Ceramide synthase 4 and *de novo* production of ceramides with specific N-acyl chain lengths are involved in glucolipotoxicity-induced apoptosis of INS-1 β-cells

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INTRODUCTION

Type 2 diabetes mellitus is a disease characterized by progressive impaired insulin secretion and peripheral insulin resistance [1–3]. The pathogenesis of β-cell failure is due to both β-cell dysfunction and reduced β-cell mass. The reduction in insulin secretory capacity and β-cell mass observed during Type 2 diabetes is thought to be caused by chronic hyperglycaemia, a phenomenon that has been termed ‘glucotoxicity’ [4]. In addition to hyperglycaemia, accumulated evidence suggests that Type 2 diabetes is often associated with abnormalities in lipid metabolism and excessive circulating lipids levels [3,5]. NEFAs [non-esterified (‘free’) fatty acids] are an important physiological fuel for islets, and act as supplemental nutrients able to potentiate insulin secretion in response to glucose [6,7]. However, chronically elevated levels of NEFAs in the circulation have been postulated to cause peripheral insulin resistance and impairment of insulin secretion from β-cells, a phenomenon that has been termed ‘lipotoxicity’ [2,8]. Indeed, palmitate, one of the most abundant NEFAs in plasma, has detrimental effects on β-cell function, including impairment of glucose-induced insulin release [9,10], defective insulin gene expression [11–13] and induction of β-cell apoptosis [14–17]. More recently, Marchetti and colleagues have provided evidence that prolonged exposure to NEFAs induced apoptosis in isolated human islets [18].

The chronic adverse effects of NEFAs on β-cell function and viability are potentiated by the presence of hyperglycaemia, a phenomenon that has been termed ‘glucolipotoxicity’ [19,20]. Indeed, inhibition of insulin gene expression by long-term treatment of β-cells with palmitate is observed only in the presence of elevated glucose concentrations [2,12]. Prentki and colleagues have shown that saturated NEFAs exhibit lower toxicity at low glucose concentrations, but NEFAs synergize with elevated glucose concentrations to promote β-cell apoptosis [17]. The molecular mechanisms underlying the pathogenesis of glucolipotoxicity in pancreatic β-cells are not completely understood. At low glucose levels, NEFAs are readily degraded by β-oxidation and are therefore non-toxic to β-cells. In contrast, when glucose and NEFAs are simultaneously elevated, glucose inhibits NEFA oxidation, raising the levels of long-chain acyl-CoA, which serve for complex lipid synthesis [2,21]. Indeed, long-chain acyl-CoA can be esterified into PLs (phospholipids) and triacylglycerols [22–25] which promote β-cell dysfunction and lipo-apoptosis [12,23]. Among these lipids, ceramides have been suggested to be important mediators of NEFA-induced β-cell dysfunction and apoptosis.

Ceramides are produced either by *de novo* biosynthesis or turnover of complex sphingolipids [24,25]. *De novo* synthesis of ceramides occurs at the endoplasmic reticulum and is initiated by the condensation of serine and...
induced apoptosis is blocked by inhibitors of ceramide synthesis [14,15].

Ceramide species are distinguishable by the length and/or saturation of their N-acyl chains [24,27]. In β-cells, despite such evidence for a role for ceramides in their dysfunction/apoptosis, nothing is known about the ceramide species produced in response to (gluco)lipotoxicity. Previous studies have reported distinct cellular functions for ceramides with specific N-acyl chain length [26,27]. Determining which ceramide species are produced under these conditions may shed light on the mechanisms involved in the ceramide-dependent β-cell glucolipotoxicity. Therefore, in the present study, we used a LC (liquid chromatography)–MS/MS (tandem MS) methodology to examine the impact of NEFA oversupply on ceramide profiles in pancreatic β-cells. Owing to the need for relatively large amount of biological material to perform sphingolipidomic analyses, we use the β-cell line INS-1 which can be expanded to quantities sufficient for diverse experimentation. These results revealed that high glucose levels potentiate palmitate-induced de novo ceramide synthesis. Importantly, we found that glucolipotoxicity induced the expression of the CerS4 (ceramide synthase 4) isoform in β-cells. This induction was associated with the formation of specific ceramide species such as C_{18:0}, C_{22:0} and C_{24:1}, which contributed to the apoptotic effect of glucolipotoxicity on β-cells.

**MATERIALS AND METHODS**

**Materials**

Tissue culture medium was from Lonza. [γ-32P]ATP was purchased from PerkinElmer. Palmitate, fatty-acid-free BSA and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] were from Sigma–Aldrich. FB1 and PPMP (D,L-threo-1-phenyl-2-palmitoylamino-3-morpholinopropan-1-ol) were from Biomol. DAG (diacylglycerol) kinase was from Calbiochem. Apo-ONE® Homogenous Caspase-3/7 Assay kit was from Promega. All solvents were from Merck Eurolab or Fisher Scientific. Ceramides, dihydroceramides, sphingosine, dihydro-sphingosine, C_{17}-ceramide and C_{17}-sphingosine were from Avanti Polar Lipids. Anti-β2A (haemagglutinin) and anti-Cer-S4 antibodies were from Covance and Abcam respectively. Mission® siRNA (short interfering RNA) against CerS4 and control siRNA were from Sigma. Lipofectamine™ LTX and RNAiMAX were purchased from Invitrogen.

**Cell culture conditions**

Rat insulinoma INS-1 cells (clone 368), kindly provided by Merck–Serono, were grown in RPMI 1640 medium buffered with 10 mM Hepes containing 10% (v/v) FBS (fetal bovine serum), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/mL 2-mercaptoethanol and 100 units/ml penicillin/streptomycin. Before each experiment, cells were plated for 48 h in 96-well plates (2.2 × 10⁴ cells/well) for MTT assay and caspase 3/7 activity, in six-well plates (10⁶ cells/well) for insulin secretion and in 50 mm plates (5 × 10⁶ cells/plate) for analysis of sphingolipids by LC–MS/MS. Palmitate was administered to the cells as a conjugate with fatty-acid-free BSA. Briefly, dried aliquots (750 μl) of palmitate in ethanol were dissolved in PBS containing 5% (w/v) BSA to obtain a 4 mM stock solution. The molar ratio of NEFAs to BSA was 5:1. The NEFA stock solutions were diluted in RPMI 1640 medium supplemented with 1% FBS to obtain a 0.4 mM final concentration at a fixed concentration of 0.5% BSA.

**Figure 1** Palmitate and high concentrations of glucose induce ceramide accumulation in INS-1 cells

(A) De novo ceramide synthesis in mammals. The de novo synthesis of ceramide starts in the endoplasmic reticulum with the condensation of serine and palmitoyl-CoA, followed by reduction of 3-oxodihydrosphingine to dihydrosphingine. The latter compound is subsequently N-acetylated by a family of ceramide synthases to form dihydroceramides. Finally, a trans double bond at C₄–C₅ is introduced by dihydroceramide desaturase to produce ceramides. Ceramides are then utilized for the formation of more complex sphingolipids. (B) Cells were incubated for 24 h without or with 0.4 mM palmitate in the presence of 5 mM (G5), 10 mM (G10) or 30 mM (G30) glucose. Ceramide levels were measured by the DAG kinase assay and expressed as the percentage of ceramide levels at 5 mM glucose. Results are means ± S.E.M. for three or four independent experiments performed in duplicate. *P < 0.05 for G30 compared with G5, and G10 compared with G5. **P < 0.05 G10 plus palmitate compared with G10, and G30 plus palmitate compared with G30.

Palmitoyl-CoA catalysed by SPT (serine palmitoyl transferase) (Figure 1). The 3-oxosphinganine formed is rapidly reduced to dihydrosphingosine, which is subsequently N-acetylated by (dihydro)ceramide synthases to form dihydroceramides [26,27]. Finally, a trans double bond at C₄–C₅ is introduced by dihydroceramide desaturase to produce ceramides [24,25]. Previous studies showed that cell-permeant analogues of ceramide impaired insulin production in pancreatic β-cells [28]. Using pharmacological approaches, de novo ceramide synthesis has been implicated in inhibition of glucose-stimulated insulin gene expression by palmitate in rodent islets [12]. In addition, FB1 (fumonisin-B1), a ceramide synthase inhibitor blocked palmitate-induced apoptosis in rodent and human islets [16,18]. Moreover, Unger and colleagues have shown that ceramides are increased in islets of obese ZDF (Zucker diabetic fatty) rats and that NEFA-
Cell transfection
The cDNA encoding human CerS4 [27] was amplified by PCR using a sequence-specific sense primer (5′-CGGGATCCAGC- AGAATGCTTCTGCAAAG-3′) containing the BamHI restriction site and the reverse primer (5′-TGGGAACA TCGTA TGGGTA TGTGGCTGTTGTGTGCCTG-3′) containing the HA sequence tag. The amplification product was subcloned in the Zero Blunt TOPO vector PCR Cloning kit (Invitrogen). Then, the CerS4 cDNA was cloned into the pcDNA5-TG vector (Invitrogen) at the level of the BamHI restriction site and its sequence was verified. INS-1 cells were transiently transfected with the empty pcDNA5-TG vector or the pcDNA5-TG containing HA-tagged CerS4 construct using Lipofectamine™ LTX. Transfection efficiencies were typically 40–70% for INS-1 cells. In some experiments, cells were transfected with 50 or 100 nmol of sequence-specific siRNA against CerS4 and control siRNA using Lipofectamine™ RNAiMAX.

Western blotting
Equal amounts of proteins were separated by SDS/PAGE (10% gels) and then transblotted on to nitrocellulose. Blots were probed with either a polyclonal anti-CerS4 antibody or a monoclonal anti-HA antibody. Immunoreactive bands were visualized by enhanced chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and SuperSignal West Pico chemiluminescent substrate (Pierce).

Measurement of MTT cell viability
At the end of each treatment, cells were treated with MTT (1 mg/ml) for 4 h. Supernatants were discarded and DMSO was added. Absorbance was measured at 560 nm using a microplate reader (Dynex-MRX). Each experimental condition tested was performed in triplicate.

Measurement of caspase 3/7 activity
Caspase 3/7 activity assays were performed using the Promega Apo-ONE® Homogenous Caspase-3/7 Assay kit. Briefly, lysis buffer containing the fluorogenic Z-DEVD-R110 (benzoylloxycarbonyl)-Asp-Glu-Val-Asp-rhodamine 110) substrate was added to each well and fluorescence was measured over a 120 min period using a Fluostar plate reader set at 485 nm excitation and 530 nm emission. Caspase 3/7 specific activity was expressed as the slope of the kinetic in arbitrary units. Each experimental condition tested was performed in triplicate.

Quantitative PCR
Total RNA was isolated from INS-1 cells using the RNeasy mini kit (Qiagen). Total RNA (4 μg) from each sample was reverse transcribed with 40 units of MMLV (Moloney murine leukaemia virus) reverse transcriptase (Invitrogen) using random hexamer primers. The primers used for PCR were derived from rat sequences and designed using OLIIGO6 (the primer sets used are described in Supplementary Table S1 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). Real-time quantitative PCR amplification reactions were carried out in a LightCycler 1.5 detection system (Roche) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). Reverse-transcribed RNA (10 ng) was used as the template for each reaction. All reactions were run in duplicate with no template control. The PCR conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. mRNA transcript levels of four housekeeping genes [encoding rpL19 (ribosomal protein L19), TBP (TATA-box-binding protein), cyclophilin A and 18S] were assayed. Since similar results were obtained with the four housekeeping genes, only TBP was retained for normalization of other transcripts.

Enzymatic measurement of ceramide levels
Ceramide levels in cellular extracts were measured by the DAG kinase enzymatic method as described previously [29]. Briefly, 200 μl aliquots of the chloroform phases from cellular lipid extracts were resuspended in 7.5% (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin (diphosphatidylglycerol) in 1 mM DETAPAC (diethylenetriaminepenta-acetic acid)/10 mM imidazole (pH 6.6). The enzymatic reaction was started by the addition of 20 mM DTT (dithiothreitol), 0.88 unit/ml Escherichia coli DAG kinase, 5 μCi/10 mM [γ-32P]ATP and the reaction buffer [100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM MgCl2 and 2 mM EGTA]. After incubation for 1 h at room temperature (20°C), lipids were extracted with chloroform/methanol/HCl (100:100:1, by vol.) and 1 M KCl. [γ-32P]Ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.) and quantified using a Storm PhosphorImager (GE Healthcare). Known amounts of bovine ceramide standards were included in each assay. Ceramide levels are expressed in fmol per nmol of PL levels. Each measurement was carried out in duplicate.

Measurement of total cellular PLs
Total PLs present in cellular lipid extracts used for ceramide analysis were quantified as described previously [30] with minor modifications. Briefly, a mixture of 10 M H2SO4/70% perchloric acid (3:1, v/v) was added to lipid extracts which were incubated overnight. After centrifugation, water and 4.2% ammonium molybdate in 4 M HCl/0.045 M Malachite Green (1:3, v/v) was added. Samples were incubated at 37°C for 30 min, and absorbance was measured at 660 nm.

Lipid extraction and sample preparation for LC–MS/MS
Cellular lipids were extracted using a modified Bligh and Dyer procedure [31] with the use of 0.1 M HCl for phase separation. C17-ceramide (30 pmol) and C17-sphingosine (30 pmol) employed as internal standards, were added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v) and aliquots were taken out to determine total PL content. Samples of 100 μl were concentrated under a stream of nitrogen, redissolved in methanol, transferred into autosampler vials and subjected to consecutive LC–MS/MS analysis.

Analysis of sphingoid bases and ceramides by LC–MS/MS
Analyses of sphingolipids were performed by LC–MS/MS as described previously [32,33]. The instrumentation employed was an API4000 triple-quadrupole mass spectrometer (Applied Biosystems) interfaced with an automated Agilent 1100 series liquid chromatograph and autosampler (Agilent Technologies). Briefly, sphingolipids were ionized via ESI (electrospray
ionization) with detection via MRM (multiple reaction monitoring) in positive-ion mode. The resolution of sphingoid bases was achieved with a Supelco Discovery C18 column (2.1 mm × 50 mm, 5 μm particle size) and a gradient from methanol/water/formic acid (61:39:0.5, by vol.) with 5 mM ammonium formate to methanol/acetonitrile/formic acid from methanol/water/formic acid (61:38:1, by vol.) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. The MRM transitions monitored for detection of sphingoid bases were as follows: $m/z$ 268$→$268 (C17:sphingosine, internal standard); $m/z$ 300$→$282 (sphingosine); and $m/z$ 302 $→$ 284 (dihydrosphingosine).

Ceramide molecular species were resolved using a 3 mm × 100 mm Waters X-Terra XDB-C8 column (3.5 μm particle size) and a gradient from methanol/water/formic acid (61:39:0.5, by vol.) with 5 mM ammonium formate to acetonitrile/chloroform/water/formic acid (90:10:0.5:0.5, by vol.) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. MRM transitions monitored for the elution of ceramide molecular species were as follows: $m/z$ 510$→$264, C14:0-ceramide; $m/z$ 538$→$264, C16:0-ceramide; $m/z$ 540$→$284, C16:0-dihydroceramide; $m/z$ 552$→$264, C17:0-ceramide (internal standard); $m/z$ 564$→$264, C18:1-ceramide; $m/z$ 566$→$284, C18:1-dihydroceramide; $m/z$ 566$→$264, C18:1-dihydroceramide; $m/z$ 568$→$284, C18:0-ceramide; $m/z$ 594$→$264, C30:0-ceramide; $m/z$ 596$→$284, C20:0-dihydroceramide; $m/z$ 624$→$284, C22:0-dihydroceramide; $m/z$ 650$→$264, C24:1-ceramide; $m/z$ 652$→$284, C24:0-dihydroceramide; $m/z$ 654$→$284, C24:0-dihydroceramide; $m/z$ 680$→$264, C26:1-ceramide; $m/z$ 682$→$264, C26:0-ceramide; $m/z$ 708$→$264, C28:1-ceramide; $m/z$ 710$→$264, C30:0-ceramide.

Standard curves for each of the sphingoid bases or ceramide molecular species were constructed via the addition of increasing concentrations of the individual analyte to 30 pmol of the structural analogues of the sphingolipid classes used as the internal standards. Linearity and the correlation coefficients of the standard curves were obtained via a linear regression analysis. The standard curves were linear over the range 0–300 pmol of each of the sphingolipid analytes with correlation coefficients ($R^2$) >0.98. Parameters of DP (declustering potential), EP (entrance potential), CE (collision energy) and CXP (collision cell exit potential) were determined for each individual analyte by the infusion of the corresponding standards. Turbo-V ion source was operated at 550°C. GS1 = 40, GS2 = 50 and curtain gas = 20. Correction for ion suppression by the matrix was controlled by creating standard curves in the presence of total lipid extract from human pulmonary artery endothelial cells (4 nmol of total lipid phosphorus).

Insulin secretion

INS-1 cells were seeded in 12-well plates and treated for 24 h with 0.4 mM palmitate in the presence of various glucose concentrations. Cells were then pre-incubated in KRHB (Krebs–Ringer/bicarbonate/Hepes) containing 0.2% fatty-acid-free BSA and 5.5 mM glucose for 1 h. Insulin secretion was measured following a 60 min incubation in KRHB containing 0.2% defatted BSA with 5.5 mM glucose or 16.7 mM glucose. The insulin concentration in the medium was determined by RIA as described previously [34].

Statistical analysis

Results are expressed as means ± S.E.M. Significance was assessed using Student’s unpaired and two-tailed t-tests. $P > 0.05$ was considered significant.

RESULTS

Palmitate and high glucose elicit ceramide accumulation

Although palmitate has been shown to mediate part of its lipotrophic effects on β-cells through ceramide synthesis [12,14], the chain length and degree of saturation of ceramides have not been determined. We therefore tested the effect of 0.4 mM palmitate, in the presence of various concentrations of glucose, on ceramide levels in INS-1 cells. In a first step, using the DAG kinase assay, which determined ceramide levels after phosphorylation to ceramide 1-phosphate [35], we found that palmitate induced a 2.2-fold increase in ceramide levels at low glucose concentrations in INS-1 cells (Figure 1B). Surprisingly, we found that glucose also raised the levels of ceramides in INS-1 cells (Figure 1B). Next, we examined the levels of ceramide molecular species by LC–MS/MS. The most abundant ceramide molecular species in INS-1 cells were very-long-chain ceramides such as C24:0 and C24:1, followed by the long-chain ceramides C22:0, C18:0 and C16:0 (see Supplementary Table S2 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). As observed with the DAG kinase assay, LC–MS/MS analysis showed that high glucose concentrations increased ceramide levels in INS-1 cells in a time-dependent manner (Figures 2A and 2B, and see Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). Indeed, increasing the concentration of glucose to 10 mM compared with low glucose levels induced the accumulation of ceramides C20:0, C22:0 and C26:0 (Figures 2A and 2B) at 24 h. However, 30 mM glucose did not increase the levels of these ceramide species further, but rather slightly increased ceramide C16:0 and C18:0 levels in INS-1 cells. At 5 mM glucose, palmitate was able to increase ceramide levels as soon as 12 h after the treatment and with a greater efficiency than high glucose levels (Figures 2C and 2D). Palmitate increased both long-chain and very-long-chain ceramides at 12 h, most obviously enhancing the ceramides C16:0 and C18:0 (Figures 2A and 2B) at 24 h. However, 30 mM glucose did not increase the levels of these ceramide species further, but rather slightly increased ceramide C16:0 and C18:0 levels in INS-1 cells. At 5 mM glucose, palmitate was able to increase ceramide levels as soon as 12 h after the treatment and with a greater efficiency than high glucose levels (Figures 2C and 2D). Palmitate increased both long-chain and very-long-chain ceramides at 12 h, most obviously enhancing the ceramides C16:0, C18:0, C20:0 and C22:0 species (3-fold increase). After 24 h of treatment, palmitate only increased the levels of long-chain ceramides (see Supplementary Figures S1C and S1D). Altogether, these results show that both palmitate and high glucose concentrations triggered ceramide accumulation in INS-1 cells.

Palmitate and glucose induce dihydroceramide accumulation

In the de novo pathway, ceramides are produced by desaturation of dihydroceramides (Figure 1A). However, ceramides could also result from catabolism of complex sphingolipids such as sphingomyelins and glycosphingolipids [24]. Therefore differences between dihydroceramide and ceramide profiles following palmitate or glucose treatment might indicate which pathway of ceramide production is regulated by these compounds. Increasing the concentrations of glucose induced a large increase in total dihydroceramides in INS-1 cells in a dose-dependent manner (Figure 3A, and see Supplementary Figure S2 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). Interestingly, high glucose levels increased dihydroceramide species bearing an N-acyl chain length similar to those incorporated into ceramides (Figure 3B). Glucose at 30 mM preferentially increased the levels of very-long-chain dihydroceramide species such as C30:0 (Figure 3B). Adding 0.4 mM palmitate in the presence of 5 mM glucose also induced an increase in the overall level of dihydroceramides after 12 h in INS-1 cells, which decreased by 24 h (Figure 3C). Looking at the dihydroceramide molecular species, we found that palmitate increased the levels of C16:0, C18:0 and C24:0 after 12 h of treatment (Figure 3D). At 24 h, similarly
Glucolipotoxicity and ceramide synthesis in INS-1 cells

Figure 2  Chain-length specificity of ceramide production in response to palmitate and high concentrations of glucose in INS-1 cells

Cells were incubated in the presence of various concentrations of glucose for 24 h (A and B) or 0.4 mM palmitate with 5 mM glucose for 12 h (C and D). Levels of N-acyl chain lengths of ceramides were determined by LC–MS/MS. Values are expressed as a fold increase over 5 mM glucose treatment. Results are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 compared with G5, G10 compared with G5, and Palmitate compared with None. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.

to what was observed in ceramides, we found that palmitate only increased the levels of long-chain dihydroceramides in INS-1 cells (see Supplementary Figure S2). The N-acyl chain length pattern of dihydroceramides was comparable with that of ceramides, supporting the idea that palmitate induced de novo synthesis of ceramide in INS-1 cells.

High glucose potentiates the accumulation of dihydrophosphcosine induced by palmitate

Increased de novo biosynthesis of ceramide is accompanied by a substantial accumulation of their precursors: dihydrophosphcosine and dihydroceramides. As shown in Figure 4(A), high glucose
concentrations induced an increase in dihydro sphingosine levels at 12 h. Dihydro sphingosine levels increased before the accumulation of dihydroceramides, supporting the observation that high glucose by itself up-regulates the de novo pathway of ceramide formation. Palmitate also increased dihydro sphingosine levels 3-fold at low glucose concentrations after 12 h of treatment. Interestingly, we found that 30 mM glucose potentiated the production of dihydro sphingosine induced by palmitate at 12 and 24 h (Figure 4A, and see Supplementary Figure S3A at http://www.BiochemJ.org/bj/438/bj4380177add.htm). Because dihydro sphingosine could potentially be formed from the hydrolysis of dihydro ceramides by ceramidases [36], we analysed the levels of another sphingoid base, i.e. sphingosine, which is produced by deacylation of ceramide pools in mammals. LC–MS/MS measurements of sphingosine indicated that neither palmitate nor high glucose alone, or together modulated the levels of this compound after 12 and 24 h of treatment (Figure 4B, and see Supplementary Figure S3B). Altogether, these results suggest that high glucose concentrations stimulate the entry of palmitate in the de novo ceramide synthesis at the level of dihydro sphingosine production in INS-1 cells.

High glucose potentiates palmitate-induced ceramide accumulation

As shown in Figures 5(A) and 5(B), 30 mM glucose increased the accumulation of dihydroceramides induced by palmitate after 12 h of treatment. However, the effect of glucose appear to be selective as it potentiated 2–3-fold the palmitate-induced production of only C₁₆:₀, C₂₂:₀, C₂₄:₀ and C₂₄:₁ species. After 24 h of treatment, 30 mM glucose still amplified the accumulation of C₁₈:₀ dihydro ceramide induced by palmitate (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). Then, we examined whether this specific production of dihydroceramides has an impact on the accumulation of ceramide species induced by palmitate with high glucose levels. Using the DAG kinase assay, we found that 30 mM glucose potentiated the production of ceramides induced by palmitate (Figure 1B). Interestingly, LC–MS/MS analysis showed that the N-acyl chain lengths of ceramide species induced by palmitate with 30 mM glucose were similar to those incorporated into dihydroceramides (Figures 5C and 5D). Indeed, accumulation of C₁₈:₀, C₂₂:₀ and C₂₄:₁ induced by palmitate at 12 h was potentiated in the presence of 30 mM glucose compared with 5 mM glucose. In contrast, accumulation of C₁₆:₀ and C₂₀:₀ was not affected in the presence of high glucose concentrations (Figures 5C and 5D). The effect of high glucose was transient since it was unable to amplify the accumulation of these ceramide species after 24 h of palmitate treatment (see Supplementary Figure S4). Altogether, these results suggest that glucolipotoxicity could mediate its apoptotic effect through the production of specific ceramide species rather than through an overall increase in ceramide in INS-1 cells.

Palmitate with high glucose induces caspase 3/7 activation through ceramide accumulation

At 5 mM glucose, palmitate exerted no toxicity towards INS-1 cells (Figure 6A) and did not alter 16.7 mM glucose-induced insulin secretion (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/438/bj4380177add.htm). In contrast, incubation of INS-1 cells with palmitate in the presence of 10 and 30 mM glucose reduced cell viability by 28 % and 51 % respectively (Figure 6A) and drastically decreased insulin secretion induced by 16.7 mM of glucose (see Supplementary Figure S5). A similar effect of palmitate on cell viability was observed when assessing the ability of cells to exclude Trypan Blue (results not shown). To confirm that the reduction of cell number was part of an apoptotic process, we analysed the effect of palmitate on caspase 3/7 activation at various concentrations of glucose. At 5 mM glucose, palmitate did not alter caspase activity. In contrast, palmitate increased caspase 3/7 activity 2- and 7-fold in the presence of 10 and 30 mM glucose respectively (Figure 6B). To confirm that caspase activation was dependent on ceramide biosynthesis, we treated the cells with FB1, the inhibitor of CoA-dependent ceramide synthases. FB1 at 25 μM reduced ceramide accumulation and caspase 3/7 activation induced by palmitate in the presence of 30 mM glucose, by 47 % and 51 % respectively (Figures 6C and 7A). Then, we used PPMP, a potent inhibitor of glucosylceramide synthase, to block the conversion of ceramide into glucosyl ceramides in the Golgi apparatus. PPMP at 10 μM induced the accumulation of several (dihydro)ceramide species (Figure 7B–7D) which increased caspase 3/7 activity at low and high glucose concentrations (Figure 6D). Moreover, PPMP potentiated the accumulation of long-chain (dihydro)ceramide species induced by palmitate with high glucose (Figures 7B–7D). Interestingly, PPMP potentiated the accumulation of long-chain ceramide in response to glucolipotoxicity. The increased accumulation of ceramide in the presence of PPMP potentiated palmitate-induced caspase 3/7 activation 14- and 53-fold at low and high glucose concentrations respectively (Figure 6D). Altogether, these results support the idea that ceramides, rather than glucosylceramides, mediate INS-1 cell apoptosis induced by glucolipotoxicity.
Figure 5  High concentrations of glucose potentiate dihydroceramide and ceramide production induced by palmitate in INS-1 cells

Cells were incubated for 12 h with 0.4 mM palmitate in the presence of 5 mM (G5) or 30 mM (G30). Levels of N-acyl chain lengths of dihydroceramides (A and B) and ceramides (C and D) were determined by LC–MS/MS. Results are expressed as a fold increase over 5 mM glucose treatment and are means ± S.E.M. for three independent experiments. *P < 0.05 for G5 + palmitate compared with G5, and G30 + palmitate compared with G30; **P < 0.05 for G30 + palmitate compared with G5 + palmitate. G5, 5 mM glucose; G5P4, 5 mM glucose + 0.4 mM palmitate; G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate.

Figure 6  Glucolipotoxicity activates caspase 3/7 through ceramide accumulation in INS-1 cells

Cells were incubated for 24 h with 0.4 mM palmitate in the presence of various concentrations of glucose. Cell viability (A) and caspase 3/7 activity (B) were determined by MTT assay and the Apo-ONE® Homogenous Caspase-3/7 Assay kit respectively. Results are expressed as the percentage of living cells at 5 mM glucose for cell viability and as arbitrary units (AU) for caspase activity and are means ± S.E.M. for four to five independent experiments performed in triplicate. *P < 0.05 for G30 compared with G5, and G10 compared with G5; **P < 0.05 for G10 + palmitate compared with G10, and G30 + palmitate compared with G30. Cells were pre-incubated without or with 25 μM FB1 (C) or 10 μM PPMP (D), then incubated for 24 h with 0.4 mM palmitate in the presence of 5 or 30 mM glucose. Caspase 3/7 activity was determined as described above. Results are means ± S.E.M. for three independent experiments performed in triplicate. *P < 0.05 for G5 + PPMP compared with G5, G30 + PPMP compared with G30, and G30 + palmitate compared with G30; **P < 0.05 for G30 + palmitate + FB1 compared with G30 + palmitate, G5 + palmitate + PPMP compared with G5 + palmitate, and G30 + palmitate + PPMP compared with G30 + palmitate. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.
**Figure 7** FB1 and PPMP inversely regulate ceramide accumulation induced by glucolipotoxicity in INS-1 cells

Cells were pre-incubated or not with 25 μM FB1 (A) or 10 μM PPMP (B, C and D), then incubated for 24 h with 0.4 mM palmitate in the presence of 30 mM glucose. (A) Ceramide levels were measured by the DAG kinase assay and expressed as a percentage of ceramide levels at 5 mM glucose. Results are means ± S.E.M. for three independent experiments performed in duplicate. Levels of N-acyl chain length of dihydroceramides (B) and ceramides (C and D) were determined by LC–MS/MS. Values are expressed as a fold increase over 30 mM glucose treatment (B and D). *P < 0.05 for G30 + palmitate compared with G30, and G30 + PPMP compared with G30; ** P < 0.05 for G30 + palmitate + PPMP or FB1 compared with G30 + palmitate. G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate.

**Palmitate with high glucose induces CerS4 expression**

The structural variety of ceramides in mammals relies on the existence of a family of enzymes: the ceramide synthases [26,27]. Six ceramide synthases have been identified and possess a characteristic substrate preference for a particular fatty acyl-CoA [26,27]. Quantitative real-time PCR experiments showed that INS-1 cells expressed CerS1–CerS5 (Figure 8A). Whereas no change in ceramide synthase expression was detected in β-cells after incubation with palmitate at 5 mM glucose (Figure 8A), palmitate increased the levels of CerS4 mRNA 3-fold in the presence of 30 mM glucose (Figure 8B). Western blot analysis revealed that palmitate with low and high glucose concentrations increased in a time-dependent manner the protein levels of CerS4 in INS-1 cells (Figure 8C). We overexpressed HA–CerS4 in INS-1 cells to determine the role of CerS4 in ceramide accumulation induced by palmitate in the presence of high glucose concentrations. Western blot analysis with anti-HA monoclonal antibody or anti-CerS4 polyclonal antibody revealed that HA-tagged CerS4 was overexpressed in INS-1 cells (Figure 9A). INS-1 cells transiently expressing HA–CerS4 for 24 h where incubated for an additional 24 h with palmitate in the presence of 30 mM glucose. Overexpressed CerS4 potentiated the accumulation of ceramide levels induced by palmitate in the presence of high glucose concentrations (Figure 9B). Looking at the ceramide molecular species, we found that transient overexpressed HA–CerS4 increased by 20% the levels of ceramides C18:0 and C22:0, but had no effect on the accumulation of ceramides C16:0 and C24:0 (Figure 9C). Altogether, these results suggest that CerS4 plays a central role in the accumulation of selective ceramide species induced by glucolipotoxicity in INS-1 cells.

**CerS4 potentiates palmitate-induced β-cell apoptosis**

Overexpressed HA–CerS4 was unable to significantly increase caspase 3/7 activation induced by palmitate at low glucose levels (Figure 10A). In contrast, the increase in ceramide levels induced by HA–CerS4 was associated with a 2-fold increase in caspase 3/7 activity induced by palmitate in the presence of 30 mM glucose (Figure 10A). Moreover, siRNA against CerS4 reduced significantly the induction of CerS4 by palmitate in the presence of high glucose concentrations (Figure 10C). Interestingly, down-regulation of endogenous CerS4 by siRNA inhibited by 39% caspase 3/7 activation induced by palmitate in the presence of high glucose concentrations (Figure 10D). CerS4 is an enzyme which shows a preference for fatty acids such as stearic, lignoceric and nervonic acids [26,27]. In agreement with this observation, overexpressed of HA–CerS4 potentiated ceramide accumulation induced by stearate (Figure 9B), a C18:0 saturated fatty acid, which is particularly toxic for β-cells [16,17]. This ceramide accumulation exacerbated caspase 3/7 activation accumulation induced by stearate with 30 mM glucose (Figure 10B). Altogether, these results suggest that CerS4 might play a critical role in β-cell apoptosis induced by glucolipotoxicity through the synthesis of specific ceramide species such as C18:0.

**DISCUSSION**

The biochemical basis of β-cell glucolipotoxicity is not completely understood, although it requires the metabolism of glucose and lipids [2,19]. Previous studies have shown that de novo synthesis of ceramides is involved in β-cell apoptosis induced by glucolipotoxicity [14,16]. In the present study, we found that palmitate at low glucose concentrations induces
Glucolipotoxicity and ceramide synthesis in INS-1 cells

Figure 8 Palmitate with high glucose levels induces CerS4 expression in INS-1 cells

Cells were incubated for different times with 0.4 mM palmitate in the presence of 5 mM or 30 mM glucose. (A and B) Ceramide synthase mRNA expression was determined after 12 h of treatment by quantitative real-time PCR. Results are means ± S.E.M. for three independent experiments performed in duplicate. *P < 0.05 for Palmitate compared with None. (C) CerS4 protein levels were determined by Western blot analysis using an anti-CerS4 antibody. G5, 5 mM glucose; G30, 30 mM glucose.

ceramide accumulation in β-cells, without inducing β-cell death. These results suggest that this production of ceramides is not qualitatively and/or quantitatively sufficient to trigger apoptosis. Another possibility is that palmitate concomitantly activates signalling pathways that counteract the apoptotic action of ceramide. Indeed, recent studies have shown that palmitate-induced stimulation of purinergic P2X7 receptors [37] and autophagy [38] reduces β-cell apoptosis triggered by the fatty acid. Moreover, El-Assaad et al. [39] have recently shown that glucolipotoxicity induces SCD-1 (stearoyl-CoA desaturase-1) in INS-1 cells. This enzyme participates in the conversion of stearate into oleate, a fatty acid which does not induce β-cell apoptosis, but rather protects β-cells from the deleterious effect of palmitate [16,40].

In agreement with previous studies [17,23], we found that palmitate was more toxic to β-cells in the presence of elevated glucose concentrations. Caspase 3/7 activation confirmed that glucolipotoxicity induced apoptosis, leading to β-cell death. High glucose concentrations potentiated ceramide accumulation induced by palmitate. Interestingly, we found that exposing INS-1 cells to high glucose alone increased ceramide production. It is known that glucose metabolism following an increase in its level in β-cells leads to the production of malonyl-CoA, a potent inhibitor of fatty acid oxidation [2,21]. Therefore chronic hyperglycaemia may channel both endogenous fatty acyl-CoA and exogenous palmitate into lipid synthesis, such as ceramide, which in turn affect β-cell fate. Accordingly, we found that both palmitate and high glucose concentrations increased the levels of dihydrosphingosine and dihydroceramide, the precursors for the de novo biosynthesis of ceramide. Importantly, high glucose concentrations potentiated palmitate-induced production of dihydrosphingosine and dihydroceramides in β-cells. The ‘salvage pathway’, a novel pathway for ceramide production, has been described in which hydrolysis of complex sphingolipids produces ceramide and eventually sphingosine which is then re-acylated to form ceramide with specific N-acyl chains [41]. In the present study, dihydrosphingosine levels accumulated, whereas sphingosine levels remained unchanged in β-cells in response to glucolipotoxicity, suggesting that ceramide accumulation induced

Figure 9 Overexpression of CerS4 enhances ceramide production induced by glucolipotoxicity in INS-1 cells

Cells were transiently transfected with the empty vector alone or the HA-tagged CerS4 pcDNA5-T0 construct. (A) Overexpressed CerS4 was determined by Western blot using an anti-HA or an anti-CerS4 antibody. INS-1/vector and INS-1/CerS4 cells were incubated for 24 h with 0.4 mM palmitate or 0.4 mM stearate in the presence of 30 mM glucose. Levels of ceramides (B) and N-acyl chain length of ceramides (C) were determined by the DAG kinase assay and LC–MS/MS respectively. Results are expressed as expressed as fmol of ceramide/nmol of PL (B) and a fold increase over 30 mM glucose treatment (C) and are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 + fatty acids compared with G30 in vector cells; **P < 0.05 for G30 + fatty acids in CerS4 cells compared with G30 + fatty acids in vector cells. G30, 30 mM glucose; G30P4, 30 glucose + 0.4 mM palmitate; G30St, 30 mM glucose + stearate.
by both compounds does not involve the salvage pathway, but is mainly due to de novo ceramide biosynthesis. Moreover, these results suggest that chronic hyperglycaemia induces incorporation of endogenous fatty acyl-CoA and added palmitate at least at the level of dihydrosphingosine synthesis, which in turn induces β-cell apoptosis by increasing ceramide levels.

Using FB1, a potent inhibitor of ceramide synthases, we confirmed that de novo ceramide production partially contributed to the apoptotic effect of glucolipotoxicity on β-cells [14,16,18]. Glycosphingolipid metabolites of ceramides have been proposed to play a role in the development of insulin resistance in vitro and in ZDF rats [42]. Interestingly, inhibition of glycosphingolipid synthesis prevented the loss of pancreatic β-cells in ZDF rats [43] suggesting a potent role of these lipids on lipotoxicosis. In the present study, we have shown that an inhibitor of glucosylceramide synthase, an enzyme which convert ceramide into glucosylceramide, potentiated the accumulation of long-chain ceramides and caspase 3/7 activation by palmitate in INS-1 cells at high glucose levels. Thus, at variance to glycosphingolipid-induced insulin resistance, it seems that ceramides rather than glycosphingolipids are responsible for the toxic effect of palmitate on β-cells. Our results are in agreement with a recent study showing that overexpression of glucosylceramide synthase in β-cells reduced apoptosis induced by glucolipotoxicity in INS-1 cells [42]. Interestingly, Shimabukuro et al. [14] have shown that NEEFs induce SPT expression in islets from normal and diabetic ZDF rats, suggesting that ceramide accumulation in β-cells relies on up-regulation of the de novo synthesis pathway. Altogether, these results suggest that palmitate and high glucose concentrations taken separately do not favour a specific ceramide profile in β-cells, but rather force the entry of available fatty acyl-CoA into ceramide synthesis.

Lipidomic analysis also showed that high glucose concentrations exhibit a certain selectivity on the production of (dihydro)ceramides induced by palmitate. Indeed, the (dihydro)ceramide species induced by palmitate plus high glucose concentrations were specifically C18:0, C22:0 and C24:1. Accumulation of these ceramide species strongly suggest that they play a critical role in β-cell apoptosis induced by palmitate with high glucose. Recently, El-Assaad et al. [39] have shown that glucolipotoxicity induced early changes in lipid partitioning in order to induce β-cell dysfunction and apoptosis. Indeed, glucolipotoxicity induced the expression of proteins which favoured fatty acid esterification such as SCD-1 through desaturation of fatty acids, and decreased the expression of enzymes involved in lipid oxidation such as the β-subunit of AMPK (AMP-activated protein kinase). However, modulation

Figure 10  Modulation of CerS4 expression regulates caspase 3/7 activation induced by glucolipotoxicity in INS-1 cells

Cells were transiently transfected with the empty vector alone or the HA-tagged CerS4 pcDNA3-TOD construct. INS-1/vector and INS-1/CerS4 cells were incubated for 24 h with 0.4 mM palmitate (A) or 0.4 mM stearate (B) in the presence of 5 mM or 30 mM glucose. Caspase 3/7 activity was determined using the Apo-ONE Homogeneous Caspase-3/7 Assay kit and is expressed as arbitrary units (AU). Results are means ± S.E.M. for three independent experiments. (C) Western blot showing the expression of CerS4 in INS-1 cells transfected with control and CerS4 siRNA and treated for 6 h and 24 h with 0.4 mM palmitate with 30 mM glucose. (D) INS-1 cells were transfected with control and CerS4 siRNA and treated for 24 h with 0.4 mM palmitate with 30 mM glucose. Caspase 3/7 activity was determined and expressed as described above. Results are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 + palmitate compared with G5 in vector and CerS4 cells, and G30 + palmitate compared with G5 + palmitate in vector cells; **P < 0.05 for G30 + palmitate in CerS4 cells compared with G30 + palmitate in vector cells. G5, 5 mM glucose; G5P4, 5 mM glucose + 0.4 mM palmitate; G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate.

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of enzymes involved in ceramide metabolism was not reported in this study. In mammals, the variety of ceramide species relies on the existence of a family of enzymes, ceramide synthases [26,27]. Six ceramide synthases have been identified and possess a characteristic substrate preference for a particular fatty acyl-CoA [26,27]. Our results revealed that, in the presence of high glucose concentrations, palmitate led to increased CerS4 mRNA and protein levels. Surprisingly, palmitate also increased CerS4 protein levels at low glucose concentrations. Recent data suggest that the level of another ceramide synthase, CerS1, is regulated via ubiquitination and proteasome by diverse stresses [48], suggesting that palmitate could also increase CerS4 protein levels by altering its turnover in β-cells. Overexpression of CerS4 in INS-1 cells potentiated ceramide production induced by glucolipotoxicity. Interestingly, transient overexpression of CerS4 selectively potentiated accumulation of C₁₈:₀ and C₂₂:₀ ceramide species, without affecting the levels of C₁₆:₀ ceramide or very-long-chain ceramides in response to glucolipotoxicity. Altogether, these results support the idea that induction of CerS4 in β-cells contributes to the selective pattern of ceramide species produced by glucolipotoxicity. Overexpressed CerS4 was unable to increase significantly apoptosis induced by palmitate at low glucose levels. This result is probably related to the elevated β-oxidation pathway at low glucose concentrations in β-cells, which do not favour fatty acid esterification into ceramide. In contrast, ceramide accumulation induced in INS-1 cells overexpressing CerS4 was associated with an enhanced apoptosis induced by glucolipotoxicity. Moreover, siRNA against CerS4 decreased partially the induction of CerS4 and caspase 3/7 activity induced by glucolipotoxicity. The slight effect of CerS4 siRNA could be related to its efficacy or the higher degree of redundancy and inter-regulation between ceramide synthases. Indeed, a recent study has shown that down-regulation of specific ceramide synthases is associated with the maintenance of ceramide levels at the expense of glycosphingolipids and up-regulation of non-targeted ceramide synthases [49]. Whereas CerS4 did not affect the sensitivity of cells to chemotherapeutic drugs [50], our results show for the first time that CerS4 could affect the sensitivity of β-cells to glucolipotoxicity. In ZDF rats, the leptin unresponsiveness is associated with an increase in palmitate-induced SPT mRNA in islets, which contributes to accumulation of ceramide and β-cell apoptosis [14,15]. Interestingly, recent data have shown that leptin-induced decreased ceramide levels are associated with a reduction of CerS2 and CerS4 mRNA expression in white adipose tissue [51]. Whether up-regulation of CerS4 expression by hyperlipidaemia occurs and contributes to apoptosis of islets from ZDF rats is a possibility that needs to be examined. Among the ceramide species produced by glucolipotoxicity, the C₁₈:₀ species contains stearate, a saturated fatty acid particularly toxic for β-cells [16,17]. Interestingly, we found that ceramide production and caspase 3/7 activation induced by stearate was drastically increased by overexpressed CerS4 in INS-1 cells. Recent studies provided evidence that fatty acid elongase 6 (Elov6-6) mRNA levels are increased by hyperglycaemia in INS-1 cells [52] and are decreased in islets of Langerhans from diabetic ZDF rats [53]. Down-regulation of Elov6-6 in INS-1 cells attenuated palmitate-induced-apoptosis whereas overexpression of Elov6-6 potentiated apoptosis [53]. Importantly, the activity of Elov6-6 is mostly involved in elongation of de novo synthesized palmitate to produce stearate. Therefore our results suggest that glucolipotoxicity could mediate β-cell apoptosis at least through ceramide C₁₈:₀ by a concomitant up-regulation of CerS4 and Elov6-6. Our results are in agreement with previous studies showing the pro-apoptotic role for ceramide C₁₈:₀. Indeed, ceramide C₁₈:₀ has been shown to contribute to chemotherapeutic drug-induced apoptosis of chronic myeloid leukaemia cells [46], whereas it was down-regulated in carcinoma tissue compared with adjacent normal tissues [54]. However, we cannot exclude a role for the other ceramide species induced by glucolipotoxicity in β-cell apoptosis. Moreover, despite any change in their expression, other ceramide synthases may play a role in the induction of β-cell apoptosis, especially CerS1 and CerS3 which can synthesize ceramide C₁₈:₀ [26,27] and are regulated by allosteric modifications in the case of CerS1 [48]. Finally, our results do not support a role for ceramide C₁₆:₀, a ceramide species considered to have pro-apoptotic properties under a broad spectrum of stimuli [45,55], in β-cell apoptosis since its levels was not increased by glucolipotoxicity. In conclusion, the present study demonstrates that glucolipotoxicity can induce apoptosis by a dual mechanism involving the rate-limiting step in the de novo ceramide biosynthesis (i.e. generation of 3-oxodihydrosphingosine and dihydrosphingosine) and the formation of cereamides with specific N-acyl chain lengths. The latter mechanism involved the induction of CerS4 by glucolipotoxicity, an enzyme which potentiated β-cell apoptosis at least through the production of ceramide C₁₈:₀. These results suggest that downstream of the lipid partitioning induced by glucolipotoxicity, synthesis of specific ceramide species could play a critical role in β-cell apoptosis.

**AUTHOR CONTRIBUTION**

Julien Véret made the initial discovery, performed the research and analysed the data; Nicolas Coant performed the research, analysed the data and wrote the paper; Nicole Therville contributed vital reagents; Anastasia Skobeleva, Irina Gorshkova and Evgeny Berdychev performed lipidomic analysis; Evgeny Berdychev, Bernard Portha and Viswanathan Narayanan contributed to a critical reading of the paper before submission and helpful discussion; Hervé Le Stunff directed the research, analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Ceramide synthase 4 and de novo production of ceramides with specific N-acyl chain lengths are involved in glucolipotoxicity-induced apoptosis of INS-1 β-cells

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Figure S1 Chain-length specificity of ceramide production in response to palmitate and high concentrations of glucose in INS-1 cells

Cells were incubated in the presence of various concentrations of glucose for 48 h (A and B) or 0.4 mM palmitate for 24 h (C and D). Levels of N-acyl chain lengths of ceramides were determined by LC–MS/MS. Results are expressed as a fold increase over 5 mM glucose treatment and are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 compared with G5, G10 compared with G5, and Palmitate compared with None; P < 0.05. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose; GSP4, glucose 5 mM + 0.4 mM palmitate.

Table S1 Description of primer sets used for quantitative PCR amplification

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Figure S2 Palmitate and high concentrations of glucose increase dihydroceramide production in INS-1 cells

Cells were incubated in the presence of various concentrations of glucose for 48 h (A and B) or 0.4 mM palmitate for 24 h (C). Levels of total (A) and N-acyl chain length of dihydroceramide (B and C) were determined by LC–MS/MS. Results are expressed as fmol/nmol of PL (A and C) or as a fold increase over 5 mM glucose treatment (B and C) and are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 compared with G5, G10 compared with G5, and Palmitate compared with None. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.

Table S2 Levels of (dihydro)ceramide species in INS-1 cells

Cells were incubated for 24 h in the presence of 5 mM glucose. Levels of N-acyl chain length of ceramides and dihydroceramides were determined by LC–MS/MS. Values are expressed as fmol/nmol of PL. Results are means ± S.E.M. for three independent experiments.

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<td>C20:1</td>
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<td>C24:0</td>
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<tr>
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Figure S3 Palmitate and high concentrations of glucose increase dihydrosphingosine production in INS-1 cells

Cells were incubated with 0.4 mM palmitate in the presence of various concentrations of glucose for 24 h. Levels of sphingosine (A) and dihydrosphingosine (B) were determined by LC–MS/MS. Results are expressed as fmol/nmol of PL and are means ± S.E.M. for three independent experiments. *P < 0.05 for G30 + palmitate compared with G30; **P < 0.05 for G30 + palmitate compared with G5. G5, 5 mM glucose; G30, 30 mM glucose.
Glucolipotoxicity and ceramide synthesis in INS-1 cells

Figure S4  High concentrations of glucose potentiates dihydroceramide and ceramide production induced by palmitate in INS-1 cells

Cells were incubated for 24 h with 0.4 mM palmitate in the presence of various concentrations of glucose. Levels of N-acyl chain lengths of dihydroceramides (A and B) and ceramides (C and D) were determined by LC–MS/MS. Results are expressed as a fold increase over 5 mM glucose treatment and are means ± S.E.M. for three independent experiments. *P < 0.05 for G5 + palmitate compared with G5, and G30 + palmitate compared with G5; †P < 0.05 for G30 + palmitate compared with G5 + palmitate. G5, 5 mM glucose; G5P4, 5 mM glucose + 0.4 mM palmitate; G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate.

Figure S5  Effect of high concentrations of glucose and palmitate on insulin secretion by INS-1 cells

Cells were incubated for 24 h with 0.4 mM palmitate in the presence of various concentrations of glucose. Cells were subsequently washed and pre-incubated for 1 h in KRBH containing 0.2 % fatty-acid-free BSA, then incubated for a further 1 h in the same buffer at 5.5 mM glucose (G5.5) or 16.7 mM glucose (G16.7). Results are expressed as a fold increase over 5.5 mM glucose treatment and are means ± S.E.M. for three independent experiments. *P < 0.05 compared with G5.5-incubated cells cultured at 5 mM glucose in the absence of palmitate; G5, 5 mM glucose; G5P4, 5 mM glucose + 0.4 mM palmitate; G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate.

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