids. These domains are mostly referred to as Detergent Resistant Membranes (DRMs) or rafts and are less well defined than caveolae. Regarding their composition, number and size, data widely differ between different cell types and the purification and identification protocols used. Apart from experimentally induced differences, there is evidence for compositional heterogeneity of rafts (for a review see 64). Isolation of DRMs using the detergent Triton X 100, the most common method, results in different outcomes regarding their content of specific proteins such as the insulin receptor. Results vary depending on the temperature at which the DRM isolation is performed and the concentration of the detergent used<sup>65</sup>. These limitations of present methodology should be taken into account when interpreting reports regarding insulin receptor detection in DRMs.

One of the earliest reports on the presence of the IR in caveolae by Gustavsson and coworkers showed co-localization of the insulin receptor with caveolin-1 by electron- and immunofluorescence microscopy in both isolated rat adipocytes and 3T3-adipocytes. They separated a caveolae-enriched membrane fraction by centrifugation methods with and without detergent and found the IR  $\beta$ -subunit to be present in this fraction<sup>66</sup>. Later studies on the presence of the IR in caveolae yielded conflicting results (see 67 for a review), most likely due to the different methods used for caveolae isolation. Recently several papers on the subject have been published. Souto and coworkers used a detergent-free caveolae isolation technique

based on mechanical homogenizing of primary rat adipocytes and gradient centrifugation of membranes. They failed to detect the IR in caveolae. They confirmed this finding by electron microscopy with immunogold labeling of the IR and caveolin-1 resulting in different staining patterns for these antigens<sup>68</sup>. In contrast, Karlsson and coworkers did show localization of IR and IRS-1 in caveolae isolated from primary human adipocytes by gradient centrifugation<sup>69</sup>. A recent report on the subject, using electron microscopy and freeze fracture analysis of 3T3-adipocytes, indicates that IR is located in the 'neck' of caveolae<sup>70</sup>. An earlier paper by Smith and coworkers also reported co-localization of IR and caveolin-1 as analyzed by immuno-electronmicroscopy of membrane fractions obtained from H35 hepatoma cells by gradient density gradient centrifugation<sup>71</sup>. Balbis and coworkers also show co-immunoprecipitation of caveolin-1 with the phosphorylated IR in freshly isolated membranes from rat hepatocytes prepared after insulin stimulation<sup>72</sup>. A study by Vainio and coworkers using a hepatoma (HuH7) cell line indicated that upon insulin stimulation, the insulin receptor is recruited to a part of the membrane that is Triton X-100 resistant<sup>73</sup>. In DRMs of insulin stimulated differentiated L6 myocytes (myotubes), the phosphorylated insulin receptor only partially co-localized with caveolin-1, indicating that in these cells the IR is found both in caveolae and DRMs not containing caveolin-1<sup>62</sup>.

Recent studies using transgenic mouse models suggested that the presence of caveolin is required for proper insulin signaling<sup>74</sup>. Caveolin may stabilize the IR protein or directly stimulate IRs. Other studies have proposed that caveolae also provide cellular microdomains for GLUT-4, but opposing views on this matter exist<sup>60</sup>. For an extensive overview of the role of caveolae and caveolin-1 in insulin-mediated glucose uptake the reader is referred to refs<sup>60,75,76</sup>.

#### 4.2 *Impact on insulin signaling*

Several studies suggest that localization of IR in caveolae/DRMs is crucial for proper signal transduction. Gustavson and coworkers showed that disruption of caveolae in rat adipocytes and 3T3-adipocytes by reduction of cellular cholesterol with  $\beta$ -cyclodextrin attenuated insulin stimulated glucose uptake. Subsequent replenishment of cholesterol restored insulin signaling<sup>66</sup>. These results were later confirmed by the same group in additional studies<sup>69,77</sup>. Others investigators have studied the effect of (glyco)sphingolipids on the IR membrane localization and signaling. A report by Grigsby *et al.* suggests that exposing 3T3-adipocytes

to TNF $\alpha$  increases caveolar ceramide concentration and impairs IR function. Prevention of ceramide accumulation using Z-VAD-FMK, a caspase inhibitor, restored IR and IRS-1 phosphorylation<sup>78</sup>. Kabayama *et al.* reported the effect of prolonged TNF $\alpha$  exposure on IR localization in 3T3-adipocytes<sup>79</sup>. In untreated 3T3 cells, IR was found in DRMs extracted with low concentration Triton X-100 or under hypertonic alkaline conditions. Exposure of cells to TNF $\alpha$ , caused a partial shift of IR to higher density fractions, coinciding with a reduction in IRS-1 phosphorylation in response to insulin. GM3 was twice as abundant in DRMs from TNF $\alpha$ -treated adipocytes, whereas cholesterol and caveolin levels were unchanged. Depletion of GM3 using 1-phenyl-2-decanoylamino-3-morpholinopropanol (d-PDMP), an inhibitor of glucosylceramide synthesis, prevented exclusion of the IR from DRMs normally induced by TNF $\alpha$ <sup>79</sup>. An earlier study by Vainio and colleagues also revealed that in liver-derived cells lacking caveolae, autophosphorylation of the endogenous IR is compromised by acute cyclodextrin-mediated cholesterol depletion or by antibody induced clustering of glycosphingolipids<sup>73</sup>.

## 5 Influence of (glyco)sphingolipid levels on insulin signaling and action

### 5.1 Ceramide

Ceramide functions as a mediator in signaling cascades that regulate apoptosis, differentiation and cell cycle arrest<sup>80</sup>. An additional role for ceramide in the insulin signaling pathway has more recently been proposed. Ceramide does not interfere at the level of IR or IRS-1 phosphorylation, but impairs insulin signaling by inhibition of PKB activation<sup>81</sup>. The inhibition of PKB by ceramide is thought to be accomplished by two mechanisms. Ceramide blocks the translocation of PKB to the plasma membrane and activates PP2A, which impairs PKB activity by removing activating phosphates<sup>81</sup>.

The role of ceramide in insulin resistance will only be dealt with briefly, for detailed reviews the reader is referred to Summers and coworkers<sup>15,82</sup>. Investigations with cultured cells first revealed an association between cellular ceramide and insulin responsiveness. Exposing cultured myotubes to high doses of the free fatty acid (FFA) palmitate increases *de novo* ceramide synthesis, followed by inhibition of PKB phosphorylation<sup>14,83,84</sup>, glucose uptake<sup>85</sup> and

glycogen synthesis<sup>86</sup>. Overexpression of acid ceramidase in these cells reverses FFA-induced ceramide accumulation and improves insulin signaling<sup>87</sup>. Addition of short-chain ceramide analogues to cultured 3T3-adipocytes was found to inhibit insulin signaling and action<sup>13,88-90</sup>. Several investigators have reported the occurrence of elevated concentrations of ceramide in plasma and tissues of animal models of obesity-induced insulin resistance and type II diabetes<sup>82,91,92</sup>. However, not all studies confirmed this. Lee and coworkers reported that male rats on a diet rich in saturated fatty acids developed insulin resistance with an increase in muscle diacylglycerol concentration, but without significant changes in muscle ceramide content<sup>93</sup>. In two other recent studies no significant abnormalities in skeletal muscle ceramide content of insulin-resistant Zucker Diabetic Fatty (ZDF) rats were noted<sup>94,95</sup>.

Planavila and coworkers showed that treatment of mice with the PPAR $\gamma$  agonist troglitazone, an established antidiabetic drug, decreased muscle ceramide<sup>96</sup>.

Recently, studies were conducted in humans on the possible role of ceramide in obesity and FFA induced insulin resistance. Two investigations reported an association between insulin resistance and elevated levels of ceramide in skeletal muscle<sup>97,98</sup>. A more recent study by Skovbro *et al.* showed no increase in muscle ceramide content in insulin-resistant and type II diabetic individuals compared to insulin sensitive individuals<sup>99</sup>. Infusion of lipid emulsion (Intralipid<sup>®</sup>, an emulsion of soy bean oil and egg phospholipids), known to decrease peripheral insulin sensitivity, was found to increase muscle ceramide content in one study<sup>100</sup>, but not in another investigation<sup>101</sup>. However, the lipid emulsion used (Intralipid), contains largely unsaturated fat and unsaturated fat has later been shown by Holland *et al* to cause insulin resistance in a ceramide independent manner<sup>92</sup>.

The discrepancies in reported ceramide concentrations in relation to insulin resistance are difficult to explain. Animal and human muscle ceramide concentrations mentioned in various publications differ greatly, pointing to crucial differences in applied analytic methods<sup>102</sup>.

## 5.2 Neutral glycosphingolipids and gangliosides

Nojiri *et al.* were the first to demonstrate a negative correlation between ganglioside concentrations and insulin responsiveness by showing ganglioside-mediated inhibition of insulin-dependent cell growth in leukemic cell lines<sup>103</sup>. Tagami and coworkers later established that addition of GM3 to cultured adipocytes suppresses phosphorylation of IR and its down-stream substrate IRS-1, resulting in reduced glucose uptake<sup>104</sup>. As described in section

4.2, Inokuchi and coworkers demonstrated that exposure of cultured adipocytes to TNF $\alpha$  increases the ganglioside GM3<sup>79</sup>.

The same investigators rendered important new insights regarding the interference of excessive GM3 on the interaction of IR with caveolin-1<sup>105</sup>. 3T3-adipocytes were exposed to TNF $\alpha$  resulting in increased cellular GM3 concentration and diminished IR signaling. They noted that in lysates from untreated 3T3-adipocytes, the IR co-precipitated with caveolin-1. In lysates from 3T3-adipocytes that had been exposed to TNF $\alpha$ , the caveolin-1-IR interaction was diminished. Direct GM3-insulin receptor interactions were demonstrated using cross-linking of GM3 followed by immunoprecipitation of the IR  $\beta$ -subunit. The basic lysine residue in the insulin receptor (K944), located just above the transmembrane domain, proved to be essential for the binding of IR to GM3. Based on their findings, Kabayama and workers have proposed that in adipocytes high GM3 concentrations displace the IR from caveolae, resulting in compromised insulin signaling<sup>105</sup>.

The findings in these in vitro experiments were strengthened by subsequent studies in rodents. GM3 synthase knockout (GM3S<sup>-/-</sup>) mice completely lack GM3 and show enhanced phosphorylation of the skeletal muscle IR after ligand binding. Insulin stimulated whole body glucose uptake and insulin mediated suppression of endogenous glucose production were increased. High fat diet did not lead to a deterioration of these parameters in the GM3S<sup>-/-</sup> mice, indicating protection from high-fat diet induced insulin resistance<sup>106</sup>. In line with this, insulin sensitivity and glucose tolerance were found improved in mice with increased expression of the GM3 degrading sialidase Neu3 in the liver<sup>107</sup>. In another study, mice with a more general Neu3 overexpression showed variable increases in GM1 and GM2 and small decreases in GM3 in various tissues. Surprisingly, the male transgenic mice became resistant to insulin and even developed diabetes<sup>108</sup>.

Data on glycosphingolipid concentrations in insulin resistant rodents, supporting a tentative role in insulin resistance are still scarce. Concentrations of glucosylceramide, the precursor of complex glycosphingolipids, were found to be slightly elevated in liver and muscle of *ob/ob* mice and ZDF rats compared to lean animals with similar background<sup>94,95</sup>. Increased GM3 synthase mRNA concentrations were found in adipose tissue from *ob/ob* mice and ZDF rats<sup>104</sup>. In a later study however, reduced levels of GM3 synthase mRNA in adipose tissue from *ob/ob* mice were found<sup>109</sup>. Using anti-GM3 antibodies, immunofluorescent staining was found to be more intense in muscle sections from ZDF rats, compared to lean

littermates<sup>95</sup>.

Very recently Inokuci and co-workers reported that serum GM3 concentrations were higher (1.4 to 1.6 fold) in hyperlipidemic individuals and type II diabetics with and without hyperlipidemia (1.4-fold) compared to normal subjects<sup>110</sup>. There are unfortunately still no supporting data on ganglioside levels in tissues of obese, insulin-resistant or type II diabetic humans. In a single study, muscle glucosylceramide concentrations in obese humans were noted to be comparable to those in lean subjects, and FFA infusion did not change muscle glucosylceramide in lean or obese subjects<sup>101</sup>. Of interest are observations made in type I Gaucher disease (GD) patients. This condition is characterized by a primary glucosylceramide accumulation and secondary elevation of gangliosides<sup>111</sup>. An euglycemic clamp revealed that insulin-mediated whole body glucose uptake in GD patients is reduced compared to healthy control subjects<sup>112</sup>. Since plasma levels of GM3 are elevated in these patients<sup>111</sup>, one could hypothesize that in GD patients altered glycosphingolipid levels in muscle and/or fat tissue result in the observed insulin resistance.

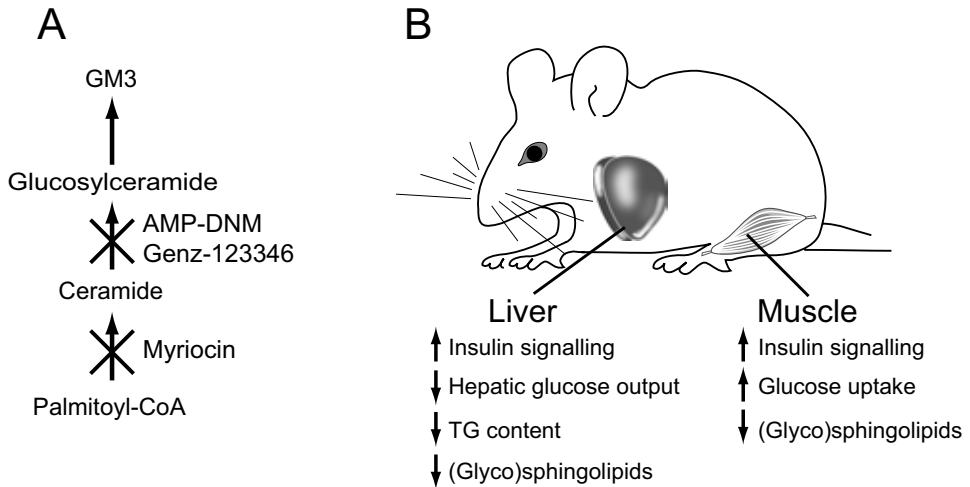
An additional role for yet another sphingolipid metabolite, sphingosine, in insulin resistance has been proposed by Samad *et al.* They observed in *ob/ob* and leptin receptor deficient (*db/db*) mice elevated plasma concentrations of ceramide<sup>109</sup>. However, in adipose tissue of the obese animals, ceramide and sphingomyelin concentrations were found to be slightly decreased. Concentrations of glycosphingolipids in plasma and tissue were unfortunately not determined in the study. Of interest, a clear increase in the ceramide metabolite sphingosine was detected in adipose tissue of obese mice. A concomitant increase was observed in mRNAs encoding enzymes involved in ceramide formation (SPT, acid and alkaline sphingomyelinase) as well enzymes responsible for the hydrolysis of ceramide to sphingosine (acid and alkaline ceramidase)<sup>109</sup>. Based on these results, it has been speculated that, via some unknown mechanism, sphingosine also influences the responsiveness to insulin of adipose tissue.

## 6 Obesity induced inflammation alters glycosphingolipid metabolism

There is a strong association between obesity-induced insulin resistance and inflammation<sup>6,8</sup>. Adipose tissue isolated from obese individuals is characterized by the presence of high numbers of macrophages that secrete pro-inflammatory cytokines and chemokines<sup>7</sup>. Storage of fat in the liver, derived from exogenous sources and produced by local lipogenesis, may also result in inflammation<sup>113</sup>. The increased cytokine levels in obesity promote sphingolipid metabolism<sup>82</sup>, which may in turn exacerbate insulin resistance. For example, binding of TNF $\alpha$  to the 55kDa TNF receptor (TNF-R55) activates endosomal/lysosomal acid sphingomyelinase (ASMase) and neutral sphingomyelinase (NSMase) through different domains of this receptor<sup>114</sup>. Activation of ASMase increases cellular ceramide derived from sphingomyelin<sup>115</sup>. TNF $\alpha$  also increases ceramide levels by stimulating *de novo* ceramide synthesis<sup>116</sup>. Consistent with this, intraperitoneal injections of TNF $\alpha$  were found to increase mRNA levels of ASMase and NSMase and SPT in adipose tissue of lean mice. In adipose tissue of obese *ob/ob* and *db/db* mice mRNA levels of these enzymes were also found to be increased<sup>109</sup>. TNF $\alpha$  is known to increase GM3 in 3T3-adipocytes (see section 5.3). Not surprisingly, mice lacking the TNF-R55 (TNFRp55<sup>-/-</sup>) have reduced ganglioside levels in various tissues<sup>117</sup>.

In analogy with TNF $\alpha$ , glucocorticoids induce insulin resistance and are known to stimulate sphingolipid synthesis. Administration of dexamethasone (a synthetic glucocorticoid) diminishes hepatic and peripheral insulin sensitivity<sup>118</sup>. In mice, dexamethasone stimulates (glyco)sphingolipid synthesis by increasing expression of genes involved in ceramide, glucosylceramide and sphingosine synthesis. Treating mice with myriocin, a potent inhibitor of SPT, prevents glucocorticoid-induced insulin resistance<sup>92</sup>. In insulin-resistant obese individuals, plasma levels of glucocorticoids are not elevated, but expression and activity levels of the enzyme involved in the conversion of inactive cortisone to active cortisol (11beta-hydroxysteroid dehydrogenase type 1) are upregulated in adipose tissue, possibly contributing to reduced insulin responsiveness<sup>119</sup>. The role of glycosphingolipid synthesis in glucocorticoid-induced insulin resistance has not yet been studied in humans.

Whilst synthesis of sphingolipids is promoted by inflammation, the lipids in turn may stimulate the expression of cytokines and chemokines, creating a vicious circle. For example, incubation of 3T3-adipocytes with short-chain ceramides, sphingosine, or GM3, was found



**Figure 3** Effects of inhibition of (glyco)sphingolipid synthesis in rodent models of insulin resistance and type II diabetes. (A) Overview of pathway and inhibited steps of synthesis. Myriocin inhibits serine palmitoyl transferase, reducing ceramide synthesis. AMP-DNM and Genz-123346 inhibit glucosylceramide synthase, reducing glucosylceramide synthesis. (B) Effects of inhibition.

to increase expression of TNF $\alpha$ <sup>109</sup>. Inhibition of glucosylceramide synthesis by treatment with the iminosugar AMP-DNM potently reduces inflammation in adipose tissue of *ob/ob* mice<sup>120</sup>.

## 7 The effect of inhibition of glyco(sphingo)lipid synthesis on insulin sensitivity

Drugs that inhibit sphingolipid formation at different steps in the synthetic pathway ameliorate insulin resistance in cultured cells and animals. Inhibition of *de novo* ceramide synthesis in cultured myotubes by myriocin, fumonisin B1 or L-cycloserine, blocks the inhibitory effect of saturated FFA on insulin signaling<sup>82</sup>. Inhibition of *de novo* glucosylceramide synthesis in cultured cells has generally been found to be beneficial. The relatively specific glucosyl-

ceramide synthase inhibitor AMP-DNM ameliorates insulin signaling in TNF $\alpha$ -treated 3T3-adipocytes<sup>94</sup>. AMP-DNM prevents TNF $\alpha$ -induced increases in GM3 and GM2 and simultaneously improves insulin receptor, IRS-1 and AKT phosphorylation<sup>94</sup>. Contradictory effects of d-PDMP, a rather non-specific inhibitor of glucosylceramide synthase, have been reported. Tagami *et al.* reported that d-PDMP reduced GM3 and enhanced insulin signaling in TNF $\alpha$ -treated 3T3-adipocytes<sup>104</sup>. In contrast, Chavez *et al.* using higher concentrations of d-PDMP, observed an inhibition of insulin signaling in the same cell model<sup>84</sup>. The conflicting outcomes of the two studies are most likely due to the poor specificity of d-PDMP. This compound inhibits glycosylation as well as transacylation of ceramide. At high concentration, the latter effect of d-PDMP may result in increased cellular ceramide that causes impaired insulin signaling<sup>121</sup>.

To study the role of sphingolipids in insulin resistance *in vivo*, Holland and coworkers studied the effect of myriocin on insulin signaling and glucose homeostasis in mice<sup>92</sup>. Insulin resistance was induced in mice by glucocorticoid or lard oil infusion. Myriocin treatment restored insulin-mediated PKB phosphorylation in liver and muscle, and improved hepatic and peripheral insulin sensitivity as measured by a hyperinsulinemic euglycemic clamp (figure 3). In ZDF rats, myriocin improved plasma glucose levels, reduced hyperinsulinemia and improved glucose tolerance and insulin sensitivity. In all models myriocin reduced liver ceramide concentrations<sup>92</sup>. Unfortunately, the effect of myriocin treatment on glycosphingolipids concentrations was not documented. It can therefore not be excluded that reductions in glycosphingolipids, resulting from a decrease in their precursor ceramide, contribute to the beneficial responses induced by myriocin.

Two unrelated inhibitors of glucosylceramide synthase, AMP-DNM and Genz-123346, have also been tested in animal models of insulin resistance and type II diabetes<sup>94,95</sup>. In *ob/ob* mice, treatment with AMP-DNM resulted in lower blood glucose levels, improved whole body glucose uptake and better suppression of endogenous glucose production by insulin as measured by a hyperinsulinemic euglycemic clamp. AMP-DNM also improved insulin-stimulated insulin receptor phosphorylation in the liver, and reduced hepatic fat accumulation (figure 3). In high fat diet-induced insulin-resistant mice and in ZDF rats, AMP-DNM improved fasting and non-fasting plasma glucose levels. Hyperinsulinemia was partially corrected and glucose tolerance was improved and glycated hemoglobin levels were reduced. In *ob/ob* mice and ZDF rats, AMP-DNM reduced hepatic and muscle glucosylceramide

content without changing ceramide concentrations. A reduction in gangliosides GM1, GM2 and GM3 in liver and muscle of ZDF rats was demonstrable, again without alterations in ceramide levels<sup>94</sup>. Inhibition of glycosphingolipid formation in ZDF rats and diet-induced insulin-resistant mice with Genz-123346 had comparable beneficial effects on glucose homeostasis: correction of hyperinsulinemia, reduction of non-fasted blood glucose levels, lowering of glycated hemoglobin, and improvement of glucose tolerance. Treatment with Genz-123346, like AMP-DNM, promoted insulin-stimulated phosphorylation of the insulin receptor in muscle<sup>95</sup> (figure 3).

## 8 Summary and future prospects

Evidence for a role for glycosphingolipids in insulin resistance is accumulating.

Firstly, a growing number of studies indicate that the insulin receptor is present in glycosphingolipid-rich caveolae/DRMs, and that this localization is essential for its function.

Excessive ganglioside content of caveolae/DRMs seems to hamper insulin signaling by displacing the insulin receptor from these domains<sup>79</sup>. Support for this model still largely comes from investigations using cultured cells and membrane fractionations. Translation of such data to the *in vivo* situation is problematic given the remaining uncertainties about the physiological relevance of isolated DRMs.

Secondly, several studies indicate that glycosphingolipids directly interfere with insulin signaling. In cultured adipocytes and myotubes, increased concentrations of ceramide and GM3 both impair insulin signaling at different levels of the signaling cascade. Ceramide has been shown to inhibit PKB, a key mediator in insulin signaling<sup>82</sup>. Glycosphingolipids, in particular the ganglioside GM3, act even further upstream, at the level of insulin receptor phosphorylation.

Thirdly, pharmacological reduction of glycosphingolipid synthesis is found to ameliorate insulin sensitivity in rodents. Inhibition of ceramide synthesis by myriocin and inhibition of glucosylceramide synthesis by two unrelated inhibitors, AMP-DNM or Genz-123346 all improve insulin signaling<sup>92,94,95</sup>. Reducing glycosphingolipid synthesis may also positively influence other consequences of obesity such as inflammation (see section 7) and atherosclerosis<sup>122-124</sup>.

Partial inhibition of glycosphingolipid synthesis is well tolerated by rodents and humans. Pharmacological reduction of glycosphingolipids is already applied clinically. Chronic administration of (3x100 mg/d) N-butyl-deoxynojirimycin (Miglustat, Zavesca), an inhibitor of glucosylceramide synthase, effectively reduces glucosylceramide storage in Gaucher disease patients, without major side effects<sup>125-128</sup>.

To establish whether pharmacological reduction of glycosphingolipid synthesis is a useful treatment for insulin resistance and type II diabetes, it seems crucial to demonstrate that elevated concentrations of glycosphingolipids indeed occur in relevant organs of insulin-resistant humans.

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