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

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Short title: Gluco-lipotoxicity and ceramide synthesis in INS-1 cells

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Abbreviations:

CPT-1, carnitine palmitoyl-transferase; Cer : ceramide ; CerS : ceramide synthase ; DHCer: Dihydroceramide ; FB1, fumonisin-B1 ; FFA, free fatty acids ; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LC-CoA, long chain acyl-coA; PL: phospholipids; PPMP, D,L-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol; SPT, serine palmitoyl-transferase; ZDF, zucker diabetic fatty.

Abstract

Pancreatic β -cell apoptosis induced by palmitate requires high glucose concentrations. Ceramides have been suggested to be important mediators of gluco-lipototoxicity-induced β -cell apoptosis. In INS-1 β -cells, 0.4 mM palmitate with 5 mM glucose increased the levels of dihydrosphingosine and dihydroceramides, two lipid intermediates in the *de novo* biosynthesis of ceramides, without inducing apoptosis. Increasing glucose concentrations to 30 mM amplified palmitate-induced accumulation of dihydrosphingosine and the formation of (dihydro)-ceramides. Of note, gluco-lipototoxicity specifically induced the formation of 18:0, 22:0 and 24:1 (dihydro)-ceramide molecular species, which was associated with the up-regulation of ceramide synthase 4 (CerS4) levels. Fumonisin-B1, a ceramide synthase inhibitor, partially blocked apoptosis induced by gluco-lipototoxicity. In contrast, apoptosis was potentiated in the presence of D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, an inhibitor of glucosyl-ceramide synthase. Moreover, over-expression of CerS4 amplified ceramide production and apoptosis induced by palmitate with 30 mM glucose whereas down-regulation of CerS4 by siRNA reduced apoptosis. CerS4 also potentiates ceramide accumulation and apoptosis induced by another saturated fatty acid, stearate. Collectively, our results suggest that gluco-lipototoxicity induced β -cell apoptosis through a dual mechanism involving the *de novo* ceramide biosynthesis and the formation of ceramides with specific N-acyl chain lengths rather than an overall increase of ceramide content.

Keywords: Type 2 diabetes; pancreatic β -cells; gluco-lipototoxicity; ceramides; apoptosis; ceramide synthases; lipidomic

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]. Free fatty acids (FFA) are important physiological fuel for islets, and act as supplemental nutrient able to potentiate insulin secretion in response to glucose [6, 7]. However, chronically elevated levels of FFA 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 FFA 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 FFA induced apoptosis in isolated human islets [18].

The chronic adverse effects of FFA on β -cell function and viability are potentiated by the presence of hyperglycaemia, a phenomenon that has been termed “gluco-lipotoxicity” [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 FFA exhibit low toxicity at low glucose concentrations but FFA synergize with elevated glucose concentrations to promote β -cell apoptosis [17]. The molecular mechanisms underlying the pathogenesis of (gluco)-lipotoxicity in pancreatic β -cells are not completely understood. At low glucose levels, FFA are readily degraded by β -oxidation and therefore are non-toxic to β -cells. In contrast, when glucose and FFA are simultaneously elevated, glucose inhibits FFA oxidation, raising the levels of long chain acyl-CoA (LC-CoA), which serve for complex lipid synthesis [2, 21]. Indeed, LC-CoA can be esterified into phospholipids and triglycerides [22-25] which promote β -cell dysfunction and lipo-apoptosis [12, 23]. Among these lipids, ceramides have been suggested to be important mediators of FFA-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 cytosolic face of the endoplasmic reticulum and is initiated by the condensation of serine and palmitoyl-CoA catalysed by serine palmitoyltransferase (SPT, Fig.1). The 3-keto-sphinganine formed is rapidly reduced to dihydrosphingosine, which is subsequently N-acylated 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 permeable 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, fumonisins-B1 (FB1), 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 Zucker diabetic fatty (ZDF) rats and that FFA-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 of ceramides in their dysfunction/apoptosis, nothing is known about the ceramide species produced in response to (gluco)-lipotoxicity. Recent 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 gluco-lipototoxicity. Therefore, in the present study, we used a LC/MS/MS methodology to examine the impact of FFA oversupply on ceramide profiles in pancreatic β -cells. Due 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 data revealed that high glucose levels potentiate palmitate-induced *de novo* ceramide synthesis. Importantly, we found that gluco-lipototoxicity induced the expression of the ceramide synthase 4 isoform in β -cells. This induction was associated with the formation of specific ceramide species such as C18:0, C22:0 and C24:1, which contributed to the apoptotic effect of gluco-lipototoxicity on β -cells.

Materials and Methods

Materials

Tissue culture media was from Lonza. [γ 32 P]ATP was purchased from Perkin Elmer. Palmitate, fatty acid-free BSA and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were from Sigma-Aldrich. FB1 and D,L-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were from Biomol. Diacylglycerol kinase was from Calbiochem. Apo-ONE homogenous caspase-3/7 assay was from Promega. All solvents were from Merck Eurolab or Fisher Scientific. Ceramides, dihydroceramides, sphingosine, dihydro-sphingosine, C17-ceramide and C17-sphingosine were from Avanti polar lipids. Anti-HA and anti-CerS4 antibodies were from Covance and Abcam, respectively. Mission[®] siRNA 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% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2- β -mercaptoethanol and 100 U/ml penicillin/streptomycin. Prior to each experiment, cells were plated for 48 h in 96-well plates (22×10^3 cells/well) for MTT assay and caspase-3/7 activity, in 6-well plates (1×10^6 cells/well) for ceramide levels, in 12-well plates (1×10^6 cells/well) for insulin secretion and in 50 mm plates (5×10^6 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 of palmitate in ethanol were dissolved in PBS containing 5% BSA to obtain a 4 mM stock solution. The molar ratio of FFA to BSA was 5:1. The FFA stock solutions were diluted in RPMI supplemented with 1% FBS (described hereafter as incubation medium) to obtain a 0.4 mM final concentration at a fixed concentration of 0.5% BSA.

Cell transfection

The cDNA encoding human CerS4 [27] was amplified by PCR using a sequence specific sense primer (5'-cgggatccagcagagaatgctgtccagt-3') containing the BamHI restriction site and the reverse primer (5'-cggaattcctaagcgtaatctggaacatcgatgggtatgtggctgtgtgtgctg-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-TO 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-TO vector or the pcDNA5-TO containing HA-tagged CerS4 construct using Lipofectamine LTX (Invitrogen). 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 (Invitrogen).

Western Blotting

Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose. Blots were probed with either a polyclonal anti-CerS4 antibody or a monoclonal anti-HA antibody, stripped, and re-probed with a polyclonal anti- β -actin antibody. Immunoreactive bands were visualized by enhanced chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Measurement of MTT cell viability

At the end of each treatment, cells were treated with MTT (1mg/ml) for 4 hr. 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 with the Promega Apo-ONE Homogeneous Caspase-3/7 Assay kit. Briefly, lysis buffer containing the fluorogenic Z-DEVD-R110 substrate was added to each well and fluorescence was measured every 6 min over a 120 min period using a Fluostar plate reader set at 37°C (excitation at 485 nm and emission at 530 nm). 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 U of M-MLV Reverse Transcriptase (Invitrogen) using random hexamer primers. The primers used for PCR were derived from rat sequences and designed using OLIGO6 (the primer sets used are described in supplemental table 1). 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 4 housekeeping genes (rpL19, Tbp, cyclophilin a, 18S) were assayed. Since similar results were obtained with the 4 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 diacylglycerol (DAG) kinase enzymatic method as previously described [29]. Briefly, aliquots of the chloroform phases from cellular lipid extracts were resuspended in 7.5% (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6). The enzymatic reaction was started by the addition of 20 mM DTT, 0.88 U/ml *E. 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, lipids were extracted with chloroform/methanol/HCl (100:100:1, v/v) and 1 M KCl. [γ -³²P]-ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a phosphorimager (Storm, Amersham). Known amounts of bovine ceramide standards were included in each assay. Ceramide levels are expressed as fmol by nmol of phospholipid (PL) levels. Each measurement was done in duplicate.

Measurement of total cellular phospholipids

Total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as described previously [30] with minor modifications. Briefly, a mixture of 10N H₂SO₄/70% perchloric acid (3:1, v/v) was added to lipid extracts which were incubated for 30 min at 210°C. After cooling, water and 4.2% ammonium molybdate in 4 N HCl/0.045% 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 by modified Bligh and Dyer procedure [31] with the use of 0.1 N HCl for phase separation. C17-Cer (30 pmol) and C17-shingosine (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 phospholipid content. Samples were concentrated under a stream of nitrogen, redissolved in methanol, transferred into autosampler vials and subjected to consecutive liquid chromatography-tandem mass spectrometry (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]. 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 electrospray ionization (ESI) with detection via multiple reaction monitoring (MRM) in positive ions mode. The resolution of sphingoid bases was achieved with a Discovery C18 column (2.1 x 50 mm, 5 μ m particle size, Supelco, Bellefonte, PA) and a gradient from methanol/water/formic acid (61:38:1, v/v) with 5 mM ammonium formate to methanol/acetonitrile/formic acid (39:60:1, v/v) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. The MRM transitions employed for detection of sphingoid bases were as follows: m/z 286>268 (C17-Sph, internal standard); m/z 300>282 (Sph); and m/z 302>284 (DHSph).

Ceramide molecular species were resolved using a 3 x 100 mm X-Terra XDB-C8 column (3.5 μ m particle size, Waters, Milford, MA) and a gradient from methanol/water/formic acid (61:39:0.5, v/v) with 5 mM ammonium formate to acetonitrile/chloroform/water/formic acid (90:10:0.5:0.5, v/v) 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, 14:0-Cer; m/z 538>264, 16:0-Cer; m/z 540>284, 16:0-DHCer; m/z 552>264, 17:0-Cer (internal standard); m/z 564>264, 18:1-Cer; m/z 566>284, 18:1-DHCer; m/z 566>264, 18:0-Cer; m/z 568>284, 18:0-DHCer; m/z 594>264, 20:0-Cer; m/z 596>284, 20:0-DHCer; m/z 624>284, 22:0-DHCer; m/z 650>264, 24:1-Cer; m/z 652>284, 24:1-DHCer; m/z 652>264, 24:0-Cer; m/z 654>284, 24:0-DHCer; m/z 680>264, 26:1-Cer; m/z 682>264, 26:0-Cer; m/z 708>264, 28:1-Cer; m/z 710>264, 28:0-Cer.

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 analogs 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 of 0.0 – 300 pmol of each of the sphingolipid analytes with correlation coefficients (R^2) >0.98. Parameters of declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) 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 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 KRBH 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 KRBH containing 0.2% defatted BSA with 5.5

mM glucose or 16.7 mM glucose. The insulin concentration in the medium was determined by radio-immunoassay as described previously [34].

Statistical analysis

Data are expressed as means \pm S.E.M. Significance was assessed by the Student's *t* test unpaired and two-tailed. *P* values less than 0.05 were considered as significant.

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Results

Palmitate and high glucose elicit ceramide accumulation

Although palmitate has been shown to mediate part of its lipotoxic effects on β -cells through ceramide synthesis [12, 14], the chain length and degree of saturation of ceramides have not been determined. Therefore, we 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 of ceramide levels at low glucose concentrations in INS-1 cells (Fig 1B). Surprisingly, we found that glucose also raised the levels of ceramides in INS-1 cells (Fig 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 (VLC) ceramides such as C24:0 and C24:1 followed by long chain (LC) ceramides C22:0, C18:0 and C16:0 (supplemental table 2). 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 (Fig. 2A and B; supplemental 1). Indeed, increasing the concentration of glucose to 10 mM compared to low glucose levels, induced the accumulation of ceramides C20:0, C24:0 and C26:0 (Fig. 2A and B) at 24 h. Thirty mM glucose did not further increase the levels of these ceramide species, but rather slightly increased ceramides 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 (Fig. 2C and D). Palmitate increased both LC- and VLC-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 LC-ceramides (supplemental 1 C and D). Altogether, these data 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 (Fig. 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 of total dihydroceramides in INS-1 cells in a dose-dependent manner (Fig. 3A; supplemental 2). Interestingly, high glucose levels increased dihydroceramide species bearing N-acyl chain length similar to those incorporated into ceramides (Fig. 3B). Thirty mM glucose preferentially increased the levels of VLC-dihydroceramide species such as DH24:0 (Fig. 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 (Fig. 3C). Looking at the dihydroceramide molecular species, we found that palmitate increased the levels of DH16:0, DH18:0 and DH24:0 after 12 h of treatment (Fig. 3D). At 24 h, similarly to what was observed in ceramides, we found that palmitate only increased the levels of LC-dihydroceramides in INS-1 cells (supplemental 2). The N-acyl chain length pattern of dihydroceramides was comparable to that of ceramides, supporting the idea that palmitate induced *de novo* synthesis of ceramide in INS-1 cells.

High glucose potentiates the accumulation of dihydrosphingosine induced by palmitate

Increased *de novo* biosynthesis of ceramide is accompanied by a substantial accumulation of their precursors, dihydrosphingosine and dihydroceramides. As shown in Fig. 4A, high glucose concentrations induced an increase in dihydrosphingosine levels at 12 h. Dihydrosphingosine 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 dihydrosphingosine levels by 3-fold at low glucose concentrations after 12 h of treatment. Interestingly, we found that 30 mM glucose potentiated the production of dihydrosphingosine induced by palmitate at 12 h and 24 h (Fig. 4A; supplemental 3A). Because dihydrosphingosine could potentially be formed from the hydrolysis of dihydroceramides by ceramidases [36], we analyzed the levels of another sphingoid base, *i.e.*, sphingosine, which is produced by de-acylation 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 h and 24 h of treatment (Fig. 4B; supplemental 3B). Altogether, these data suggest that high glucose concentrations stimulate the entry of palmitate in the *de novo* ceramide synthesis at the level of dihydrosphingosine production in INS-1 cells.

High glucose potentiates palmitate-induced ceramide accumulation

As shown in Fig. 5A and 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 by 2 to 3-fold the palmitate-induced production of only DH18:0, DH22:0, DH24:0 and DH24:1 species. After 24 h of treatment, 30 mM glucose still amplified the accumulation of DH18:0 induced by palmitate (supplemental 4). 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 (Fig. 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 (Fig. 5 C and D). 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 to 5 mM glucose. In contrast, accumulation of C16:0 and C20:0 was not affected in the presence of high glucose concentrations (Fig. 5C and D). 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 (supplemental 4). Altogether, these data suggest that gluco-lipototoxicity could mediate its apoptotic effect through the production of specific ceramide species rather than an overall increase of 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 (Fig. 6A) and did not alter 16.7 mM glucose-induced insulin secretion (supplemental 5). In contrast, incubation of INS-1 cells with palmitate in the presence of 10 mM and 30 mM glucose, reduced cell viability by 28% and 51%, respectively (Fig. 6A) and drastically decreased insulin secretion induced by 16.7 mM of glucose (supplemental 5). A similar effect of palmitate on cell viability was observed when assessing the ability of cells to exclude trypan blue (data not shown). To confirm that the reduction of cell number was part of an apoptotic process, we analyzed 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 by 2- and 7-fold in the presence of 10 mM and 30 mM glucose, respectively (Fig. 6B). To confirm that caspase activation was dependent on ceramide biosynthesis, we treated the cells with FB1, the inhibitor of CoA-dependent ceramide synthases. Twenty five μ M FB1 reduced ceramide accumulation and caspase-3/7 activation induced by palmitate in the presence of 30 mM glucose, by 47 and 55%, respectively (Fig. 6C and 7A). Then, we used D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, a potent inhibitor of glucosyl-ceramide synthase to block the conversion of ceramide into

glucosyl-ceramide in the Golgi apparatus. Ten μM of PPMP induced accumulation of several (dihydro)-ceramide species (Fig. 7B-D) which increased caspase3/7 activity at low and high glucose concentrations (Fig. 6D). Moreover, PPMP potentiated the accumulation of long chain (dihydro)-ceramide species induced by palmitate with high glucose (Fig. 7B-D). Interestingly, PPMP potentiated the accumulation of long chain ceramide in response to gluco-lipototoxicity. The increased accumulation of ceramide in the presence of PPMP potentiated palmitate induced-caspase-3/7 activation by 14- and 53-fold at low and high glucose concentrations, respectively (Fig. 6D). Altogether, these data support the idea that ceramides rather than glucosyl-ceramides mediate INS-1 cell apoptosis induced by gluco-lipototoxicity.

Palmitate with high glucose induces ceramide synthase 4 expression

The structural variety of ceramides in mammals relies on the existence of a family of enzymes, the ceramide synthases (CerS) [26, 27]. Six CerS have been identified and possess a characteristic substrate preference for particular fatty acyl-CoA [26, 27]. Quantitative real-time PCR experiments showed that INS-1 cells expressed CerS1-5 (Fig. 8A). Whereas no change in CerS expression was detected in β -cells after incubation with palmitate at 5 mM glucose (Fig. 8A), palmitate increased the levels of CerS4 mRNA by 3-fold in the presence of 30 mM glucose (Fig. 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 (Fig. 8C). We over-expressed 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 mAb or anti-CerS4 antibodies revealed HA-tagged CerS4 over-expressed in INS-1 cells (Fig. 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. Over-expressed CerS4 potentiated accumulation of ceramide levels induced by palmitate in the presence of high glucose concentrations (Fig. 9B). Looking at the ceramide molecular species, we found that transient over-expressed HA-CerS4 increased by 20% the levels of ceramide C18:0 and C22:0 but had no effect on the accumulation of ceramide C16:0 and C24:0 (Fig. 9C). Altogether, these results suggest that CerS4 plays a central role in the accumulation of selective ceramide species induced by gluco-lipototoxicity in INS-1 cells.

Ceramide synthase 4 potentiates palmitate-induced β -cell apoptosis

Over-expressed HA-CerS4 was unable to significantly increase caspase3/7 activation induced by palmitate at low glucose levels (Fig. 10A). In contrast, the increase of ceramide levels induced by HA-CerS4 was associated with a 2-fold increase of caspase3/7 activity induced by palmitate in the presence of 30 mM glucose (Fig. 10A). Moreover, siRNA against CerS4 reduced significantly the induction of CerS4 induction by palmitate in the presence of high glucose concentrations (Fig. 10C). Interestingly, down-regulation of endogenous CerS4 by siRNA inhibited by 39% caspase3/7 activation induced by palmitate in the presence of high glucose concentrations (Fig. 10D). CerS4 is an enzyme which show preference for fatty acids such as stearic, lignoceric, and nervonic acids [26, 27]. In agreement, with this observation, over-expressed of HA-CerS4 potentiated ceramide accumulation induced by stearate (Fig. 9B), a C18:0 saturated fatty acid, which is particularly toxic for β -cells [16, 17]. This ceramide accumulation exacerbated caspase3/7 activation accumulation induced by stearate with 30 mM glucose (Fig. 10B). Altogether, these results suggest that CerS4 might play a critical role in β -cell apoptosis induced by gluco-lipototoxicity through the synthesis of specific ceramide species such as C18:0.

Discussion

The biochemical basis of β -cell gluco-lipotoxicity 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 gluco-lipotoxicity [14, 16]. In the present study, we found that palmitate at low glucose concentrations induces 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-Assad et al. (2010) have recently shown that gluco-lipotoxicity induced stearoyl-CoA desaturase-1 (SCD-1) in INS-1 β -cells. This enzyme participates in the conversion of stearate to oleate, a fatty acid which does not induce β -cell apoptosis but rather protects β -cells from the deleterious effect of palmitate [16, 39].

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 gluco-lipotoxicity 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 of 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. Recently, 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 [40]. In this study, dihydrosphingosine levels accumulated whereas sphingosine levels remained unchanged in β -cells in response to gluco-lipotoxicity, suggesting that ceramide accumulation induced 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 induced incorporation of endogenous fatty acyl-CoA and added palmitate at least at the level of dihydrosphingosine synthesis, which in turn induced β -cell apoptosis by increasing ceramide levels.

Using FBI, a potent inhibitor of ceramide synthases, we confirmed that *de novo* ceramide production partially contributed to the apoptotic effect of gluco-lipotoxicity 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 Zucker diabetic fatty acid (ZDF) rats [41]. Interestingly, inhibition of glycosphingolipid synthesis prevented the loss of pancreatic β -cells in ZDF rats [42] suggesting a potent role of these lipids on lipo-apoptosis. Here, we show that an inhibitor of glucosyl-ceramide synthase, an enzyme which convert ceramide into glucosyl-ceramide, 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 over-expression of glucosyl-ceramide synthase in β -cells reduced apoptosis induced by palmitate at low glucose concentrations [43].

Although a high glucose concentration on its own increased ceramide levels, 24 h of treatment was not associated with β -cell death, in agreement with previous studies [17, 38]. Recent studies have reported distinct cellular functions for ceramide with specific N-acyl chain length [44, 45]. Therefore, we examined whether palmitate and high glucose concentrations alone or together induced specific ceramide species in these cells, which would affect differentially β -cell fate. LC/MS/MS analysis showed that high glucose concentrations increased the mass of all (dihydro)-ceramide acyl chain species, most obviously enhancing C24:0, the predominant species in INS-1 cells. In agreement, it has been shown that high glucose concentrations induced *de novo* lipid synthesis in INS-1 cells [46] which will provide the necessary fatty acids for ceramide synthesis. Lipidomic analysis revealed that palmitate at low glucose increased all ceramide species, most notably enhancing C16:0, C18:0, C20:0 and C22:0. Interestingly, Shimabukuro and co-authors [14] have shown that FFA increases 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, Prentki and co-workers have shown that gluco-lipototoxicity induced early changes in lipid partitioning in order to induce β -cell dysfunction and apoptosis [47]. Indeed, gluco-lipototoxicity 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 AMP-kinase. However, modulation 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 (CerS) [26, 27]. Six CerS have been identified and possess a characteristic substrate preference for particular fatty acyl-CoA [26, 27]. Our data 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. Over-expression of CerS4 in INS-1 potentiated ceramide production induced by gluco-lipototoxicity. Interestingly, transient over-expression 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 gluco-lipototoxicity. Altogether, these results support the idea that induction of CerS4 in β -cells contributes to the selective pattern of ceramide species produced by gluco-lipototoxicity.

Over-expressed 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 over-expressing CerS4 was associated with an enhanced apoptosis induced by gluco-lipototoxicity. Moreover, siRNA against CerS4 decreased partially the induction of CerS4 and caspase3/7 activity induced by gluco-lipototoxicity. The slight effect of siCerS4 could be related to its efficacy or the higher degree of redundancy and inter-regulation between CerS. Indeed, a recent study have shown

that down regulation of specific CerS is associated with the maintenance of ceramide levels at the expense of glycosphingolipids and up-regulation of non-targeted CerS [49]. Whereas CerS4 did not affect the sensitivity of cells to chemotherapeutic drugs [50], our data show for the first time that CerS4 could affect the sensitivity of β -cells to gluco-lipototoxicity. In ZDF rats, the leptin unresponsiveness is associated with an increase of 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 hyperlipidemia occurs and contributes to apoptosis of islets from ZDF rats is a possibility that needs to be examined.

Among the ceramide species produced by gluco-lipototoxicity, 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 over-expressed CerS4 in INS-1 cells. Recent studies provided evidence that fatty acid elongase 6 (Elovl-6) mRNA levels are increased by hyperglycaemia in INS-1 [52] and are decreased in islets of Langerhans from diabetic ZDF rats [53]. Down-regulation of Elovl-6 in INS-1 cells attenuated palmitate induced-apoptosis whereas over-expression of Elovl-6 potentiated apoptosis [53]. Importantly, activity of Elovl-6 is mostly involved in elongation of *de novo*-synthesized palmitate to produce stearate. Therefore, our results suggest that gluco-lipototoxicity could mediate β -cell apoptosis at least through ceramide C18:0 by a concomitant up-regulation of CerS4 and Elovl-6 gene. 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 [45] whereas it was down-regulated in carcinoma tissue compared to adjacent normal tissues [54]. However, we cannot exclude a role for the other ceramide species induced by gluco-lipototoxicity in β -cell apoptosis. Moreover, despite any change in their expression, other CerS 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 data do not support a role for ceramide C16:0, a ceramide species considered to have pro-apoptotic properties under a broad spectrum of stimuli [44, 55], in β -cell apoptosis since its levels was not increased by gluco-lipototoxicity.

In conclusion, the present study demonstrates that gluco-lipototoxicity can induce apoptosis by a dual mechanism involving the rate-limiting step in the *de novo* ceramide biosynthesis (*i.e.*, generation of 3-keto-dihydrosphingosine and dihydrosphingosine) and the formation of ceramides with specific N-acyl chain lengths. The latter mechanism involved the induction of CerS4 by gluco-lipototoxicity, an enzyme which potentiated β -cell apoptosis at least through the production of Cer-18:0. These results suggest that downstream the lipid partitioning induced by gluco-lipototoxicity, synthesis of specific ceramide species could play a critical role in β -cell apoptosis.

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References

- 1 Kahn, C. R. (1994) Banting Lecture. Insulin action, diabetogenesis, and the cause of type II diabetes *Diabetes* **43**, 1066-84
- 2 Poitout, V. and Robertson, R. P. (2008) Glucolipotoxicity: fuel excess and beta-cell dysfunction *Endocr Rev* **29**, 351-66
- 3 Kahn, S. E. (2003) The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes *Diabetologia* **46**, 3-19
- 4 Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A. and Butler, P. C. (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes *Diabetes* **52**, 102-10
- 5 Carey, D. G., Jenkins, A. B., Campbell, L. V., Freund, J. and Chisholm, D. J. (1996) Abdominal fat and insulin resistance in normal and overweight women: Direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM *Diabetes* **45**, 633-8
- 6 Crespin, S. R., Greenough, W. B., 3rd and Steinberg, D. (1969) Stimulation of insulin secretion by infusion of free fatty acids *J Clin Invest* **48**, 1934-43
- 7 Stein, D. T., Stevenson, B. E., Chester, M. W., Basit, M., Daniels, M. B., Turley, S. D. and McGarry, J. D. (1997) The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation *J Clin Invest* **100**, 398-403
- 8 Boden, G. and Shulman, G. I. (2002) Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction *Eur J Clin Invest* **32 Suppl 3**, 14-23
- 9 Sako, Y. and Grill, V. E. (1990) A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation *Endocrinology* **127**, 1580-9
- 10 Zhou, Y. P. and Grill, V. E. (1994) Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle *J Clin Invest* **93**, 870-6
- 11 McGarry, J. D. and Dobbins, R. L. (1999) Fatty acids, lipotoxicity and insulin secretion *Diabetologia* **42**, 128-38
- 12 Kelpe, C. L., Moore, P. C., Parazzoli, S. D., Wicksteed, B., Rhodes, C. J. and Poitout, V. (2003) Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis *J Biol Chem* **278**, 30015-21
- 13 Ritz-Laser, B., Meda, P., Constant, I., Klages, N., Charollais, A., Morales, A., Magnan, C., Ktorza, A. and Philippe, J. (1999) Glucose-induced preproinsulin gene expression is inhibited by the free fatty acid palmitate *Endocrinology* **140**, 4005-14
- 14 Shimabukuro, M., Higa, M., Zhou, Y. T., Wang, M. Y., Newgard, C. B. and Unger, R. H. (1998) Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression *J Biol Chem* **273**, 32487-90
- 15 Shimabukuro, M., Zhou, Y. T., Levi, M. and Unger, R. H. (1998) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes *Proc Natl Acad Sci U S A* **95**, 2498-502
- 16 Maedler, K., Spinas, G. A., Dyntar, D., Moritz, W., Kaiser, N. and Donath, M. Y. (2001) Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function *Diabetes* **50**, 69-76
- 17 El-Assaad, W., Buteau, J., Peyot, M. L., Nolan, C., Roduit, R., Hardy, S., Joly, E., Dbaibo, G., Rosenberg, L. and Prentki, M. (2003) Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death *Endocrinology* **144**, 4154-63

- 18 Lupi, R., Dotta, F., Marselli, L., Del Guerra, S., Masini, M., Santangelo, C., Patane, G., Boggi, U., Piro, S., Anello, M., Bergamini, E., Mosca, F., Di Mario, U., Del Prato, S. and Marchetti, P. (2002) Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated *Diabetes* **51**, 1437-42
- 19 Prentki, M., Joly, E., El-Assaad, W. and Roduit, R. (2002) Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes *Diabetes* **51 Suppl 3**, S405-13
- 20 Weir, G. C., Laybutt, D. R., Kaneto, H., Bonner-Weir, S. and Sharma, A. (2001) Beta-cell adaptation and decompensation during the progression of diabetes *Diabetes* **50 Suppl 1**, S154-9
- 21 Prentki, M. and Corkey, B. E. (1996) Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* **45**, 273-83
- 22 Roche, E., Farfari, S., Witters, L. A., Assimacopoulos-Jeannet, F., Thumelin, S., Brun, T., Corkey, B. E., Saha, A. K. and Prentki, M. (1998) Long-term exposure of beta-INS cells to high glucose concentrations increases anaplerosis, lipogenesis, and lipogenic gene expression *Diabetes* **47**, 1086-94
- 23 Briaud, I., Harmon, J. S., Kelpke, C. L., Segu, V. B. and Poitout, V. (2001) Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids *Diabetes* **50**, 315-21
- 24 Hannun, Y. A. and Obeid, L. M. (2008) Principles of bioactive lipid signalling: lessons from sphingolipids *Nat Rev Mol Cell Biol* **9**, 139-50
- 25 Zheng, W., Kollmeyer, J., Symolon, H., Momin, A., Munter, E., Wang, E., Kelly, S., Allegood, J. C., Liu, Y., Peng, Q., Ramaraju, H., Sullards, M. C., Cabot, M. and Merrill, A. H., Jr. (2006) Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy *Biochim Biophys Acta* **1758**, 1864-84
- 26 Mizutani, Y., Kihara, A. and Igarashi, Y. (2005) Mammalian Lass6 and its related family members regulate synthesis of specific ceramides *Biochem J* **390**, 263-71
- 27 Pewzner-Jung, Y., Ben-Dor, S. and Futerman, A. H. (2006) When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis *J Biol Chem* **281**, 25001-5
- 28 Sjöholm, A. (1995) Ceramide inhibits pancreatic beta-cell insulin production and mitogenesis and mimics the actions of interleukin-1 beta *FEBS Lett* **367**, 283-6
- 29 Le Stunff, H., Galve-Roperh, I., Peterson, C., Milstien, S. and Spiegel, S. (2002) Sphingosine-1-phosphate phosphohydrolase in regulation of sphingolipid metabolism and apoptosis *J Cell Biol* **158**, 1039-49
- 30 Van Veldhoven, P. P. and Mannaerts, G. P. (1987) Inorganic and organic phosphate measurements in the nanomolar range *Anal Biochem* **161**, 45-8
- 31 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification *Can J Biochem Physiol* **37**, 911-7
- 32 Berdyshev, E. V., Gorshkova, I. A., Usatyuk, P., Zhao, Y., Saatian, B., Hubbard, W. and Natarajan, V. (2006) De novo biosynthesis of dihydrosphingosine-1-phosphate by sphingosine kinase 1 in mammalian cells *Cell Signal* **18**, 1779-92
- 33 Berdyshev, E. V., Gorshkova, I. A., Garcia, J. G., Natarajan, V. and Hubbard, W. C. (2005) Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry *Anal Biochem* **339**, 129-36

- 34 Dolz, M., Bailbe, D., Giroix, M. H., Calderari, S., Gangnerau, M. N., Serradas, P., Rickenbach, K., Irminger, J. C. and Portha, B. (2005) Restitution of defective glucose-stimulated insulin secretion in diabetic GK rat by acetylcholine uncovers paradoxical stimulatory effect of beta-cell muscarinic receptor activation on cAMP production *Diabetes* **54**, 3229-37
- 35 Perry, D. K., Bielawska, A. and Hannun, Y. A. (2000) Quantitative determination of ceramide using diglyceride kinase *Methods Enzymol* **312**, 22-31
- 36 Mao, C. and Obeid, L. M. (2008) Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate *Biochim Biophys Acta* **1781**, 424-34
- 37 Glas, R., Sauter, N. S., Schulthess, F. T., Shu, L., Oberholzer, J. and Maedler, K. (2009) Purinergic P2X7 receptors regulate secretion of interleukin-1 receptor antagonist and beta cell function and survival *Diabetologia* **52**, 1579-88
- 38 Choi, S. E., Lee, S. M., Lee, Y. J., Li, L. J., Lee, S. J., Lee, J. H., Kim, Y., Jun, H. S., Lee, K. W. and Kang, Y. (2009) Protective role of autophagy in palmitate-induced INS-1 beta-cell death *Endocrinology* **150**, 126-34
- 39 Flowers, J. B., Rabaglia, M. E., Schueler, K. L., Flowers, M. T., Lan, H., Keller, M. P., Ntambi, J. M. and Attie, A. D. (2007) Loss of stearoyl-CoA desaturase-1 improves insulin sensitivity in lean mice but worsens diabetes in leptin-deficient obese mice *Diabetes* **56**, 1228-39
- 40 Kitatani, K., Idkowiak-Baldys, J. and Hannun, Y. A. (2008) The sphingolipid salvage pathway in ceramide metabolism and signaling *Cell Signal* **20**, 1010-8
- 41 Aerts, J. M., Ottenhoff, R., Powlson, A. S., Grefhorst, A., van Eijk, M., Dubbelhuis, P. F., Aten, J., Kuipers, F., Serlie, M. J., Wennekes, T., Sethi, J. K., O'Rahilly, S. and Overkleeft, H. S. (2007) Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity *Diabetes* **56**, 1341-9
- 42 Zhao, H., Przybylska, M., Wu, I. H., Zhang, J., Siegel, C., Komarnitsky, S., Yew, N. S. and Cheng, S. H. (2007) Inhibiting glycosphingolipid synthesis improves glycemic control and insulin sensitivity in animal models of type 2 diabetes *Diabetes* **56**, 1210-8
- 43 Boslem, E., Macintosh, G., Preston, A. M., Bartley, C., Busch, A. K., Fuller, M., Laybutt, D. R., Meikle, P. J. and Biden, T. J. (2011) A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking *Biochem J* **435**, 267-76
- 44 Kroesen, B. J., Pettus, B., Luberto, C., Busman, M., Sietsma, H., de Leij, L. and Hannun, Y. A. (2001) Induction of apoptosis through B-cell receptor cross-linking occurs via de novo generated C16-ceramide and involves mitochondria *J Biol Chem* **276**, 13606-14
- 45 Senkal, C. E., Ponnusamy, S., Rossi, M. J., Bialewski, J., Sinha, D., Jiang, J. C., Jazwinski, S. M., Hannun, Y. A. and Ogretmen, B. (2007) Role of human longevity assurance gene 1 and C18-ceramide in chemotherapy-induced cell death in human head and neck squamous cell carcinomas *Mol Cancer Ther* **6**, 712-22
- 46 Nyblom, H. K., Nord, L. I., Andersson, R., Kenne, L. and Bergsten, P. (2008) Glucose-induced de novo synthesis of fatty acyls causes proportional increases in INS-1E cellular lipids *NMR Biomed* **21**, 357-65
- 47 El-Assaad, W., Joly, E., Barbeau, A., Sladek, R., Buteau, J., Maestre, I., Pepin, E., Zhao, S., Iglesias, J., Roche, E. and Prentki, M. (2010) Glucolipotoxicity alters lipid partitioning and causes mitochondrial dysfunction, cholesterol, and ceramide deposition and reactive oxygen species production in INS832/13 ss-cells *Endocrinology* **151**, 3061-73

- 48 Sridevi, P., Alexander, H., Laviad, E. L., Pewzner-Jung, Y., Hannink, M., Futerman, A. H. and Alexander, S. (2009) Ceramide synthase 1 is regulated by proteasomal mediated turnover *Biochim Biophys Acta* **1793**, 1218-27
- 49 Mullen, T. D., Spassieva, S., Jenkins, R. W., Kitatani, K., Bielawski, J., Hannun, Y. A. and Obeid, L. M. (2011) Selective knockdown of ceramide synthases reveals complex interregulation of sphingolipid metabolism *J Lipid Res* **52**, 68-77
- 50 Min, J., Mesika, A., Sivaguru, M., Van Veldhoven, P. P., Alexander, H., Futerman, A. H. and Alexander, S. (2007) (Dihydro)ceramide synthase 1 regulated sensitivity to cisplatin is associated with the activation of p38 mitogen-activated protein kinase and is abrogated by sphingosine kinase 1 *Mol Cancer Res* **5**, 801-12
- 51 Bonzon-Kulichenko, E., Schwudke, D., Gallardo, N., Molto, E., Fernandez-Agullo, T., Shevchenko, A. and Andres, A. (2009) Central leptin regulates total ceramide content and sterol regulatory element binding protein-1C proteolytic maturation in rat white adipose tissue *Endocrinology* **150**, 169-78
- 52 Green, C. D., Ozguden-Akkoc, C. G., Wang, Y., Jump, D. B. and Olson, L. K. (2010) Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species *J Lipid Res* **51**, 1871-7
- 53 Green, C. D. and Olson, L. K. (2011) Modulation of palmitate-induced endoplasmic reticulum stress and apoptosis in pancreatic β -cells by stearoyl-CoA desaturase and Elovl6 *Am J Physiol Endocrinol Metab* **300**, E640-9
- 54 Koybasi, S., Senkal, C. E., Sundararaj, K., Spassieva, S., Bielawski, J., Osta, W., Day, T. A., Jiang, J. C., Jazwinski, S. M., Hannun, Y. A., Obeid, L. M. and Ogretmen, B. (2004) Defects in cell growth regulation by C18:0-ceramide and longevity assurance gene 1 in human head and neck squamous cell carcinomas *J Biol Chem* **279**, 44311-9
- 55 Thomas, R. L., Jr., Matsko, C. M., Lotze, M. T. and Amoscato, A. A. (1999) Mass spectrometric identification of increased C16 ceramide levels during apoptosis *J Biol Chem* **274**, 30580-8

Figure Legends

Fig. 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-keto-dihydro-sphingosine to dihydro-sphingosine. The latter compound is subsequently N-acylated by a family of ceramide synthases to form dihydro-ceramides. Finally, a trans double bond at C4-C5 is introduced by dihydro-ceramide desaturase to produce ceramides. Ceramides are then utilized for 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) of glucose. Ceramide levels were measured by the diacylglycerol kinase assay and expressed as a percent of ceramide levels at 5 mM glucose. Data are means \pm SEM of 3-4 independent experiments performed in duplicate. * G30 vs G5 and G10 vs G5, $p < 0.05$. ** G10 plus palmitate vs G10 and G30 plus palmitate vs G30, $p < 0.05$.

Fig. 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 measurement as described in Materials and Methods. Values are expressed as a fold increase over 5 mM glucose treatment. Data are means \pm SEM of 3 independent experiments. * G30 vs G5 and G10 vs G5, Palmitate vs None, $p < 0.05$. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.

Fig. 3. Palmitate and high concentrations of glucose increase dihydro-ceramide production 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 total (A and C) and N-acyl chain length of dihydro-ceramide (B and D) were determined by LC-MS/MS measurement as described in Materials and Methods. Values are expressed as fmol/nmol of PL (A and C) or as a fold increase over 5 mM glucose treatment (B and D). Data are means \pm SEM of 3 independent experiments. * G30 vs G5 and G10 vs G5, Palmitate vs None, $p < 0.05$. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.

Fig. 4. Palmitate and high concentrations of glucose increase dihydro-sphingosine production in INS-1 cells. Cells were incubated with 0.4 mM palmitate in the presence of 5 mM (G5) or 30 mM (G30) of glucose for 12 h. Levels of dihydro-sphingosine (A) and sphingosine (B) were determined by LC-MS/MS measurement as described in Materials and Methods. Values are expressed as fmol/nmol of PL. Data are means \pm SEM of 3 independent experiments. * G5+ palmitate vs G5 and G30 + palmitate vs G30, $p < 0.05$. ** G30 + palmitate vs G5, $p < 0.05$.

Fig. 5. High concentrations of glucose potentiate dihydro-ceramide 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 measurement as described in Materials and Methods. Values are expressed as a fold increase over 5 mM glucose treatment. Data are means \pm SEM of 3 independent experiments. * G5 + palmitate vs G5 and G30 + palmitate vs G5, $p < 0.05$. ** G30 + palmitate vs G5 + palmitate, $p < 0.05$. G5, 5 mM glucose, G5P4, 5 mM glucose + 0.4 mM palmitate, G30, 30 mM glucose, G30P4, 30 mM glucose + 0.4 mM palmitate.

Fig. 6. Gluco-lipotoxicity 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 the Apo-ONE Homogeneous Caspase-3/7 Assay kit, respectively. Values are expressed as a percent of living cells at 5 mM glucose for cell viability and as arbitrary units (AU) for caspase activity. Data are mean \pm SEM of 4-5 independent experiments performed in triplicate. * G30 vs G5 and G10 vs G5, $p < 0.05$. ** G10 + palmitate vs G10 and G30 + palmitate vs G30, $p < 0.05$. Cells were pre-incubated without or with 25 μ M FB1 (C) or 10 μ M 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. Data are means \pm SEM of 3 independent experiments performed in triplicate. * G5 + PPMP vs G5, G30 + PPMP vs G30 and G30 + palmitate vs G30, $p < 0.05$. ** G30 + palmitate + FB1 vs G30 + palmitate, G5 + palmitate + PPMP vs G5 + palmitate and G30 + palmitate + PPMP vs G30 + palmitate, $p < 0.05$. G5, 5 mM glucose; G10, 10 mM glucose; G30, 30 mM glucose.

Fig. 7. Fumonisin B1 and PPMP inversely regulate ceramide accumulation induced by gluco-lipotoxicity in INS-1 cells. Cells were pre-incubated or not with 25 μ M FB1 (A) or 10 μ M 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 diacylglycerol kinase assay and expressed as a percent of ceramide levels at 5 mM glucose. Data are means \pm SEM of 3 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 measurement as described in Materials and Methods. Values are expressed as a fold increase over 30 mM glucose treatment (B and D). * G30 + palmitate vs G30 and G30 + PPMP vs G30, $p < 0.05$. ** G30 + PPMP vs G30 and G30 + palmitate + PPMP or FB1 vs G30 + palmitate, $p < 0.05$. G30, 30 mM glucose, G30P4, 30 mM glucose + 0.4 mM palmitate.

Fig. 8. Palmitate with high glucose levels induces ceramide synthase 4 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 synthases (CerS) mRNA expression was determined after 12 h of treatment by quantitative real-time PCR. Data are means \pm SEM of 3 independent experiments performed in duplicate. * G30 + palmitate vs G30, $p < 0.05$. (C) CerS4 protein levels were determined by western-blot using an anti-CerS4 antibody.

Fig. 9 : Over-expression of ceramide synthase 4 enhances ceramide production induced by gluco-lipotoxicity in INS-1 cells. Cells were transiently transfected with the empty vector alone or the HA-tagged CerS4 pcDNA5-TO construct. (A) Over-expressed CerS4 was determined by western blot using an anti-HA or an 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 diacylglycerol kinase assay and LC-MS/MS measurement, respectively. Values are expressed as expressed as fmol of ceramide by nmol of PL (B) and a fold increase over 30 mM glucose treatment (C). Data are means \pm SEM of 3 independent experiments. * G30 + fatty acids vs G30 in vector cells, $p < 0.05$; ** G30 + fatty acids in CerS4 cells vs G30 + fatty acids in vector cells, $p < 0.05$. G30, 30 mM glucose; G30P4, 30 mM glucose + 0.4 mM palmitate; G30St, 30 mM glucose + stearate.

Fig. 10 : Modulation of ceramide synthase 4 expression regulates caspase-3/7 activation induced by gluco-lipotoxicity in INS-1 cells. Cells were transiently transfected with the

empty vector alone or the HA-tagged CerS4 pcDNA5-TO 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 were determined the Apo-ONE Homogeneous Caspase-3/7 Assay kit and expressed as arbitrary units (AU). Data are means \pm SEM of 3 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. Data are means \pm SEM of 3 independent experiments. * G5 + palmitate vs G5 in vector and CerS4 cells and G30 + palmitate vs G5 + palmitate in vector cells, $p < 0.05$; ** G30 + palmitate in CerS4 cells vs G30 + palmitate in vector cells, $p < 0.05$. 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 1

A

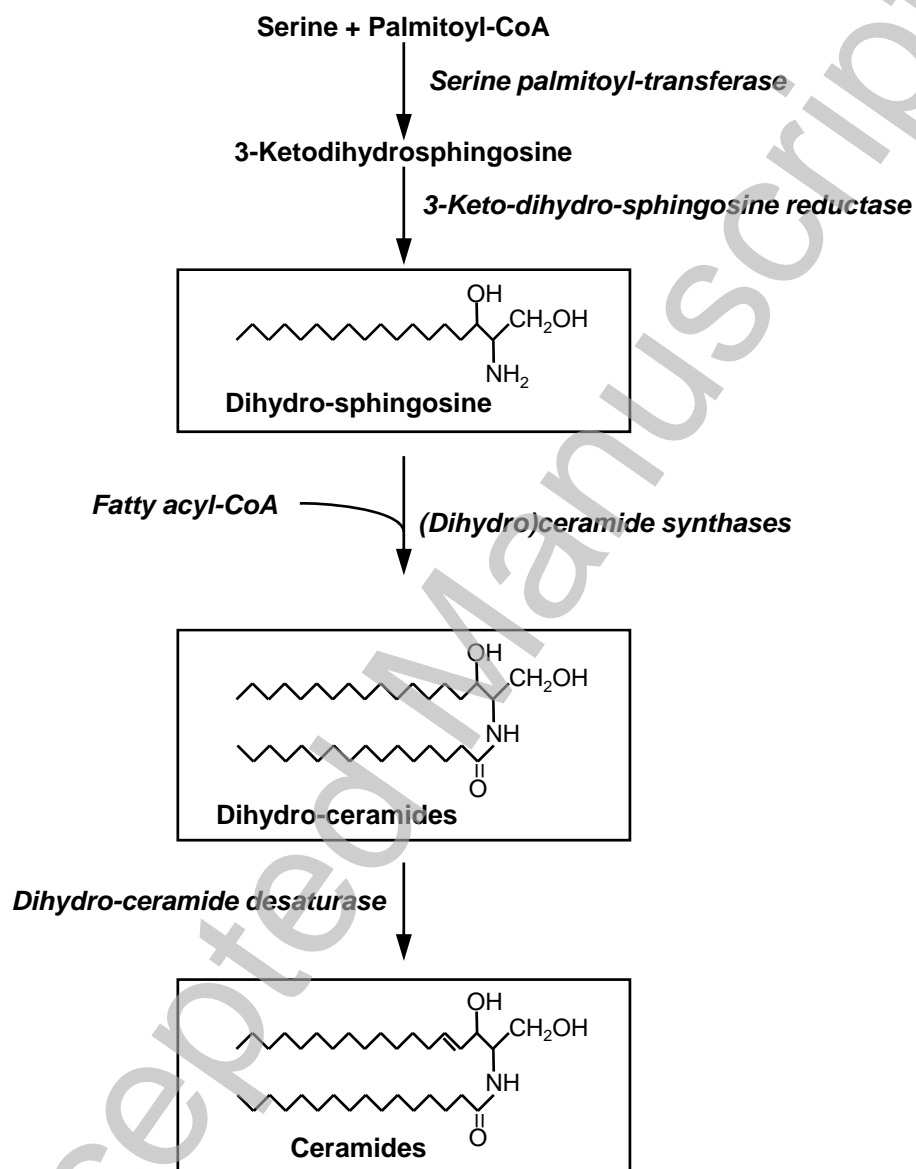


Figure 1

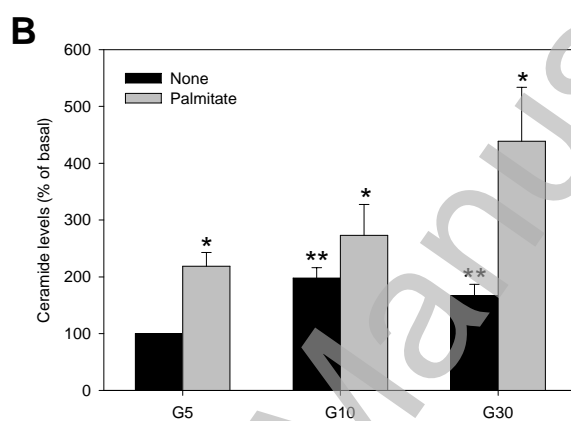
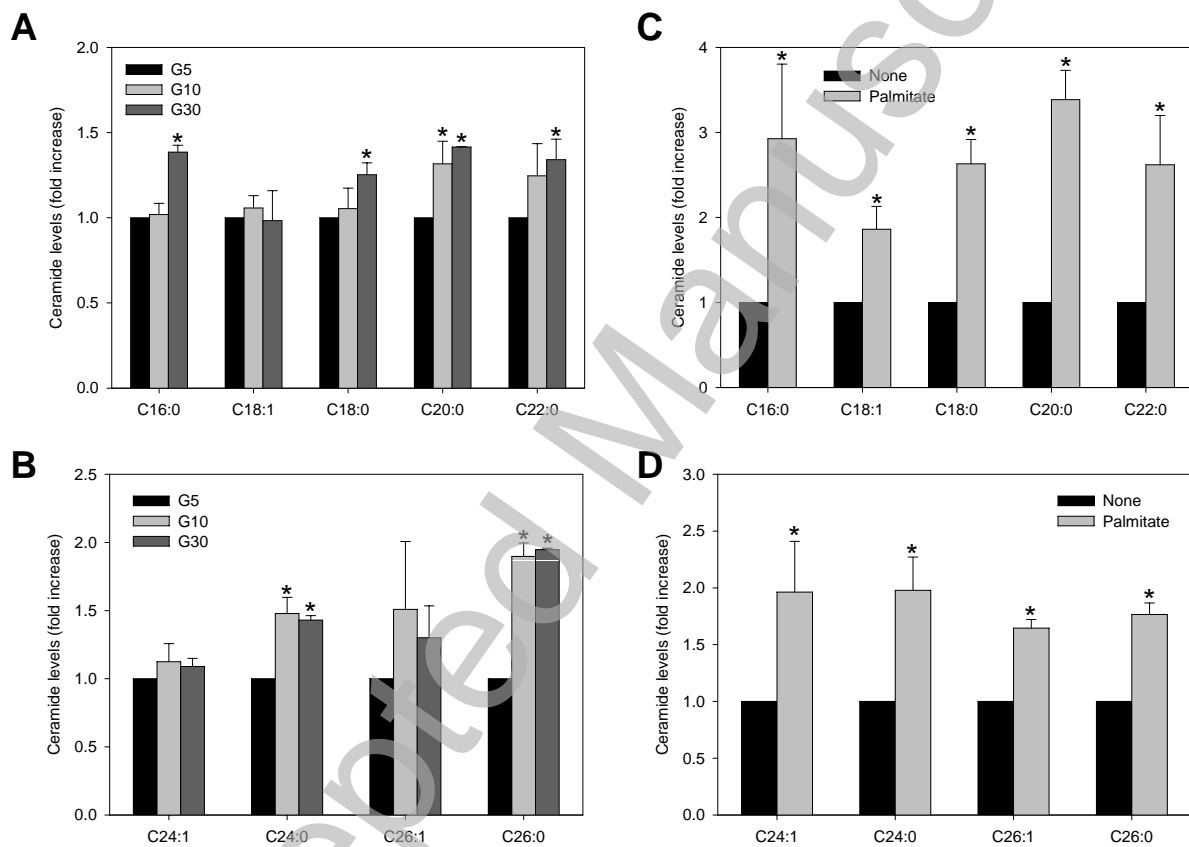


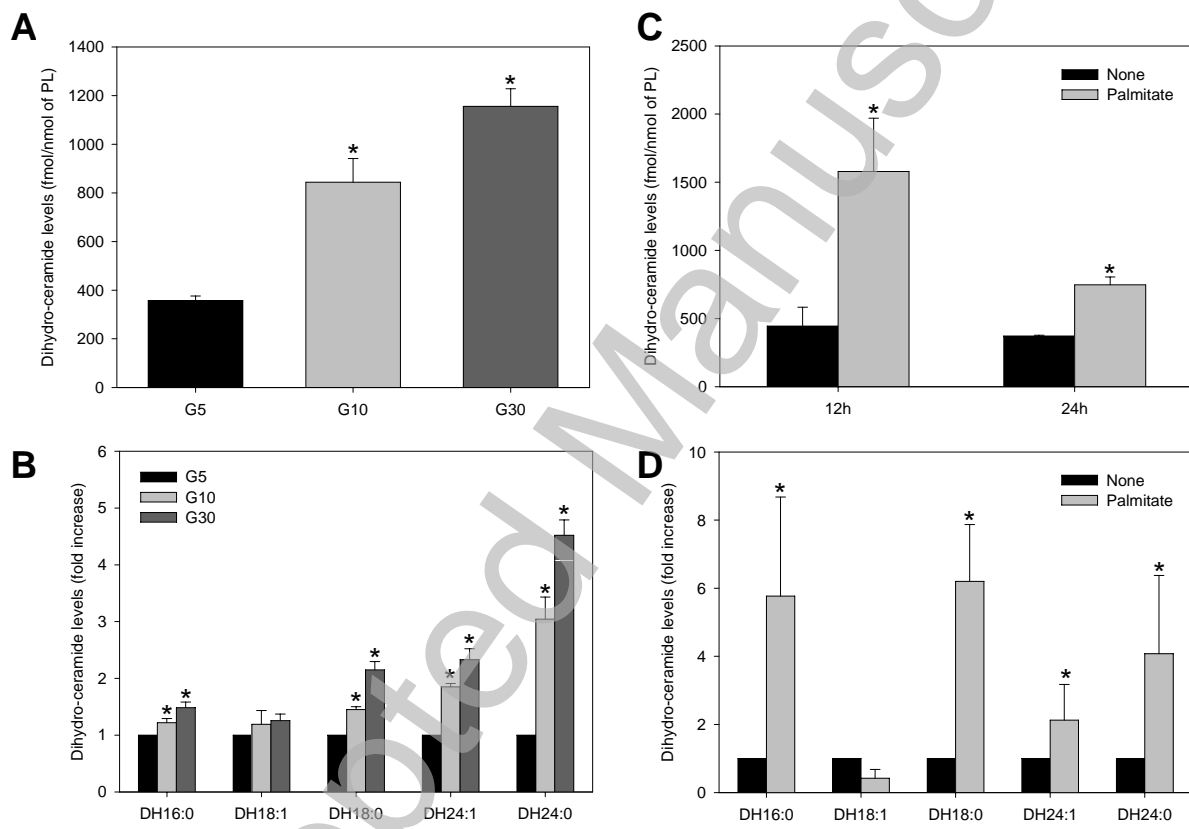
Figure 2



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Figure 3



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Figure 4

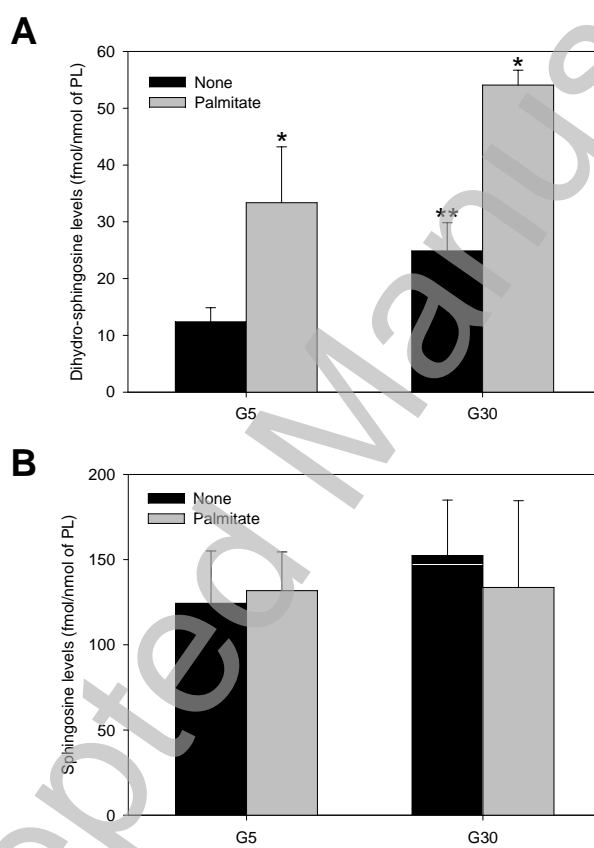
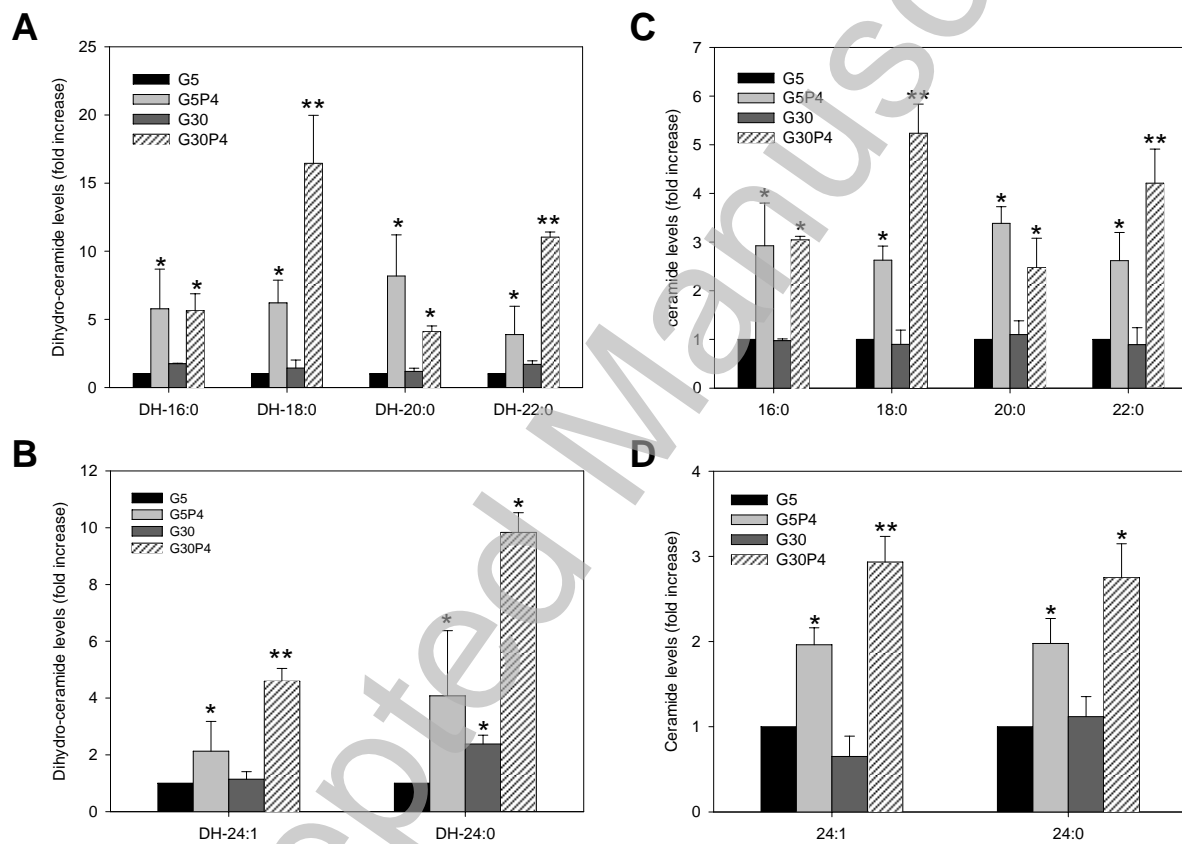
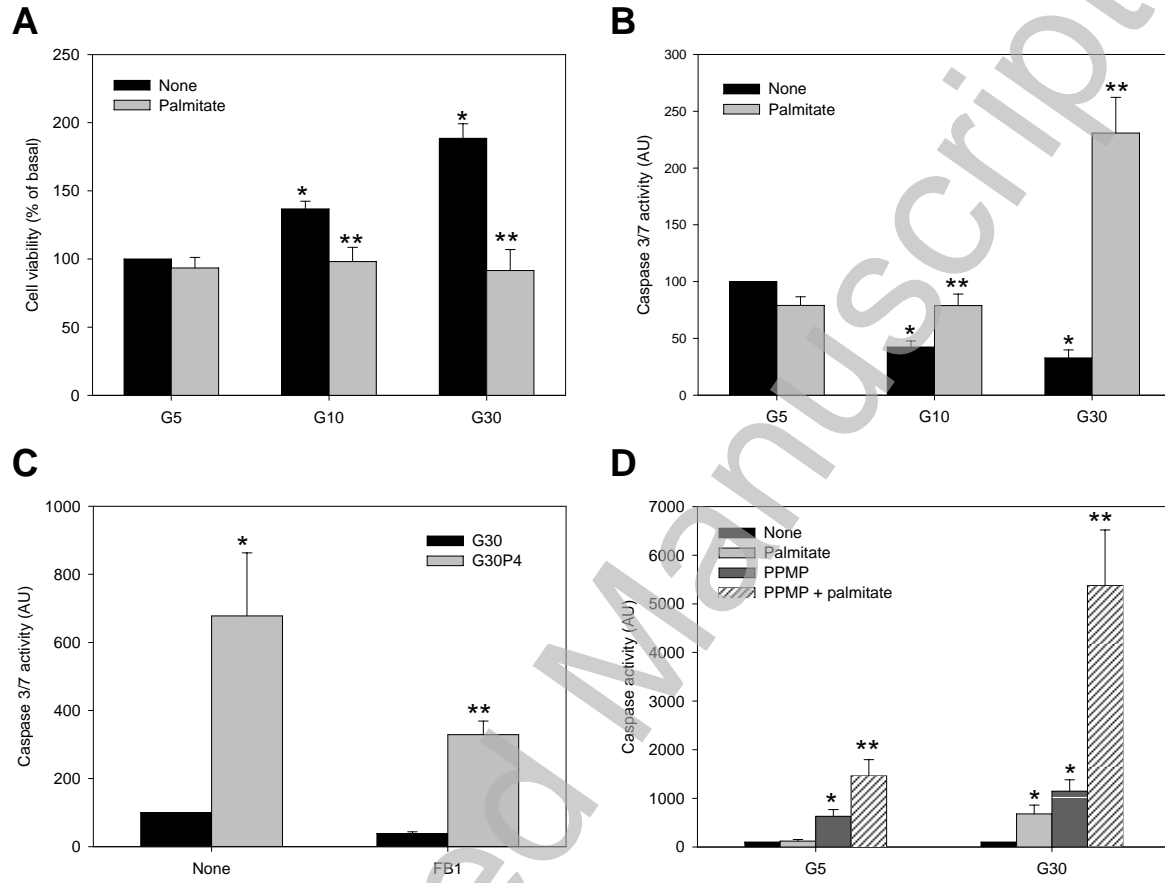


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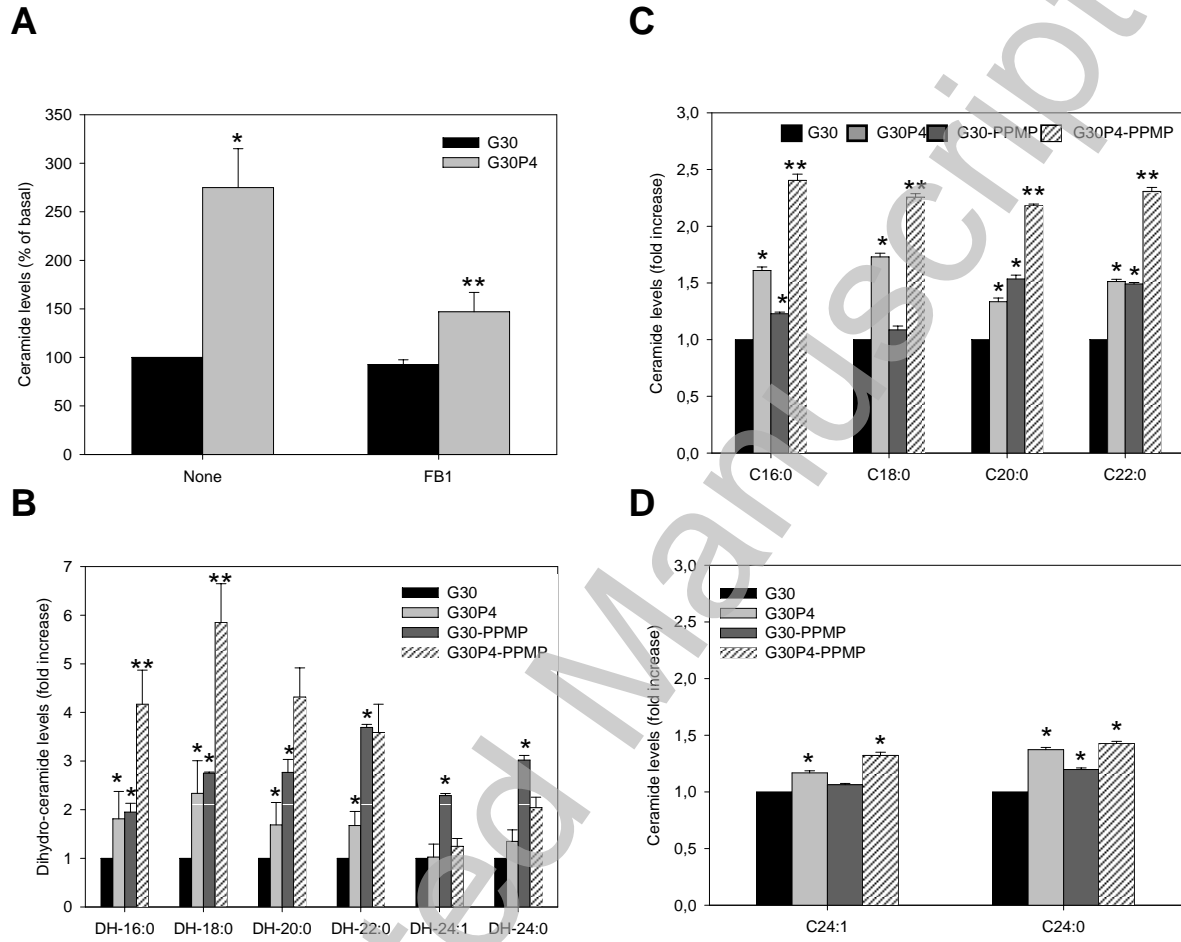
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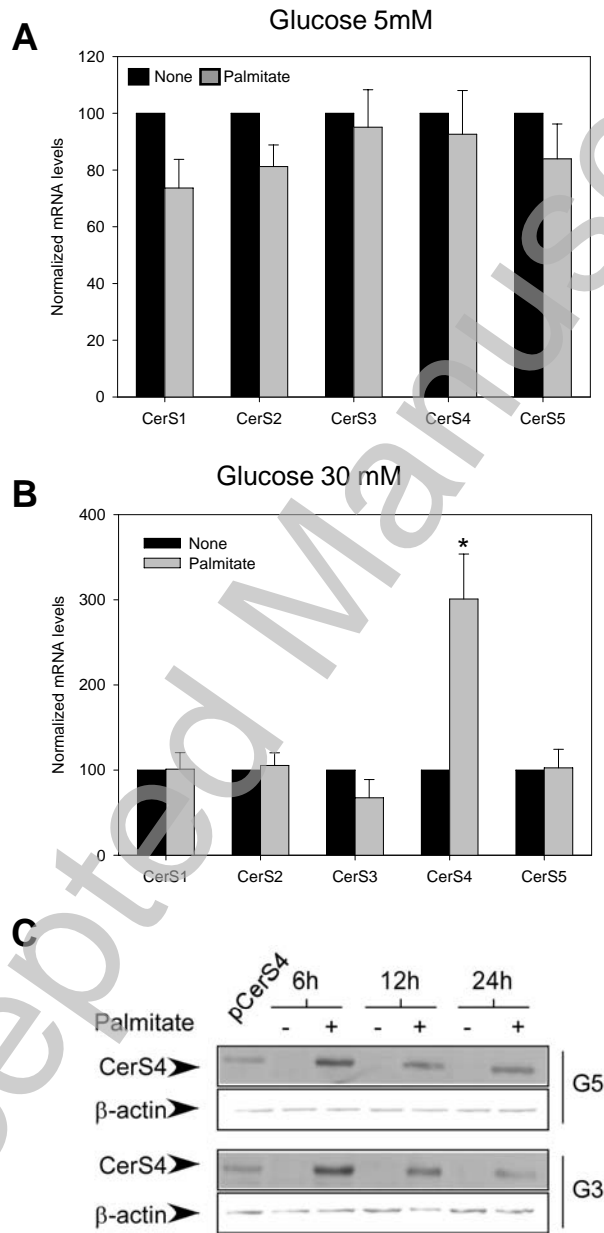
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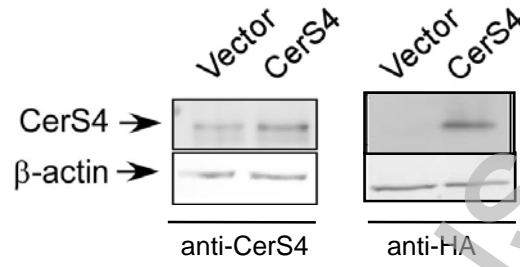
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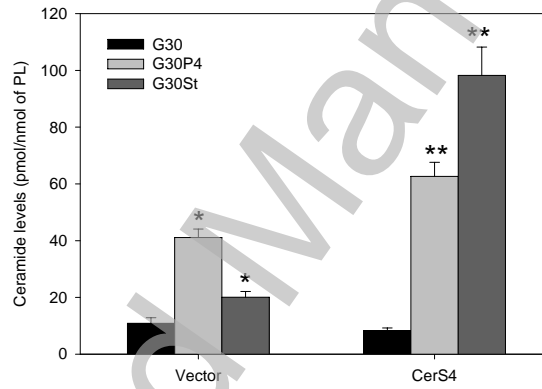
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Figure 9

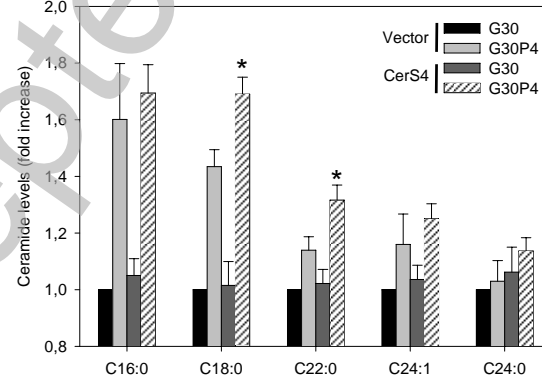
A



B

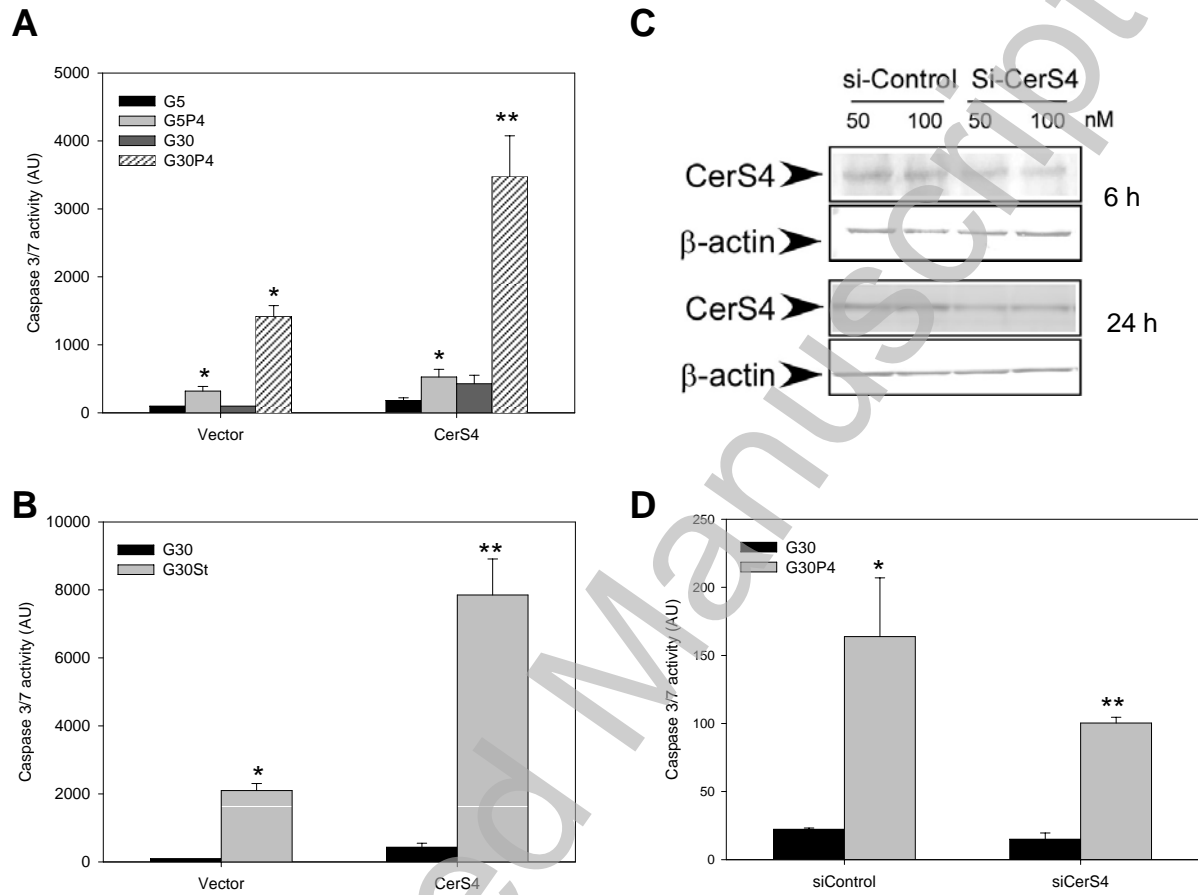


C



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Figure 10



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