Original Article

Increased Fatty Acid Desaturation and Enhanced Expression of Stearoyl Coenzyme A Desaturase Protects Pancreatic β-Cells from Lipoapoptosis

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Increased availability of fatty acids causes cell death and dysfunction in β-cell lines, isolated islets, and animal models of diabetes. From the MIN6 β-cell line, we selected two subpools that are resistant to palmitate-induced apoptosis. Protection was not universal because palmitate-resistant cells remained sensitive to cytokine- and streptozotocin-induced apoptosis. Palmitate oxidation and incorporation into cholesterol ester (but not triglycerides) were significantly higher in palmitate-resistant cells than in control cells. Consistent with these findings, transcript profiling revealed increased expression in palmitate-resistant cells of several β-oxidation genes as well as a 2.8-fold upregulation of stearoyl-CoA desaturase 1 (SCD1). Correspondingly, the oleate-to-palmitate ratio of palmitate-resistant cells was double that of palmitate-pretreated control cells. At least some of this additional oleate in palmitate-resistant cells was incorporated into cholesterol ester stored in the form of large cytosolic lipid bodies. However, blocking cholesterol ester formation did not render palmitate-resistant cells sensitive to palmitate-induced apoptosis. On the other hand, an inhibitor of SCD1, 10,12-conjugated linoleic acid, dose dependently overcame the resistance of palmitate-resistant cells to lipoapoptosis. Our results suggest that desaturation per se is more important in protecting β-cells from the cytotoxic effects of palmitate than is the nature of neutral lipid storage pool thus generated. ***Diabetes*** **54:2917–2924, 2005**

Type 2 diabetes is characterized by insulin resistance in peripheral tissues as well as a progressive decline in the function of the pancreatic β-cell. The latter defect is manifested as insufficient insulin secretion in response to glucose and as a relative loss of β-cell mass (1–4). Obesity is an important risk factor in the development of type 2 diabetes, and hyperlipidemia is strongly associated with insulin resistance. Increased insulin secretion from the β-cell can compensate for insulin resistance in most instances, but in some individuals there is a progressive loss of β-cell function leading to decreased insulin secretion, hyperglycemia, and diabetes. The fact that many obese and insulin-resistant individuals never develop type 2 diabetes suggests that a defect in β-cell function or adaptation is essential in the development of the disease (5). Given the strong genetic component in type 2 diabetes, it is probable that allelic variation or alterations in expression of β-cell genes contribute to this defect.

Chronic exposure of β-cells to fatty acids in vitro results in secretory alterations and apoptosis similar to the defects observed in type 2 diabetes (6–11). Fatty acid–induced cell death (lipoapoptosis) is also seen in some other cell types including CHO cells (12), cardiomyocytes (13), and breast cancer cells (14). In most instances, the degree of saturation of the fatty acids seems to be important for the cytotoxic effect, because saturated fatty acids (such as palmitate) cause marked apoptosis, whereas unsaturated fatty acids (such as oleate) are much less cytotoxic (8,11,13,15). Unsaturated fatty acids have been shown to protect against the proapoptotic effects of saturated fatty acids (8,12–14). The suggested mechanisms by which fatty acids mediate apoptosis include nitric oxide (NO) synthesis (6), suppression of antiapoptotic factors such as bcl-2 (7,16), accumulation of intracellular triglycerides (7,17), generation of reactive oxygen species (ROS) (18), activation of nuclear factor-κB (19), decreased synthesis of the mitochondrial phospholipid cardiolipin (14,20), and de novo synthesis of ceramide (6–8,12). Despite intense investigation, the relative contributions of these various mechanisms to lipoapoptosis in β-cells remain controversial.

In this study, we sought to reinvestigate these issues by taking advantage of the adaptive mechanisms by which β-cells might protect themselves against lipoapoptosis. This was achieved by selecting two palmitate-resistant subpools of the murine β-cell line MIN6 by continuous culturing in a high concentration of the fatty acid. By comparing lipid metabolic profiles and global gene expression in the palmitate-resistant versus MIN6 cells, we demonstrate that the capacity for fatty acid desaturation is a critical determinant of β-cell survival

RESEARCH DESIGN AND METHODS

Culture media and the Superscript Choice system were from Gibco BRL (Gaithersburg, MD). The Cell Death Detection ELISA^{PLUS} kit was purchased from Roche Diagnostics (Penzberg, Germany). 3-[4,5-Dimethylthiazol-2-yl]-2,5-

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ACAT, acyl-CoA:cholesterol acyltransferase; CLA, conjugated linoleic acid; CPT1, carnitine palmitoyl transferase 1; DMEM, Dulbecco's modified Eagle's medium; ROS, reactive oxygen species; SCD1, stearoyl-CoA desaturase 1; SPT1, serine palmitoyltransferase-1.

diphenyl-tetrazolium bromide (MTT), 98% fatty acid–free BSA, palmitate sodium salt, Nile Red, and butylated hydroxytoluene were from Sigma-Aldrich (St. Louis, MO). The bicinchoninic acid protein assay kit was from Pierce. RNeasy mini kits were obtained from Qiagen (Melbourne, Australia), and the High Yield RNA transcript labeling kits were from Enzo Biochem (New York, NY). U74A, U74B version 2, and U74C version 2 microarrays were from Affymetrix (Santa Clara, CA). Kits for radioimmunoassay of rat insulin were obtained from Linco Research (St. Louis, MO). [U-^{14}C]palmitate was from PerkinElmer (Boston, MA). Silica Gel 60Å thin-layer chromatography plates (layer thickness, 250 μm) and glass fiber filters (grade DE81) were from Whatman. [γ-^{32}P]ATP and the *sn*-1,2-Diacylglycerol Biotrak assay reagents system were purchased from Amersham Biosciences. Immunofluore was from ICN. CI976 was a gift from Professor T.Y. Chang (Dartmouth Medical School). 10,12-conjugated linoleic acid (10,12-CLA) and 9,11-conjugated linoleic acid (9,11-CLA) were from Matreya (Pleasant Gap, PA).

Cell culture and general experimental treatment. Palmitate-resistant cell pools were selected and routinely passaged in 75-cm^2 flasks with 20 ml Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose, 24 mmol/l $NaHO_3$, 10 mmol/l HEPES, 10% (vol/vol) FCS, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 1 mmol/l palmitate coupled to 2.3% BSA (wt/vol). This selection procedure was carried out twice, giving rise to two independent pools of palmitate-resistant cells. Although both pools were used for transcript profiling experiments, functional studies used only the lower passage pool of palmitate-resistant cells. Control MIN6 cells were taken through the same selection process but using BSA-medium without palmitate. Cells were seeded in DMEM as follows. Cell death detection ELISA and MTT cell viability assays: 3×10^4 cells per well in 96-well dishes (0.2 ml); [^{14}C]palmitate labeling and protein measurement: 2×10^5 cells per well in 24-well dishes (0.5 ml); microarray experiments: 5×10^6 cells per 25-cm^2 flask (5 ml); and lipid composition analysis: 6×10^6 cell per 10-cm dish (15 ml). For confocal experiments cells were seeded on cover slips placed in 24-well dishes: 1×10^5 cells/well (0.5 ml). At 48 h before the experiments (24 h after seeding), the medium was replaced with DMEM (as above but with 5 mmol/l glucose) supplemented with either BSA alone or BSA coupled to palmitate. Couplings were prepared in DMEM (25 or 5 mmol/l glucose) as described previously (21). The couplings were diluted 1:8 (for culture and for experiments), giving a final concentration of 1 mmol/l palmitate to 2.3% BSA (wt/vol), corresponding to a molar ratio of 3:1.

Apoptosis assay. Apoptosis was measured using an ELISA kit (Roche), which determines the amount of apoptotic mono- and oligonucleosomes in a sample. The culture medium was removed and replaced with 0.2 ml of supplied lysis buffer. After 30 min incubation at room temperature, the lysate was spun at 200*g* for 10 min. The assay was performed using 20 μl of supernatant in the ELISA according to the manufacturer's instructions. For measurement of the total amount of DNA in each sample, cells were seeded and treated in parallel as above, except for an additional sonication step to extract all DNA.

Cell viability (MTT) assay. Cultured cells in 96-well dishes were washed twice with 0.2 ml Krebs-Ringer bicarbonate buffer containing 16.8 mmol/l glucose and 0.25% BSA (wt/vol) and then incubated with 0.1 ml of the same buffer containing 100 μg/ml MTT for 4 h at 37°C. Then 0.1 ml 10% (vol/vol) dimethylformamide in 20% (wt/vol) sodium dodecyl sulfate was added, and the reactions were left overnight at 37°C. The absorbance of the dissolved formazan was measured at 550 nm in a multiwell plate reader (model 3,550-UV; Bio-Rad). The background value in the reaction solution alone (without cells) was subtracted from the sample absorption values before data analysis.

Transcript profiling. Total RNA (15–40 μg) was isolated, and samples were prepared for microarray analysis as previously described (21). Affymetrix Mouse genome U74A, U74B version 2, and U74C version 2 were used, and data were analyzed by MAS 5.0. The raw data were further analyzed using Phenzomix, a data management tool developed in-house for microarray analysis and previously described (21).

Incorporation of palmitate into cellular lipids. Cells were treated for 48 h as indicated in the figure legends. Two hours before the end of the treatment 2.5 μCi [^{14}C]palmitate in ethanol was added to each well (24-well dish, 0.5 ml culture medium). At the end of the treatment, the medium was removed and used for determination of fatty acid oxidation (see below). The labeled cells were put on ice, washed in 3×0.5 ml ice-cold 1× PBS, scraped off the dish in 1 ml cold 1× PBS, and spun for 10 min at 3,000*g*. The supernatant was discarded, and lipids in the cell pellet were extracted overnight in 1 ml chloroform:methanol (2:1, vol/vol). The extractions were washed in 250 μl H_2O, followed by an additional wash in 125 μl H_2O, and the resulting lipid-containing, organic phase was dried under a stream of nitrogen. The lipids were redissolved in 50 μl chloroform:methanol (2:1, vol/vol), and a small fraction was used to determine the total amount of counts in the extracted lipids. The rest of the sample was spotted onto silica plates (K6; Whatman); and lipids were separated by thin layer chromatography in petroleum ether: diethyl ether:methanol:acetic acid (180:14:4:1 by volume). Spots comigrating with triglyceride and cholesterol ester standards were individually scraped and counted by liquid scintillation spectrometry. Protein was measured in parallel using a bicinchoninic acid protein assay (Pierce).

Fatty acid β-oxidation. Culture medium from [^{14}C]palmitate-labeled cells (see above) was transferred to Erlenmeyer flasks. A glass fiber filter (Whatman) was dipped in 1 mol/l KOH and suspended over the medium, and the flasks were closed with rubber septae. The medium was then acidified by injection of 6% (by volume) perchloric acid through the septae, and the flasks were placed at 37°C with mild agitation for 2 h. The amount of radioactively labeled CO_2 released from the medium and captured on the filters was then measured by liquid scintillation spectrometry.

Quantitative PCR. Real-time PCR was carried out as previously described (21) using the following primers (forward and reverse): CAATCTCTGGATGC GGTAGAAAAG and GGACTTGTCAAACCACCTGTCG (CPT-1); AAGGGCAG TTCTGAGGTGATTAGAG and ATGGCAGTGGGTAGGTAGTCTTGC (SCD1); and TCTGCTGGCAAAGTTCCACC and TCTGTTCCTCCACACAAGACGAC (Acaa2).

Confocal microscopy. Cells were fixed with 4% paraformaldehyde in 1× PBS for 1 h and permeabilized for 30 min in 1× PBS; 0.2% saponin, before staining with a 1:1,000 dilution of a saturated Nile Red stock solution in acetone. Cells were mounted, after washing in 1× PBS and water, in Immuno-fluore. Slides were examined using Leica laser scanning confocal microscopes (Leica Microsystems, Wetzlar, Germany). Nile Red was excited with the appropriate excitation wavelength. Each image represents a single ~0.8-μm "Z " optical section. Images were processed using the Leica confocal software and Adobe Photoshop v7 (San José, CA) for Apple Computer.

Analysis of cellular fatty acid composition. Total cellular lipids were extracted as described above using chloroform:methanol (2:1, vol/vol) containing 0.01% (wt/vol) butylated hydroxytoluene as an antioxidant. Fatty acid composition was determined by the direct transesterification method of Lepage and Roy (22). Briefly, total cellular lipids were transmethylated with methanol:toluene (4:1, vol/vol) and acetyl chloride at 100°C for 1 h, and the fatty acid methyl esters were separated by gas-liquid chromatography on a Shimadzu GC-17A gas chromatograph with a Restek FAMEWAX capillary column. Individual fatty acids were identified by comparing the retention time of each peak with those of external standards.

Statistical analysis. Unless otherwise indicated, results are expressed as the means ± SE. Statistical significance was determined with unpaired Student's *t* test.

RESULTS

Selection of palmitate-resistant MIN6 cells. To investigate the mechanisms whereby chronic exposure of pancreatic β-cells to the saturated fatty acid palmitate (16:0) causes apoptosis, we adopted a selection strategy in which MIN6 cells were continuously cultured in the presence of this fatty acid coupled to BSA. The few cells surviving two to three passages under these conditions were subsequently expanded in palmitate-containing medium. These palmitate-resistant cells had growth rates comparable with control MIN6 cells cultured in parallel in BSA alone (data not shown). In terms of secretory responsiveness to glucose, sensitivity to lipoapoptosis, and lipid metabolic parameters, these control cells used for all direct comparisons with palmitate-resistant cells were essentially similar to MIN6 cells passaged without BSA (not shown). Palmitate-resistant cells showed an interesting secretory profile: glucose responsiveness was robust but absolutely dependent on the co-provision of palmitate (not shown). This suggests that the exocytotic machinery is normal in the cells but that nutrient recognition is perturbed consistent with alterations in lipid metabolism described below. This secretory phenotype is being pursued in other studies and is not further investigated here.

Palmitate-resistant cells are selectively resistant to palmitate. We initially investigated the sensitivity of palmitate-resistant cells to different cytotoxic insults. After 48 h, exposure to palmitate apoptosis was increased fourfold in native MIN6 cells but was unaffected in palmi-

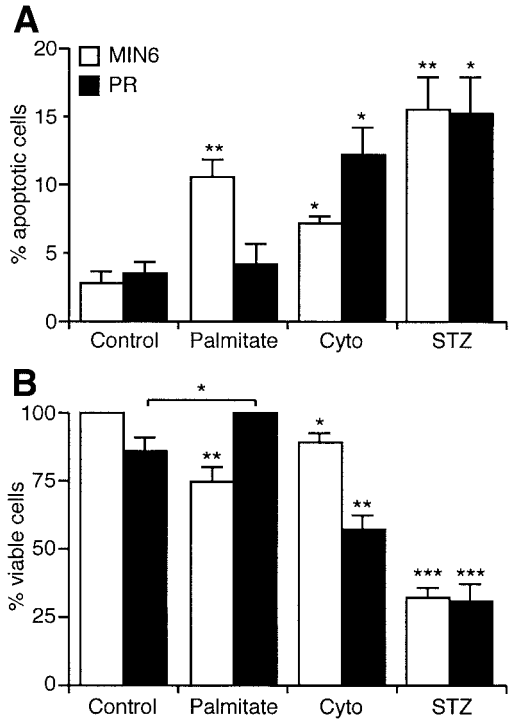

FIG. 1. Effect of 48 h of treatment with palmitate, cytokines, or streptozotocin on apoptosis and cell viability. Cells were treated for 48 h in medium supplemented with 2.3% BSA alone (Control) or 1 mmol/l palmitate/2.3% BSA (Palmitate), a cocktail of cytokines (2 ng/ml interleukin-1β, 10 ng/ml tumor necrosis factor-α, and 100 ng/ml interferon-γ) (Cyto), or 1 mg/l streptozotocin (STZ) as indicated. Apoptosis (*A*) and cell viability (*B*) were measured as described in RESEARCH DESIGN AND METHODS. Cell viability is expressed as percentage of viability in cells cultured in 2.3% BSA alone for MIN6 cells and 1 mmol/l palmitate/2.3% BSA for palmitate-resistant cells (the usual culture conditions of the cells). Results are means ± SE of three separate experiments done in duplicate (*A*) or quadruplicate (*B*). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the equivalent value in control-treated cells or as indicated.

tate-resistant cells, thereby confirming their lack of sensitivity to lipoapoptosis (Fig. 1*A*). However, these cells were equally or even more sensitive than MIN6 cells to 48-h pretreatment with the β-cell toxin streptozotocin or a mixture of cytokines. These features were confirmed when cell viability was independently determined using the MTT assay (Fig. 1*B*). Neither PR nor MIN6 cells underwent apoptosis when pretreated with the unsaturated fatty acid oleate (results not shown). Collectively, these data indicate that palmitate-resistant cells retain sensitivity to most apoptotic stimuli but have been selected for adaptations in lipid metabolism rendering them specifically less sensitive to the effects of saturated fatty acid.

β-Oxidation and incorporation of fatty acid into cholesterol ester, but not triglyceride, is increased in palmitate-resistant cells. To investigate the potential metabolic adaptations undergone by palmitate-resistant cells, we compared the incorporation of [^{14}C]palmitate into neutral lipids over a 2-h period in palmitate-resistant and MIN6 cells. There was no difference in [^{14}C]palmitate uptake per protein unit between the two cell populations, irrespective of the culture condition (data not shown), suggesting that palmitate resistance in palmitate-resistant cells is not merely due to reduced fatty acid transport. In MIN6 cells, 48 h of palmitate treatment resulted in a 1.7-fold increase in [^{14}C]palmitate incorporation into triglycerides (Fig. 2*A*). However, this level was indistinguishable from that of palmitate-resistant cells, suggesting that altered triglyceride synthesis is not responsible for the protection against palmitate-induced cell death in palmitate-resistant cells. Pretreatment of control cells for 48 h with palmitate resulted in a two- to threefold increase in palmitate channelling into cholesterol ester (Fig. 2*B*). A similar fold increase was seen in palmitate-resistant cells but from a baseline that was more than double that of MIN6 cells. Thus, in pretreated palmitate-resistant cells, cholesterol ester accounted for a [^{14}C]palmitate incorporation similar to that of triglyceride, thereby indicating that cholesterol ester can act as a major store of neutral lipid under these conditions (Fig. 2*B*). Fatty acid oxidation was also augmented in both cell populations by prior treatment with palmitate (Fig. 2*C*). However, palmitate oxidation was threefold higher across the board in palmitate-resistant versus MIN6 cells, irrespective of the pretreatment conditions. Taken together, these results indicate that MIN6 and palmitate-resistant cells similarly partition palmitate into triglycerides, but that palmitate resistance is associated with an increased capacity to oxidize this saturated fatty acid and to channel it into cholesterol esters.

Genes of fatty acid β-oxidation and desaturation are upregulated in palmitate-resistant cells. Alterations in gene transcription, potentially contributing to the observed alterations in lipid metabolism, were investigated by transcript profiling of palmitate-cultured palmitate-resistant cells. This revealed that expression of 27 genes was significantly altered 1.9-fold or more in both cell lines compared with the corresponding control cells that had been cultured in 2.3% BSA alone (results not shown). This is much fewer than the number of genes similarly altered in native MIN6 cells pretreated with palmitate (21,23),

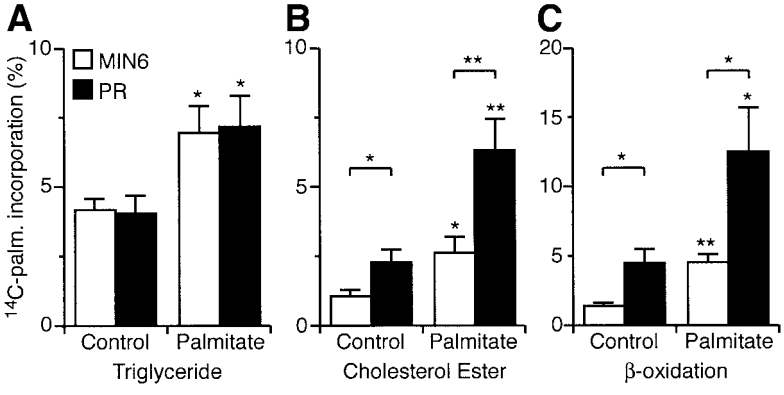

FIG. 2. Palmitate oxidation and incorporation of palmitate into triglycerides and cholesterol ester. Cells were treated for 48 h in medium supplemented with either 2.3% BSA alone (Control) or 1 mmol/l palmitate/2.3% BSA (Palmitate). For the last 2 h of the treatment, [^{14}C]palmitate was added to the medium. Incorporation into neutral lipids and β-oxidation was analyzed as described in RESEARCH DESIGN AND METHODS and expressed as percentage of counts incorporated into total lipids. Results are means ± SE of five independent experiments done in duplicate. *P < 0.05 and **P < 0.01 compared with control-treated cells or as indicated.

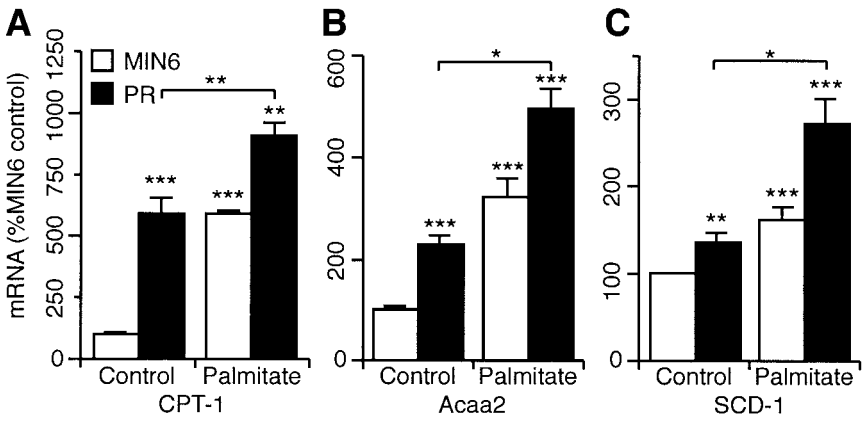

FIG. 3. mRNA levels of genes involved in β-oxidation and fatty acid desaturation. Cells were treated as described in Fig. 2. Total RNA was extracted and analyzed by real-time PCR. Results are means ± SE of five to seven independent experiments and are expressed as percentage of the mRNA levels in control-treated MIN6 cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with control-treated MIN6 cells or as indicated.

suggesting that the selection process undergone by the palmitate-resistant cells is something more than a global alteration in gene expression due to changes in nutrient supply. Of the genes altered in palmitate-resistant cells, nearly 35% were involved in lipid metabolism, including the three showing the greatest fold change: carnitine palmitoyl transferase 1 (CPT1), 3-ketoacyl-CoA thiolase (Acaa2), and stearoyl-CoA desaturase 1 (SCD1). Expression of these genes was confirmed by RT-PCR (Fig. 3). The expression profiles of CPT1 and Acaa2 are very similar to the relative rates of palmitate oxidation under equivalent treatment conditions (Fig. 2*C*). This would be consistent with the roles of these enzymes as key controllers of β-oxidation (24). SCD1 expression showed a different profile (Fig. 3), reminiscent of the pattern for [^{14}C]palmitate partitioning into cholesterol ester (Fig. 2*B*), because the most pronounced increases in either case were seen in palmitate-pretreated palmitate-resistant cells.

Extracellular palmitate is incorporated into cholesterol ester as a fatty acyl CoA side chain. We next sought to investigate the potential relationship between fatty acid desaturation by SCD1 and cholesterol ester formation. The terminal step of cholesterol ester synthesis is catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT), which prefers an unsaturated fatty acyl-CoA, usually oleoyl-CoA, for condensation with cholesterol (25,26). Incorporation of [^{14}C]palmitate into cholesterol ester via this route would therefore require desaturation of palmitoyl CoA, consistent with a role for SCD1. Alternatively, in a route not dependent on desaturation, palmitoyl CoA might be metabolized by mitochondrial β-oxidation to form acetyl CoA that could then be used for de novo synthesis of cholesterol. To determine which of these two routes predominates in our system, we used lovastatin, an inhibitor of cholesterol biosynthesis, and mevalonate, a metabolite formed downstream of the site of lovastatin inhibition. Lovastatin significantly reduced incorporation of [^{14}C]palmitate into cholesterol ester in each cell population, in the presence and in the absence of palmitate (Fig. 4). This was rescued in all cases by addition of nonradioactive mevalonate, which would not be expected if the main route for incorporation [^{14}C]palmitate into cholesterol ester was via biosynthesis of the cholesterol moiety. Rather, these data suggest that the major route is via desaturation and the subsequent provision of unsaturated fatty acyl-CoA substrates for condensation with cholesterol.

SCD1 inhibition diminishes neutral lipid accumulation. To confirm whether desaturation of palmitate was required for its incorporation into cholesterol ester, we used *cis*-10,12-conjugated linoleic acid (10,12-CLA), an inhibitor of SCD1 function (27). As shown in Fig. 5*A*, 10,12-CLA inhibited [^{14}C]palmitate incorporation into cholesterol ester in control MIN6 cells by ~70%, whereas a structural analog, 9,11-CLA, which does not inhibit SCD1, was without effect. Moreover, in palmitate-pretreated cells, the enhanced tracer incorporation seen under these conditions was markedly diminished by the inhibitor, especially in palmitate-resistant cells where it approached levels equivalent to those in MIN6 cells. Again the inactive analog was ineffective. CLA exerted less obvious effects on [^{14}C]palmitate incorporation into triglyceride (Fig. 5*B*). No effect was seen in control-treated cells, but the inhibitor did abolish the increase in triglyceride incorporation observed upon pretreatment of MIN6 cells with palmitate. These results suggest that a major feature of palmitate-

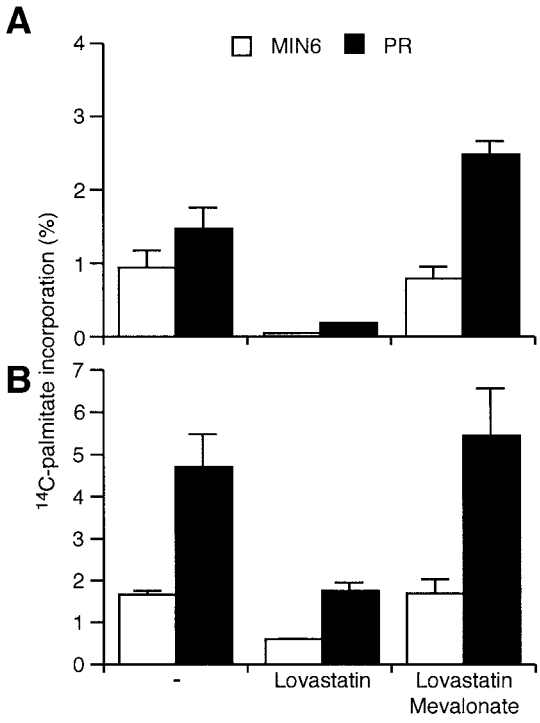

FIG. 4. Blocking cholesterol biosynthesis does not prevent incorporation of [^{14}C]palmitate into cholesterol ester. Cells were treated for 48 h in medium supplemented with either 2.3% BSA alone (*A*) or 1 mmol/l palmitate/2.3% BSA (*B*) in combination with 25 μmol/l lovastatin and 200 μmol/l mevalonate as indicated. Incorporation of [^{14}C]palmitate into cholesterol ester was analyzed and expressed as described in Fig. 2. Results are means ± range from a representative of three independent experiments done in duplicate.

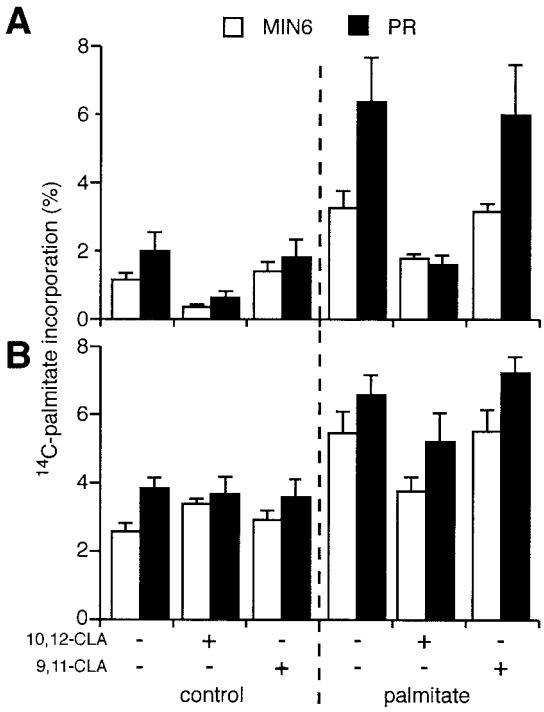

FIG. 5. Inhibition of SCD1 diminishes incorporation of palmitate into neutral lipids. Cells were treated for 48 h with either 2.3% BSA or 1 mmol/l palmitate/2.3% BSA in combination with 40 μmol/l 10,12-CLA or the inactive control compound 9,11-CLA as indicated. Incorporation of [^{14}C]palmitate into cholesterol ester (*A*) and triglyceride (*B*) was analyzed and expressed as described in Fig. 2. Results are means ± SE of three independent experiments done in duplicate.

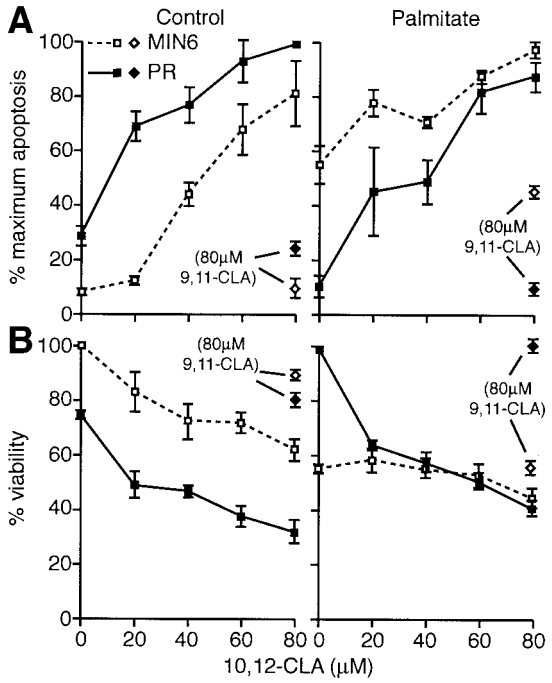

FIG. 6. Inhibition of SCD-1 renders PR3 cells sensitive to the cytotoxic effects of palmitate. Cells were treated for 48 h in medium supplemented with the indicated concentrations of 10,12-CLA and either 2.3% BSA alone (Control) or 1 mmol/l palmitate/2.3% BSA (Palmitate). Apoptosis (*A*) and cell viability (*B*) was measured as described in RESEARCH DESIGN AND METHODS. The effect of 80 μmol/l of the inactive control compound 9,11-CLA is also indicated. Results are means ± SE of four independent experiments done in duplicate (*A*) or quadruplicate (*B*). *$P < 0.05$ and ***$P < 0.01$ compared with similarly treated MIN6 cells.

resistant cells is their enhanced removal of palmitate by increased activity of SCD1 and the deposition of the resultant unsaturated fatty acid metabolites into neutral lipid, especially cholesterol ester.

Palmitate-resistant cells are enriched in unsaturated fatty acid species. As an additional confirmation of the functional consequences of upregulated SCD1 expression, we have analyzed fatty acid composition in our two cell populations (Table 1). Under control conditions, MIN6 and palmitate-resistant cells displayed essentially similar fatty acid profiles. Perhaps not surprisingly, pretreatment of MIN6 cells for 48 h tended to increase the proportion of palmitate incorporated into total cellular lipids at the expense of oleate. This is most clearly seen as the palmitate-to-oleate ratio, which doubled after palmitate pretreatment. In marked contrast, palmitate-resistant cells retained their fatty acid profile irrespective of whether they had been preexposed to palmitate. Thus, there was no significant difference in the palmitate-to-oleate ratio under these conditions. Moreover, the amount of oleoyl species (18:1n9 and 18:1n7) incorporated into total cellular lipids after chronic palmitate exposure was significantly higher in the palmitate-resistant versus MIN6 cells (Table 1).

TABLE 1
Fatty acid composition of total cellular lipids

Fatty acid	MIN6 control	MIN6 palmitate	PR3 control	PR3 palmitate
16:0	24.7 ± 3.4	35.2 ± 3.6	25.7 ± 2.0	28.0 ± 2.2
16:1n7	3.0 ± 1.1	9.8 ± 1.9	4.0 ± 1.3	7.4 ± 1.3
18:0	22.8 ± 3.3	19.0 ± 1.4	19.5 ± 1.9	16.5 ± 1.4
18:1n9	21.3 ± 1.4	14.9 ± 0.6	22.6 ± 0.8	19.8 ± 0.4*
18:1n7	7.4 ± 0.9	5.1 ± 0.5	9.5 ± 0.8	11.1 ± 1.9†
20:4n6	6.4 ± 1.1	6.3 ± 0.4	8.6 ± 0.2	7.7 ± 0.3†
16:0/18:1 ratio	0.88 ± 0.18	1.78 ± 0.20	0.80 ± 0.07	0.91 ± 0.08†

Data are percentage of total fatty acids in the total lipid extracts and are means ± SE of three independent experiments done in duplicate. Cells were treated for 48 h in medium supplemented with 2.3% BSA alone (control) or 1 mmol/l palmitate/2.3% BSA (palmitate). *$P <$ 0.01 and †$P < 0.05$ compared with palmitate-treated MIN6 cells.

Desaturation of fatty acids protects against lipoapoptosis. To determine whether the observed increase in SCD1 function was necessary for protecting palmitate-resistant cells against lipoapoptosis, we again made use of the SCD1 inhibitor 10,12-CLA. This caused a dose-dependent increase in apoptosis under all treatment conditions, approaching an upper limit of 15–20% with a dose of 80 μmol/l 10,12-CLA in the palmitate-resistant cells, against which all other responses were therefore normalized for comparison (Fig. 6). When added for 48 h in the absence of palmitate (Fig. 6*A*, *left panel*), 10,12-CLA augmented apoptosis dose dependently in both cell populations, although toxicity was correspondingly higher in the palmitate-resistant cells at each concentration used. The inactive analog 9,11-CLA, which does not inhibit SCD1, was without effect even at 80 μmol/l. As shown above, palmitate pretreatment increased apoptosis in MIN6 but not palmitate-resistant cells, and this was further increased by 10,12-CLA (Fig. 6*A*, right panels). However, the effects of the compound were more pronounced on palmitate-resistant cells, enhancing apoptosis at 20 μmol/l, and by 60 μmol/l, restoring palmitate sensitivity to an extent indistinguishable from that of MIN6 cells treated under the

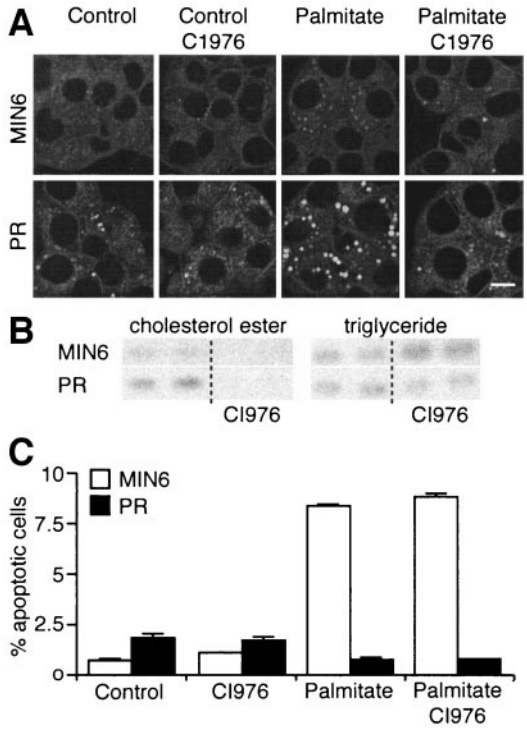

FIG. 7. Large lipid bodies formed in palmitate-resistant cells are sensitive to inhibition of cholesterol ester formation. Cells were treated for 48 h with either 2.3% BSA (Control) or 1 mmol/l palmitate/2.3% BSA in combination with 10 μmol/l CI976 as indicated. *A*: Cells stained with Nile Red to visualize lipid bodies were examined by confocal microscopy. *B*: Incorporation of [^{14}C]palmitate into cholesterol ester and triglyceride was measured in cells labeled with [^{14}C]palmitate as described in Fig. 2. *C*: Level of apoptosis was determined as described in RESEARCH DESIGN AND METHODS. Results are means ± range from a representative of three independent experiments done in duplicate.

same conditions. These effects were not reproduced by 9,11-CLA. Supporting results were also obtained by assessing cell viability (Fig. 7*B*). In summary, 10,12-CLA was toxic to MIN6 and palmitate-resistant cells, but the latter were more sensitive in the absence and presence of palmitate pretreatment. These findings are consistent with a greater reliance of the palmitate-resistant cells on removal of excess palmitate by desaturation.

Lipid bodies in palmitate-resistant cells contain cholesterol ester. Excess neutral lipid is usually stored intracellularly in the form of lipid bodies, which can be visualized by staining with the hydrophobic dye Nile Red. As shown by confocal microscopy, lipid bodies were readily apparent in palmitate-resistant cells and were clearly larger and more abundant than those in MIN6 cells, either pretreated or not with palmitate (Fig. 7*A*). Pretreatment for 48 h with the ACAT inhibitor CI976 (25,26) inhibited formation of these lipid bodies, confirming that they are major sites of cholesterol ester deposition in palmitate-resistant cells (Fig. 7*A*). The specificity of CI976 was confirmed independently by its abolition of [^{14}C]palmitate incorporation into cholesterol ester (Fig. 7*B*). Partitioning into triglyceride was not affected under these conditions.

Cholesterol ester formation is not necessary for protection against palmitate-induced apoptosis in palmitate-resistant cells. We next tested whether blocking cholesterol esterification with the ACAT inhibitor CI976 affected the level of lipoapoptosis in the palmitate-resistant cells. However, as demonstrated in Fig. 7*C*, pretreatment for 48 h with CI976 did not increase the level of apoptosis in either control or palmitate-resistant cells, even in the presence of palmitate. This was despite the accompanying inhibition of lipid droplet formation (Fig. 7*A*) and partitioning of palmitate into cholesterol ester (Fig. 7*C*). These results suggest that the protection against palmitate in the palmitate-resistant cells lies at a step between fatty acid desaturation and incorporation of unsaturated fatty acid into neutral lipid.

DISCUSSION

The mechanisms by which saturated fatty acids promote apoptosis of pancreatic β-cells are the subject of active investigation because of their potential relevance to type 2 diabetes. Previous studies using β-cells as well as other cell types have focused on the hypothetical involvement of neutral lipid deposition (6,7,17,28), ROS generation (18), and de novo ceramide synthesis (6–8,15,29,30). However, a consensus has been difficult to reach. Because of this ongoing controversy, we used an alternative to testing candidate pathways, which involved a selection strategy to generate and characterize a subpool of palmitate-resistant MIN6 cells. By undertaking a metabolic and transcript profiling of these cells, we hoped to determine mechanisms whereby protection against lipoapoptosis was afforded and, by extension, to shed light on the actual cytotoxic pathways themselves. Our results using this model clearly point to the capacity for intracellular fatty acid desaturation as a critical determinant of cellular survival.

Deposition of excess fatty acid as triglyceride has been positively and negatively correlated with fatty acid–induced death of β-cells (6,7,17,28). In the Zucker diabetic fatty (ZDF) rat, an adipogenic animal model of diabetes, extreme accumulation of triglyceride in islets has been suggested to cause β-cell apoptosis by providing an expanded source of palmitoyl-CoA for de novo synthesis of ceramide and a subsequent rise in NO (6,29). However, we (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data) and others (7,28) have not seen a rise in NO in pure β-cell populations. More recent studies have pointed toward a protective effect of triglyceride accumulation in β-cells (28) as well as CHO (12) and breast cancer cells (14) possibly by diverting excess fatty acids away from cytotoxic pathways and into inert neutral lipid stores. However, we did not observe any differences between control and palmitate-resistant cells in either the expression of triglyceride synthesis genes (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data) or metabolic flux from [^{14}C]palmitate into triglycerides. This argues against increased partitioning of fatty acids into triglyceride as being a major mechanism for protecting β-cells from lipoapoptosis. Nor do we believe that the enhancement of β-oxidation observed in palmitate-resistant cells is a major contributor to their protection. Oxidation of [^{14}C]palmitate was further enhanced in the presence of 10,12-CLA in palmitate-treated PR but not MIN6 cells (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data), but this was associated with an increase rather than a decrease in lipoapoptosis.

Cholesterol ester has been less extensively studied than triglyceride as a neutral lipid storage depot in the β-cell. Our results suggest that cholesterol ester makes a quantitatively important contribution to the neutral lipid reserve

in palmitate-pretreated cells, even in control MIN6 cells in which flux of [^{14}C]palmitate was augmented to total levels of about one-third that of triglyceride. The conversion of palmitate into cholesterol ester was even more pronounced in palmitate-resistant cells, resulting in formation of large cytosolic lipid droplets. This in itself is a novel finding because previous studies of lipid droplet formation in pancreatic β-cells are rare, focused on triglyceride accumulation, and interpreted as impacting either positively or negatively on cell survival (28,31,32). Our results, showing that blockade of palmitate desaturation using 10,12-CLA inhibited cholesterol ester formation and overcame resistance to lipoapoptosis in the palmitate-resistant cells, might be interpreted as a protective role for cholesterol ester deposition. However, blocking cholesterol esterification downstream of oleoyl CoA provision, by using CI976 to inhibit ACAT, did not restore sensitivity to lipoapoptosis in the palmitate-resistant cells. These two results can be reconciled as indicating that it is the desaturation of excess palmitate that is protective against lipoapoptosis; what happens to the bulk of the desaturated fatty acid subsequently is not particularly important. This consideration also helps resolve the conflicting data showing that accumulation of neutral lipid storage products can be positively or negatively correlated with β-cell death.

The crucial role of SCD1 activity in regulating β-cell survival has not previously been reported. However, this finding is broadly consistent with data demonstrating that co-provision of oleate reduces the cytotoxicity of even an excess of palmitate (8,28), a result we have additionally seen in MIN6 cells (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data). Moreover β-cells are known to express a number of saturases whose expression is downregulated by 24-h pretreatment with the unsaturated fatty acids linoleate and arachidonate (33). Our data showed slight up or down modulation of SCD1 with palmitate and oleate, respectively, in MIN6 cells (21) but a much more pronounced increase in the palmitate-resistant cells. Most importantly, we demonstrated using the inhibitor 10,12-CLA that SCD1 function was necessary for the protection against lipoapotosis. This extends a single previous study using CHO cells, showing that SCD overexpression was sufficient to overcome palmitate-induced cytotoxicity (12). Perhaps not surprisingly, however, the mechanisms underlying palmitate-resistance in the two models appeared to differ, because SCD1 affected mainly palmitate incorporation into triglyceride in CHO cells but not cholesterol ester, as we observed. This reinforces the conclusion that it is desaturation per se, rather than the nature of the neutral lipid storage product, that contributes most to the protection from lipoapoptosis.

In the CHO cell study, it was proposed that unsaturated fatty acid channeled palmitate away from toxic pathways such as ceramide generation (12). However, an earlier study had shown that inhibitors of de novo ceramide synthesis had little effect on lipoapoptosis in CHO cells and that ROS generation appeared to be the underlying cause of cytotoxicity (18). We found no difference in ROS production between lipid-pretreated MIN6 and palmitate-resistant cells, and inhibitors of de novo ceramide synthesis only very slightly protected against lipoapoptosis (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data). Moreover, in our [^{14}C]palmitate labeling experiments, an undetectably low level of counts was channeled into ceramide in MIN6 and palmitate-resistant cells (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data), despite pronounced alterations in other measured intermediates under these conditions. We are aware of only one β-cell study in which partitioning of palmitate into ceramide has actually been demonstrated (6). This was in islets of ZDF rats, which appear to incorporate as much palmitate tracer into ceramide as they oxidize. This suggests that the ZDF model has an unusually active de novo synthetic pathway, probably explained by its enhanced expression of serine palmitoyltransferase-1 (SPT1) (29), the first committed step of ceramide synthesis. However, our transcript profiling of MIN6 cells revealed that this gene was not altered by 48 h of lipid pretreatment (A.K.B., E.G., D.V.C., M.S., G.S.D., D.R.L., W.E.H., T.J.B., unpublished data). Therefore, in our model, a stoichiometric switching of palmitate away from ceramide metabolism and toward neutral lipid synthesis would appear to be an overly simplistic explanation for the protection observed in palmitate-resistant cells. However, ceramide metabolism is complex, and we cannot exclude involvement of pathways other than de novo synthesis at this stage. But it is also possible that alterations in lipid metabolites other than ceramide make important contributions to the balance between death and survival of lipid-pretreated β-cells. From studies in other cell types, hypothetical cases could be made for key roles of cardiolipin, phosphatidylcholine, and phosphatidylserine (14,34,35), but in principle, other unidentified metabolites could equally be involved. Further profiling of the palmitate-resistant cell model should be helpful in identifying and assessing the contributions of such metabolites.

Although our findings of a key role for SCD1 in protecting β-cells from lipoapoptosis are consistent with data generated in CHO cells, the situation in skeletal muscle appears very different (36). In this case, ablation of SCD1 reduces the partitioning of palmitate into ceramide due to adaptive alterations in the expression of SPT1 (decreased) and CPT1 (increased). However, in palmitate-resistant cells, enhanced expression of SCD1 was accompanied by increases in CPT1 and no change in SPT1. These differences highlight the drawbacks in attempting to interpret β-cell lipoapoptosis by extrapolating from data obtained in other cell types. More importantly, our data showing a beneficial role for SCD1 in β-cells, raises a note of caution concerning the use of SCD1 inhibitors for treatment of type 2 diabetes and the metabolic syndrome. Although loss of function of this enzyme is clearly beneficial in peripheral tissues, by reducing adiposity and improving insulin sensitivity (37), our results would suggest that such benefits might come at the expense of a loss in β-cell mass.

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