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SHORT COMMUNICATION

Ceramide accumulation in L6 skeletal muscle cells due to increased activity of ceramide synthase isoforms has opposing effects on insulin action to those caused by palmitate treatment

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Abstract
Aims/hypothesis An accumulation of ceramides has been implicated in the generation of insulin resistance in skeletal muscle upon an oversupply of fatty acid. Different ceramide species are generated through the actions of ceramide synthases (CerSs), which incorporate specific acyl side chains. We tested whether particular CerS isoforms promoted insulin resistance through the generation of more inhibitory ceramide species, thus representing potential targets for intervention.
Methods CerS isoforms CerS1, CerS2, CerS4, CerS5 and CerS6 were overexpressed in L6 myotubes using adenovirus, and cells were treated with palmitate and stimulated with insulin. Alternatively, CerS isoforms were knocked down using siRNAs. Sphingolipids were examined by mass spectrometry and tracer incorporation. Phosphorylation of IRS1 and Akt was measured by immunoblotting, while glucose disposal was assessed by measuring GLUT4 translocation and the incorporation of [^{14}C]glucose into glycogen.
Results Palmitate treatment increased the levels of several ceramides but reduced the levels of sphingomyelins, while insulin had no effect. The fatty acid also inhibited insulin-stimulated Akt phosphorylation and glycogen synthesis. Overexpression of CerS isoforms increased specific ceramides. Unexpectedly, the overexpression of CerS1 and CerS6 promoted insulin action, while no isoform had inhibitory effects. CerS6 knockdown had effects reciprocal to those of CerS6 overexpression.
Conclusions/interpretation Palmitate may increase intracellular ceramide levels through sphingomyelin hydrolysis as well as de novo synthesis, but no particular species were implicated in the generation of insulin resistance. The modulation of ceramides through an alteration of CerS expression does not affect the action of insulin in the same way as ceramide generation by palmitate treatment. Conversely, certain isoforms promote insulin action, indicating the importance of ceramides in cell function.

Keywords Akt · Ceramide · Fatty acid · Glycogen synthesis · Insulin resistance · IRS1 · L6 muscle cell · Palmitate · Sphingomyelin

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Abbreviations

CerS	Ceramide synthase
ER	Endoplasmic reticulum

FA Fatty acid
LacZ β-Galactosidase
siRNA Small interfering RNA

Introduction

Insulin resistance in skeletal muscle has a major impact on whole-body glucose homeostasis and is strongly linked to an oversupply of lipid. One of the mechanisms by which lipids induce insulin resistance involves the accumulation of intracellular ceramide, which in turn interferes with insulin signal transduction and glucose disposal [1]. In addition to the sphingoid backbone derived from palmitate during de novo synthesis, ceramide molecules comprise acyl side chains of varying length, which are incorporated through the action of ceramide synthase (CerS). Six mammalian CerS isoforms have been described with differing fatty acid (FA) substrate preferences [2]; these also participate in the remodelling of ceramide species by the salvage pathway [2].

With the more recent ability to measure the levels of different ceramide species by mass spectrometry, it has been suggested that some ceramide species are more strongly associated with the inhibition of glucose disposal than others, in which case certain CerS isoforms may make a greater contribution to the generation of lipid-induced insulin resistance [2–5]. To investigate the effects of a range of ceramide species on insulin action in skeletal muscle cells, and the roles of CerS isoforms, we overexpressed or knocked down these enzymes in lipid-treated L6 skeletal muscle cells and examined insulin signalling and glucose disposal.

Methods

Recombinant adenovirus Differentiated L6 myotubes were infected with viruses mediating the expression of CerS1, CerS2, CerS4, CerS5 and CerS6, or β-galactosidase (LacZ) as a control, 24 h before lipid pretreatment. Viruses were generated and characterised, including the analysis of mRNA expression by RT-PCR, as described in the Supplementary Methods (see the electronic supplementary material [ESM]).

siRNA-mediated knockdown of CerS isoforms Myotubes were transfected with siRNAs targeting CerS1, CerS2, CerS4, CerS5 or CerS6 48 h before lipid pretreatment, as described in the ESM Supplementary Methods.

FA pretreatment and insulin stimulation Myotubes were pretreated for 20 h with 0.375 mmol/l palmitate. Cells were stimulated with 100 nmol/l insulin prior to the analysis of insulin signal transduction, GLUT4 translocation, glycogen synthesis and cellular respiration, as described in the ESM Supplementary Methods.

Ceramide and sphingomyelin determination Electrospray ionisation mass spectrometric analysis of ceramides and sphingomyelins was performed on a QTRAP 2000 triple quadrupole linear ion trap mass spectrometer. Flux through the de novo synthesis and salvage pathways was examined by [^{3}H]serine or sphingosine incorporation. Details are provided in the ESM Supplementary Methods.

Statistical analysis Results are shown as means ± SEM of independent experiments. Factorial ANOVA was performed using StataSE v9.2 (Stata Corporation, TX, USA).

Results

The profile of ceramides observed in extracts from L6 myotubes infected with control virus mediating the expression of β-galactosidase (LacZ) is shown in Fig. 1a. For seven of the 15 species detected, the treatment of cells with palmitate caused a significant increase in the ceramide levels observed, especially the major C16:0 ceramide. Acute insulin stimulation had no effect.

The characterisation of viruses mediating the expression of the CerS isoforms previously detected in muscle [6] is shown in ESM Fig. 1. We overexpressed each isoform at a level that led to an increase in specific ceramides in the myotubes that was similar in magnitude to the effects of a low dose of palmitate, so that the effects on insulin signalling and glucose metabolism could be examined. The results of lipidomic analyses are shown in Fig. 1b–h (major and total ceramides) and ESM Fig. 2a (minor ceramides).

In agreement with the reported substrate specificities of the different CerS isoforms [7], overexpression led to significant increases in distinct ceramides, especially upon palmitate treatment. C16:0 ceramide was increased by an overexpression of CerS6, and to a lesser extent of CerS5, while C18:0 ceramide was increased by an overexpression of CerS1 (Fig. 1b, c). CerS2 overexpression led to an elevation in C22:0, C24:0 and C24:1 ceramides, whereas CerS4 expression was associated with increased levels of C20:0 and C22:0 ceramides (Fig. 1d–g). Although particular ceramide species were raised by CerS overexpression to a similar degree as that seen with palmitate treatment, it should be noted that total ceramides were not altered to the same extent (Fig. 1h). In addition to these expected changes, CerS6 also significantly reduced the levels of C18:0, C24:0 and C24:1 ceramides (Fig. 1c, f, g).

We also determined the effects of CerS overexpression, palmitate treatment and insulin stimulation on sphingomyelins

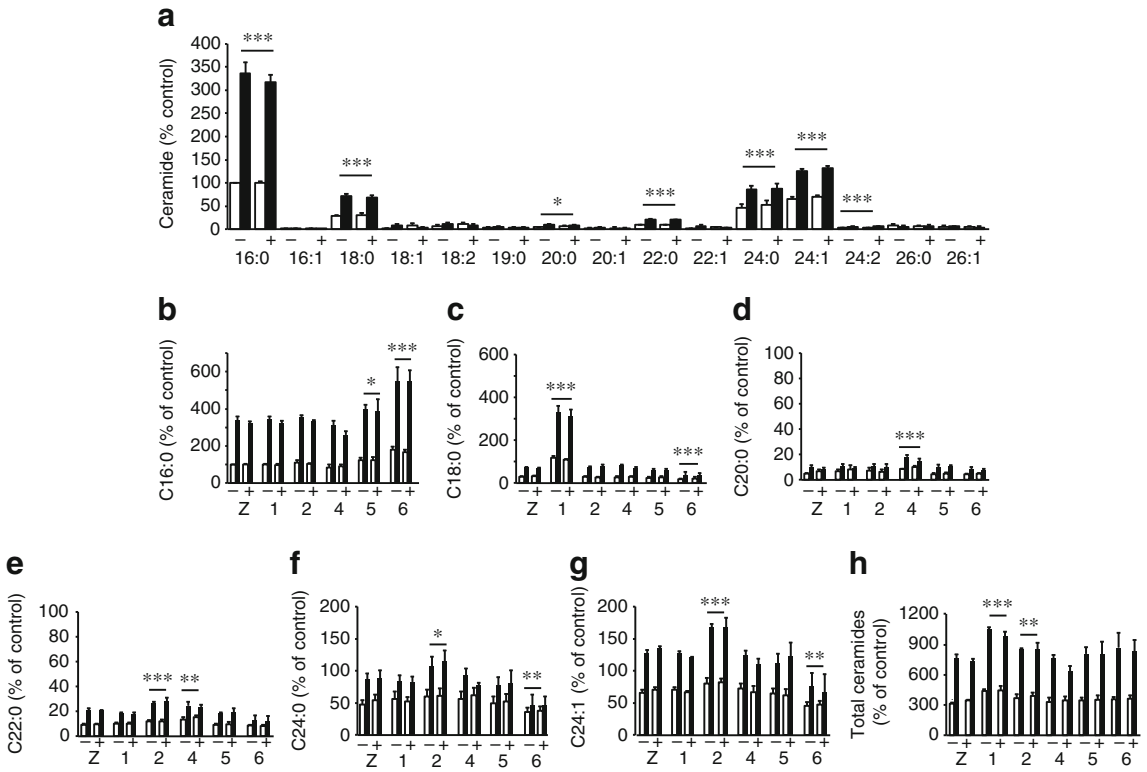

Fig. 1 The effects of CerS isoform overexpression on major ceramide species in L6 myotubes. (**a**) Myotubes infected with control adenovirus were pretreated with palmitate (black bars) or untreated (white bars) and stimulated with insulin (+) or not stimulated (−). Ceramide levels in lipid extracts from cells were analysed by mass spectrometry, and the species indicated by chain length and saturation. ANOVA: ***$p<0.001$ for the effect of palmitate vs control (± insulin). (**b–h**) The levels of the most abundant ceramide species in basal (−) and insulin-stimulated (+) L6 myotubes overexpressing the CerS isoforms indicated (1, 2, 4, 5 or 6) or LacZ (Z) as the control and pretreated without (white bars) or with (black bars) palmitate. ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$ for the effect of CerS isoform vs the LacZ control (with or without insulin, and with or without palmitate). The results shown are the means from three independent experiments carried out in triplicate. All values are expressed relative to C16:0 ceramide in basal cells infected with LacZ-expressing virus but not treated with palmitate (control)

(ESM Fig. 2b, c). Insulin had no effect, while palmitate treatment caused a significant decrease in nine of 22 species, including the predominant C16:0 species. Overexpression of CerS isoforms had limited effects, although CerS1 expression increased both C18 species detected, CerS4 increased C20:1 sphingomyelin and CerS6 increased C16:1 sphingomyelin. CerS1 and CerS6 also reduced a broad range of other species (ESM Fig. 2c), including C22 and C24 sphingomyelins.

CerS overexpression at these levels had minimal effects on the expression of genes involved in sphingolipid metabolism, including other CerS isoforms, serine–palmitoyl transferases and regulatory Orm proteins. The exception was the downregulation of endogenous CerS1 and minor upregulation of endogenous CerS5 by CerS1 overexpression (ESM Fig. 3). Importantly, there was no compensatory downregulation of de novo sphingolipid synthesis, as determined by [^{3}H]serine incorporation into total sphingomyelin (ESM Fig. 4a). CerS1, CerS4 and CerS6 increased flux through the salvage pathway (ESM Fig. 4b–e), as measured by the incorporation of [^{3}H]sphingosine into other sphingolipids.

We next determined whether the changes in sphingolipid profiles caused by CerS isoform overexpression led to alterations in insulin signalling or glucose disposal. Unexpectedly, overexpression of CerS1 or CerS6 caused a significant increase in IRS1 phosphorylation (Fig. 2a), while other isoforms had no effects. These beneficial effects were also observed at the level of Akt serine phosphorylation (Fig. 2b), and CerS1 also increased the level of GLUT4 at the cell surface (ESM Fig. 5). Finally, CerS1 and CerS6 also promoted glycogen synthesis (Fig. 2c). FA treatment had inhibitory effects on insulin-stimulated Akt phosphorylation and glycogen synthesis, as previously reported [8], but importantly, these were not potentiated by the overexpression of any CerS isoform.

We also tested whether CerS knockdown affected the action of insulin. Measurement of endogenous mRNA levels of the different CerS isoforms demonstrated that we were able to achieve 40–60% knockdown in each case (ESM Fig. 6a), and this was confirmed at the protein level in the case of CerS6 (ESM Fig. 6b). Knockdown of any isoform did not affect the mRNA levels of other CerS enzymes (data not shown). We

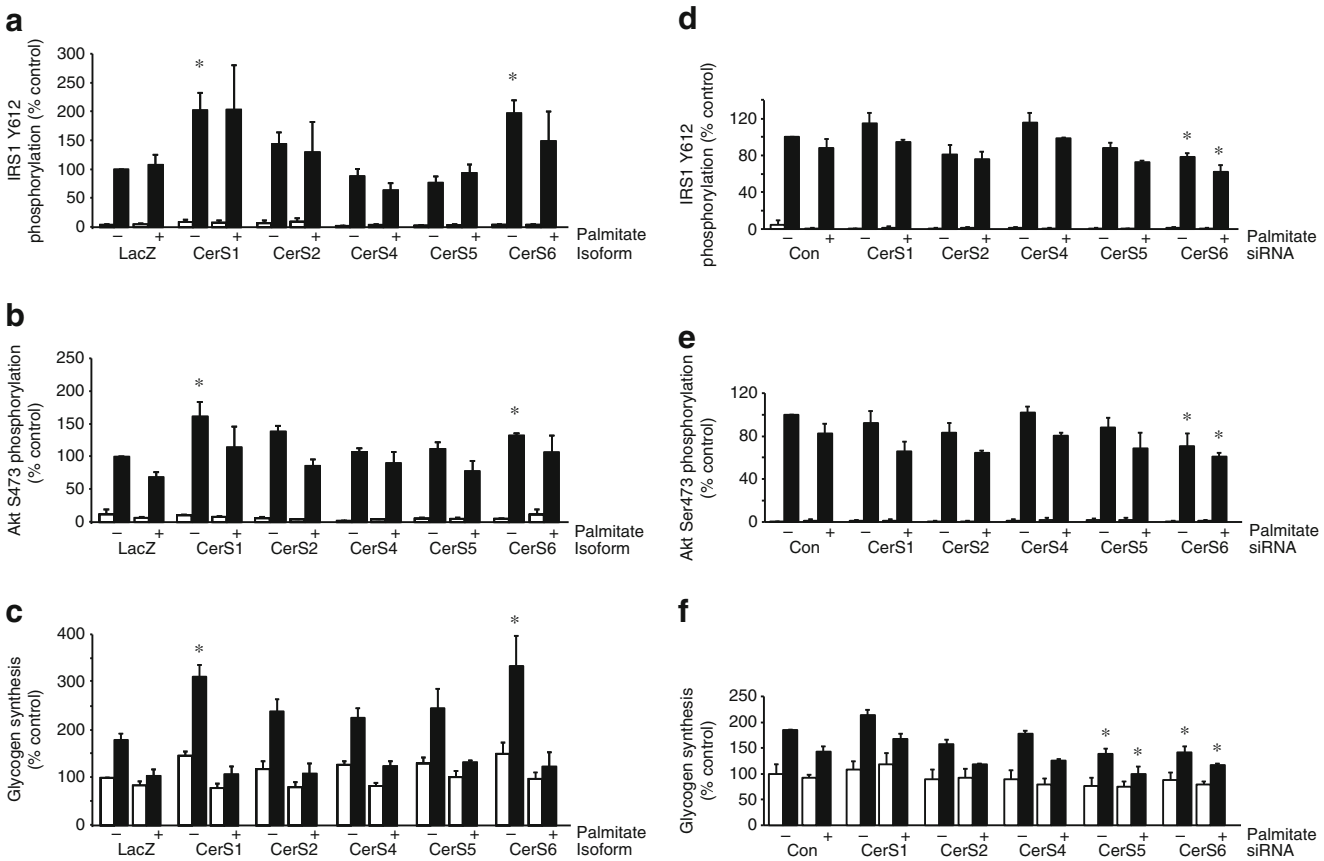

Fig. 2 The effect of CerS isoform overexpression and knockdown on insulin signalling and glycogen synthesis in L6 myotubes. (**a**–**c**) Cells were infected with adenoviruses mediating the expression of CerS isoforms or LacZ (Z) as control, and treated or not treated with palmitate as indicated. Cells were subsequently stimulated (black bars) or not stimulated (white bars) with insulin. Cells were harvested and the lysates subjected to immunoblotting with antibodies against phospho-IRS1 Y612 (**a**) or phospho-Akt S473 (**b**). Alternatively, control and palmitate-treated myotubes incubated with [^{14}C]glucose in the absence or presence of insulin were extracted in KOH for the determination of glycogen synthesis (**c**). Conversely, cells were transfected with siRNAs mediating knockdown of the CerS isoforms indicated (**d**–**f**), and treated with palmitate and insulin as above. Cells were harvested and lysates subjected to immunoblotting with antibodies against phospho-IRS1 Y612 (**d**) or phospho-Akt S473 (**e**). Glycogen synthesis was determined in myotubes incubated with [^{14}C]glucose (**f**). Results are expressed relative to control values (% LacZ-expressing, insulin-stimulated cells [**a, b, d, e**] or % LacZ-expressing, basal cells [**c, f**]). ANOVA: $*p<0.05$ for effect of CerS isoform vs control virus or siRNA under the same conditions. Results shown are the means from three (**a**–**e**) or five (**f**) independent experiments carried out in triplicate

then carried out similar experiments to those described above. In the case of CerS1, CerS2 and CerS4, a reduction in isoform expression had no effect on insulin-stimulated IRS1 tyrosine phosphorylation, Akt serine phosphorylation or glycogen synthesis (Fig. 2e, f). However, CerS5 knockdown did reduce glycogen synthesis in the absence or presence of palmitate (Fig. 2f). Importantly, knockdown of CerS6 had a reciprocal effect to that observed with CerS6 overexpression, reducing both insulin signalling at the levels of IRS-1 and Akt and also insulin-stimulated glycogen synthesis in the absence or presence of palmitate (Fig. 2e, f).

CerS isoforms are located primarily at the endoplasmic reticulum (ER), but although ER stress has been linked to insulin resistance, we did not observe significant levels of ER stress markers in myotubes treated with palmitate (not shown). Because changes in mitochondrial ceramides have been reported to affect the respiratory chain [9], which may in turn modulate insulin action, we examined whether the altered cellular profiles of sphingolipids induced by the overexpression of CerS1 or CerS6 altered mitochondrial function. The oxygen consumption rate of intact myotubes was reduced by approximately 25% by palmitate treatment, whereas overexpressing CerS1 or CerS6 had no effect (ESM Fig. 7).

Discussion

We were able to demonstrate that different CerS isoforms altered the ceramide profile in L6 myotubes in a highly specific manner when they were overexpressed. However, contrary to our hypothesis, no isoform was found to inhibit insulin signalling or glycogen synthesis per se, or to potentiate the inhibitory effect of palmitate. Indeed, we showed that overexpression of CerS1 or CerS6 was beneficial, while

knockdown of CerS6 had reciprocal effects on signalling and glucose disposal, underscoring the positive role that this isoform plays in this model. These findings indicate that the effects of ceramide accumulation on insulin sensitivity are complex, and it does not appear that particular ceramide species inhibit insulin signalling or glucose disposal in a more potent manner when elevated by the action of CerS.

A possible explanation for the differences between the effects of palmitate and CerS overexpression on the action of insulin may involve the novel finding that FA reduces the levels of sphingomyelins. This suggests that palmitate also generates ceramide by promoting the hydrolysis of sphingomyelin, potentially through an inflammatory pathway leading to the activation of sphingomyelinase [10]. If this contributes significantly to ceramide accumulation and hence insulin resistance, it could partly explain why the modulation of CerS activity, which is not involved in this route of ceramide production, has contrasting effects on insulin action. The positive actions of CerS1 and especially CerS6 may involve the remodelling of ceramides, perhaps at different cellular locations from the ceramide released from sphingomyelin, to promote effects that are distinct from those of palmitate excess. However, further complexity is indicated by the observation that CerS1 or CerS6 overexpression also induced reductions in the levels of several sphingomyelins.

Although unexpected, our results indicating positive roles for certain CerS isoforms in insulin action in muscle cells complement recent findings from CerS2 null mice, which indicated a beneficial role for this isoform in insulin receptor membrane localisation in the liver [4]. Overall, it is therefore unlikely that targeting CerS activity would be a useful approach to improving insulin sensitivity in obesity and diabetes.

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Contribution statement GF, BD, B-QML, JTS, NJH, TWM and CS-P all designed and performed studies and analysed data. CS-P directed the study, interpreted the data and wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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