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Fatty Acid-Induced Oxidation and Triglyceride Formation Is Higher in Insulin-Producing MIN6 Cells Exposed to Oleate Compared to Palmitate

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ABSTRACT

Palmitate negatively affects insulin secretion and apoptosis in the pancreatic β-cell. The detrimental effects are abolished by elongating and desaturating the fatty acid into oleate. To investigate mechanisms of how the two fatty acids differently affect β-cell function and apoptosis, lipid handling was determined in MIN6 cells cultured in the presence of the fatty acids palmitate (16:0) and oleate (18:1) and also corresponding monounsaturated fatty acid palmitoleate (16:1) and saturated fatty acid stearate (18:0). Insulin secretion was impaired and apoptosis accentuated in palmitate-, and to some extent, stearate-treated cells. Small or no changes in secretion or apoptosis were observed in cells exposed to palmitoleate or oleate. Expressions of genes associated with fatty acid esterification (SCD1, DGAT1, DGAT2, and FAS) were augmented in cells exposed to palmitate or stearate but only partially (DGAT2) in palmitoleate- or oleate-treated cells. Nevertheless, levels of triglycerides were highest in cells exposed to oleate. Similarly, fatty acid oxidation was most pronounced in oleate-treated cells despite comparable up-regulation of CPT1 after treatment of cells with the four different fatty acids. The difference in apoptosis between palmitate and stearate was paralleled by similar differences in levels of markers of endoplasmic reticulum (ER) stress in cells exposed to the two fatty acids. Palmitate-induced ER stress was not accounted for by ceramide de novo synthesis. In conclusion, although palmitate initiated stronger expression changes consistent with lipid accumulation and combustion in MIN6 cells, rise in triglyceride levels and fatty acid oxidation was favored specifically in cells exposed to oleate. J. Cell. Biochem. 111: 497–507, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: CERAMIDE; APOPTOSIS; ENDOPLASMIC RETICULUM STRESS; TRIGLYCERIDE; LIPOTOXICITY; PALMITATE; OLEATE

Free fatty acids (FFAs) have pleiotropic effects in the pancreatic β-cell involving signaling, insulin secretion, fuel metabolism, and membrane composition [Prentki et al., 1992; Itoh and Hinuma, 2005; Nolan et al., 2006]. The fatty acid-induced effects on the cell depend both on which FFA the cell is exposed to and if the exposure is short or long term. In most studies, the acute effects of FFAs on glucose-stimulated insulin secretion (GSIS) are stimulatory with saturated fatty acid palmitate being more potent than mono-unsaturated fatty acid oleate [Stein et al., 1997; Gravena et al., 2002]. The mechanisms of this insulinotropic effect of acute administration has been linked to the intracellular pool of long chain acyl-CoAs, which serve as coupling factors and are needed for optimal GSIS by affecting the activities of the exocytotic machinery [Deeney et al., 2000], PKC [Yaney et al., 2000], mitochondria and ATP/ADP ratio [Woldegiorgis et al., 1981], K_{ATP} channels [Larsson et al., 1996], and endoplasmic reticulum (ER) Ca^{2+}ATPase [Deeney et al., 1992]. In addition, FFAs are ligands to the β-cell expressed GPR40 [Briscoe et al., 2003; Itoh et al., 2003], which has further added to the understanding how acute effects of FFAs are translated into enhanced GSIS via PLC-coupled rise of cytoplasmic Ca^{2+} [Fujiwara et al., 2005].

Abbreviations used: BiP, Ig heavy chain binding protein; BSA, bovine serum albumin; CHOP, C/EBP homologous transcription factor; CPT1, carnitine palmitoyltransferase 1; DGAT, acyl-CoA:diacylglycerol acyltransferase; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; FAS, fatty acid synthase; FBS, fetal bovine serum; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; SCD, stearoyl-CoA desaturase.

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When β-cells are exposed to FFAs for longer time periods, negative effects on both function and mass are observed [El-Assaad et al., 2003; Maedler et al., 2003; Boucher et al., 2004]. In contrast to the acute effects of FFAs on GSIS, saturated fatty acids cause more severe negative effects on insulin secretory capacity and rates of apoptosis compared to monounsaturated fatty acids [Maedler et al., 2001]. In addition, the detrimental effects on β-cell function and mass were abolished when monounsaturated fatty acids and saturated fatty acids were administered together [Maedler et al., 2003]. Different mechanisms for how saturated fatty acids but not monounsaturated fatty acids exert negative effects on β-cell function and mass have been proposed including increased apoptosis [Maedler et al., 2003], mitochondrial dysfunction [Busch et al., 2002], ER stress activation [Karaskov et al., 2006; Laybutt et al., 2007; Sargsyan et al., 2008], ceramide formation [Maedler et al., 2003], dissociation of Ca^{2+} channels and secretory granules [Hoppa et al., 2009], and signaling via GPR40 [Zhang et al., 2007].

Given the importance of chain length and saturation for the long-term effects of the fatty acids palmitate and oleate on β-cell function and mass, the present study focused on determining triglyceride formation and fatty acid oxidation as well as expression of genes involved in lipid storage in β-cells cultured in the presence of the prevalent circulating fatty acids palmitate (16:0) and oleate (18:1) and also the corresponding monounsaturated fatty acid palmitoleate (16:1) and saturated fatty acid stearate (18:0). The results indicate that whereas expression of genes involved in lipid combustion and storage was particularly promoted in cells exposed to palmitate, actual fatty acid oxidation, and triglyceride formation was accentuated in cells exposed to oleate.

MATERIALS AND METHODS

CELL CULTURE

Mouse MIN6 cells, a kind gift from Professor Jun-Ichi Miyazaki, Osaka University, Japan, were maintained in DMEM supplemented with 15% FBS, 100 units/ml of penicillin, 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA), and 55 μM β-mercaptoethanol at 37°C and 5% CO_2. All experiments with MIN6 cells were performed between passages 21 and 28.

FATTY ACID PREPARATION AND CELL TREATMENT

Stock solutions containing palmitate (Sigma P-9767, St. Louis, MO), stearate (Sigma S-3381), or oleate (Sigma O-7501) were prepared by dissolving the fatty acids in 50% ethanol to a final concentration of 100 mM. Stock solution of palmitoleate (Sigma 76169) was prepared by dissolving the fatty acid in 100% ethanol to a concentration of 200 mM. The stock solutions were then diluted in culture medium with 0.5% fatty acid-free BSA (Boehringer Mannheim GmbH, Mannheim, Germany) to a final concentration of 0.5 mM. Cells were exposed to the fatty acids for 48 h at which point the cell confluence was 60–70%.

GLUCOSE-STIMULATED INSULIN SECRETION MEASUREMENTS

GSIS was determined in MIN6 cells after 48 h fatty acid treatment. The cells were first incubated for 60 min in standard culture medium but with 2 mM glucose. Subsequently, the medium was changed to KRBH buffer consisting of (in 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 (fraction V, Boehringer Mannheim GmbH). The cells were maintained for 30 min before medium was changed to the same type of buffer but with either 2 or 20 mM glucose. The cells were then incubated for another 30 min. After incubation, aliquots of buffer were taken for later determination of released insulin. Cells were then washed in PBS, lysed in MilliQ H_2O, and frozen for later determination of DNA content. Released insulin was determined with an ELISA as previously described [Bergsten and Hellman, 1993].

APOPTOSIS MEASUREMENTS

Apoptosis in MIN6 cells after fatty acid treatment was assayed with the 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. The apoptosis measurements were related to DNA content and compared to the average value obtained from untreated cells.

MEASUREMENTS OF mRNA EXPRESSION

Total mRNA was isolated from MIN6 cells using the RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions and reverse-transcribed with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). 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 used for the amplification are shown in Table I. PCR products were quantified fluorometrically using SYBR Green, and normalized to the housekeeping gene β-actin and relative to the control according to the following formula: target amount $= 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \{[C_t \text{ (target gene)} - C_t \text{ (β-actin)}] - [C_t \text{ (control)} - C_t \text{ (β-actin control)}]\}$.

TRIGLYCERIDE MEASUREMENTS

MIN6 cells were grown for 48 h in the absence or presence of fatty acids after which the cells were trypsinized and collected into glass tubes. A small sample of the cells was taken for protein measurements. Lipids were then extracted according to Folch as described previously [Folch et al., 1957]. After the extraction, solvent was evaporated under a stream of nitrogen and the lipids were redissolved in chloroform and spotted on a TLC plate. The plate was run in a toluene:diethyl ether:ethyl acetate:glacial acetic acid

TABLE I. Primers Used for Real-Time PCR

Target	Forward primer	Reverse primer
β-actin	GTTACAGGAAGTCCCTCACC	GGAGACCAAAGCCTTCATAC
SCD1	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG
SCD2	GCCCACTTAAACAAATGTGA	TGGTACACCCTTAACGACAG
DGAT1	TCGTCTGCAAGATTCTTTGT	CCAGGATGCCATACTTGATA
DGAT2	CCTCTTCTCCTCTGACACCT	CATGGTACAGGTCGATGTCT
CPT1	GCACTCCTGGAAGAAGAAGT	TCTAGGGTCCGATTGATCTT
FAS	TGCATTGGTAGGGTACAAAG	TGCTCCCTTGAGTCAGTAAA

(400:50:50:1) solvent system, allowed to dry and then sprayed with 2,7-DCF (Sigma). Spots were visualized with excitation (465 nm) and emission (535 nm) filters and quantified with Quantity One software (Bio-Rad, Hercules, CA). The results were normalized to protein amount.

FATTY ACID OXIDATION MEASUREMENTS

Reaction mixture was prepared by adding 2 μCi ^{3}H-palmitate per milliliter culture media containing 0.5% fatty acid-free BSA. Unlabelled palmitate was added to make the final concentration 0.5 mM. MIN6 cells exposed to fatty acids or not were washed with PBS. Cells were then incubated for 2 h with the reaction mixture after which the media was collected. Radioactive water was separated from the radioactive palmitate in the media by three subsequent Folch extractions [Folch et al., 1957]. During extraction, proteins were isolated from the cells and the concentration was measured. After the last extraction, 10 ml scintillation liquid was mixed with the water phase and the mixture was counted in a scintillation counter. The results were normalized to protein amount in the corresponding wells.

WESTERN BLOT MEASUREMENTS

Samples for western blotting were prepared from MIN6 cells by washing the cells twice with PBS followed by lysing the cells on ice with a buffer composed of 150 mM NaCl, 20 mM Tris, 1% Triton X100, 0.25% Na-deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 2 mM EDTA, and 1% protease inhibitor cocktail (Sigma P-8340) for 30 min. After lysis, the preparations were collected and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were transferred to new tubes and the total protein concentration was determined by the DC protein assay (BioRad, Hercules, CA) according to the manufacturer's instruction. Samples were mixed with SDS–PAGE sample buffer containing Tris–HCl (pH 6.8), SDS, glycerol, and DTT and boiled for 5 min. Samples (25 μg per well) were then subjected to SDS–PAGE. After electrophoresis, proteins were transferred onto PVDF membrane. Immunoblot analyses were performed with antibodies towards phosphorylated eIF2α (p-eIF2α) and eIF2α (Cell Signaling, Beverly, MA), BiP (Abcam, Cambridge, UK), and CHOP (Santa Cruz Biotechnology, Santa Cruz, CA). Immuno-reactive bands were imaged with Fluor-S MultiImager MAX (Bio-Rad, Hercules, CA) and quantified with Quantity One software (Bio-Rad). After imaging, the PVDF membranes were stained with Coomassie and later de-stained with 50% methanol. The blots were then scanned in a standard table-top scanner and quantified with Quantity One software. The expression level of each protein was normalized to the Coomassie-stained blot with the exception of p-eIF2α, which was normalized to total eIF2α content.

DATA ANALYSIS

Results are presented as means ± SEM. Statistical significance between two conditions was analyzed by the Student's paired *t*-test and between several groups using one-way ANOVA with Tukey post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

FATTY ACID-INDUCED IMPAIRMENT OF GSIS DEPENDS ON FATTY ACID SATURATION BUT NOT CHAIN LENGTH

The impact of prolonged exposure of palmitate and oleate and corresponding monounsaturated fatty acid palmitoleate and saturated fatty acid stearate on GSIS was determined in insulin-producing MIN6 cells. The cells were treated with 0.5 mM palmitate (16:0), stearate (18:0), palmitoleate (16:1), or oleate (18:1) for 48 h. After culture, insulin secretion at basal (2 mM) and stimulating (20 mM) glucose levels was measured. In MIN6 cells cultured in the absence of fatty acids insulin release rose fourfold ($P < 0.05$), when the glucose concentration was increased (Fig. 1). Addition of either palmitate or stearate during culture decreased insulin secretion at both basal and stimulatory glucose concentrations (Fig. 1). The reductions were almost identical for the two saturated fatty acids resulting in a twofold rise ($P < 0.05$) in GSIS from MIN6 cells cultured in the presence of either of the saturated fatty acids. In contrast, when cells were cultured in the presence of either palmitoleate or oleate, basal secretion was increased. Stimulated release was unaffected in cells cultured in the presence of palmitoleate and slightly decreased in oleate-treated cells. The resulting GSIS rose about twofold ($P < 0.05$). Thus, the modulating effects of the two monounsaturated fatty acids on insulin release were similar. It was concluded that whereas saturation of the fatty acid was a major determinant for both basal and stimulated insulin release from MIN6 cells cultured in the presence of fatty acids, fatty acid chain length was not.

FATTY ACID-INDUCED RISE IN APOPTOSIS DEPENDS ON FATTY ACID SATURATION AND SATURATED FATTY ACID CHAIN LENGTH

The influence of prolonged exposure of MIN6 cells to saturated fatty acids and monounsaturated fatty acids with different chain lengths

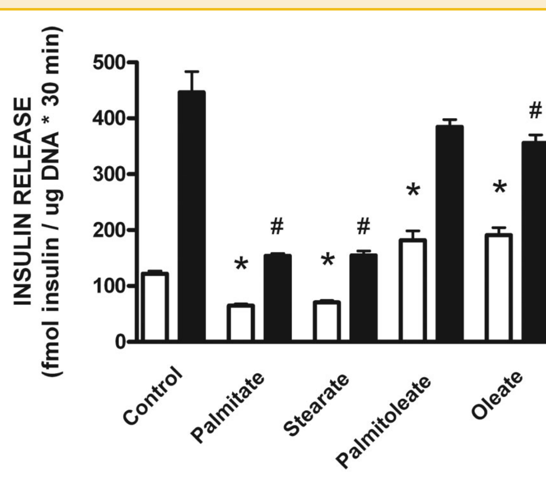

Fig. 1. Glucose-stimulated insulin secretion from MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated fatty acids. After culture, cells were stimulated with 2 mM (white bars) or 20 mM (black bars) glucose. Results are means ± SEM from six individual experiments. *$P < 0.05$ and #$P < 0.05$ denote effects compared to levels at control 2 and 20 mM glucose, respectively.

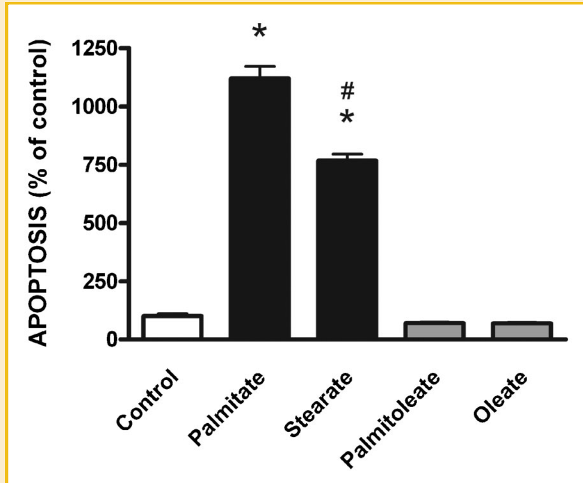

Fig. 2. Apoptosis in MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated saturated (black bars), mono-unsaturated (gray bars) fatty acids or under control conditions (white bar). After culture, apoptosis was measured and normalized to DNA content. Results are means ± SEM from four individual experiments. $^{*}P < 0.05$ denotes effect compared to control and $^{\#}P < 0.05$ compared to palmitate.

on apoptosis was next determined. Whereas palmitate and stearate caused several-fold increased apoptosis, palmitoleate and oleate did not affect apoptosis compared to control after 48 h culture of MIN6 cells (Fig. 2). The magnitude of apoptosis was affected by the chain length of the saturated fatty acid. Whereas palmitate increased the apoptosis 11-fold over control, stearate caused 7-fold increase. Based on the insulin release and apoptosis results, we hypothesized that expression of enzymes involved in intracellular lipid handling would be of importance for β-cell function and mass after fatty acid exposure.

FATTY ACID-INDUCED CHANGES IN SCD EXPRESSION DEPENDS ON FATTY ACID SATURATION BUT NOT CHAIN LENGTH

Saturated fatty acids can undergo desaturation in the cell via stearoyl-CoA desaturase (SCD) to produce its monounsaturated fatty acid counterpart [Miyazaki et al., 2006]. As indicated in Figure 2, oleate and palmitoleate do not induce apoptosis whereas their saturated fatty acid counterparts do. We therefore measured levels of SCD to see if there was a link between apoptosis and SCD levels. SCD exists in four different isoforms in the mouse. When their expression levels were determined in the absence of fatty acids, SCD2 demonstrated 17.8 ± 1.2 higher levels than SCD1, while SCD3 and SCD4 levels were <0.01 of SCD1 levels (Supplementary Fig. 1). To determine if the differences in the observed apoptosis (Fig. 2) could be explained by variations in SCD expression levels, the transcriptional response of the two major isoforms of SCD in the mouse β-cell, SCD1, and SCD2 were measured. MIN6 cells treated with palmitate or stearate showed up-regulation of SCD1, while levels of SCD2 were unchanged when compared with control (Fig. 3A,B). No differences in expression between cells treated with palmitate and stearate were found. As expected, culture in the presence of palmitoleate or oleate caused a major decrease in levels

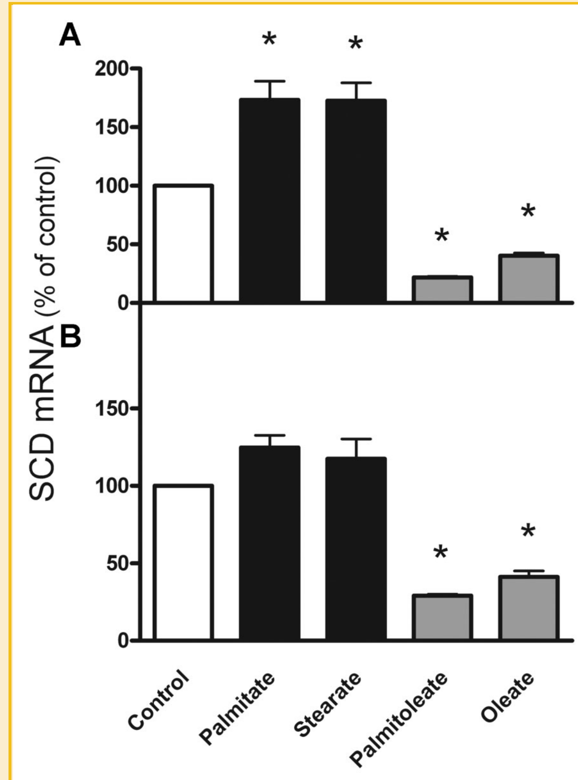

Fig. 3. SCD levels in MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated saturated (black bars), mono-unsaturated (gray bars) fatty acids or under control conditions (white bar). After culture, SCD1 (A) and SCD2 (B) transcript levels were measured. Results are means ± SEM from four to five individual experiments. $^{*}P < 0.05$ denotes effect compared to control.

of both SCD1 and SCD2 compared with control (Fig. 3A,B). There was no difference in down-regulations caused by palmitoleate or oleate. In conclusion, regulation of the desaturases was largely dependent of saturation of the fatty acid where exposure to palmitate or stearate caused increased levels while cells exposed to palmitoleate or oleate had reduced levels.

ELONGASE EXPRESSION IS AFFECTED NEITHER BY FATTY ACID SATURATION NOR CHAIN LENGTH

Fatty acids undergo chain extension via elongases to produce fatty acids with chain length increased by multiples of two carbons [Jakobsson et al., 2006]. We next sought to determine if elongases were differentially regulated when exposed to fatty acids potentially explaining differences in β-cell function and mass (Figs. 1 and 2). Elongases exist in seven isoforms, where ELOVL1 and 6 would elongate palmitate or palmitoleate to stearate or oleate [Jakobsson et al., 2006]. When their expression levels were determined under control conditions, ELOVL4 expression was 0.3 ± 0.1, ELOVL5 expression 3.2 ± 0.8, and ELOVL6 expression 0.6 ± 0.2 compared to expression level of ELOVL1 (Supplementary Fig. 2). Very low expression was evident for ELOVL2, 3, and 7. When MIN6 cells were cultured in the presence of palmitate, stearate, palmitoleate, or oleate, expression levels of ELOVL1, 4, 5, and 6 were not affected

(Supplementary Fig. 3). Although ELOVL3 was induced 3.5-fold by palmitate and ELOVL7 2-fold by palmitate or palmitoleate, the resulting expression levels were still very low (Supplementary Figs. 2 and 3). Since elongase expression was modulated to a minor extent, it was concluded that elongation is not a major lipid handling pathway up-regulated in response to long chain fatty acids in the MIN6 cell.

TRIGLYCERIDE FORMATION DEPENDS ON FATTY ACID SATURATION AND CHAIN LENGTH

Accumulation of complex lipids in β-cells has been associated with decline in β-cell function and mass [Unger and Zhou, 2001]. The formation of triglycerides may, however, serve a protective function since triglyceride content was demonstrated to be inversely correlated to β-cell apoptosis [Cnop et al., 2001; Listenberger et al., 2003]. It was suggested that formation of triglycerides could protect β-cells from apoptosis by storing the fatty acids in a non-reactive form. To test the hypothesis that the observed differences in apoptosis (and GSIS) depended on triglyceride formation, we measured triglycerides in MIN6 cells cultured in the presence of the different fatty acids. Cellular levels of triglycerides increased when cells were exposed to either fatty acid compared to control-treated cells (Fig. 4A). No difference in triglyceride levels was detected between cells treated with palmitate, stearate, or palmitoleate. However, cells exposed to oleate had two- to threefold higher levels than cells exposed to palmitate, stearate, or palmitoleate. In conclusion, exposure to any of the fatty acids increased levels of triglycerides. There was a dependence on both saturation and chain length of the triglyceride formation, however, as exposure to oleate increased levels more than any of the other fatty acids.

FATTY ACID-INDUCED RISE IN DGAT EXPRESSION DEPENDS ON FATTY ACID SATURATION BUT NOT CHAIN LENGTH

The enzyme acyl-CoA:diacylglycerol acyl-transferase (DGAT) controls the last acylation in the triglyceride formation and is rate-limiting for the formation [Bell and Coleman, 1980]. We measured the transcript levels of the two isoforms DGAT1 and DGAT2 after exposure to the different fatty acids. In the absence of external application of fatty acids, MIN6 cells express equal amounts of DGAT1 and DGAT2 (data not shown). Based on triglyceride formation (Fig. 4A), we hypothesized a major rise in DGATs in cells exposed to oleate but less in cells exposed to palmitoleate or the saturated fatty acids. When fatty acids were included during culture of MIN6 cells, palmitate and stearate up-regulated DGAT1 by approximately 50% and DGAT2 by threefold compared to control (Fig. 4B,C). There was no difference between DGAT expression levels in the presence of the two saturated fatty acids. When MIN6 cells were cultured in the presence of palmitoleate or oleate, DGAT1 expression was not affected (Fig. 4B). In contrast, palmitoleate and oleate induced the expression of DGAT2 albeit to a lesser extent than saturated fatty acids (twofold; Fig. 4C). No differences in expression of the DGATs depending on chain length of the monounsaturated fatty acids were found. In conclusion, expression of DGATs was dependent on fatty acid saturation. The

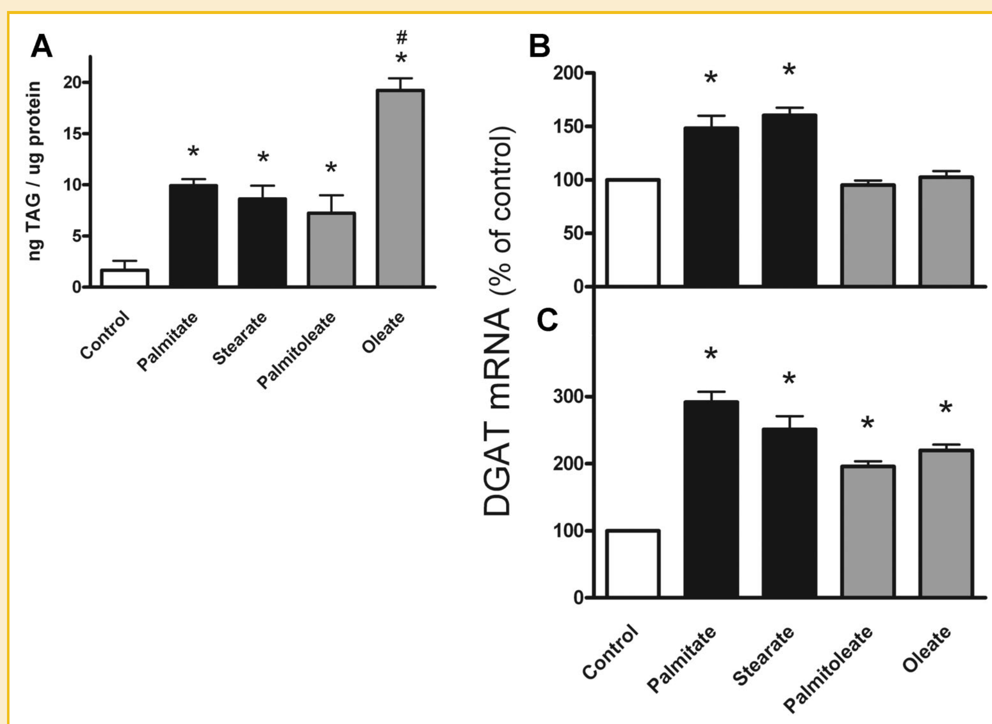

Fig. 4. Triglyceride and DGAT levels in MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated saturated (black bars), mono-unsaturated (gray bars) fatty acids or under control conditions (white bar). After culture, levels of triglycerides (A), or DGAT1 (B), and DGAT2 (C) transcript levels were measured. Results are means ± SEM from four to five individual experiments. $^{*}P < 0.05$ denotes effect compared to control and $^{\#}P < 0.05$ compared to palmitate.

levels of DGATs were only partially correlating with levels of triglycerides, however (Fig. 4A).

FATTY ACID OXIDATION DEPENDS ON SATURATION BUT NOT CHAIN LENGTH

In addition to storage, cellular handling of fatty acids includes the disposal by combustion, a pathway connected with amelioration of negative effects induced by fatty acids [Hellemans et al., 2007]. We therefore hypothesized that cells with varying degrees of apoptosis (Fig. 2) and impaired GSIS (Fig. 1) would show corresponding variation in fatty acid oxidation as a result of exposure to the different fatty acids. When fatty acid oxidation was determined, MIN6 cells cultured in the presence of oleate showed higher fatty acid oxidation than cells treated with palmitate or stearate (Fig. 5A). No difference in level of oxidation was detected between palmitate, stearate, and palmitoleate. Fatty acid oxidation was increased in cells exposed to saturated fatty acids or palmitoleate when compared to cells cultured in absence of fatty acids, however.

FATTY ACID-INDUCED RISE IN CPT1 DEPENDS NEITHER ON FATTY ACID SATURATION NOR CHAIN LENGTH

Fatty acid oxidation depends on import of fatty acids into the mitochondria, where they are β-oxidized. Based on the differences in fatty acid oxidation in MIN6 cells exposed to the different fatty acids (Fig. 5A), we hypothesized that levels of the main mitochondrial fatty acid importer carnitine-palmitoyltransferase 1 (CPT1) would show corresponding variations. In addition, since decreased fatty acid import has been shown to promote apoptosis [Paumen et al., 1997], differences in expression levels of CPT1 could also contribute to explain the differences in apoptosis observed in MIN6 cells exposed to the different fatty acids (Fig. 2). When CPT1 mRNA levels were measured in MIN6 cells cultured in the presence of palmitate, stearate, palmitoleate, or oleate, CPT1 levels rose more than sixfold irrespective of which fatty acid was applied (Fig. 5B). Furthermore, the up-regulations provoked by the different fatty acids were equal. Preferential CPT1-dependent mitochondrial import and subsequent combustion of certain fatty acids, that could potentially affect β-cell function and mass, was thus not evident at the transcript level of the fatty acid mitochondrial transporter.

FATTY ACID-INDUCED RISE IN FAS EXPRESSION DEPENDS ON FATTY ACID SATURATION AND CHAIN LENGTH

When β-cells are exposed for prolonged periods to situations where energy is plentiful, cataplerosis of citrate occurs and through a series of reactions malonyl-CoA is formed [Farfari et al., 2000]. Increased levels of malonyl-CoA inhibit CPT1, thereby decreasing the import of fatty acids into the mitochondria and subsequently decreasing β-oxidation [Prentki et al., 1992]. The discrepancy between the observed fatty acid oxidation (Fig. 5A) and CPT1 levels (Fig. 5B) may thus be caused by elevated levels of malonyl-CoA resulting from the prevailing high culture glucose concentration. Malonyl-CoA is also the building block in fatty acid de novo synthesis performed by the enzyme complex fatty acid synthase (FAS). We determined FAS expression levels as a measure of de novo synthesis in the presence of the different fatty acids. FAS levels were not affected in MIN6

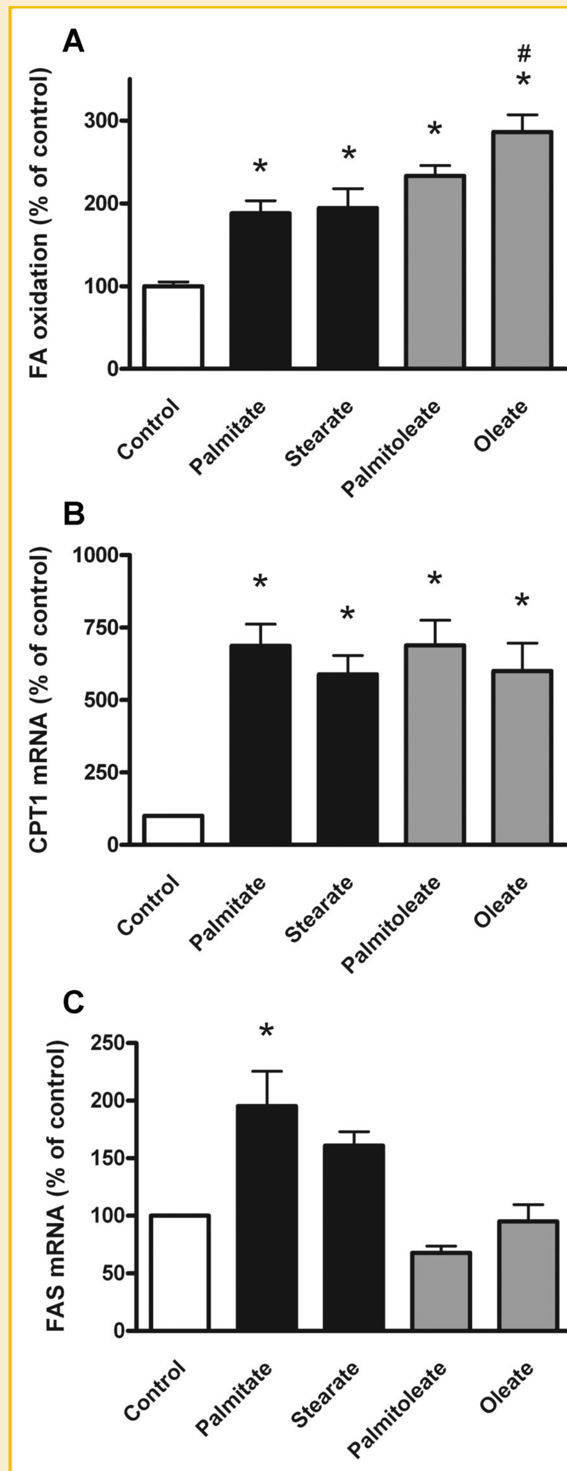

Fig. 5. Fatty acid oxidation, CPT1 and FAS levels in MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated saturated (black bars), mono-unsaturated (gray bars) fatty acids or under control conditions (white bar). After culture, fatty acid oxidation (A), or CPT1 (B) and FAS (C) transcript levels were measured. Results are means ± SEM from four to five individual experiments. $^{*}P < 0.05$ denotes effect compared to control and $^{\#}P < 0.05$ compared to palmitate.

cells cultured in the presence of palmitoleate or oleate (Fig. 5C). Palmitate caused enhanced FAS expression, indicating an increase in de novo synthesis. Stearate-treated cells also had a tendency towards increased levels of FAS, although this was not significant.

FATTY ACID-INDUCED ER STRESS RESPONSE DEPENDS ON FATTY ACID SATURATION AND CHAIN LENGTH

Prolonged exposure to palmitate but not oleate has previously been shown to induce the ER stress response, leading to increased levels of phosphorylated eIF2α and CHOP, thereby causing cells to enter an apoptotic state [Karaskov et al., 2006; Laybutt et al., 2007; Sargsyan et al., 2008]. To determine if the difference in apoptosis observed between palmitate, stearate, palmitoleate, and oleate in this study can be explained by the ability of the fatty acids to induce ER stress, levels of phosphorylated eIF2α (p-eIF2α), BiP, and CHOP protein were measured. P-eIF2α was increased in cells cultured in the presence of palmitate or stearate (Fig. 6A). The increase in phosphorylation was notably higher in cells cultured the presence of palmitate than in stearate-treated cells. Whereas CHOP protein levels were highly elevated in cells cultured in the presence of palmitate, stearate failed to induce CHOP translation (Fig. 6B). With regard to protein levels of BiP, both saturated fatty acids failed to induce translation of the chaperone (Fig. 6C). Neither of the monounsaturated fatty acids increased eIF2α phosphorylation, CHOP, or BiP protein levels (Fig. 6A–C). In conclusion, induction of ER stress after exposure to any of the fatty acids correlated with the apoptosis data (Fig. 2), indicating a role for ER stress in induction of apoptosis. The less pronounced ability of stearate to induce apoptosis in comparison to palmitate may account for the differences in apoptosis seen between these fatty acids.

CERAMIDE DE NOVO SYNTHESIS IS NOT INVOLVED IN PALMITATE-INDUCED APOPTOSIS, IMPAIRED GSIS OR ER STRESS

Palmitate is the pre-cursor for de novo synthesis of ceramide, a lipid second messenger which has been shown to be involved in β-cell dysfunction and apoptosis [Maedler et al., 2001; Lupi et al., 2002; Maedler et al., 2003]. Since only palmitate and not stearate can be used for this de novo synthesis, we hypothesized that the observed accentuated palmitate-induced apoptosis (Fig. 2) depended on ceramide formation. To address this issue, MIN6 cells cultured in the presence of palmitate for 48 h were exposed to ISP1, an inhibitor of serine palmitoyltransferase (SPT) [Miyake et al., 1995]. Apoptosis was not altered by inclusion of the SPT inhibitor (data not shown). The observed phosphorylation of eIF2α and induction of CHOP observed in MIN6 cells cultured in the presence of palmitate were not affected by inclusion of the inhibitor (Fig. 7A,B). Also, BiP levels determined in MIN6 cells exposed to palmitate were not affected by the inhibitor (Fig. 7C). The possible role of ceramide synthesis for palmitate-induced reduction in GSIS (Fig. 1) was also investigated by including ISP1 during culture of MIN6 cells in the presence of

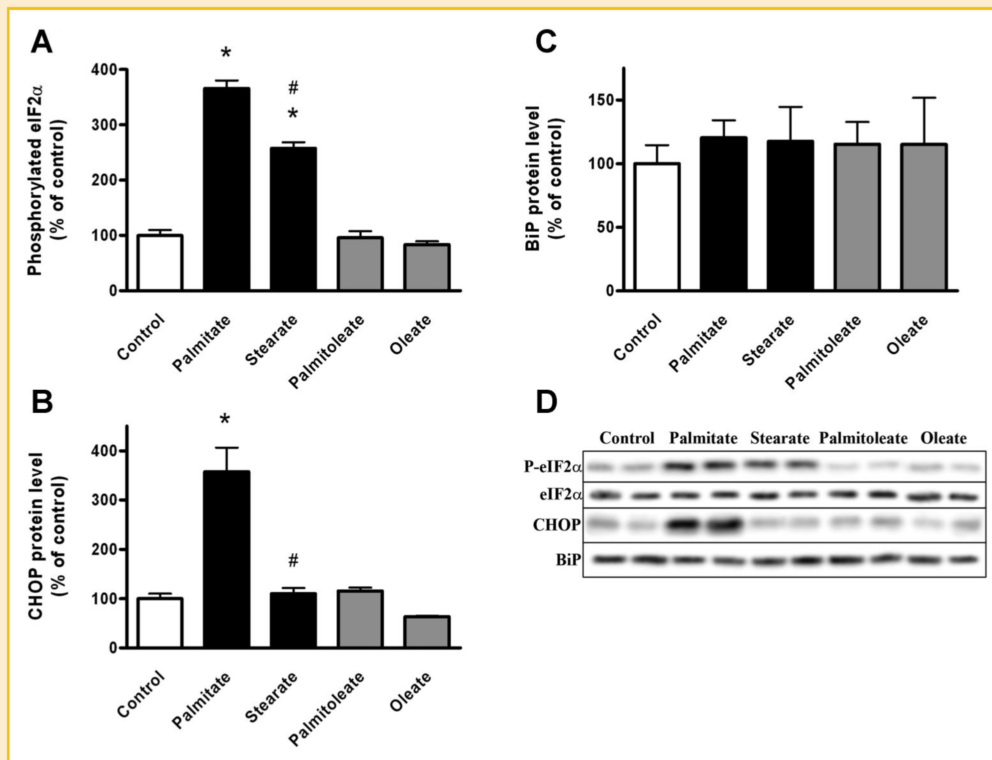

Fig. 6. Endoplasmic reticulum stress markers in MIN6 cells exposed to different fatty acids. MIN6 cells were cultured for 48 h in the presence of the indicated saturated (black bars), mono-unsaturated (gray bars) fatty acids or under control conditions (white bar). After culture, phosphorylation of eIF2α (A), and protein levels of CHOP (B) and BiP (C) were measured. Representative blots are shown (D). Results are means ± SEM from four individual experiments. $^{*}P < 0.05$ denotes effect compared to control and $^{\#}P < 0.05$ compared to palmitate.

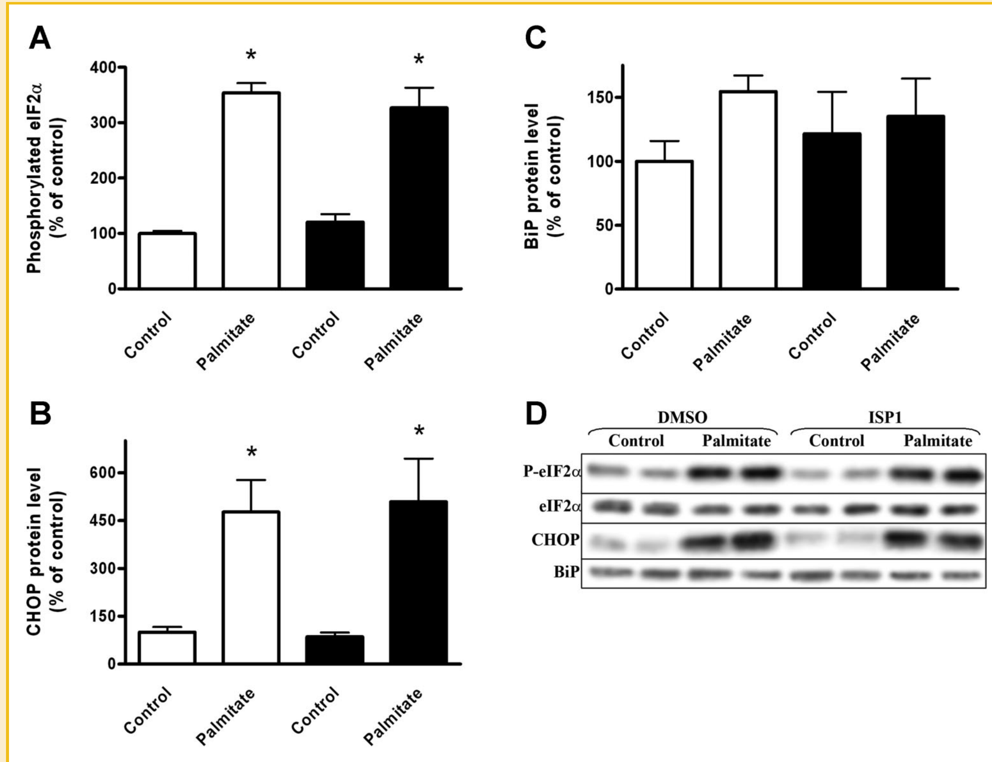

Fig. 7. Endoplasmic reticulum stress markers in MIN6 cells exposed to palmitate and inhibitor of ceramide de novo synthesis. Phosphorylation of eIF2α (A), protein levels of CHOP (B), and BiP (C) of MIN6 cells cultured for 48 h in the presence of palmitate without (white bars) or with (black bars) 1 μM ISP1. Representative blots are shown (D). Results are means ± SEM from four individual experiments. $^{*}P < 0.05$ denotes effect compared to control in the respective group.

palmitate. No alteration in GSIS was observed when ISP1 was included (data not shown). In conclusion, de novo synthesis of ceramide does not affect neither cellular function nor apoptosis in palmitate-treated MIN6 cells.

DISCUSSION

Prolonged elevated levels of the saturated fatty acid palmitate have negative effects on β-cell mass and function [Maedler et al., 2001; El-Assaad et al., 2003; Boucher et al., 2004]. The effects on β-cell function and mass are much less accentuated when palmitate is replaced by the monounsaturated fatty acid oleate [Maedler et al., 2001; Karaskov et al., 2006; Laybutt et al., 2007]. Several mechanisms have been proposed to explain the differences between the two fatty acids. In particular, differences in expression of SCDs and ELOVLs have been observed in β-cells cultured in the presence of palmitate [Busch et al., 2002]. Indeed, the report implied that the β-cell would be able to reduce the detrimental effects of palmitate by converting the fatty acid to oleate via intermediates palmitoleate or stearate. We hypothesized that inadequate desaturation or elongation in the β-cell may be hampering conversion from palmitate to oleate. To address the hypothesis, in addition to palmitate and oleate, we exposed MIN6 cells to stearate or palmitoleate, which would only require β-cell desaturation or elongation, respectively, to form oleate. The results indicate that the detrimental effects of palmitate compared to oleate on β-cell function and mass reside in differences in fatty acid oxidation and triglyceride formation rather than inadequate desaturation or elongation.

Fatty acid desaturation is performed by the SCDs, which are a family of enzymes catalyzing the introduction of a Δ9 double bond in saturated fatty acids. Four SCD isoforms have been described [Miyazaki et al., 2006]. SCD1 has been reported to be expressed in MIN6 cells [Busch et al., 2005]. In the present study, we also report SCD2 expression in these cells. Indeed, the SCD2 expression was 17-fold higher than SCD1. Palmitate increased SCD1 expression [Busch et al., 2005], which has been connected with improved function. The elongases (ELOVLs) add two-carbon units to fatty acyls and are responsible for fatty acid elongation. They constitute a group of seven enzyme isoforms with different substrate specificity, where ELOVL1 and six would elongate palmitate to stearate [Jakobsson et al., 2006]. These isoforms were, however, not differentially regulated after fatty acid exposure, indicating that this pathway does not play a major role under these conditions in the MIN6 cells.

GSIS from MIN6 cells cultured in the presence of stearate was very similar to GSIS from palmitate-treated cells. In contrast, apoptosis was lower in stearate-treated cells. However, no change in desaturation was evident at the transcript level. Hence, conversion of stearate to oleate via desaturation is less plausible as an explanation to the lower level of apoptosis found after stearate treatment. However, desaturation of stearate may be more accentuated than for palmitate despite these similar expression

levels since SCD1 and SCD2 have higher conversion rates for stearate than palmitate [Miyazaki et al., 2006]. When MIN6 cells were exposed to palmitoleate GSIS and apoptosis was similar to results obtained in the presence of oleate. Based on these results it is not likely that MIN6 cells use conversion of palmitate to palmitoleate or oleate as a major salvage mechanism to avert palmitate-induced toxicity. Also, the results imply that although palmitoleate and oleate have similar effects on β-cell function and mass these effects are mediated via different mechanisms.

Palmitate-induced toxicity has been linked to a redirection of lipids from combustion to formation of complex lipids [Paumen et al., 1997; Moffitt et al., 2005]. Indeed, we also found significantly higher fatty acid oxidation in β-cells exposed to oleate compared to cells exposed to palmitate. When CPT1 was used as a marker of fatty acid oxidation, no difference in the fatty acid mitochondrial transporter was observed between MIN6 cells exposed to any of the four fatty acids. From the oxidation results it is evident that CPT1 levels do not reflect β-oxidation, which may be explained by the fact that CPT1 can be inhibited by malonyl-CoA. This product is formed under cataplerosis from the mitochondria, and thereafter functions as a precursor for palmitate de novo synthesis. Since expression of FAS, the enzyme responsible for fatty acid de novo synthesis, is up-regulated after palmitate treatment, levels of malonyl-CoA may very well be increased and β-oxidation decreased despite high levels of CPT1. The sensitivity of CPT1 to malonyl-CoA can also be modulated by the fluidity of the outer mitochondrial membrane [Faye et al., 2005]. Since palmitate may cause alterations in membrane fluidity [Leekumjorn et al., 2009], this may contribute to the differences in β-oxidation after palmitate and oleate exposure. Interestingly, fatty acid oxidation in β-cells exposed to palmitoleate was not different from that observed in palmitate or stearate. Discrepancy between oleate and palmitoleate, despite the similar GSIS and apoptosis, was also evident when triglyceride formation was measured. It is noteworthy that when expression of DGATs was used as indicators of triglyceride formation, expression was mainly elevated in the presence of palmitate and stearate and not palmitoleate or oleate. When triglycerides were measured, channeling of the fatty acids into triglycerides was especially high in cells exposed to oleate, however. The absence of correlation between levels of DGAT and incorporation of fatty acids into triglycerides probably reflects the substrate specificity of the DGAT enzymes. In agreement with our data, it has previously been shown that palmitate is poorly incorporated into triglycerides compared to oleate when administered separately [Montell et al., 2001; Moffitt et al., 2005]. The results indicate that cells exposed to palmitate have both reduced fatty acid oxidation and triglyceride formation in comparison with cells cultured in the presence of oleate. Further, down-regulation of glycolytic enzymes in insulin-producing β-cells by palmitate but not oleate was recently demonstrated [Hovsepyan et al., 2010]. Although glucose oxidation was not measured the results indicate that palmitate but not oleate inhibits the rate of glycolysis, that is, shows Randle effect [Randle et al., 1963]. It appears that, when insulin-producing β-cells are exposed for prolonged periods to palmitate, they not only fail to handle the fatty acid by adequate up-regulation of β-oxidation and triglyceride formation but also inhibit glucose oxidation, which further aggravates the insulin secretory capacity [Grant et al., 1980]. In this context it should be noted that increased metabolic rate was observed in the SCD1 (−,−) mouse [Ntambi et al., 2002; Rahman et al., 2003]. It appears that the enzyme has a more complex role than mere desaturation activity [Busch et al., 2005]. A mechanism for such an additional role was recently offered when it was demonstrated that the lipogenic transcription factor sterol regulatory element binding protein 1-c (SREBP-1c) together with PPAR-γ coactivator-1b (PGC-1b) were not activated in the SCD1 (−,−) mouse [Sampath et al., 2007]. In these mice, palmitate and stearate stimulate lipid oxidation rather than causing lipid accumulation. Thus, it could be speculated that the beneficial effects of oleate compared to palmitate on β-cell function and mass documented in this study and other studies [Busch et al., 2002; Karaskov et al., 2006; Laybutt et al., 2007] reflect balanced lipid accumulation and oxidation, where the decrease in SREBP-1c and downstream targets including SCD1 plays a pivotal role. In agreement with these observations in the SCD1 (−,−) mouse, MIN6 cells in the present study with low SCD1 levels after exposure to monounsaturated fatty acids also had low levels of DGAT1 and FAS, two other SREBP-1c target genes [Ikeda et al., 2002; Wang et al., 2003]. It is concluded that SCD1 down-regulation induced by monounsaturated fatty acids could reflect modulation of SREBP-1c, favoring lipid oxidation. This is also in agreement with studies in man, where subjects given an oleate-rich diet had higher levels of fatty acid oxidation than those given a palmitate-rich diet [Kien and Bunn, 2008]. However, this is not the only explanation for the differences in cytotoxicity between saturated and monounsaturated fatty acids. When cells were exposed to palmitoleate, which was associated with low cytotoxicity, levels of triglycerides and β-oxidation were similar to those observed in cells exposed to saturated fatty acids.

In the present study, MIN6 cells exposed to palmitate had high levels of apoptosis, which was associated with ER stress activation. Indeed, the specific up-regulation of FAS by palmitate could accentuate the ER stress by increase in fatty acid load. Stearate induced apoptosis to a lesser extent, however. In addition, the ER stress activation in response to stearate only involved eIF2α phosphorylation. Since palmitate is the exclusive precursor of ceramide, it was tested if generation of the sphingolipid contributed to the accentuated detrimental effects observed in MIN6 cells exposed to palmitate. Increased ceramide de novo synthesis has been proposed to induce apoptosis [Lupi et al., 2002; Maedler et al., 2003]. When de novo synthesis of ceramide was halted in the present study ER stress was not altered, however. This is in agreement with other studies showing that inhibition of ceramide de novo synthesis does not influence cell death [Listenberger et al., 2001; Wei et al., 2006].

It is concluded that insulin-producing β-cells exposed to palmitate initiate expression changes consistent with lipid accumulation and combustion, which cause minor rise in triglyceride levels and fatty acid oxidation, however (Fig. 8). In contrast, cells exposed to oleate show enhanced lipid combustion and storage despite small changes in expression of lipid handling genes. These differences in oxidation and storage of fatty acids in cells exposed to palmitate or oleate probably contribute to explain the changes in

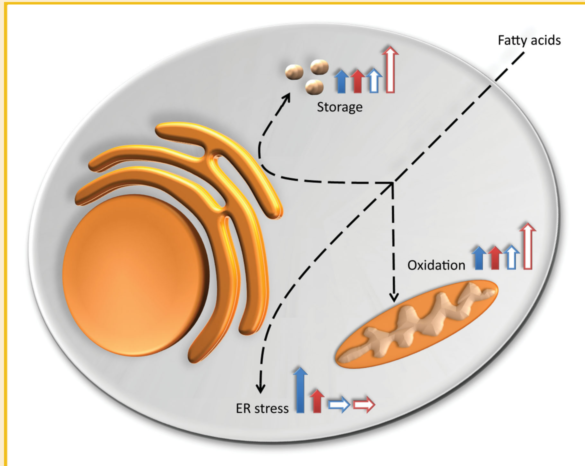

Fig. 8. Cellular fate of fatty acids. The scheme depicts metabolism of palmitate (closed blue arrow), stearate (closed red arrow), palmitoleate (open blue arrow), and oleate (open red arrow). Arrow length indicates magnitude of fatty acid-induced change compared to control conditions where horizontal arrows indicate no change compared to control. Fatty acids are modulated on the ER by DGAT, ELOVL, and SCD to be stored as triglycerides and/or imported via CPT1 into the mitochondria to be β-oxidized. Fatty acids may also induce ER stress, which can be translated into apoptosis.

β-cell function and mass in these cells. However, the different effects on ER stress and apoptosis in cells exposed to palmitate, palmitoleate, or stearate despite the equal low ability of these fatty acids to promote lipid combustion and storage demonstrates that other fatty acid chain- and saturation-dependant effects are critical for β-cell function and mass.

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