Palmitate-induced impairments of beta-cell function are linked with generation of specific ceramide species via acylation of sphingosine

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Prolonged exposure to palmitate impairs beta-cell function and mass. One of the proposed mechanisms is alteration in ceramide generation. In the present study, exposure to palmitate induced the level of palmitoyl transferase and ceramide synthases, enzymes of the ceramide *de novo* and salvage pathways, and doubled total ceramide levels, which was associated with decreased insulin secretion and augmented apoptosis in MIN6 cells and human islets. By inhibiting enzymes of the pathways pharmacologically with ISP-1 or fumonisin B1 or by siRNA we showed that Cer(14:0), Cer(16:0), Cer(20:1) and Cer(24:0) species, generated by the salvage pathway, are linked to the harmful effect of palmitate on beta-cells. Oleate attenuates negative effects of palmitate on beta-cells. When oleate was included during culture of MIN6 cells with palmitate the palmitate-induced up-regulation of the enzymes of the *de novo* and salvage pathways was prevented resulting in normalized levels of all ceramide species except Cer(20:1). Our data suggest that enhanced ceramide generation in response to elevated palmitate levels involves both *de novo* and salvage pathways. However, the negative effects of palmitate on beta-cells are attributed to generation of ceramide species Cer(14:0), Cer(16:0) and Cer(24:0) via acylation of sphingosine.

Meal-related rises in circulating free fatty acid (FFA) levels promote insulin release from the insulin-producing β-cell (1). In contrast, prolonged elevated FFA levels lead to β-cell functional impairment and eventual decline in insulin secretion (2, 3). In addition to exposure time, the degree of saturation of the fatty acids plays a role. Thus, saturated fatty acids exert more negative effects on the β-cell than unsaturated fatty acids (4–6). Among the saturated fatty acids palmitate is one of the most prevalent in the circulation and has been linked to functional impairment of the β-cell via several mechanisms (7, 8). One of the proposed mechanisms is formation and accumulation of ceramide (Cer) (4, 9, 10). However, the reports concerning the role of Cer metabolism in fatty acid-induced effects on β-cells are still contradictory. In one study, exposure of insulin-producing MIN6 cells to palmitate caused no changes in the levels of ceramide (11). However, it increased the level of glucosylceramide, the product of Cer glycosylation. In another study, palmitate treatment of insulin-producing INS-1 cells increased the level of dihydroceramide, the precursor of Cer (12). Studies on Zucker diabetic fatty (ZDF) rat, an animal model of obesity-induced type 2 diabetes mellitus (T2DM) with elevated levels of FFAs, showed that their islets contain elevated levels of Cer (13, 14).

Exposure to palmitate leads to the generation of Cer via two pathways, de novo synthesis and acylation of sphinganine. De novo synthesis starts with condensation of palmitate and L-serine, a reaction catalyzed by serine palmitoyl transferase (SPT) (15). This step and the subsequent reaction catalyzed by 3-ketodehydrosphinganine reductase (Kdsr) lead to the formation of dihydrosphin-

Abbreviations:

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gosine (sphinganine). Sphinganine generated by the de novo pathway can later be acylated into dihydroceramide and subsequently reduced to Cer. Cer can also be formed by direct acylation (salvage) of sphingosine (the unsaturated sphinganine) (16). Sphingosine is generated by degradation of complex sphingolipids and deacetylation of ceramide in endolysosomal system (17). Acylation of sphinganine and sphingosine is catalyzed by ceramide synthases (CerSs). There are six known isoforms of CerS (Lass1-Lass6) and each isoform has specificity for fatty acids with different chain length (18). Lass1 catalyzes formation of Cer with C18 and C26 acyl-CoAs; Lass2 and Lass3 form Cer with C18 and C24; Lass4 forms Cer with C18 and C20; Lass5 forms Cer with C16 and Lass6 forms Cer with C14 and C16. To what extent these different Cer species have different biological effects have not been examined, however.

The role of the de novo synthesis and salvage pathways in palmitate-induced effects on β-cells has been addressed in a number of studies. In human and mouse islets, it has been demonstrated that the SPT inhibitor myriocin (ISP-1) and the nonselective inhibitor of CerSs fumonisin B1 (FB1) protect β-cells from palmitate-induced apoptosis (19, 20). FB1 has also been shown to ameliorate FFA-induced β-cell apoptosis in ZDF rat islets (21). Interestingly, in vivo administration of ISP-1 to mice on a high fat diet markedly improved insulin sensitivity in muscle tissue (22).

Here, we aimed to define the contribution of the de novo and salvage pathways of Cer synthesis in the negative effects of palmitate on β-cells and identify Cer species, whose formation is detrimental to β-cells exposed to palmitate. Also, we addressed if the protective effect of oleate on palmitate-exposed β-cells involves changes in Cer metabolism.

Materials and Methods

Culture and treatment of MIN6 cells and human islets

Mouse MIN6 insulinoma cells were cultured (passage 20–28) in DMEM supplemented with 15% FBS, 100 U/ml of penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol at 37°C and 5% CO_2. Culture medium and supplements were purchased from Invitrogen (Carlsbad, CA). Cells cultured to 60%-70% confluence were treated with 0.25 mM palmitate or 0.25 mM oleate (Sigma, St. Louis, MO) for 48 hours. Fatty acids were complex with 0.5% BSA (Boehringer Mannheim GmbH, Mannheim, Germany) in culture medium for 30 minutes. When fatty acids were combined 1% BSA was used. In addition, control and palmitate-exposed MIN6 cells were treated with 10 μM inhibitor of SPT, ISP-1 (Enzo Life Sciences, Farmingdale, NY) or 10 μM nonselective inhibitor of CerSs, FB1 (Sigma). MIN6 cells were also treated with 50 or 100 μM ISP-1 or FB1.

Human islets were obtained from the Islet Transplantation Unit at Uppsala University from nondiabetic individuals. Human islets were cultured in CMRL 1066 medium containing 5.5 mM glucose and supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. Ethical permission to use human islets isolated from healthy individuals have been obtained from the Regional Ethical Review Board in Uppsala (date: 2010–02-10; number 2010/006). Human islets were treated with 0.5 mM palmitate for 7 days in the absence or presence of 20 μM ISP-1 or FB1. Human islets were also treated with 50 or 100 μM ISP-1 or FB1.

Measurement of glucose-stimulated insulin secretion

MIN6 cells or human islets were initially incubated for 60 minutes in culture medium containing 2 mM glucose. Subsequently, cells were incubated for another 60 minutes in KRBH buffer consisting of (mM): glucose 2, NaCl 130, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 5.0, and HEPES 10, titrated to pH 7.4 with NaOH and supplemented with 1 mg/ml of BSA. The buffer was changed to KRBH with either 2 or 20 mM glucose and the incubation continued for another 30 minutes after which aliquots of the buffer were taken for later determination of released insulin. Cells or human islets were then washed in PBS. MIN6 cells were lysed in H_2O and used for measurements of DNA content. Total protein in human islet samples was measured by Lowry (23). Released insulin was determined with an ELISA as previously described (24).

Extraction of ceramides

Total Cer fraction was extracted essentially as described previously (25). In short, MIN6 cells were detached by scraping in PBS. The extraction solvent methanol/chloroform (2:1, v/v) mixture was added at 7.5 times volume of the PBS volume. Sphingolipid internal standard mixture (Avanti Polar Lipids, Alabaster, AL) was added to each sample in the initial step of lipid extraction. Subsequently, samples were sonicated and then incubated for 18 hours at 48°C. After incubation, samples were cooled to room temperature and chloroform/water (1:2, v/v) mixture was added at 3 times volume of the sample volume. Samples were mixed to avoid the formation of an emulsion and centrifuged at 2000 g for 5 minutes. The organic layer was carefully transferred to new tube and dried under nitrogen stream. Samples were resuspended in the mobile phase.

Analysis of ceramide species

Cer analysis was performed by LC-MS/MS using Agilent 1100 LC system (Agilent, Sweden) and Kinetex C18 (2.1 × 100 mm x 2.6 μm) LC column (Phenomenex, Torrance, CA). Ceramides were identified by ABI 3200 Q trap (linear) mass spectrometer (Applied Biosystems, CA) with positive ESI mode. Standards of Cer (Avanti Polar Lipids) were used at a final concentration of 1 μg/μl. Samples (5 μl) were injected and separated by gradient chromatography at room temperature. The injected sample first entered into the precolumn followed by the analytical column and was then delivered to the MS/MS. Sample vials were kept at 6°C in a cooled auto sampler. The mobile phases consisted of 10 mM ammonium acetate, 0.1% formic acid in water (eluent A) and 10 mM ammonium acetate, 0.1% formic acid in acetonitrile/isopropanol (4:3, v/v) (eluent B). Gra-

dient elution was begun with 65% B (2 minutes), then raised to 75% in 0.5 minutes and followed to 100% in 15 minutes and held for 5 minutes. Equilibration was then reduced to 65% B in 0.1 minute and held there for 10 minutes before the injection of the next sample. The flow rate was set at 200 μl/min. To minimize contamination of the MS, the column flow was directed only from 1.0 to 4.0 minutes into the MS using a diverter valve. Analytes were monitored in the multiple reaction monitoring (MRM) mode and mass transitions were obtained from Bielawski et al (25).

Measurement of palmitate incorporation into ceramides

To measure palmitate incorporation into Cer, 2 μCi [^{3}H] palmitate (Perkin Elmer, Waltham, MA) per ml was added during culture of MIN6 cells. After treatment, lipids from cultured MIN6 cells were extracted as described above. Samples were resuspended in chloroform/methanol (2:1, v/v) and subsequently applied to TLC-plates made of silica gel 60 with concentrating zone 20 $\times$ 10 cm (Merck, Darmstadt, Germany). Chloroform/methanol/acetic acid (190:9:1, v/v/v) was used as mobile phase. Cer(18:0) and Cer(24:0) (Larodan Fine Chemicals) were used as standards. After drying, the plates were sprayed with a 10% copper sulfate and 8% phosphoric acid solution and were then charred by heating at 180°C for 15 minutes. Subsequently, Cer spots were identified and scraped into vials with chloroform/methanol (2:1, v/v) solution. Scintillation fluid (10 ml; Ultima-Gold™, Chemical Instruments AB, Sollentuna, Sweden) was added to extracted Cer and radioactivity determined by a liquid-scintillation spectrometer (Wallac System 1400™ PerkinElmer, Boston, MA).

Down-regulation of Kdsr, Lass5 and Lass6 in MIN6 cells

Knockdown of genes was performed using siRNAs (Qiagen, Valencia, CA) with the following targeting sequences: Kdsr - AAGCACTCTATTAATGACAAA; Lass5 – CTGAGGAGAATCAAAGAAATA and Lass6 – CCCGAAGAACTCACTGCAATA. Control cells were transfected with mock siRNA (Qiagen). Transfection was performed in 24-well plates by adding 100 000 cells, 50 nM siRNA and 2 μl FuGENE HD (Roche NimbleGen, Madison, WI) to 200 μl Opti-MEM (Invitrogen) according to manufacturer's instructions. After 24 hours, cells were treated or not with 0.25 mM palmitate for 48 hours. The validation of knockdown was performed using RT-qPCR as described below.

Measurement of mRNA expression

After culture, total RNA was isolated from MIN6 cells or human islets using Trizol (Invitrogen) according to the manufacturer's instructions and reverse transcribed with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) using Oligo-dT primers. The real-time PCR was performed in 10 μl volume containing ~20 ng RNA equivalent, 0.5 μM forward and reverse primers and 5 μl Dynamo Capillary SYBR green qPCR kit (Finnzymes, Espoo, Finland). Primers were checked for specificity with the Basic Local Alignment Search Tool (BLAST). Primers used for the amplifications are shown in Table 1. PCR products were quantified fluorometrically using SYBR Green, and normalized to the housekeeping gene actin and relative to the control according to the following formula: target amount = 2-$\Delta\Delta$Ct, where $\Delta\Delta$Ct={[Ct (target gene) - Ct (β-actin)] - [Ct (control) - Ct (β-actin control)]}. The cycle number range was 20–28 for expressed genes. Specificity of products was checked on the gel.

Measurements of protein levels by western blot

Western blotting was performed as previously described (26). PVDF membranes were incubated with primary antibodies towards Lass5, Lass6 (Abcam, Cambridge, MA), cleaved caspase-3 and GAPDH proteins (Cell Signaling, MA). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences GE, Little Chalfont, UK) and imaged with ChemiDoc XRS+ (Bio-Rad, Hercules, CA). Signals were quantified with Image Lab 4.0.1 (Bio-Rad).

Measurement of apoptosis

Apoptosis in MIN6 cells was measured by cleaved caspase-3 levels (see above) and by using Cell Death Detection kit ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The ELISA measures cytoplasmic oligonucleosomes that increase after apoptosis-associated DNA degradation. Apoptosis was normalized to protein content.

Statistical analysis

All values are expressed as means $\pm$ SD. Student's t test and one-way ANOVA with Bonferonni's post hoc test was used for statistical analysis. Statistical significance was set to $P < .05$.

Table 1. Primers for enzymes of sphingolipid metabolism used for real-time PCR

Gene	5′(sense) primer	3′(antisense) primer	Gene accession number
Actb	GTTACAGGAAGTCCCTCACC	GGAGACCAAAGCCTTCATAC	NM_007393
Sptlc1	TGCCAGAACATCCATAAGTC	AGGCACTTCTCTTCCTTGTC	NM_009269
Sptlc2	ATACAGCAGTTGGCTGAGAA	CTTTGGTATGAGCTGCTGAC	NM_011479
Sptlc3	GCCAGCACCAGAAATGAAAT	GCACCTGAGAGTCGAGATCC	NM_175467
Kdsr	AGAGGGTCAGGTTTGGAGGT	TCATCTGAGGCACTTGTTGC	NM_027534
Lass1	CGTAAGGACTCGGTGGTCAT	CTGACGTCATGCAGGAAGAA	NM_138647
Lass2	CAGGCCAAGAAAGAAAGCTG	CGCCGAAATATCAGGTCCTA	NM_029789
Lass3	CTCTGGGAGGTTTGGAATGA	CAGGTGGTGGATGACATGAG	DQ646881
Lass4	CAAGGACCAATAAAACGTCA	CTGAGGAGCAGGGAGTATCT	NM_026058
Lass5	CTCTTTGAAAGCTGGGAGAT	TTGTTTTTGTGGGTTGTCTC	NM_028015
Lass6	ACTGGAGAATAGCAGCAACA	GTCTAGTCCTCGGCTCATCT	NM_172856

Results

De novo and salvage pathways are involved in the generation of ceramide in palmitate-exposed MIN6 cells

First, we examined if palmitate altered the transcript and/or protein levels of key enzymes involved in the de novo and salvage pathways of Cer generation. The study was performed in MIN6 cells in order to measure Cer level specifically in β-cells.

The initial steps of the de novo pathway were examined by measuring the rate-limiting enzyme SPT, which was expressed in the β-cell in two isoforms, Sptlc1 and Sptlc2. Palmitate exposure caused 30% increase in Sptlc2 but no changes in Sptlc1 mRNA level (Figure 1A). The next en-

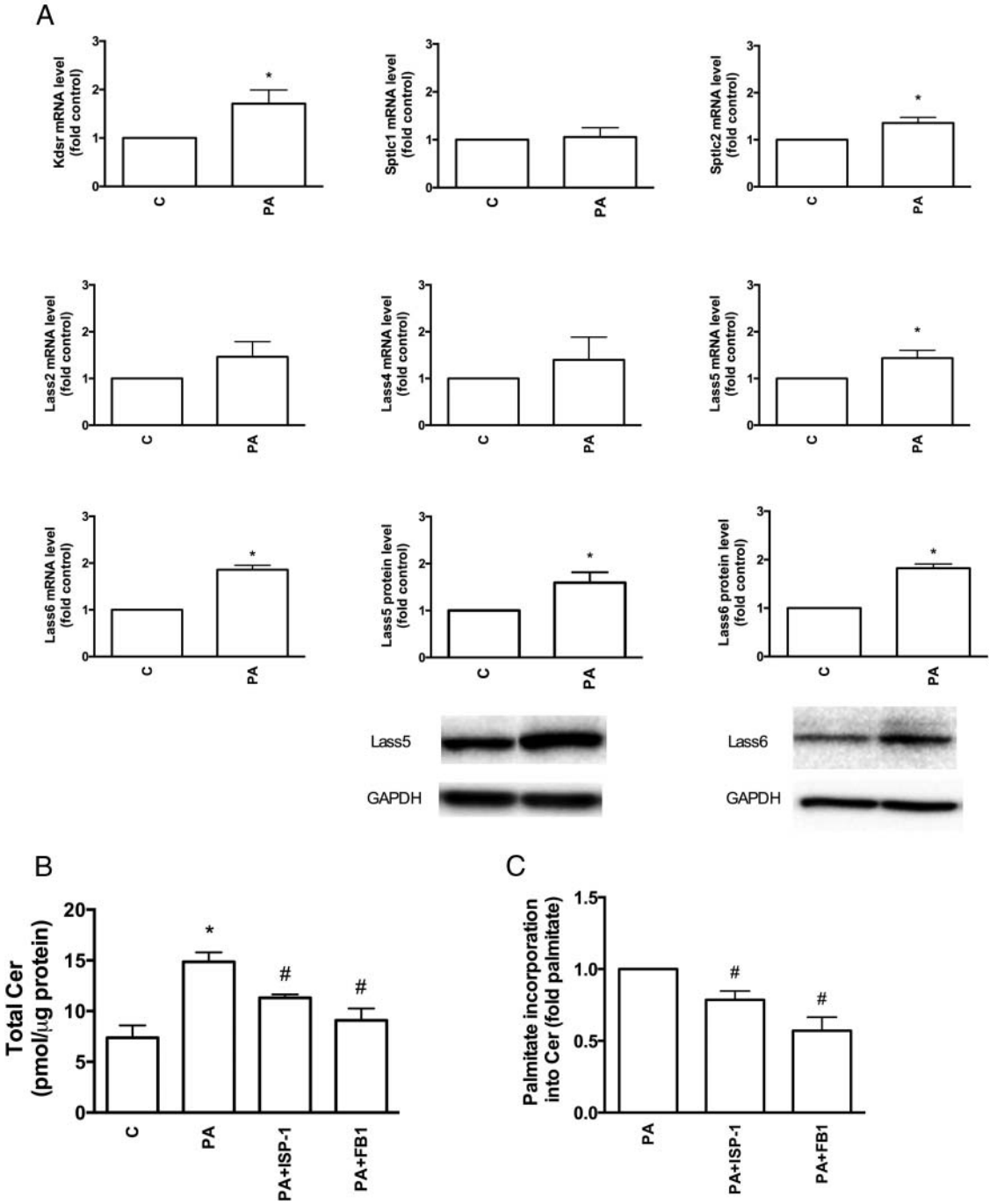

Figure 1. mRNA and/or protein levels of enzymes of de novo Cer generation and CerSs (panel A), total Cer levels (panel B) and incorporation of external palmitate into the total Cer fraction (panel C) in MIN6 cells cultured in the absence (C) or presence of palmitate (PA) for 48 hours. Results are means ± SD for 4 or 5 experiments. *$P < .05$ compared to control cells, #$P < .05$ compared to cells cultured in the presence of palmitate alone.

zyme in the de novo pathway is 3-ketodihydrosphingosine reductase (Kdsr). Kdsr mRNA level was increased by 70% after 48 hours exposure to palmitate (Figure 1A).

CerSs are involved in Cer generation both via the de novo and the salvage pathways. Six isoforms have been described (18, 27). We found that four isoforms, Lass2, Lass4, Lass5 and Lass6, were expressed in MIN6 cells. When cells were exposed to palmitate, mRNA levels of Lass2 and Lass4 showed only a tendency to rise, whereas mRNA levels of Lass5 and Lass6 increased significantly by 50 and 80%, respectively (Figure 1A). Lass5 and Lass6 use palmitate as a substrate and were analyzed also at the protein level (27). Protein levels of these enzymes were also increased by 50 and 80%, respectively (Figure 1A).

Next, we examined to what extent the de novo and salvage pathways contributed to the generation of Cer in palmitate-exposed MIN6 cells. In control MIN6 cells total Cer levels were 7.4 ± 1.2 pmol/μg protein (Figure 1B). When MIN6 cells were cultured in the presence of palmitate for 48 hours, total Cer levels rose 2-fold to 14.9 ± 0.9 pmol/μg protein. Inhibition of the early steps of the de novo pathway with ISP-1 curtailed palmitate-induced rise in Cer levels to approximately 50%. Inhibition of the later steps of the de novo and salvage pathways with FB1 reduced Cer levels in palmitate-exposed MIN6 cells to those observed in control cells. In line with this, incorporation of externally applied palmitate into Cer during 48 hours was reduced by 21% in the presence of ISP-1 and by 43% in the presence of FB1 (Figure 1C). Our results suggest about equal contribution of the de novo and sphingosine salvage pathway to Cer generation in MIN6 cells exposed to elevated levels of palmitate.

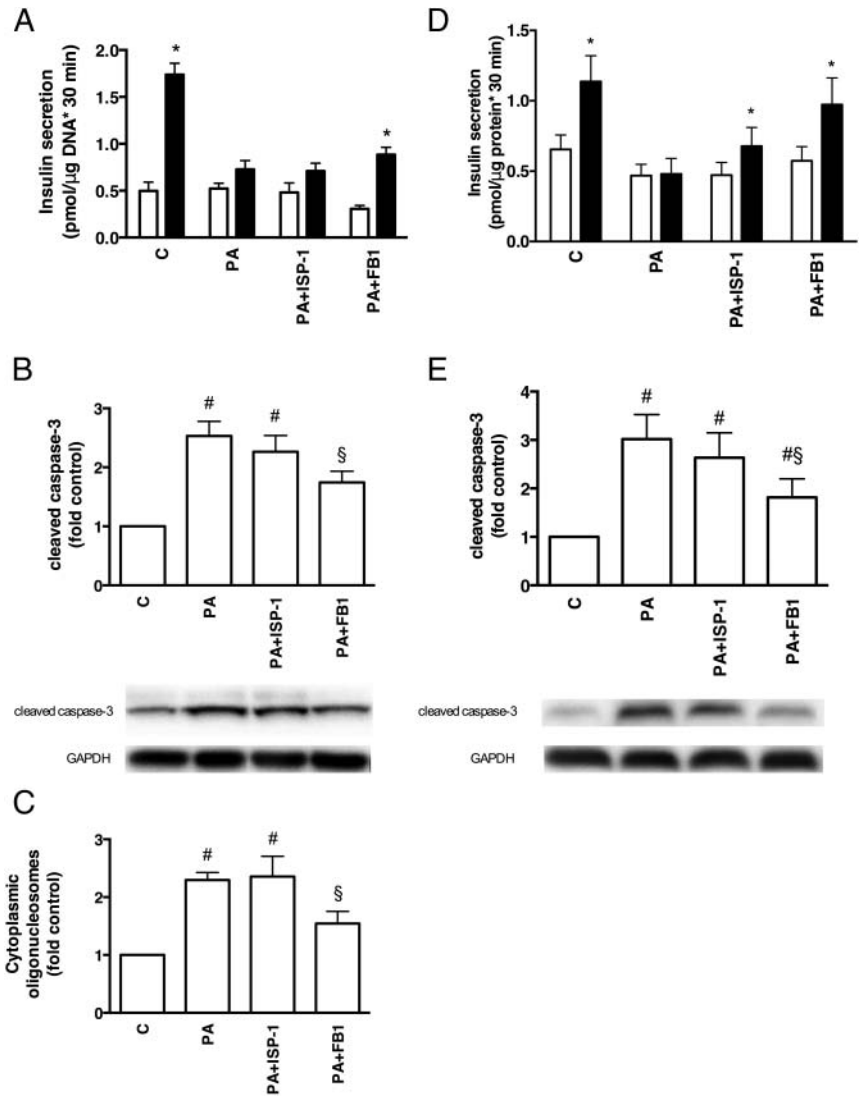

Figure 2. Glucose-stimulated insulin secretion and apoptosis from MIN6 cells (panels A, B and C) and human islets (panels D and E) cultured in the absence (C) or presence of palmitate (PA) and inhibitors of Cer metabolism (ISP-1, FB1) for 48 hours for MIN6 cells and 7 days for human islets. Insulin release was measured at 2 (white bars) and 20 (black bars) mM glucose. Results are means ± SD for 3–5 experiments. *$P < .05$ compared to 2 mM glucose, #$P < .05$ compared to control, §$P < .05$ compared to cells cultured in the presence of palmitate alone.

Inhibition of the salvage pathway but not de novo synthesis of ceramide prevents long-term negative effects of palmitate on MIN6 cells and human islets

The role of the de novo and salvage pathways of Cer generation in the negative effects of palmitate on β-cells was addressed by exposing MIN6 cells to palmitate in the presence or absence of the SPT inhibitor ISP-1 or the nonselective CerSs inhibitor FB1. After 48-hour treatments GSIS and apoptosis were measured. In control MIN6 cells GSIS was 3.5-fold (Figure 2A). Palmitate exposure decreased insulin release at 20 mM glucose resulting in suppressed GSIS. Inclusion of ISP-1 during culture of MIN6 cells with palmitate showed no improvements in GSIS (Figure 2A and Supplemental Figure 1A). In contrast, administration of FB1 to the cells cultured with palmitate improved GSIS by lowering insulin release at 2 mM glucose and slightly increasing insulin release at 20 mM glucose (Figure 2A and Supplemental Figure 1A).

Apoptosis was determined by

measuring levels of cleaved caspase-3 and oligonucleosomes in the cytoplasm. Palmitate treatment increased both cleaved caspase-3 levels and the level of oligonucleosomes in the cytoplasm by about 2.5 times (Figure 2B and C). Inclusion of ISP-1 had no effect on palmitate-induced apoptosis (Figure 2B and C and Supplemental Figure 1B). Presence of FB1 reduced palmitate-induced apoptosis to 1.6-fold of control (Figure 2B and C). Noteworthy, high concentrations of FB1 were rather toxic than protective for MIN6 cells (Supplemental Figure 1B). GSIS and apoptosis were not affected when cells were treated with the compounds alone (results not shown).

In human islets, impaired GSIS and increased apoptosis were observed after 7-day treatment with 0.5 mM palmitate (Figure 2D and E) (28). When ISP-1 was included during culture, only slight changes in GSIS and no changes in apoptosis were detected irrespective of ISP-1 concentration (Figure 2D and E, Supplemental Figure 1C and D). Inclusion of FB1 during palmitate treatment improved both GSIS (Figure 2D and Supplemental Figure 1C) and apoptosis (Figure 2E and Supplemental Figure 1D).

The contribution of different pathways of Cer generation in the negative effects of palmitate on MIN6 cells was also addressed by siRNA approach. Whereas de novo synthesis of Cer was inhibited by knocking down Kdsr, the salvage pathway of Cer generation was inhibited by knocking down Lass5 and Lass6. mRNA levels of Kdsr, Lass5 and Lass6 were reduced by 40%, 35 and 42%, respectively (Figure 3A). Silencing of Kdsr did not improve impaired GSIS and increased apoptosis in β-cells exposed to palmitate, however (Figure 3B and C). In contrast, silencing of Lass5 and Lass6 improved GSIS reduced cleaved caspase-3 by 25% in the palmitate-exposed cells (Figure 3B and C).

Salvage pathway leads to generation of Cer(14:0), Cer(16:0), Cer(20:1) and Cer(24:0) in palmitate-exposed MIN6 cells

To address why the salvage pathway but not the de novo synthesis of Cer is deleterious upon palmitate treatment we determined Cer species in control and palmitate-treated MIN6 cells in the absence or presence of ISP-1 and FB1. Nine Cer species were identified. In control cells the two most abundant Cer species were Cer(16:0) and Cer(24:1) with 2.3 $\pm$ 0.3 and 2.2 $\pm$ 0.3 pmol/μg protein, respectively (Table 2). These two species accounted for more than 60% of the total Cer content. Two species, Cer(14:0) and Cer(20:1), were present at very low concentrations 0.04 $\pm$ 0.03 and 0.05 $\pm$ 0.03 pmol/μg protein, respectively and accounted for about 1% of the total Cer content.

When MIN6 cells were exposed to palmitate for 48 hours, the observed 2-fold increase in total Cer (Figure 1B) was accounted for by corresponding rise of most Cer species (Figure 4). Cer(16:0) was one of these species showing 2.1-fold rise. The most accentuated fold-changes were observed in Cer(14:0) and Cer(22:1), which increased more than 5-fold (Figure 4).

When ISP-1 was added to palmitate-treated MIN6 cells, Cer(20:0), Cer(22:0) and Cer(24:1) levels were normalized. The remaining six Cer species were not affected by the SPT inhibitor (Figure 4). When FB1 was added, Cer(14:0), Cer(16:0), Cer(20:0), Cer(20:1), Cer(22:0), Cer(24:0) and Cer(24:1) levels were normalized. The CerS inhibitor did not affect levels of Cer(18:0) and Cer(22:1) (Figure 4). Our results imply that in palmitate-treated MIN6 cells Cer(14:0), Cer(16:0), Cer(20:1) and Cer(24:0) are generated by salvage pathway but not de novo synthesis.

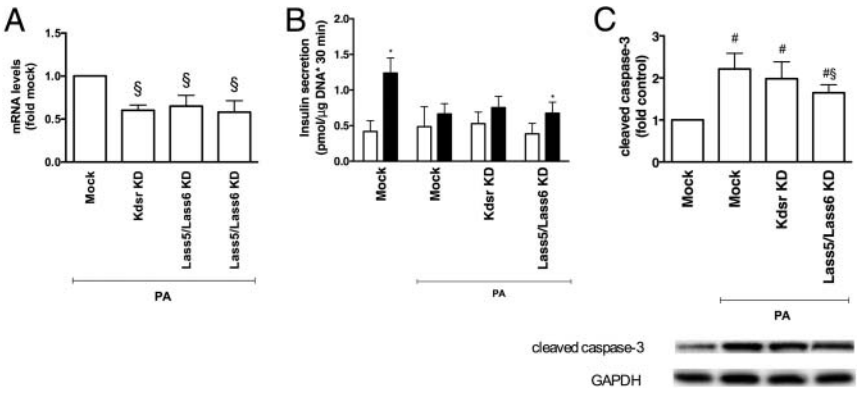

Figure 3. mRNA levels of Kdsr, Lass5 and Lass6 enzymes (panel A), glucose-stimulated insulin secretion (panel B) and cleaved caspase-3 (panel C) from MIN6 cells transfected with siRNA targeted towards Kdsr, Lass5 and Lass6 or with nonsense siRNA (mock) in the presence of palmitate for 48 hours. Insulin release was measured at 2 (white bars) and 20 (black bars) mM glucose. Results are means $\pm$ SD for 5 experiments. *$P < .05$ compared to 2 mM glucose, #$P < .05$ compared to mock-transfected control, §$P < .05$ compared to mock-transfected cells cultured in the presence of palmitate.

Improvement of β-cell function in palmitate-treated MIN6 cells by oleate involves reduction in ceramide generation via the de novo and salvage pathways

Unsaturated fatty acid oleate showed no harmful effects on MIN6 cells exposed to the fatty acid during 48 hours (Figure 5A and B). Moreover, oleate improved the impaired GSIS and elevated apoptosis observed in palmitate-treated cells (Figure 5A and B), which is in agreement with previous reports (4, 5). We decided to address if the protective effect of unsaturated fatty acid oleate on palmitate-exposed β-cells in-

Table 2. Ceramide species in MIN6 cells. MIN6 cells were cultured in the absence (control) or presence of 0.25 mM palmitate for 48 h. After culture, Cer species were extracted from the cells and quantified by LC-MS/MS. Results are means ± SD for 4 experiments. *$P <$ 0.05 compared to control cells

	Control		Palmitate	
	pmol/μg protein	% of total Cer	pmol/μg protein	Fold control
Cer(14:0)	0.04 ± 0.03	0.5	0.21 ± 0.10	5.3*
Cer(16:0)	2.34 ± 0.27	31.7	4.99 ± 0.21	2.1*
Cer(18:0)	0.53 ± 0.11	7.2	1.01 ± 0.06	1.9*
Cer(20:0)	0.49 ± 0.17	6.6	0.84 ± 0.03	1.7*
Cer(20:1)	0.05 ± 0.03	0.7	0.11 ± 0.01	2.2*
Cer(22:0)	1.13 ± 0.57	15.3	0.15 ± 0.32	1.9*
Cer(22:1)	0.13 ± 0.11	1.8	0.69 ± 0.12	5.3*
Cer(24:0)	0.50 ± 0.08	6.8	1.28 ± 0.32	2.6*
Cer(24:1)	2.18 ± 0.33	29.5	3.58 ± 0.27	1.6*
Total	7.37 ± 1.22	100	14.86 ± 0.94	2.0*

volved changes in Cer metabolism. To this end, we measured mRNA and/or protein level of enzymes involved in the generation of Cer and determined the amount of total Cer in MIN6 cells exposed to oleate alone or to the combination of palmitate and oleate. Oleate alone showed a tendency to reduce expression level of enzymes involved in the generation of Cer both via the de novo and salvage pathways (Figure 5C). When both fatty acids were combined expression levels of the genes were not different from control cells, ie, oleate normalized rise in expression levels induced by palmiate (Figure 5C). Total Cer amount in cells treated with oleate alone or with both fatty acids was not different from the amount in control cells (Figure 5D). Finally, we addressed how different Cer species were affected by oleate. None of the nine identified Cer species was different from control condition in the presence of oleate alone. In cells cultured in the presence of both oleate and palmitate all palmitate-induced changes were normalized but one, Cer(20:1), which remained elevated (Figure 6).

Discussion

Individuals with obesity and obesity-related T2DM have elevated levels of palmitate (29), which is detrimental for

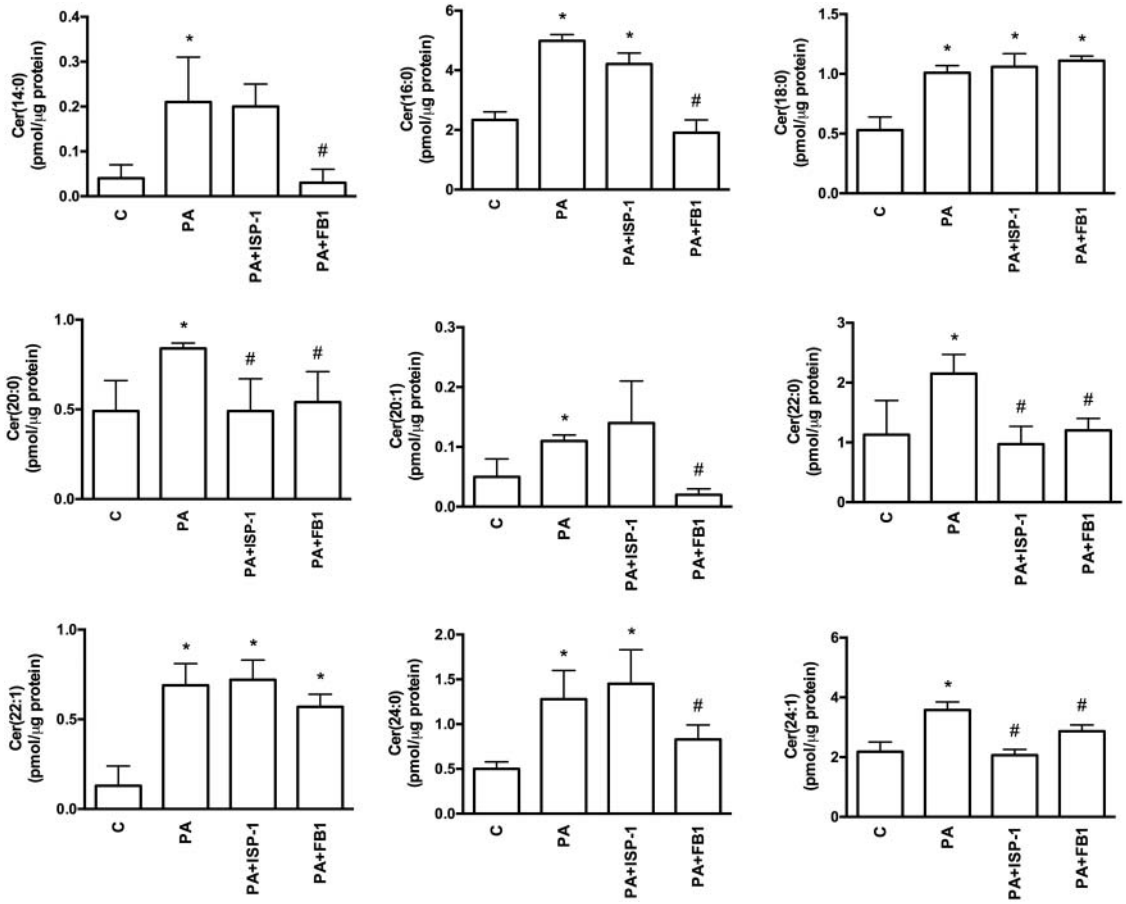

Figure 4. Levels of ceramide species in MIN6 cells cultured in the absence (C) or presence of palmitate (PA) and inhibitors of Cer metabolism (ISP-1, FB1) for 48 hours. Results are means ± SD for 4 experiments. *$P <$ .05 compared to control cells, #$P <$ .05 compared to cells cultured in the presence of palmitate alone.

β-cell function (7, 8). Alterations in β-cell function and apoptosis have been associated with elevated Cer levels since the fatty acid is a key building block in Cer synthesis (4, 7, 8, 23). Studies demonstrated that generation of Cer from palmitate may occur both via de novo synthesis and via direct interaction of palmitate with already existing sphingosine/sphinganine (16). Indeed, increased Cer levels by palmitate in insulin-producing cells have previously been observed in most (12, 30, 31) but not all (11) studies. In the present study we addressed how generation of Cer via the de novo and the salvage pathways were contributing to the deleterious effects on palmitate on insulin-producing MIN6 cells and isolated human islets. We inhibited Cer generation pathways in palmitate-treated MIN6 cells by using pharmacological or RNAi approaches. After long-term treatment palmitate doubled the amount of Cer in β-cells. Inhibition of the de novo synthesis during exposure to palmitate resulted in 50% decrease in the generation of Cer but did not alleviate the negative effects of palmitate on GSIS and apoptosis. Slight improvement in GSIS observed after inhibition of de novo synthesis in human islets could be explained by the heterogeneity of human islet cells. When both pathways were inhibited, Cer levels were normalized and GSIS and apoptosis were significantly improved. Less improvement in GSIS and apoptosis by knocking-down Lass5 and Lass6 compared to the improvement by FB1 may be explained by transfection efficiency and/or by activity of Lass2 and Lass4. Our results suggest that Cer generated via de novo synthesis and acylation of sphinganine is not as deleterious as Cer generated via acylation of sphingosine. The preferential reduction in apoptosis by FB1 but not ISP-1 has also been reported by others (32). In line with this, increased apoptosis was observed in cells expressing high levels of Cer synthase Lass6 (33).

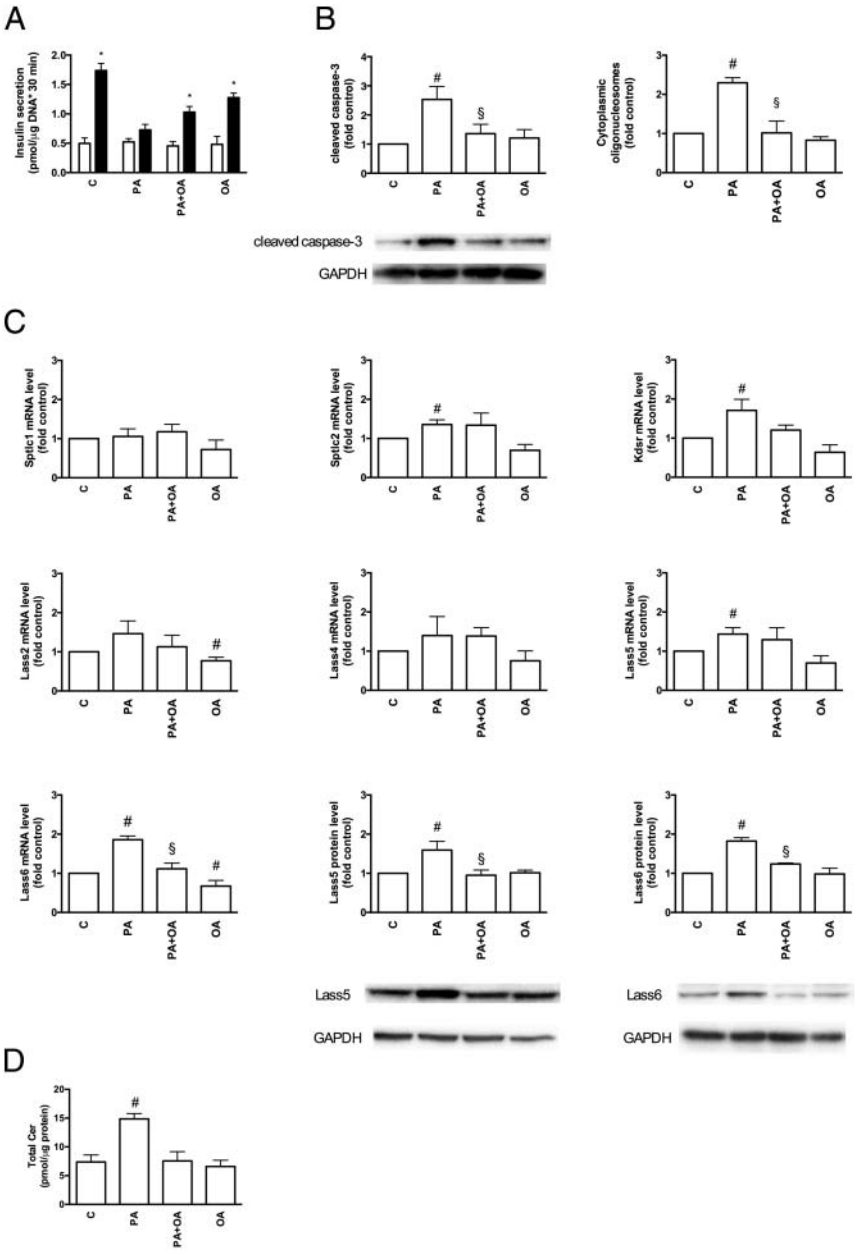

Figure 5. Glucose-stimulated insulin secretion (panel A), apoptosis (panel B), mRNA and/or protein levels of enzymes of de novo Cer generation and CerSs (panel C) and total Cer levels (panel D) in cells cultured in the absence (C) or presence of palmitate (PA) and oleate (OA) for 48 hours. Insulin release was measured at 2 (white bars) and 20 (black bars) mM glucose. Results are means ± SD for 5 experiments. *$P < .05$ compared to 2 mM glucose. #$P < .05$ compared to control cells, §$P < .05$ compared to cells cultured in the presence of palmitate alone.

To address the mechanism for why Cer generated by the two pathways has different biological effects, we determined which Cer species were formed via each of the pathways. Using MS-based analysis, we identified nine Cer species in insulin-secreting MIN6 cells. Not surprisingly, the most abundant species accounting for about 30% of total Cer was Cer(16:0). Three species, Cer(14:0), Cer(20:1) and Cer(22:1), were present at very low levels and determined for the first time in insulin-producing MIN6 cells. After 48-hour palmitate treatment, most Cer species were proportionally elevated by about 2-fold, except Cer(14:0) and Cer(22:1), which showed prom-

inent 5-fold rise. Our study is in agreement with a previous report, where six species were detected and all were raised after palmitate treatment (12). Of note, in another study, exposure to palmitate showed no effect on the level of the three measured Cer species including Cer(16:0) (11). Interestingly, rise in Cer(18:0) was not affected by either ISP-1 or FB1 indicating that Cer(18:0) might be induced via alternative mechanisms such as sphingomyelin hydrolysis.

By using inhibitors of the de novo and the salvage pathways and MS-based quantitative determinations of Cer species, we found that the de novo pathway triggers generation of Cer(20:0), Cer(22:0) and Cer(24:1), whereas the salvage pathway leads to formation of Cer(14:0), Cer(16:0), Cer(20:0), Cer(20:1), Cer(22:0), Cer(24:0) and Cer(24:1). Since inhibition of the de novo pathway results in reduced formation of sphinganine we assume that Cer(20:0), Cer(22:0) and Cer(24:1) are preferentially generated by acylation of sphinganine whereas the remaining four species are preferentially generated by acylation of sphingosine. Taking into consideration that the negative effects of palmitate are mediated via acylation of sphingosine, we concluded that elevation of Cer(14:0), Cer(16:0) Cer(20:1) and Cer(24:0) may be particularly harmful for β-cell function and viability.

Oleate prevents the negative effect of palmitate on β-cells (4, 6, 7). We tested the hypothesis if the protective effect involves inhibition of palmitate-induced generation of Cer. Therefore, we measured the amount of Cer in the presence of oleate alone or in combination with palmitate. Oleate alone or in combination with palmitate maintained Cer at the same level as control. This was due to lack of changes in the level of all species except Cer(20:1). Of note, the specie accounts for less than 1% of total Cer level. The mechanism of protection by oleate was addressed by measuring mRNA and/or protein level of key enzymes of

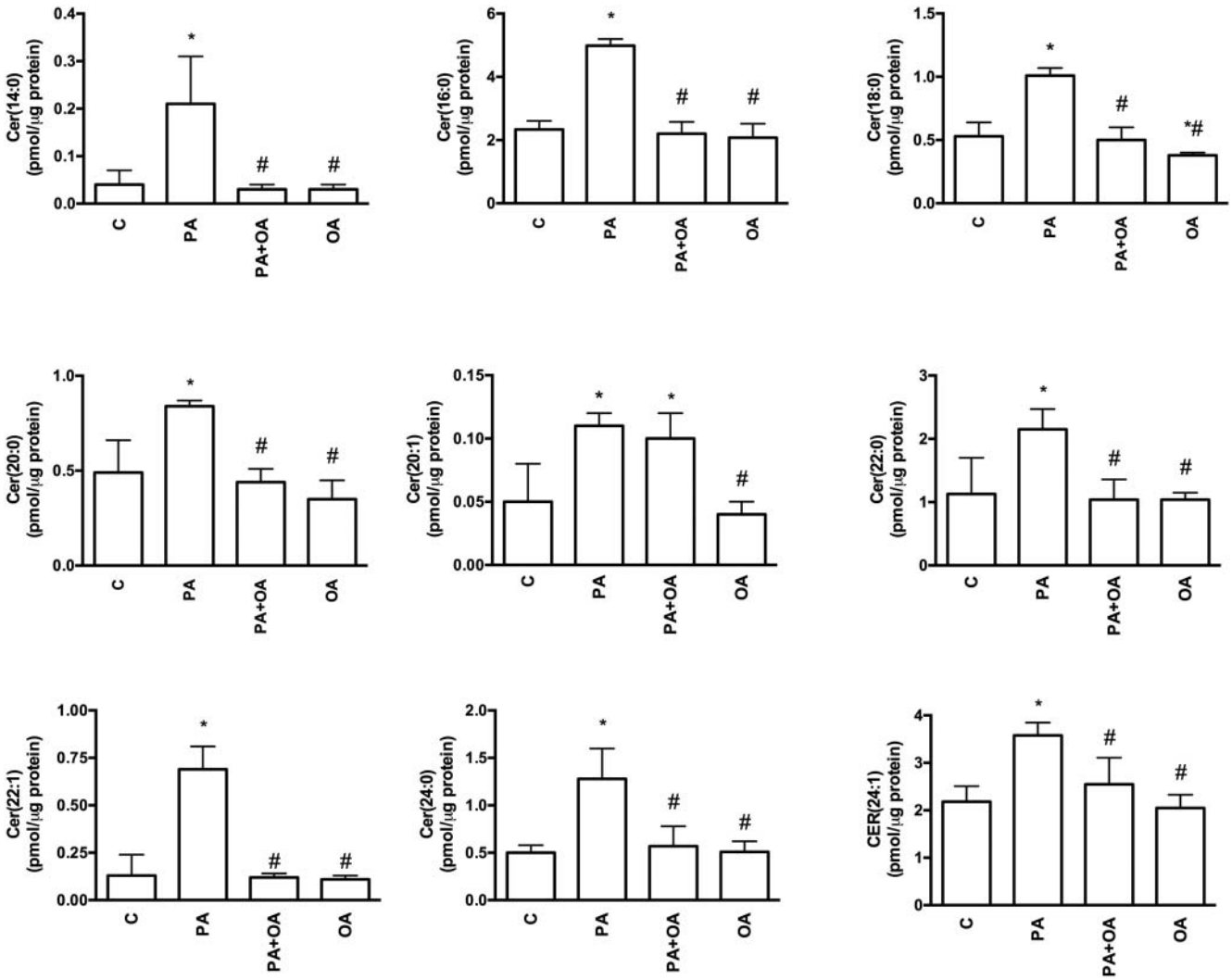

Figure 6. Levels of ceramide species in MIN6 cells cultured in the absence (C) or presence of palmitate (PA) and oleate (OA) for 48 hours. Results are means ± SD for 4 experiments. *$P < .05$ compared to control cells, #$P < .05$ compared to cells cultured in the presence of palmitate alone.

Cer de novo synthesis (Sptlc1, Sptlc2 and Kdsr) and acylation (Lass2, 4, 5 and 6). Expression levels of all the enzymes were elevated in the presence of palmitate alone and normalized when oleate was also present suggesting that oleate lowers Cer generation via inhibition of both de novo synthesis and acylation. Protective effect of oleate on β-cells is greater than the protective effect of FB1. The most plausible explanation is that mechanism of oleate action includes not only normalization of Cer level but also other pathways whereas FB1 affects specifically Cer generation. We have previously demonstrated that oleate directs fatty acids towards nontoxic pathways such as β-oxidation or formation of triglycerides (23).

In conclusion, we show that formation of Cer(14:0), Cer(16:0) and Cer (24:0) via sphingosine acylation is linked with detrimental effects on the β-cells and may represent one of the mechanisms by which palmitate impairs GSIS and increases apoptosis. We also conclude that the protective effect of oleate on palmitate-exposed β-cells involves inhibition of Cer formation and normalization of Cer levels.

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