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Defect of insulin signal in peripheral tissues: Important role of ceramide

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Abstract

In healthy people, balance between glucose production and its utilization is precisely controlled. When circulating glucose reaches a critical threshold level, pancreatic β cells secrete insulin that has two major actions: to lower circulating glucose levels by facilitating its uptake mainly into skeletal muscle while inhibiting its production by the liver. Interestingly, dietary triglycerides are the main source of fatty acids to fulfill energy needs of oxidative tissues. Normally, the unconsumed fraction of excess of fatty acids is stored in lipid droplets that are localized in adipocytes to provide energy during fasting periods. Thus, adipose tissue acts as a trap for fatty acid excess liberated from plasma triglycerides. When the buffering action of adipose tissue to store fatty acids is impaired, fatty acids that build up in other

tissues are metabolized as sphingolipid derivatives such as ceramides. Several studies suggest that ceramides are among the most active lipid second messengers to inhibit the insulin signaling pathway and this review describes the major role played by ceramide accumulation in the development of insulin resistance of peripherals tissues through the targeting of specific proteins of the insulin signaling pathway.

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Key words: Diabetes; Insulin resistance; Lipids; Insulin signaling; Triglycerides; Palmitate; Sphingolipid; Akt; Ceramide synthase; Protein phosphatase 2A; Protein kinase C ζ/λ

Core tip: Muscle and liver represent major sites for insulin-mediated glucose metabolism. The ability of insulin to promote its peripheral action is reduced significantly by excess of saturated fat that stimulates intracellular production of second-messenger lipids such as ceramide. Studies suggest that ceramide could be important contributors to lipotoxicity, as the inhibition of early steps its biosynthesis pathway has large beneficial effects in rodent models of obesity and diabetes. In this review, we describe mechanisms by which ceramide acts on insulin-sensitive tissues and we propose that targeting this lipid family could be an interesting approach to fight diabetes.

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DIABETES EPIDEMIC

Diabetes has become a serious public health problem in

both developed and developing countries. Indeed, there is a dramatic increasing incidence of diabetes in most of these countries. In 2005, 217 million people worldwide had diabetes, and the World Health Organisation predicts that it will increase to 366 million in 2030^[1]. In 2050, 33% of the population of the United States will suffer from diabetes^[2]. One consequence is that over the years, diabetes has become life-threatening, with increased risk of cardiovascular diseases, retinopathy, kidney failure, and nerve and artery damages^[3]. Diabetes is one of the first causes of haemodialysis, of blindness and of non-traumatic amputation of the legs. Another consequence is the increasing of health spending due to diabetes. For example, in the United States, diabetes costing is actually evaluated to more than \$174 billion per year and it's expected to increase in subsequent years^[2].

PATHOPHYSIOLOGY OF TYPE 2 DIABETES

There are different types of diabetes: (1) type 1 diabetes or maturity onset diabetes of the young associated to impairment of insulin production; and (2) type 2 diabetes, corresponding to 85%-90% of all diabetes, with both insulin secretion defects and peripheral insulin resistance. Type 2 diabetes is associated with obesity and although genetic factors play a role in the pathophysiology of this disease, other environmental factors such as diet and physical activity both play large roles. Several mechanisms have been proposed to explain both insulin resistance and insulin secretion defects observed in type 2 diabetes. Lipotoxicity, glucotoxicity, low grade systemic inflammation, oxidative stress and endoplasmic reticulum stress^[4-6] correspond to different mechanisms that converge on a common pathway to induce insulin resistance. In this review we will focus on cellular lipid toxicity, *i.e.*, lipotoxicity.

LIPOTOXICITY

Systemic lipid imbalances are common in metabolic syndrome, in pre-diabetes and in type 2 diabetes and it is now clear that lipotoxicity can induce glucose dysregulation and participate to the pathophysiology of type 2 diabetes^[7-9]. For example, prospective epidemiological studies performed in population with low or high risk to develop type 2 diabetes have shown that high free fatty acid (FFA) concentrations in plasma are associated with the risk of incident type 2 diabetes^[10-12].

A major characteristic of type 2 diabetes is the loss of the ability of pancreatic β cells to increase insulin secretion to maintain normoglycemia in the face of insulin resistance^[13]. Because of genetic predisposition, β cells could be unable to compensate the insulin resistance induced by FFA, but chronic exposition of β cells to high levels of FFA could equally explain defects in β cell function and decreased mass observed in type 2 diabetes. Indeed, *in vitro* studies have shown that FFA are associated with a decrease of insulin expression, synthesis and pro-

cessing^[14-16]. Another mechanism that can explain insulin secretion dysfunction in type 2 diabetes is that high FFA levels in islets induce β cell death^[17]. In this review, we will not deal with this topic but we will rather focus our message on lipid-induced peripheral insulin resistance. To more information on lipotoxicity in pancreatic beta cells, confer to the excellent review of Boslem *et al*^[18].

Since skeletal muscle constitutes 40% of human body mass and is quantitatively the most important tissue in regard to insulin-stimulated glucose disposal, it is considered the main cellular target in the development of insulin resistance. Thus, most of the studies investigating mechanisms of lipotoxicity induced insulin resistance were mostly performed in muscle tissue.

In 1963, Randle *et al*^[19] have postulated that a competition between glucose and fatty acids for their oxidation and uptake is responsible for the onset of insulin resistance in muscle and adipose tissue. *In vivo* studies performed in both rodents and humans confirmed such insulin resistance obtained after lipid infusion but they also demonstrated that, in opposite to Randle's hypothesis, insulin resistance induced by lipids was not secondary to decreased glycolysis^[20]. Indeed, lipids act directly on insulin signaling, resulting in an inhibition of the translocation of the insulin sensitive glucose transporter GLUT4 to the plasma membrane in response to the hormone, with subsequent reduced glucose uptake^[21-25]. In human, data clearly show a strong correlation between lipid intramuscular content and insulin resistance^[26-28] and a cross-sectional analysis performed in young, normal weight and non-diabetic adults reveals that a better correlation exists between muscle insulin sensitivity, assessed by the hyperinsulinaemic-euglycaemic clamp technique, and intramyocellular lipid content rather than with circulating lipid levels, body mass index, fasting blood glucose and age^[29].

Liver is another important organ implicated in insulin resistance and, like in muscle indirect data also suggest an inverse relationship between lipid liver content and insulin sensibility. Indeed, ectopic lipid accumulation in the liver, termed nonalcoholic fatty liver disease (NAFLD), is associated with insulin resistance. Interestingly, in an animal model of lipodystrophy with steatosis, but without increased visceral fat, lipid liver content is associated with insulin resistance. Insulin resistance is reversed after reduction of steatosis with liver transplantation or recombinant leptin treatment^[30]. Such association between steatosis and insulin resistance has also been observed in patients with severe lipodystrophy with equally a good response to recombinant leptin therapy^[31]. Similarly, hepatic specific overexpression of lipoprotein lipase leads specifically to hepatic steatosis and hepatic insulin resistance^[32,33]. During type 2 diabetes, reduction of steatosis by caloric restriction, or gastric bypass, is associated with increased insulin sensibility independently of visceral fat mass reduction^[34,35].

Strong evidence exists between ectopic lipid accumulation and insulin resistance. However, in some cases, like in the "athlete's paradox", there is a lack of correlation

between ectopic lipid accumulation and peripheral insulin resistance. Indeed, athletes display high insulin sensitivity but also present increased levels of intramuscular fatty acids^[36]. Thus, it seems that ectopic accumulation of fatty acids in non-adipose tissues can only be used as markers for the onset of insulin resistance but cannot be considered as a direct cause. Even if they do not seem to be directly involved, fatty acids contribute to insulin resistance as they lead to the synthesis of many lipid derivative intermediates such as diacylglycerol (DAG) and ceramide.

Over the years, studies have provided conclusive proof that ceramide plays a key role in the progression of insulin resistance in insulin sensitive tissues, targeting and inhibiting specific actors of the insulin signaling pathway.

INSULIN SIGNALING PATHWAY AND METABOLIC FUNCTIONS

Insulin is a polypeptide hormone whose major physiological role is to control glucose homeostasis by stimulating glucose uptake into insulin sensitive tissues (skeletal muscle and adipose tissue) and by inhibiting glucose output from the liver^[37]. Insulin consists of two polypeptide chains, a α chain of 21 amino acid residues linked by two disulfide bonds to a β chain of 30 amino acid residues. Insulin is produced in the β cells of the Islets of Langerhans found in the pancreas. It is initially synthesized as an immature single polypeptide chain of 110 amino acids called pre-proinsulin. Pre-proinsulin contains an N-terminal domain of 24 amino acids that acts to direct the polypeptide to the endoplasmic reticulum during translation. This domain is later cleaved to yield proinsulin. Proinsulin is transported to the secretory vesicles of the pancreatic β cells, where a proteolytic enzyme removes the central 35 residues of the peptide (termed the C-peptide) that connect α and β chains to produce insulin. Insulin is then released into the blood stream by exocytosis. Secretion of the hormone is regulated by the glucose abundance in the plasma.

In skeletal muscle, insulin promotes the uptake of glucose and its conversion into glycogen. This tissue is an important target of the hormone, representing the major site of glucose disposal *in vivo*^[37] and is reported to mediate 70%-80% of whole body insulin-stimulated glucose transport^[38]. In the liver, insulin stimulates the synthesis of glycogen while inhibiting gluconeogenesis and glycogenolysis, halting hepatic glucose output. In adipocytes, insulin promotes the uptake of glucose and its conversion into a glycerophosphate of which can be esterified by 3 fatty acids, allowing to form triglycerides for long term storage, whereas simultaneously inhibiting the lipolytic pathway^[39]. In addition to glucose metabolism, insulin also regulates many other cellular processes including amino acid transport, lipogenesis, protein synthesis and mitogenesis.

The first step in the activation of the insulin signaling pathway is the binding of insulin with its membrane receptor, the insulin receptor (IR). IR is a heterotetrameric

complex of two subunits: α -subunit, and β -subunit that possess a transmembrane domain and an intracellular part. Binding of insulin to α subunits of IR induces a rapid conformational change in the receptor. This in turn stimulates the intrinsic tyrosine kinase activity of the β subunit resulting in trans-autophosphorylation of tyrosine residues in the intracellular region of the β subunits^[40]. As a result of this autophosphorylation, the IR becomes catalytically active and promotes the tyrosine phosphorylation of a number of cellular proteins including the IR Substrate (IRS) proteins.

IRS proteins are major physiological targets of the activated insulin receptor kinase. Six different IRS isoforms have been identified so far^[41]. In skeletal muscle and adipose tissue, IRS1 is the isoform that mediate insulin signaling. In the liver, however, IRS2 is the one that drives insulin metabolic functions. In the pancreas, IRS2 is an important regulator of cell growth and regeneration^[41]. Studies have also shown that both IRS3 and IRS4 can be activated in response to insulin and insulin-like growth factor 1 (IGF1)^[42] and that IRS3 can mediate insulin signaling in adipocytes^[42]. Mice lacking either IRS3 or IRS4, however, display no major phenotype, suggesting that neither isoform plays a direct role in controlling glucose metabolism^[43,44] but may rather act as negative regulators of the IGF1 signaling pathway by suppressing the function of other IRS isoforms^[45].

One key molecule that is activated by the IRSs in response to insulin is phosphoinositide-3-kinase (PI3K). PI3K is a lipid kinase, which phosphorylates the D3 position of the inositol ring within inositol lipids resulting in the generation of 3-phosphoinositides (*e.g.*, PI-3P, PI-3,4P₂, and PI-3,4,5P₃). Eight mammalian isoforms of PI3K exist and they are grouped into three classes on the basis of their substrate specificity and structure: class I, class II, and class III. Only class I can phosphorylate phosphatidylinositol, 4, 5-bisphosphate (PIP₂)^[46]. Following PI3K activation, PIP₃ is generated from the substrate PIP₂. PIP₃ binds a protein displaying a PH domain and called the 3-phosphoinositide-dependent protein Kinase 1 (PDK1). Activated-PDK1 triggers downstream targets such as protein kinase B (PKB/Akt)^[47].

PKB/Akt also called Akt is the third central node activated by insulin. It plays a crucial role in mediating signaling effects on metabolism, cell growth and cell cycle^[48,49]. PKB/Akt has three isoforms: PKB α /Akt1, ubiquitously expressed, PKB β /Akt2 mostly present in insulin responsive tissues (liver, adipose tissue and muscle), and PKB γ /Akt3 predominant in the brain. PKB β /Akt2 is the isoform implicated in the regulation of glucose metabolism since neither PKB α /Akt1 nor PKB γ /Akt3 ablation affects glucose metabolism^[50].

PKB/Akt is activated through PI3K-produced PIP₃ which binds its PH domain. Then, PKB/Akt is recruited to the plasma membrane where it is activated by phosphorylation on two critical sites: threonine 308 (T308) in the activation loop and serine 473 (S473) in the hydrophobic motif^[51]. PDK1 phosphorylates PKB/Akt on T308.

The kinase that phosphorylates the S473 site is the complex mammalian target of rapamycin complex 2, a regulator of cell growth and proliferation^[52].

PKB/Akt is highly activated within minutes following cell exposure to insulin to mediate the metabolic effects of the hormone^[49,53].

Indeed, principle roles of PKB/Akt in insulin sensitive tissues are to: (1) Stimulate glucose uptake in muscle and adipose tissue; (2) Trigger glucose storage as glycogen in muscle and in the liver; (3) Stimulate the conversion of glucose excess into lipids in the liver; (4) Induce protein synthesis in muscle; (5) Inhibit glycogen breakdown in both muscle and liver; (6) Suppress liberation of free fatty acids from adipose tissue; (7) Inhibit *de novo* production of glucose in the liver; and (8) Impede protein breakdown in muscle (Figure 1).

Considering the crucial role PKB/Akt plays in mediating insulin metabolic actions in cells, impairing PKB/Akt activity represents the best way to compromise the whole system.

LIPID SECOND MESSENGER AND LOSS OF INSULIN SENSITIVITY

In pathological situations such as obesity and type 2 diabetes that are characterized by insulin resistance, ectopic fatty acid accumulation is increased due to reduced mitochondrial fatty acid oxidation and to enhanced fatty acid uptake^[54-57]. This increased fat content inversely correlates with insulin sensitivity in skeletal muscle, liver and adipocytes^[58-61].

Interestingly and depending on the degree of saturation, free fatty acid may exert different effects on insulin signaling. Studies have demonstrated that saturated fatty acids such as palmitate (16:0) and stearate (18:0) impair insulin sensitivity in muscle^[62,63], whereas mono-unsaturated fatty acids or poly-unsaturated fatty acids have no effect or even enhance insulin action^[64-66]. Although the exact reasons behind these differences are unclear, studies have suggested that unsaturated fatty acids may be preferentially targeted for triglyceride synthesis and storage, whilst saturated fatty acids may be used for synthesis of critical lipid intermediates such as DAG and ceramide. These two lipid second messengers have been demonstrated to mediate deleterious actions of saturated fatty acids on insulin signaling.

DAG AND INSULIN RESISTANCE

DAG is a glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule. DAG, intermediate of both triglyceride and phospholipid metabolism, is an important second messenger involved in intracellular signaling^[67].

DAG has been shown to accumulate in insulin resistant liver^[68,69] and studies have shown that intra-hepatic DAG is an important mediator of hepatic insulin resistance in obese people with nonalcoholic fatty liver

disease^[70,71]. Elevated DAG content and activation of protein kinase C (PKC) ϵ has been associated with hepatic insulin resistance and the involvement of this “lipid-activated pathway” has been validated through the use of antisense oligonucleotide against PKC ϵ in rats. Knocking down PKC ϵ expression in liver protected rats from lipid-induced hepatic insulin resistance, despite increase in hepatic lipid content^[72].

Several studies have decrypted the mechanism by which DAG-activated PKCs inhibit insulin signaling in liver. They show that IRS proteins are likely to be PKC's preferential targets. DAG-activated PKCs inhibit IRSs activity through their phosphorylation on several serine residues, preventing consequently insulin activation of IRSs through their phosphorylation on tyrosine residues^[73-75].

In muscle, however, data are contradictory. Itani *et al.*^[76] were the first to point out the positive association between DAG content and muscle insulin resistance by comparing a group of subject receiving a lipid infusion to a control group. Lipid infusion resulted in a 3-fold increase in total DAG content in muscle, and reduced insulin sensitivity. Strackowski *et al.*^[77] observed that total muscle DAG concentrations were higher in obese compared to lean controls and lean offspring type 2 diabetics, and this increased DAG content was inversely related to insulin sensitivity. Other studies have also confirmed this correlation^[78,79].

However, the association between DAG and muscle insulin resistance is still questioned. Indeed, Vistisen *et al.*^[80] performed muscle biopsies during glucose clamps and they observed a reduction in insulin sensitivity after lipid infusion, without any changes in muscle DAG content. These results were confirmed by Anastasiou *et al.*^[81] that compared obese type 2 diabetic patients to non-diabetics subjects and found no difference in muscle DAG content between the groups. Similarly, Perreault *et al.*^[82] compared insulin resistant obese patients to glucose tolerant obese patients and again found no difference in DAG content between the groups. Even more intriguing, Amati *et al.*^[83] observed a two-fold increase in DAG content in insulin sensitive human muscle biopsies compared to insulin resistant human muscle biopsies. More recently, the same group showed no difference in muscle DAG content between lean subjects compared to obese insulin resistance patients^[84].

Altogether, and in opposite to liver, it seems that DAG does not appear to be a crucial player in the onset of insulin resistance in muscle, and maybe more investigations are needed to really be able to conclude.

CERAMIDE AND INSULIN RESISTANCE

Ceramide biosynthesis

One of the main sphingolipid that has been demonstrated to play a crucial role in insulin resistance is ceramide. During obesity, ceramide is mainly generated from long chain fatty acyl-CoAs^[85,86], and has been shown to be toxic lipid when it accumulates in tissues during obesity^[87-89].

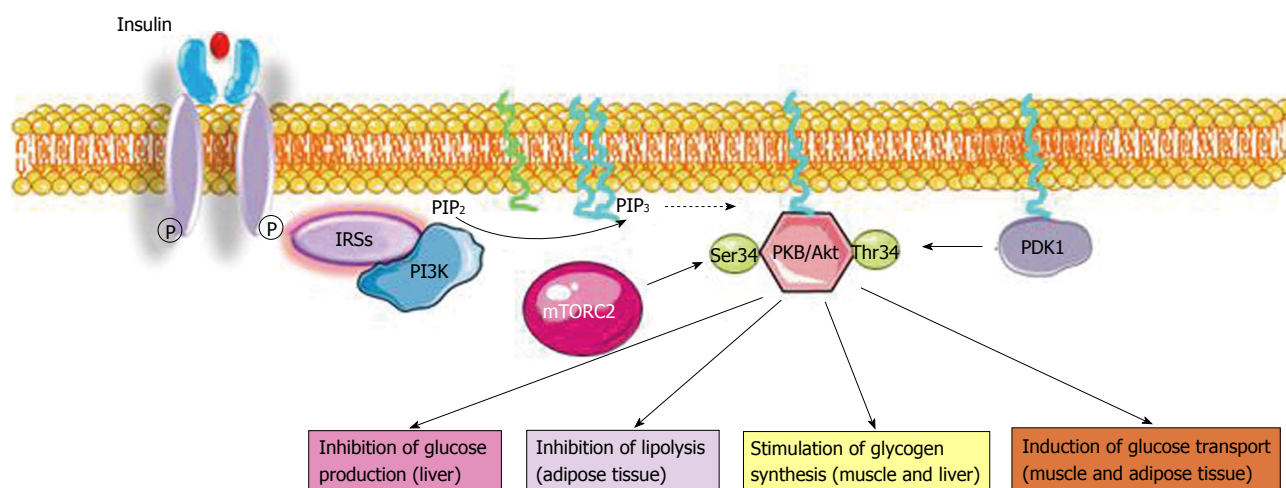


Figure 1 Insulin signaling pathway. Insulin binds with insulin receptor (IR) that activates the IR substrates (IRSs), the phosphoinositide-3-kinase (PI3K), and protein kinase B/Akt (PKB/Akt). Activated PKB/Akt mediates insulin metabolic effects and regulates nutrients homeostasis. PIP2: Phosphorylate phosphatidylinositol, 4, 5-bisphosphate; PDK1: 3-phosphoinositide-dependent protein kinase 1; mTORC2: Mammalian target of rapamycin complex 2.

Ceramide is a bioactive sphingolipid that has been implicated in mediating or regulating many cellular processes, including cell cycle arrest, proliferation, apoptosis, senescence, and stress responses. Ceramide plays also an important role in cell membrane structure^[90].

Formation of ceramide can be induced by different stimuli such as tumor necrosis factor- α , heat stress, oxidative stress, ionizing radiation, and chemotherapeutics^[91].

Multiple metabolic pathways converge to ceramide (Figure 2): (1) The *de novo* synthesis pathway from saturated fatty acids that takes place in the endoplasmic reticulum; (2) The sphingomyelinase pathway that uses sphingomyelinase to break down sphingomyelin in the cell membrane to release ceramide; and (3) The salvage pathway in lysosomes that occurs through breakdown of complex sphingolipids to give sphingosine, which is then rescued by reacylation to form ceramide.

In time of fatty acid plethora, the *de novo* ceramide biosynthesis pathway is the pathway that is likely to be most harnessed to synthesize ceramide. It occurs in the leaflet membrane of the endoplasmic reticulum where ceramide is synthesized through a series of reactions^[92,93]. *De novo* synthesis of ceramide begins with the condensation of palmitate and serine to form 3-keto-dihydrosphingosine (Figure 2). This reaction is catalyzed by serine palmitoyl transferase (SPT) and is the rate-limiting step of the pathway. In turn, 3-keto-dihydrosphingosine is reduced to dihydrosphingosine, which is then followed by acylation by ceramide synthases (CerS) to produce dihydroceramide. In mammals, six CerS isoforms are expressed and are called CerS 1 to 6. They carry out the same chemical reaction, but display distinct specificities for the acyl-CoA chain length they use for N-acylation^[94]. Thus, CerS isoforms are responsible for the fatty acid composition of ceramide. Interestingly, several studies have shown distinct cellular functions for ceramides with different N-acyl chain length^[95,96]. The final reaction to produce ceramide is catalyzed by dihydroceramide desaturase.

Inverse relationship between ceramide content and insulin sensitivity

Studies in animal and models: One of the early studies that analyzed ceramide content in obese Zucker fa/fa rats (rats homozygous for truncated, non-functional leptin receptor) was Turinsky *et al.*^[97] in 1990. The authors found that these rats present an increase in ceramide content in both muscle and liver. Increased ceramide content was also detected in insulin resistant models of rodents, as in ob/ob mice, mice fed on high fat diet, and in intra-lipid infused mice^[85,98,99]. Altogether these reports illustrate the inverse relationship between ceramide and insulin sensitivity in rodent muscle. This association was also confirmed *in vitro* in cultured C2C12 and L6 myotubes, as well as in adipocytes^[99-101]. Exposing cultured muscle cells to saturated fatty acids (like palmitate) attenuates insulin activation of glycogen synthesis and glucose transport concomitantly with increasing intracellular ceramide amounts^[63,99]. Additionally, incubation of muscle cells and adipocytes with analogues of ceramide mimics the inhibitory effects of FFAs on insulin signaling and suppresses insulin-stimulated glycogen synthesis and glucose transport^[100,101].

Studies in human subjects: In accordance with data obtained in rodents, studies in human subjects also support the inverse relationship between ceramide accumulation and insulin sensitivity. It has been shown that under basal conditions, total amount of ceramide in skeletal muscle is increased in obese subjects compared to lean ones^[83,84,87]. Another study performed in human skeletal muscle of lean normoglycemic subjects revealed again an inverse relationship between muscle ceramide accumulation and insulin sensitivity^[102]. The same authors show in another study a ceramide accumulation in muscle of type 2 diabetic patient offsprings compared to muscle of control subjects^[77]. Furthermore, the group of Goodpaster demonstrated that physical exercise reduces ceramide

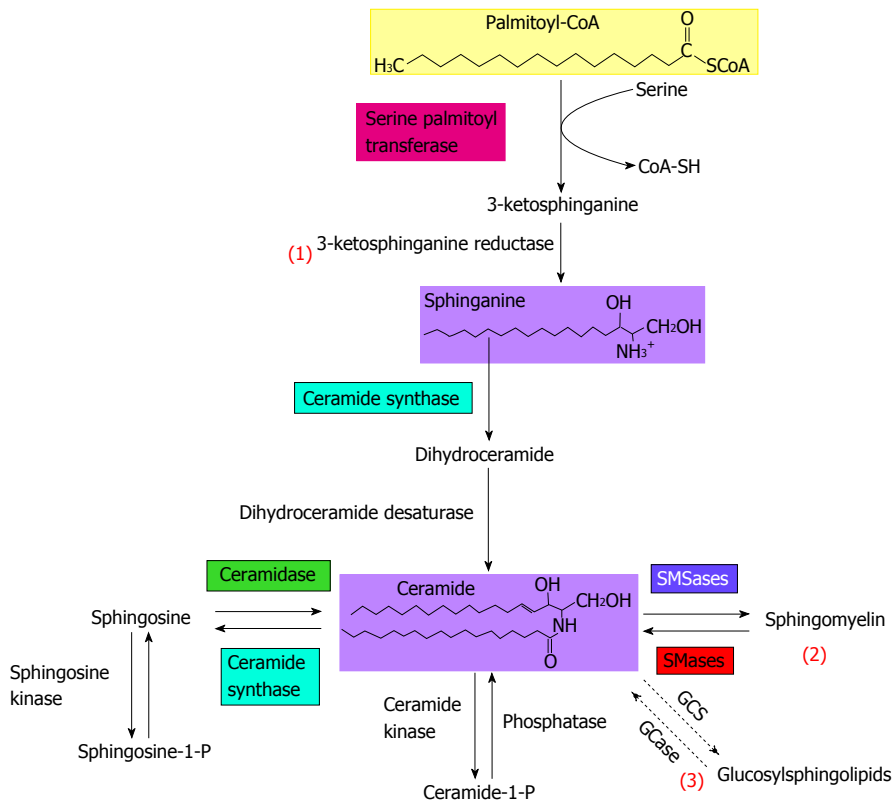


Figure 2 Sphingolipid metabolism. Ceramide can either be newly synthesized in *de novo* ceramide synthesis pathway (1), or it can be the product of complex sphingolipids degradation, including sphingomyelin hydrolysis (2). The degradation of glycosylsphingolipids constitutes the salvage pathway (3). GCase: Glucosyl ceramidase; GCS: Glucosylceramide synthase; SMases: Sphingomyelinases; SMSases: Sphingomyelin synthases.

content in obese and insulin resistant subjects, and this was correlated with improved insulin sensitivity^[83,103]. Like in muscle, accumulation of ceramide content in human adipocytes has also been demonstrated to be related to insulin resistance^[104,105].

Altogether, these studies prove a solid association between insulin resistance and an increase in ceramide content in both muscle and adipocytes.

Unlike in muscle and adipose cells, a role of ceramide in the onset of hepatic insulin resistance is more debated. Indeed, some studies see no ceramide accumulation in fatty liver^[68,70,71], making improbable these lipids as mediators hepatic insulin resistance. This is in contradiction with another study showing increases in hamster hepatic ceramide levels in response to lipopolysaccharide administration^[106]. In addition, Longato *et al.*^[107] saw a dys-regulated ceramide metabolism in high fat diet-induced hepatic steatosis.

Interestingly, and in opposite to muscle and adipose tissue, ceramide cannot accumulate in the liver. Indeed, very recently, Watt *et al.*^[108] have shown that lipid infusion in healthy subjects resulted in a rapid hepatic secretion of ceramide in the circulation, primarily within very low-density lipoprotein^[109,110], thereby protecting the liver from the deleterious effects of their intracellular accumulation. It would be interesting, however, to assess whether lipid-induced ceramide secretion is affected in fatty liver (steatosis).

Altogether, if ceramide does not seem to accumulate

in liver during lipotoxic conditions, its secretion into the circulation could be deleterious for other peripheral tissues such as pancreatic β cells and muscle cells.

Implication of ceramide in the progression of insulin resistance

Two methods were used to validate the implication of ceramide in impaired insulin sensibility: the first one was to inhibit ceramide production, and the second was to enhance ceramide metabolism towards less harmful sphingolipid species.

Inhibition of ceramide production improves insulin sensitivity

One method used to demonstrate the role of ceramide in the onset of insulin resistance was to inhibit ceramide biosynthesis. The most commonly studied molecular target involved in suppressing ceramide production is the enzyme SPT, enzyme that catalyzes the initial rate-limiting step in *de novo* ceramide synthesis (Figure 3)^[90]. Several potent inhibitors of SPT have been documented, although the most widely used is myriocin, a naturally occurring fungal metabolite isolated from *Myriococcum albomyces*^[111]. In studies carried out *in vivo*, administration of myriocin was found to attenuate PKB/Akt 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^[112-114]. As expected, these beneficial effects of myriocin were associated with reduced

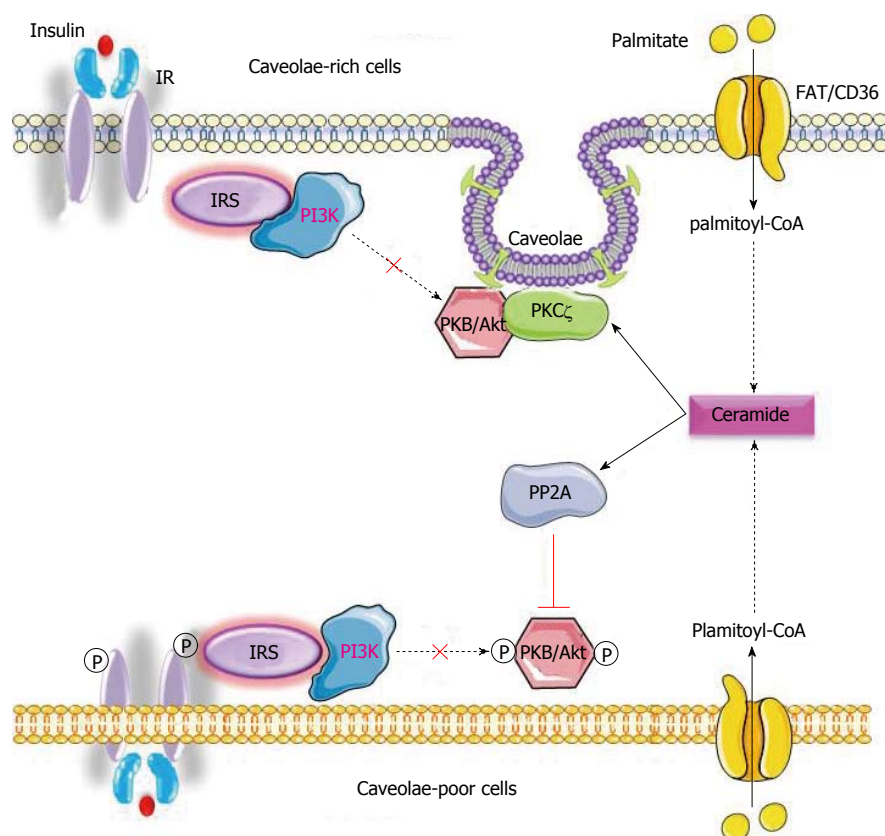


Figure 3 Ceramide inhibits insulin-induced activation of protein kinase B via two distinct mechanisms. Ceramide inhibits insulin-activation of protein kinase B (PKB/Akt) either by activating atypical PKC (PKC ζ) or by stimulating the phosphatase PP2A. Activation of either mechanism depends on plasma membrane enrichment with caveolae: (1) Ceramide-activated PKC ζ phosphorylates the PH domain of PKB/Akt on Thr/Ser34, changing the recognition site of PKB/Akt, and disabling its activation by PIP3; (2) PP2A dephosphorylates PKB/Akt, inhibiting its kinase activity. IR: Insulin receptor; IRS: IR substrates; PI3K: Phosphoinositide-3-kinase; PP2A: Protein phosphatase 2A.

levels of ceramide and were reproduced when alternative inhibitors of *de novo* ceramide synthesis such as L-cycloserine (which also inhibits SPT) and Fenretinide (dihydroceramide synthase inhibitor) were used^[63,115].

Studies performed *in vitro* in myotubes confirmed what was observed *in vivo*. They demonstrated that acute inhibition of SPT using myriocin ameliorates the loss in insulin-stimulated PKB/Akt activation in cultured L6 or C2C12 myotubes caused by palmitate-driven ceramide synthesis^[62,63].

Interestingly, a very recent study shows that inhibition of the *de novo* synthesis of ceramide using myriocin reduces hepatic lipid accumulation in liver of rats with NAFLD^[116]. This inhibition of ceramide biosynthesis is accompanied with decreased in both DAG and triglyceride contents, resulting in amelioration of hepatic insulin resistance and improvement of glucose homeostasis^[116].

Stimulation of ceramide conversion into less harmful sphingolipids improves insulin sensibility: The degradation of ceramide is initiated by the action of ceramidase that produces sphingosine, which is then phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase^[117]. S1P is the final metabolic product of sphingolipid degradation and can function as an

intracellular second messenger or in an autocrine and/or paracrine manner to activate and signal through S1P receptors^[118]. Interestingly, S1P itself opposes the effects of ceramide on intracellular signaling. S1P has been shown to ameliorate insulin-stimulated glucose uptake, possibly through the activation of PKB/Akt^[118-121]. Therefore, studies have aimed at finding ways to enhance ceramide metabolism into S1P in muscle in order to restore their insulin sensitivity. Bruce *et al.*^[122] used transgenic mice overexpressing sphingosine kinase. They show that high fat fed transgenic mice display improved insulin sensitivity compared to control mice. In addition, they used a drug called FTY720 which inhibits ceramide synthase activity and decrease ceramide accumulation in skeletal muscle^[123]. As expected, they saw an improvement of insulin sensitivity. FTY720 prevented muscle ceramide accumulation in high fat fed mice and subsequently improved glucose homeostasis^[124]. Other studies show that overexpression of ceramidase (converting ceramide to sphingosine) protects from lipid-induced muscle insulin resistance in C2C12 myotubes^[125].

Altogether, these results demonstrate that preventing the aberrant accumulation of ceramide by promoting its metabolism into sphingosine and sphingosine-derivatives might restore normal insulin sensitivity and glucose me-

tabolism in models of insulin resistance.

Ceramide inhibitory effect on the insulin signaling pathway

Several studies have reported that ceramide may attenuate insulin-stimulated glucose transport and glycogen synthesis by antagonizing early events in insulin signaling such as activation of IRS-1^[126] and possibly PI3K^[127]. However, these results are controversial, as several groups reported no defects in the activation of these molecules upon challenging cells with ceramide^[100,101]. In contrast, a number of groups suggested that PKB/Akt is the target of ceramide, and that inhibition of this kinase may account for reduced glucose transport and apoptosis observed in ceramide treated cells^[99-101,128]. Consistent with this, defects in PKB/Akt activation have been noted in a variety of ceramide-treated cell types, including 3T3-L1 adipocytes^[101], foetal brown adipocytes^[129], L6 rat and C2C12 mouse skeletal muscle^[99,100], A75R5 smooth muscle cells^[130], and MCF7 breast cancer cells^[131].

Furthermore, the inhibition of PKB/Akt by ceramide is not limited to experiments using exogenously supplied lipids. The hormonal activation of PKB/Akt is also blunted in muscle cells treated with free fatty acids in a manner which is dependent on the intracellular conversion of palmitate to ceramide^[62,63,99]. Taken together these results suggest that ability of ceramide to impair PKB/Akt activity may be an important determinant of insulin sensitivity.

A key issue is the mechanism by which ceramide inhibits PKB/Akt activity. Depending on the cell enrichment in caveolin-enriched domain^[132], ceramide inhibits the insulin-stimulated PKB/Akt either through the protein phosphatase 2A (PP2A), or *via* the atypical PKC (aPKC) pathway (Figure 3).

PP2A depended inhibition of insulin-induced activation of PKB/Akt: PP2A is a cytoplasmic serine/threonine phosphatase ubiquitously expressed that plays an important role in the regulation of diverse cellular processes, including metabolic enzymes, hormone receptors, kinase cascades, and cell growth^[133]. It has been shown that insulin inhibits PP2A in physiologic conditions^[134]. In contrast, several groups demonstrated that ceramide activates PP2A to promote the de-phosphorylation of PKB/Akt^[62,135,136]. Two different inhibitors of PP2A activity, okadaic acid or SV40 small T antigen that binds with PP2A^[137] were used to demonstrate the role of ceramide-induced PP2A inactivation of PKB/Akt. The presence of either inhibitor in cells treated with palmitate or short chain ceramide analogue (C2-ceramide), alleviated inhibition on PKB/Akt and re-established a normal, insulin signaling^[62,128]. Therefore, one way for ceramide to inhibit PKB/Akt activity is by promoting its dephosphorylation at Thr308 and Ser473 through activation of PP2A.

Atypical PKCs another ceramide-stimulated protein altering PKB/Akt activation: The second mechanism

of inactivation of PKB/Akt by ceramide requires the activation of aPKCs (PKC ζ/λ). There is mounting evidence in the literature suggesting that aPKC may regulate PKB/Akt signaling and that the relationship between the two kinases may be subject to modulation by ceramide. It is 20 years since investigators first demonstrated that PKC ζ/λ could associate with PKB/Akt in COS-7 fibroblasts^[138]. It has also been demonstrated that PKC ζ interacts directly with PKB/Akt in other cells types such as Chinese hamster ovary cells and COS-1 cells^[139], as well as the BT-549 human breast cancer cell line^[140].

In pathological conditions, ceramide-activated aPKCs impair insulin signaling. aPKCs phosphorylate PKB/Akt on its Thr34/Ser34 residue (Thr34 in PKB α and PKB β , Ser34 in PKB γ), thus preventing PIP₃ to bind the kinase on its PH domain, and to translocate to the plasma membrane and its subsequent activation in response to insulin^[132,141,142]. Based on these observations, it was proposed that an increase in intracellular ceramide leading to the activation of aPKCs promotes the stabilization of the aPKC-PKB/Akt complex and attenuates the recruitment of PKB/Akt to the plasma membrane as a result of disrupted PIP₃ binding (Figure 3).

CERAMIDE, A THERAPEUTIC TARGET?

Mechanisms by which saturated fatty acids act on insulin signaling are now getting clearer. They involve several lipid and protein intermediates that play an essential role to mediate the deleterious effects of accumulated saturated lipids in insulin sensitive tissues. Thus, two main options exist to counteract the action of these fatty acids on insulin signaling: (1) acting on ceramide downstream signaling targets (aPKCs or PP2A); or (2) modulating directly ceramide content^[143]. Considering the large involvement of both aPKCs and PP2A in numerous paths^[144,145], it would be more logical to try to directly inhibit the accumulation of ceramides in tissues. Several problems would arise with a complete inhibition of ceramide biosynthesis since these bioactive sphingolipids are in the center of sphingolipid metabolism. Indeed, ceramide signaling has been directly or indirectly involved in the diverse functions such as regulation of cell growth, differentiation, senescence, necrosis, proliferation, and apoptosis^[90]. Therefore, inhibiting completely ceramide biosynthesis would be likely to be very harmful to the cells. Targeting specific ceramides species would be more appropriate since it has been shown that specific ceramide species could be associated with different functions, depending upon the cell type^[94].

Concretely, it will be important to determine which ceramide species accumulate under lipotoxic conditions and then to evaluate whether these identified ceramide species enhance or reduce the deleterious effects of lipotoxicity in insulin sensitive tissues.

Interestingly, data existing already suggest that ceramide with distinct acyl chain-length are associated with different cell dysfunction in lipotoxic conditions. The

enzyme responsible of generating different ceramide acyl chain-length is the CerS. Six mammalian CerS have been described, with each utilizing fatty acyl CoAs of relatively defined chain lengths for ceramide synthesis^[94]. In pancreatic β -cells, C18:0, C22:0 and C24:1 ceramides induce apoptosis, and inhibition of the CerS (CerS4) responsible for their synthesis blocks this phenomenon^[146]. In the liver, CerS1 and CerS6, producing mainly C16:0 and C18:0 ceramides are associated with insulin resistance^[147], whereas C22:0 and C24:0 ceramides produced through CerS2 are rather protective^[148].

In muscle cells, however, no definitive and conclusive investigation has been carried out to date. The expression of C16:0, C18:0 and C24:0 ceramide species are increased in myotubes of type 2 diabetic patients compared to lean donors^[149]. However, one recent paper shows that over-expression of each CerS isoform in L6 muscle cells does not point out any ceramide species in the generation of insulin resistance^[150]. Since the implication of ceramide in the onset of insulin resistance in muscle has been convincingly demonstrated both *in vivo* and *in vitro* (see previous chapters), more investigations are needed before to make any conclusion in this tissue.

In summary, deciphering the mechanisms by which ceramides act negatively on insulin signaling has already been a step forward. However, the identification of the putative ceramide species that mediates lipotoxicity in cells or pushing ceramides to be converted into less toxic lipids remains the priority in order to find a way to counteract ceramide negative actions.

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