

Sphingolipids: agents provocateurs in the pathogenesis of insulin resistance

C. Lipina · H. S. Hundal

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Abstract Obesity is a major risk factor for a variety of chronic diseases, including diabetes mellitus, and comorbidities such as cardiovascular disorders. Despite recommended alterations in lifestyle, including physical activity and energy restriction, being the foundation of any anti-obesity therapy, this approach has so far proved to be of little success in tackling this major public health concern. Because of this, alternative means of tackling this problem are currently being investigated, including pharmacotherapeutic intervention. Consequently, much attention has been directed towards elucidating the molecular mechanisms underlying the development of insulin resistance. This review discusses some of these potential mechanisms, with particular focus on the involvement of the sphingolipid ceramide. Various factors associated with obesity, such as saturated fatty acids and inflammatory cytokines, promote the synthesis of ceramide and other intermediates. Furthermore, studies performed in cultured cells and in vivo associate these sphingolipids with impaired insulin action. In light of this, we provide an account of the research investigating how pharmacological inhibition or genetic manipulation of enzymes involved in regulating sphingolipid synthesis can attenuate the insulin-desensitising effects of these obesity-related factors. By doing so, we outline potential therapeutic targets that may prove useful in the treatment of metabolic disorders.

Keywords Adipose tissue · Caveolae · Ceramide · Gangliosides · GM3 · Skeletal muscle · Sphingomyelin · PKB/Akt

C. Lipina · H. S. Hundal (✉)
Division of Cell Signalling and Immunology, Sir James Black Centre,
College of Life Sciences, University of Dundee,
Dundee DD1 5EH, Scotland, UK
e-mail: h.s.hundal@dundee.ac.uk

Abbreviations

aPKC	Atypical protein kinase C
AMPK	AMP-activated protein kinase
CEM	Cholesterol-enriched (membrane) microdomain
DRM	Detergent-resistant (membrane) microdomain
GM3	Ganglioside monosialo 3
IMTG	Intramuscular triacylglycerol
LCFA	Long-chain saturated fatty acid
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
SPT	Serine palmitoyltransferase
TLR	Toll-like receptor

Introduction

The increasing occurrence of obesity and type 2 diabetes mellitus, attributable to a large extent to sedentary living and excess energy intake, has fuelled a rapidly expanding area of research examining the link between lipid metabolism and the pathogenesis of insulin resistance. Evidence for such a relationship has been acquired from numerous studies involving obese and diabetic humans as well as rodent models exhibiting metabolic dysfunction [1–4]. What has become clear from these investigations is that obesity is a major contributing factor towards the development of the metabolic syndrome and that impairment of insulin action is associated with excess lipid accumulation [3–7].

At the molecular level, a key mediator of insulin action is protein kinase B (PKB; also known as Akt), which acts to regulate a number of physiological processes, resulting in an overall hypoglycaemic response [8, 9]. Insulin promotes a reduction in circulating glucose, to a large extent by accelerating its uptake into peripheral tissues such as skeletal muscle and adipose tissue, as well as by stimulating its conversion into energy storage molecules such as glycogen. It is well documented that insulin-stimulated glucose transport into these tissues is significantly attenuated in obese individuals and people with type 2 diabetes [10, 11].

Mechanistically, activation of PKB in response to insulin involves its phosphorylation on two regulatory sites, namely Thr308 in the activation loop and Ser473 in the hydrophobic C-terminal regulatory domain (both sites corresponding to PKB α /Akt1). Phosphorylation of both residues is required for full activation [12]. This process relies upon the generation of the membrane phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) by the enzyme phosphoinositide 3-kinase, resulting in the pleckstrin homology domain-dependent translocation of PKB to the plasma membrane. The high-affinity binding of the N-terminal pleckstrin homology domain of PKB to PIP₃ facilitates a conformational change that allows subsequent phosphorylation at Thr308 by 3-phosphoinositide-dependent protein kinase 1 (PDK1) [13]. The mechanism mediating Ser473 phosphorylation remains controversial although several distinct kinases, including mammalian target of rapamycin complex 2 (mTORC2), have been proposed to be involved, depending on cell type and stimulus [14].

The activation of PKB in response to insulin can be markedly impaired by stimuli such as TNF α , glucocorticoids or prolonged exposure to long-chain saturated fatty acids (LCFAs; e.g. palmitate), all of which have been implicated in the development of insulin resistance [15, 16]. A common feature of these insulin-desensitising agents is their ability to promote the accumulation of sphingolipids such as ceramide [16–18]. Through the use of cell-permeant analogues of ceramide or inhibition of its intracellular synthesis, it is now widely acknowledged that this particular sphingolipid intermediate can regulate a diverse array of cellular processes, including cell survival and apoptosis, inflammatory responses, mitochondrial function and insulin sensitivity [19–21]. It is the last of these, that is, the ability of ceramide to modulate insulin-mediated responses and the potential mechanisms involved, that will be the focus of this review.

Intracellular ceramide synthesis

Ceramides belong to a group of lipid-derived molecules that consist of a sphingosine base linked to a fatty acid

moiety. Although previously thought of as purely structural elements of the membrane lipid bilayer, it is now clear that they also act as important signalling molecules and are implicated in a variety of cellular and physiological processes [22–24]. Ceramides can accumulate in cells via two main routes: the hydrolysis of the membrane phospholipid sphingomyelin, which is coordinated by the enzyme sphingomyelinase [22], or its de novo synthesis from LCFAs such as palmitate, which involves a multi-step biosynthetic pathway, as illustrated in Fig. 1 [22, 23]. A number of different factors, including TNF α , endotoxins and various stress stimuli, have been reported to activate sphingomyelinase leading to the generation of ceramide [22, 25].

The rate-limiting step of de novo synthesis of ceramide is the condensation of a fatty acyl-CoA, usually palmitoyl-CoA, with serine, which is catalysed by the enzyme serine palmitoyltransferase (SPT), to form 3-ketosphinganine (Fig. 1). The activity of SPT is stimulated in the presence of LCFAs but not unsaturated fatty acids [16, 26]. The final two steps of the pathway involve the generation of dihydroceramide from sphinganine by the enzyme dihydroceramide synthase and its subsequent conversion into ceramide by dihydroceramide desaturase (Fig. 1). Enhanced ceramide production arising from de novo synthesis has been reported in response to the stimulation of SPT and/or dihydroceramide synthase [27–29]. In addition, ceramide can be further modified into alternative forms, including glucosylceramide and ceramide 1-phosphate, or converted into other metabolites such as sphingosine 1-phosphate (Fig. 1), some of which can induce distinct or even opposite biological effects [22, 30, 31].

The role of ceramide in the pathogenesis of insulin resistance

So why is ceramide considered to be a contributing factor in the development of insulin resistance? The evidence underpinning this view originates from several important observations. First, sustained exposure of skeletal muscle to non-esterified fatty acids results in the accumulation of intramuscular triacylglycerol (IMTG) and fatty acid-derived molecules such as diacylglycerol and ceramide [3, 5, 19, 29, 32]. However, although the accumulation of IMTG is normally associated with a loss in skeletal muscle insulin sensitivity, the observation that muscle from endurance-trained athletes remains insulin sensitive despite elevated IMTG content (known as the athlete's paradox), initially suggested that increases in intramuscular lipid alone may not be sufficient to explain lipid-induced insulin resistance [7, 33]. Importantly, with respect to this finding, we and others have shown that

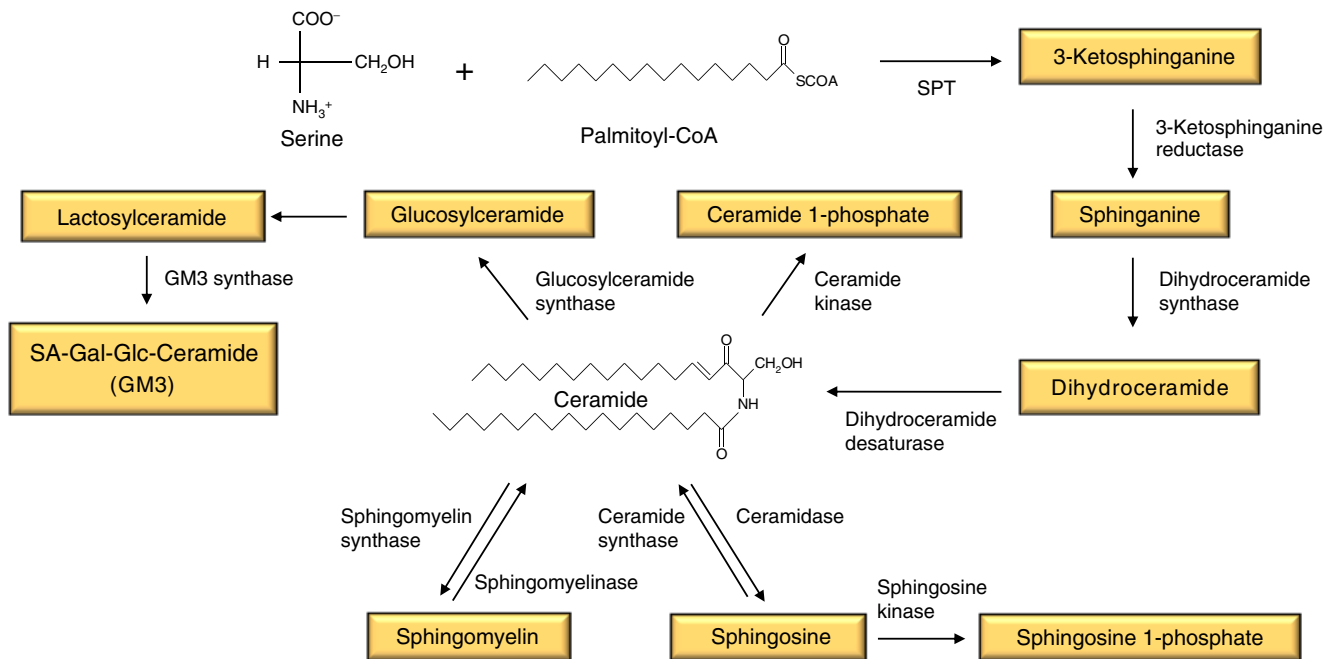


Fig. 1 An overview of the major pathways involved in the synthesis of ceramide and its derivatives. Ceramide can be generated in a number of different ways, including its de novo synthesis involving several key enzymes, such as serine palmitoyl transferase (SPT), or by the hydrolysis of sphingomyelin by the action of sphingomyelinases.

Both these processes, which take place at the plasma membrane, can be induced in response to various stimuli (as discussed in the text). The modification of ceramide (e.g. by its phosphorylation or glycosylation) produces molecular species with similar or distinct biological activities. Gal, galactose; Glc, glucose; SA, sialic acid

sustained exposure of skeletal muscle to palmitate, the predominant circulating saturated fatty acid, results in markedly impaired insulin-stimulated PKB activation concomitant with inhibition of associated processes such as glucose uptake and glycogen synthesis [8, 34, 35].

Second, given the fact that the de novo synthesis of ceramide can be driven solely by the availability of LCFAs, it has now been demonstrated in a variety of cell lines and in isolated human myoblasts that the insulin-desensitising effects of palmitate can also be mimicked by the acute application of ceramide analogues [15, 17–19, 36, 37]. In addition, under those circumstances whereby elevating levels of circulating lipids in rodents either by lipid infusion or high-fat feeding causes insulin resistance, a number of studies have now demonstrated an associated increase in ceramide content within peripheral tissues such as skeletal muscle and adipose tissue [20, 23, 38, 39]. Consistent with this, prolonged incubation of cultured skeletal muscle C2C12 and L6 myotubes or human myoblasts with palmitate also leads to an increase in ceramide content in accordance with impaired insulin-stimulated PKB activity [17, 19, 29, 37, 40]. With regards to human physiology, elevations in skeletal muscle ceramide and/or plasma ceramide levels have been reported in obese insulin-resistant and diabetic individuals, as well as in response to lipid infusion [20, 40, 41]. Furthermore, intramuscular ceramide accumulation is also known to

occur in certain animal models of insulin resistance, including Zucker (*fa/fa*) rats [42, 43].

Third, in line with being a measure of obesity prevention, the participation of insulin-resistant rodents and humans in prolonged exercise regimens has been shown to markedly improve insulin sensitivity in association with reduced intramuscular ceramide [7, 20, 44]. Finally, and perhaps most importantly, the inhibition of ceramide synthesis can counteract the antagonistic effects of saturated fatty acids on insulin-regulated PKB signalling (discussed below) [15, 17, 20]. Collectively, these observations strongly suggest that ceramide plays a key role in the development of insulin resistance, primarily through its ability to inhibit PKB [17, 19, 34].

Since initial work demonstrated that ceramide does not directly interact with PKB or inhibit upstream signalling events such as the activation of phosphoinositide 3-kinase by hormonal stimuli [19, 20, 45], researchers undertook alternative approaches in an attempt to explain its suppressive effect. Studies performed over the past decade have identified two distinct mechanisms that may be involved. The evidential basis of these proposed mechanisms, as well as the potential involvement of ceramide-rich membrane lipid rafts known as caveolae, will be discussed in the following sections. It must be noted, however, that although the mechanisms are treated separately (as depicted in Fig. 2b), their occurrence may not necessarily be mutually exclusive.

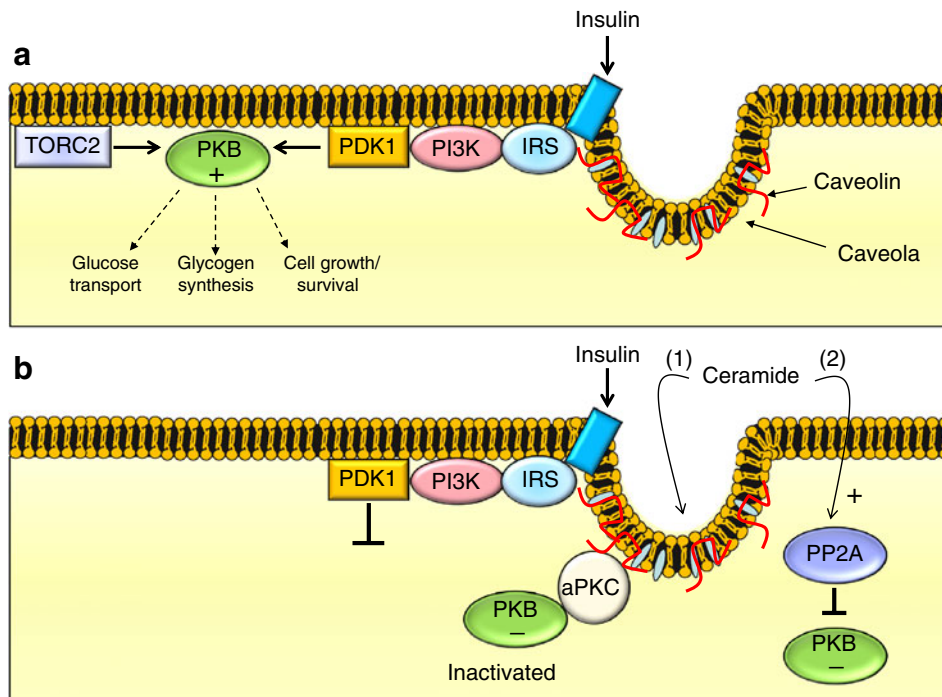


Fig. 2 Proposed mechanisms by which ceramide inhibits PKB. Normally, insulin binds to its receptor at the plasma membrane promoting the activation of PKB through the tyrosine phosphorylation of IRS proteins and the subsequent activation of phosphoinositide 3-kinase (PI3K). The associated increase in PIP₃ levels leads to the recruitment of PKB to the plasma membrane, where it is activated by the upstream kinases 3-phosphoinositide-dependent protein kinase 1 (PDK1) and TORC2. **a** The activation of PKB results in the stimulation of insulin-regulated processes such as glucose transport and glycogen synthesis. **b** In the presence of increased intracellular levels of ceramide, the ability of insulin to stimulate PKB is impaired

Involvement of phosphatase activity in ceramide-mediated inhibition of PKB

Initial evidence implicating a role for protein phosphatases in mediating the inhibitory effects of ceramide on insulin-stimulated PKB activity was obtained from studies performed using C2C12 myotubes and brown adipocytes treated with the protein phosphatase inhibitor okadaic acid [18, 46]. Although okadaic acid does not selectively target one particular protein phosphatase isoform (i.e. inhibits protein phosphatases 1 [PP1] and 2A [PP2A]), it has subsequently been demonstrated, through use of small t antigen (which displaces regulatory subunits of PP2A), that this inhibitor can prevent ceramide-mediated inhibition of PKB by specifically inhibiting PP2A, a serine/threonine phosphatase implicated in the dephosphorylation of PKB [20]. To support this, ceramide has been shown to act as a positive allosteric activator of PP2A [47, 48]. Therefore, one way that ceramide can act to inhibit PKB activity is by promoting its dephosphorylation at Thr308 and Ser473 through activation of PP2A (see Fig. 2b). However, in certain cell types, such as L6 myotubes and white adipose,

by one of two established mechanisms. In the first scenario (1), the increase in caveolar ceramide content results in the activation of aPKC λ/ζ and promotes the association of aPKC λ/ζ and PKB within these membrane microdomains. As a result of this interaction, PKB remains in a repressed state and is unable to transduce signalling to downstream components. Alternatively (2), the intracellular ceramide generated leads to the direct activation of PP2A, causing the dephosphorylation and inactivation of PKB. In both cases (1 and 2), the inhibition of PKB is associated with the impairment of key insulin-regulated responses

the ceramide-mediated inhibition of PKB cannot be antagonised by co-treatment with okadaic acid alone, thereby suggesting the involvement of an alternative mechanism(s) [19, 34].

The role of atypical protein kinase C isoforms in ceramide-induced insulin resistance

The second way that ceramide is known to promote insulin resistance involves the disruption in membrane translocation and reduced phosphorylation of PKB by a process dependent on the activation of atypical protein kinase C (aPKC) λ/ζ isoforms. A reciprocal relationship between PKC activity and insulin action was initially established in human muscle strips that were incubated in the presence of pharmacological PKC activators or inhibitors [49]. From this study and others, it has emerged that whilst activation of aPKC λ/ζ may be involved in suppressing insulin-stimulated PKB activity and associated processes, inhibition of aPKCs is able to promote an insulin-sensitising effect [49–52]. In addition, pharmacological inhibition of PKC

activity has also been shown to prevent the ceramide-induced loss of insulin-stimulated PKB activation in both isolated human adipocytes and L6 myotubes [51, 53].

To investigate how ceramide blocks the translocation of PKB to the plasma membrane, initial work by Summers' group demonstrated that whilst ceramide analogues prevent the membrane localisation of the pleckstrin homology domain of PKB, they do not alter the accumulation of 3'-phosphoinositides [45]. This therefore suggested that the inhibitory effect of ceramide occurs independently of 3'-phosphoinositide production.

Subsequent studies demonstrated that ceramide can directly activate aPKC λ/ζ isoforms *in vitro* by binding to the putative ceramide-binding region of the protein, also known as the cysteine-rich domain [50]. Importantly, aPKC λ/ζ also interacts with and suppresses the activity of PKB [50, 51, 54]. Follow-up work from our group established that the interaction between PKC λ/ζ and PKB requires the PKB pleckstrin homology domain, and that ceramide-activated PKC λ/ζ phosphorylates a threonine or serine residue at site 34 (dependent on PKB isoform) within this region [51]. As a result, reduced affinity of the pleckstrin homology domain towards PIP₃ prevents the recruitment of PKB to the plasma membrane and its subsequent activation. Based on these observations it has been proposed that increases in intracellular ceramide leading to the activation of aPKC λ/ζ promotes the stabilisation of the aPKC λ/ζ -PKB complex and attenuates the recruitment of PKB to the plasma membrane as a result of disrupted PIP₃ binding (Fig. 2b) [51, 53].

Influence of caveolae in mediating the effects of ceramide

It is now widely acknowledged that the plasma membrane is not a homogenous structure but instead consists of microdomains (or rafts), each with their own characteristic lipid composition [55]. Indeed, lipid-derived molecules such as cholesterol or sphingolipids can segregate into such microdomains, which are more ordered and less fluid in nature compared with the rest of the plasma membrane [56]. Detergent treatment of cell membranes allows the isolation of detergent-resistant microdomains (DRMs) that are enriched in sphingolipids, including ceramide [57]. This particular subset of lipid rafts, termed cholesterol-enriched membrane microdomains (CEMs), are characterised by the presence of integral membrane proteins termed caveolins (namely, caveolin-1, -2, -3 and -4) [57].

The caveolins themselves are cholesterol-binding proteins that associate to form the flask-like invaginations that characterise caveolae and, importantly, are capable of acting as scaffolding proteins onto which signalling complexes

assemble. For this reason, CEMs play an important role in numerous signalling pathways, including insulin-mediated responses [57, 58]. Our previous work has demonstrated that acute ceramide treatment (or prolonged incubation with palmitate) promotes the recruitment of both aPKC λ/ζ and PKB to these CEMs [53]. By doing so, aPKC λ/ζ would not only be exposed to a ceramide-enriched environment, rendering it active, but would simultaneously be co-localised with PKB, thereby suppressing its activity.

Intriguingly, disrupting these microdomains using a cholesterol-depleting agent prevents the recruitment of these kinases to DRMs and ameliorates ceramide-mediated loss in PKB activity [53]. Although a direct physical association between aPKC λ/ζ and PKB has yet to be established within CEMs, ceramide has been shown to promote both aPKC λ/ζ -PKB and caveolin-1-aPKC λ/ζ interactions in 3T3-L1 adipocytes, suggesting that caveolins may act to bridge the association between aPKC λ/ζ and PKB [53]. This may be at least partly due to regions within caveolin proteins, known as caveolin scaffolding domains, that allow them to interact directly with aPKC λ/ζ [59].

In addition to promoting the recruitment of PKB and aPKC λ/ζ , ceramide exposure can also significantly elevate levels of phosphatase and tensin homologue deleted on chromosome ten (PTEN) in caveolin-enriched DRM fractions [53, 60]. The tumour suppressor PTEN acts as a phosphoinositide phosphatase and its increased abundance in CEMs would likely result in reduced localised levels of 3'-phosphoinositides. Together with the presence of activated aPKC λ/ζ , the CEMs create a highly repressive environment for PKB under such circumstances. Furthermore, the fact that ceramide does not suppress total insulin-stimulated production of 3'-phosphoinositides at the plasma membrane further supports the idea that this repressive environment is unlikely to be extended to the bulk planar membrane and is rather more focused in nature [51].

Intriguingly, a recent study by Blouin et al. demonstrated that the mechanism by which ceramide inhibits PKB may itself depend on cellular CEM abundance [48]. In cells that are abundant in caveolae, such as differentiated 3T3-L1 adipocytes, ceramide predominantly acts to inhibit PKB by recruiting aPKC λ/ζ and PKB to these membrane domains. In contrast, in CEM-deficient 3T3-L1 preadipocytes, a mechanism dependent on PP2A activity occurs, and this can be prevented by cotreatment with okadaic acid [48]. Furthermore, it has been shown that ceramide-activated PP2A is able to dephosphorylate a cytosolic insulin-stimulated PKB construct lacking its pleckstrin homology domain, thereby excluding a role for the membrane translocation of PKB in this process [20]. To complement these findings, the study by Blouin et al. also demonstrated that the ceramide-activated PP2A pathway is found to dominate in fibroblasts with a low

CEM abundance but switches to a $\text{aPKC}\lambda/\zeta$ -dependent pathway following overproduction of caveolin-1 [48]. Although the mechanism responsible for promoting this switch is not fully understood, work performed in prostate cancer cells determined that caveolin-1 can interact with and suppress PP2A [61]. This observation suggests that disruption of caveolae or suppressed caveolin abundance may cause the ‘release’ of PP2A into an unbound state in the cytosol where it would be more susceptible to activation by ceramide.

Despite the implication of caveolins and caveolae in the development of insulin resistance, the reader must also bear in mind the important role that these membrane proteins play in maintaining proper signal transduction. For example, because caveolins act as scaffolding proteins involved in organising the assembly of signalling complexes, it comes as no surprise that caveolin deficiency causes impaired insulin responses as well as a wide range of phenotypes and disorders [62]. In addition, a recent study has shown that caveolins may also confer a protective effect against elevated fatty acid levels [63]. Consequently, the role of caveolins and caveolae in insulin signalling can be viewed in two different ways—either as an aid to preserving insulin-regulated processes or as membrane microstructures, which, under certain circumstances, may contribute to the development of impaired insulin action.

The potential of modulating sphingolipid/ceramide-associated signalling as a therapeutic intervention

(1) Inhibition of SPT activity Work investigating the mechanisms involved in ceramide-induced insulin resistance has led to a number of potential therapeutic targets being proposed to tackle obesity-related diseases. However, it would be reasonable to speculate that preventing ceramide accumulation in response to elevated levels of circulating lipids or other stimuli that promote insulin resistance may be beneficial in ameliorating any insulin-desensitising effects. With this in mind, the most commonly studied molecular target involved in suppressing ceramide production is the enzyme SPT which, as mentioned above, catalyses the initial rate-limiting step in de novo ceramide synthesis (Fig. 1) [64]. Several potent inhibitors of SPT have been documented, although the most widely used is myriocin, a naturally occurring fungal metabolite isolated from *Myriococcum albomyces* [64]. Studies performed by our group and others have demonstrated that acute inhibition of SPT using myriocin ameliorates the loss in insulin-stimulated PKB activation in cultured L6 or C2C12 myotubes caused by palmitate-driven ceramide synthesis [15, 29, 35, 37]. With regards to equivalent studies carried out in vivo, administration of myriocin is also found to

attenuate PKB inhibition in response to lipid infusion or high-fat feeding, as well as improving glucose tolerance and peripheral insulin sensitivity in obese *ob/ob* mice and Zucker Diabetic Fatty rats [16, 23, 65]. As expected, these beneficial effects of myriocin are associated with reduced levels of circulating ceramide and are reproduced when alternative inhibitors of de novo ceramide synthesis such as L-cycloserine (which also inhibits SPT) and fumonisins B1 (dihydroceramide synthase inhibitor) are used [15, 16, 35].

Surprisingly, however, a recent study demonstrated that, in contrast to acute inhibition of SPT, its chronic suppression in L6 myotubes by pharmacological means or short-hairpin RNA-mediated silencing, fails to prevent the insulin-desensitising effects of palmitate [29]. Although initially unexpected, a possible explanation for this apparent lack of effect may involve the channelling of palmitate into other lipid intermediates that can contribute towards insulin resistance. Consistent with this idea, the same study was able to show a comparable significant increase in diacylglycerol content, which was coupled with a loss in insulin sensitivity, in palmitate-treated L6 cells containing a stable loss of SPT expression and in L6 cells treated over a longer period (7 days) with myriocin. Intracellular diacylglycerol accumulation itself has been linked to the activation of novel and conventional PKCs (such as $\text{PKC}\theta$) that are implicated in lipid-induced insulin resistance [66]. Although the full spectrum of serine sites that may be targeted by different PKC isoforms on IRS proteins remains poorly defined, $\text{PKC}\theta$ has been suggested to directly promote phosphorylation of Ser1101 of IRS-1, resulting in reduced insulin sensitivity [67]. Therefore, in this case a PKC-mediated increase in IRS-1 or insulin receptor serine phosphorylation may underlie palmitate-induced insulin resistance under conditions where SPT expression/activity becomes chronically suppressed [29].

Importantly, based on the observations made in this particular study, although inhibiting SPT with pharmacological inhibitors can improve insulin sensitivity, at least in the short term, any apparent long-term benefits would need to be carefully assessed in light of the possible accumulation of other fatty acid-derived metabolites with the potential to induce insulin resistance. The fact that these observations regarding the sustained inhibition of SPT activity are not fully consistent with studies performed in vivo, the possibility of greater efficacy of SPT inhibitors in tissues other than skeletal muscle cannot be discounted and requires further investigation. In addition, there is the possibility that myriocin treatment may act to simultaneously reduce levels of other sphingolipids derived from ceramide (e.g. glycosphingolipids), thereby contributing to its beneficial effects. However, such consequential responses of myriocin administration have not yet been reported. Furthermore, although such studies commonly

support a role for SPT in modulating insulin sensitivity, its effect on the regulation of body weight and energy expenditure still remain unclear based on discrepancies presented in literature [16, 23, 65].

As well as targeting SPT directly, there is also evidence to suggest that manipulating the activity of molecular targets or pathways that do not directly participate in the de novo synthesis of ceramide, may also result in the modulation of SPT activity and/or ceramide production. For example, overproduction of stearoyl-CoA desaturase, an enzyme involved in the conversion of saturated fatty acids into monounsaturated fatty acids in L6 myotubes leads to both improved insulin sensitivity and the attenuation of ceramide accumulation in response to palmitate exposure [68]. Furthermore, it has recently been demonstrated that adiponectin, an insulin-sensitising adipocyte-derived secretory factor, can act through its receptors (AdipoR1 and AdipoR2) to lower ceramide levels by stimulating the activity of ceramidase, an enzyme that catalyses the degradation of ceramide [69].

It is widely acknowledged that certain exercise regimens can alleviate diet-induced obesity and insulin resistance. Indeed, a number of independent studies have demonstrated that exercise training is associated with suppression of circulating and/or tissue ceramide content [33, 44, 70]. For example, in a study by Bruce et al., obese humans who participated in 8 weeks of exercise training showed a decrease of approximately 40% in skeletal muscle ceramide content and an associated improvement in insulin sensitivity [44]. One potential contributing factor to exercise-induced suppression of ceramide generation may involve the activation of AMP-activated protein kinase (AMPK) [71]. AMPK activation by pharmacological means has been shown to prevent palmitate-induced SPT activity [72], thereby suggesting the possibility of a similar response to exercise-stimulated AMPK in vivo [73]. Furthermore, the activation of AMPK, which has a stimulatory effect on mitochondrial β -oxidation of fatty acids, acts to shunt palmitate away from ceramide synthesis towards oxidation [44, 74, 75]. Indeed, accelerating the rate of fatty acid import into and its subsequent oxidation in mitochondria can alleviate high-fat diet-induced insulin resistance [76]. Consistent with this idea, a recent study showed that preincubating C2C12 myotubes with the monounsaturated fatty acid oleate or introducing a mutant active form of carnitine palmitoyltransferase 1 (a key positive regulator of LCFA β -oxidation), can prevent palmitate-induced ceramide accumulation and insulin resistance by increased channelling of the saturated fatty acid towards triacylglycerol and/or through its enhanced oxidation [75]. This therefore raises the possibility that glucose-lowering pharmacological activators of AMPK (or exercise mimetics) and/or consumption of diets with a higher proportion of

unsaturated fatty acids, may act to prevent diet-induced obesity and impaired metabolic function, at least in part by restricting ceramide generation.

(2) *Inhibition of sphingomyelinase* As well as through de novo synthesis, ceramide can also be produced by the action of sphingomyelinase enzymes that are activated in response to stimuli such as TNF α [77]. Sphingomyelinase generates ceramide through the hydrolysis of sphingomyelin (Fig. 1). Interestingly, the abundance and/or activity of either neutral or acid sphingomyelinases has been reported to be elevated in the adipose tissue of *ob/ob* and high-fat diet-induced obese mice as well as in liver in response to high-fat feeding [38, 78, 79]. Furthermore, a recent study demonstrated that exposure of C2C12 myotubes to exogenous sphingomyelinase can elevate intracellular ceramide levels; however, effects on insulin sensitivity were not determined in this particular study [80].

It is therefore plausible that increased levels and/or activity of sphingomyelinase-stimulating stimuli or secreted serum sphingomyelinase may also contribute to ceramide accumulation in peripheral tissues [80, 81]. Indeed, it has been shown that genetic loss of acid sphingomyelinase can prevent diet-induced obesity, hyperglycaemia and insulin resistance in mice lacking the LDL receptor (*Ldlr*^{-/-}), which are prone to metabolic disease when placed on high-fat diets [82]. Furthermore, a recent study by Bioni et al. demonstrated that treatment of mice with the acidic sphingomyelinase inhibitor amitriptyline attenuates high-fat diet-induced elevations in plasma ceramide and improves insulin sensitivity [78].

An alternative mechanism by which saturated fatty acids may lead to the activation of sphingomyelinase could involve Toll-like receptor (TLR) signalling. Toll-like receptors are a family of proteins that play an important role in the innate immune system [83]. Although normally activated in response to pathogens and cell stress, it is now apparent that saturated fatty acids may themselves act as ligands for two TLR isoforms in particular, namely TLR2 and TLR4, both of which have been linked to the development of obesity and insulin resistance [84, 85]. Intriguingly, activation of TLR2/4-mediated signalling by lipopolysaccharide, an endotoxin isolated from Gram-negative bacteria, has been shown to promote the accumulation of ceramide [86]. Therefore, together with its involvement in the production of cytokines such as TNF α and IL-6, regulation of TLR2/4-mediated signalling may play an additional role in modulating ceramide generation.

To summarise, although studies determining the effects of sphingomyelinase activity on insulin sensitivity and metabolic efficiency are still comparatively few in number, the findings from them so far hold promise that sphingomyelinase enzymes may play a significant role in the

pathogenesis of type 2 diabetes and are therefore a potential therapeutic target.

(3) The ganglioside GM3 as a potential mediator of insulin resistance Another class of ceramide-derived sphingolipids that have been implicated as modulators of transmembrane signalling are the gangliosides. Gangliosides are complex sialic acid-containing glycosphingolipids consisting of a ceramide moiety linked to an oligosaccharide chain [87]. One of the gangliosides in particular, GM3 (ganglioside monosialo 3), has been shown to modulate insulin-mediated signalling. The synthesis of GM3 involves the glucosylation of ceramide (see Fig. 1), and important enzymes in this process include glucosylceramide synthase, which catalyses the initial glycosylation step, and GM3 synthase, an intracellular sialyltransferase that promotes the conversion of lactosylceramide to GM3 (Fig. 1).

Data from several studies now suggest that GM3 may be involved in mediating insulin-desensitising effects and, in particular, that of pro-inflammatory cytokines such as TNF α [26, 88, 89]. First, insulin resistance induced by TNF α in 3T3-L1 adipocytes has been associated with elevated GM3 levels caused by increased GM3 synthase abundance and activity [88]. Second, pharmacological inhibitors of glucosylceramide synthase, which deplete cellular GM3, can prevent the inhibitory effects of TNF α on insulin signalling in cultured 3T3-L1 adipocytes [88, 90]. Furthermore, GM3 itself can mimic the effects of TNF α by inhibiting tyrosine phosphorylation of the insulin receptor and IRS-1, as well as suppressing insulin-stimulated glucose uptake [89, 91]. Concomitantly, improved tyrosine phosphorylation of the insulin receptor has

been reported in response to pharmacological inhibition of glucosylceramide synthase and in GM3 synthase-deficient mice [90, 92]. Furthermore, studies by Kabayama et al. demonstrated that GM3 can directly interact with the insulin receptor and by doing so, causes its displacement from caveolae leading to attenuated insulin signalling [93]. This observation implies that GM3 may regulate insulin signalling via its effects on insulin receptor membrane localisation and activity [89, 93].

Further supporting a role for GM3 in the development of insulin resistance in vivo, administration of glucosylceramide synthase inhibitors [*N*-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) and Genz-123346] has been shown to improve both glucose tolerance and insulin sensitivity in skeletal muscle and liver of *ob/ob* mice and Zucker diabetic rats, as well as in diet-induced obese mice, without any significant alteration in food intake or loss of body weight [90, 92]. Similar effects are observed in mice lacking GM3 (due to GM3 synthase deficiency), which display protection against high-fat diet-induced insulin resistance [91]. Interestingly, insulin sensitivity is found to be improved in mice with elevated production of the GM3 degrading sialidase Neu3 in liver [94]. However, to our knowledge there are no data showing whether changes in Neu3 abundance and/or activity is regulated by obesity.

To date, one pilot study has reported increased circulating levels of GM3 in obese type 2 diabetic individuals as well as in individuals displaying hyperglycaemia and/or hyperlipidaemia [95]. This is in agreement with the elevated GM3 levels present in skeletal muscle from certain obese insulin resistant murine models, although as yet there

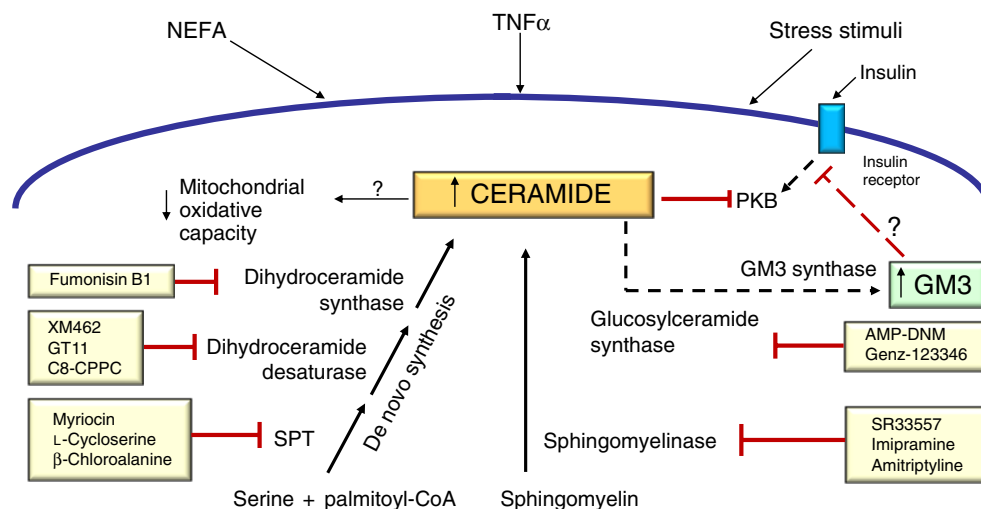


Fig. 3 Schematic representation of potential targets for glucose-lowering therapies involved in sphingolipid metabolism. Intracellular ceramide can accumulate in response to various cellular stimuli via two main routes: de novo synthesis or hydrolysis of sphingomyelin. Potential therapeutic targets for the treatment and/or prevention of

obesity-related disorders that are involved in the synthesis of ceramide and the ganglioside GM3 are presented, including the identities of some known pharmacological inhibitors used to regulate the different components. C8-CPPC, C8 cyclopropenylceramide

is no supporting evidence in tissues obtained from obese or diabetic humans [88]. Intriguingly, individuals with type I Gaucher's disease, a lysosomal storage disorder caused by an impairment in glucosylceramide breakdown leading to elevated levels of GM3, are also found to exhibit insulin resistance [87]. Collectively, these observations open up the possibility that GM3 may serve as a diagnostic marker for metabolic-related disorders, and that therapeutic interventions aimed at reducing GM3 levels in vivo may prove to be a useful strategy for combating insulin resistance.

Summary and concluding remarks

In summary, a number of studies have demonstrated an inverse relationship between ceramide content and insulin sensitivity. However, as work in our lab and others has demonstrated, ceramide should be viewed as one of multiple factors involved in impaired insulin action. The reader must also be aware that, although the pharmacological and genetic evidence presented in this review strongly implicates ceramide or its derivatives in the development of insulin resistance, some counter-evidence exists to suggest that it may not be the key or sole intermediate involved. For example, certain studies have reported no significant differences in human muscle ceramide content in individuals with varying insulin sensitivity or adiposity [3, 96, 97]. Also, lipid infusion in rodents (depending on lipid composition) can promote insulin resistance without a significant change in tissue ceramide content [96, 98]. In addition, some animal models displaying marked elevations in hepatic ceramide content do not show a corresponding impairment of insulin-mediated signalling in the liver [6, 99].

However, despite the lack of dramatic changes in total ceramide levels, one cannot rule out the possibility that alterations in the type of ceramide species present may also be an important factor in determining insulin sensitivity. Crucially, it must be noted that the methods used to determine ceramide content in these studies do not discriminate between ceramide and biologically inactive dihydroceramide. Therefore, significant differences in the levels of the inactive analogue may have a considerable effect on any results obtained. Another major issue is the relatively small sample sizes employed in the studies performed to date.

It is also possible that ceramide may affect whole body metabolism in other ways. For example, elevated levels of ceramide, which have been shown to promote mitochondrial dysfunction, could if sustained over a period of time, lead to reduced oxidative capacity in metabolically active tissues such as skeletal muscle [21]. In either case, if ceramide does play a crucial role in the

pathogenesis of the metabolic syndrome, the fact that ceramide (and its derivatives) can be generated in a number of different ways suggests that, as outlined in Fig. 3, targeting multiple pathways may be the most effective strategy for the treatment of metabolic disorders and associated complications [78, 80, 100].

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