Monounsaturated Fatty Acids Prevent the Deleterious Effects of Palmitate and High Glucose on Human Pancreatic β-Cell Turnover and Function

Kathrin Maedler,[1] José Oberholzer,[2] Pascal Bucher,[2] Giatgen A. Spinas,[1] and Marc Y. Donath[1]

Glucotoxicity and lipotoxicity contribute to the impaired β-cell function observed in type 2 diabetes. Here we examine the effect of saturated and monounsaturated fatty acids at different glucose concentrations on human β-cell turnover and secretory function. Exposure of cultured human islets to saturated fatty acid and/or to an elevated glucose concentration for 4 days increased β-cell DNA fragmentation and decreased β-cell proliferation. In contrast, the monounsaturated palmitoleic acid or oleic acid did not affect DNA fragmentation and induced β-cell proliferation. Moreover, each monounsaturated fatty acid prevented the deleterious effects of both palmitic acid and high glucose concentration. The cell-permeable ceramide analogue C_2-ceramide mimicked both the palmitic acid–induced β-cell apoptosis and decrease in proliferation. Furthermore, the ceramide synthetase inhibitor fumonisin B1 blocked the deleterious effects of palmitic acid on β-cell turnover. In addition, palmitic acid decreased Bcl-2 expression and induced release of cytochrome c from the mitochondria into the cytosol, which was prevented by fumonisin B1 and by oleic acid. Finally, each monounsaturated fatty acid improved β-cell secretory function that was reduced by palmitic acid and by high glucose. Thus, in human islets, the saturated palmitic acid and elevated glucose concentration induce β-cell apoptosis, decrease β-cell proliferation, and impair β-cell function, which can be prevented by monounsaturated fatty acids. The deleterious effect of palmitic acid is mediated via formation of ceramide and activation of the apoptotic mitochondrial pathway, whereas Bcl-2 may contribute to the protective effect of monounsaturated fatty acids.
***Diabetes* 52:726–733, 2003**

Normoglycemia is maintained in the face of insulin resistance until the pancreatic β-cells become unable to produce sufficient quantities of insulin, at which point type 2 diabetes begins. A major factor in the amount of insulin that can be secreted is the β-cell mass itself. In animal models of diabetes, the failure of β-cell mass expansion is due to an imbalance between β-cell proliferation and/or neogenesis on the one hand and β-cell apoptosis on the other (1–9). In vitro studies have shown that elevated concentrations of glucose or of free (nonesterified) fatty acids (FFA) may be directly involved in the mechanisms responsible for the defective adaptation of β-cell turnover. Indeed, elevated glucose concentrations induce β-cell apoptosis in cultured islets from diabetes-prone *Psammomys obesus* (3), from human islets (10–13), and at higher concentrations in rodent islets (3,14,15). Investigations of β-cell proliferation revealed induction of proliferation by glucose in rat (15,16), *Psammomys obesus* (3), and human islets (10). However, unlike the long-lasting effect in rat islets, only a transient and reduced proliferative response was observed in *Psammomys obesus* and human islets. FFA-induced β-cell apoptosis coupled with reduced proliferation capacity was observed in rodents islets (9,15,17). It is not known whether FFA can also inhibit β-cell turnover in human islets.

Recent studies indicate that signal transduction through the ceramide pathway activates apoptosis in various cell types (18), including islets from the ZDF rats (9) and from normal rats (15). Ceramide is synthesized from long-chain fatty acids, and the ceramide synthetase inhibitor fumonisin B1 blocks the deleterious effects of FFA on β-cell viability (9,15). Therefore, the effect of FFA on β-cell turnover is mediated via de novo ceramide formation in rodent islets. The possible role of ceramide on human β-cell turnover has not been investigated so far.

A critical event that leads to apoptosis involves changes in mitochondrial membrane, which culminates in the release of apoptogenic factors, such as cytochrome c, from the mitochondrial intermembrane space to the cytosol (19,20). Bcl-2 is located predominantly in the outer mitochondrial membrane and inhibits the release of cytochrome c from mitochondria, thereby blocking the apoptotic process (21,22). In rat islets, the saturated palmitic acid but not the monounsaturated palmitoleic acid induces release of cytochrome c from the mitochondria into the

From the [1]Division of Endocrinology and Diabetes, University Hospital, Zurich, Switzerland; and the [2]Division of Surgical Research, Department of Surgery, University of Geneva Medical Center, Geneva, Switzerland.

Address correspondence and reprint requests to Marc Y. Donath, MD, Division of Endocrinology and Diabetes, Department of Medicine, University Hospital, CH-8091 Zurich, Switzerland. E-mail: marc.donath@dim.usz.ch.

Received for publication 13 June 2002 and accepted in revised form 21 November 2002.

FFA, free fatty acids; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

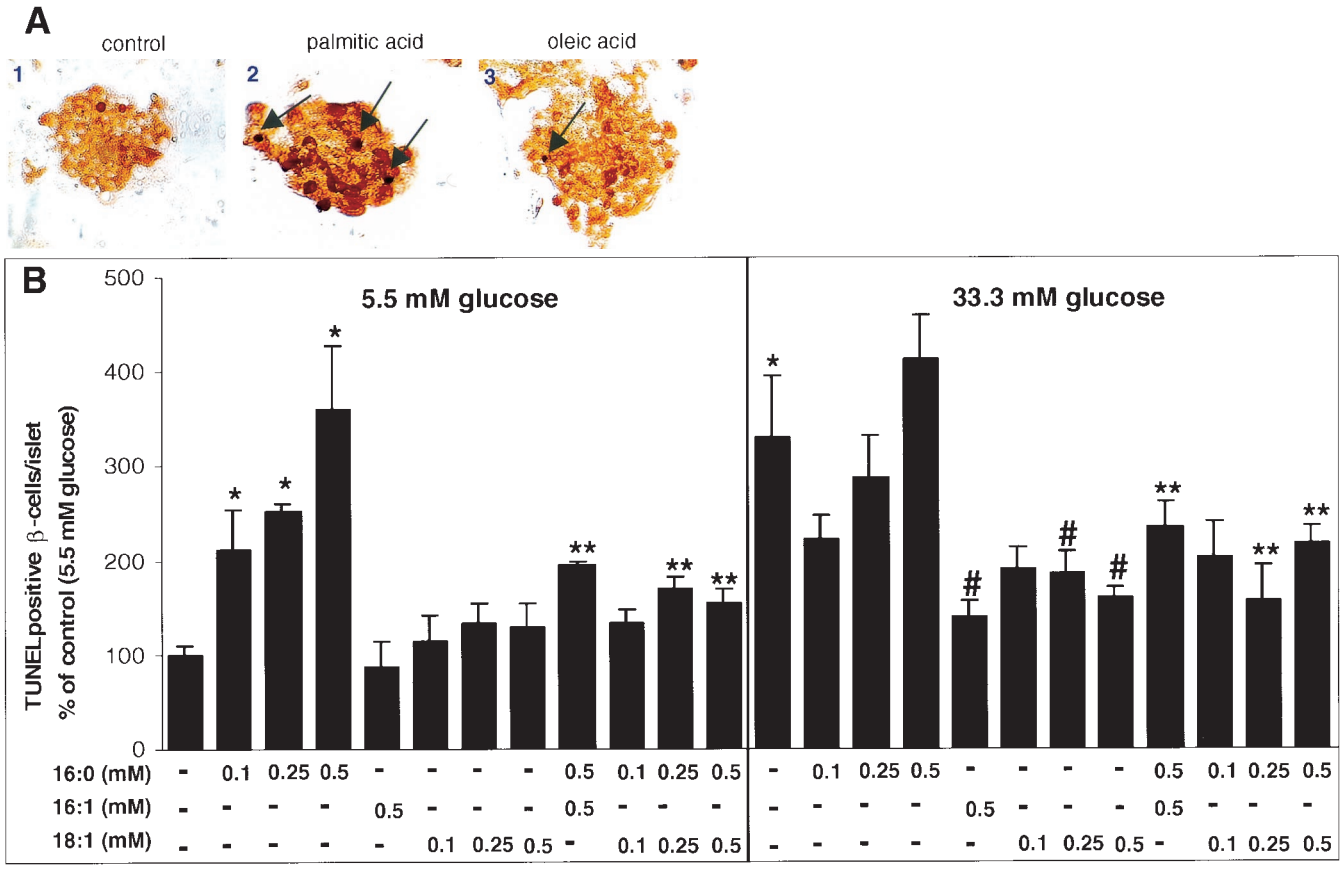

FIG. 1. Palmitoleic and oleic acids protect human β-cells from glucose- and palmitic acid–induced apoptosis. *A*: Islets were plated on extracellular matrix–coated dishes and exposed for 4 days to media containing 5.5 mmol/l glucose alone (*1*) and with 0.5 mmol/l palmitic (*2*) or oleic acid (*3*). Double immunostaining for insulin (orange) and DNA fragmentation by the TUNEL assay (black). The arrows mark β-cells nuclei stained positive for the TUNEL reaction (light microscopy ×250). *B*: Relative number of TUNEL-positive β-cells per islets after 4-day culture in 5.5 or 33.3 mmol/l glucose in the absence or presence of 0.1, 0.25, or 0.5 mmol/l palmitic (16:0), palmitoleic (16:1), or oleic (18:1) acid or a mixture of palmitic and palmitoleic acid (0.5 mmol/l each) or a mixture of palmitic and oleic acid (0.1, 0.25, or 0.5 mmol/l each). Results are means ± SE of the relative number of TUNEL-positive β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value: 0.43 TUNEL-positive β-cells per islet, corresponding to 0.33% TUNEL-positive β-cells). The mean number of islets scored from each donor was 40 (range 21–78) for each treatment condition. Islets were isolated from nine organ donors. *$P < 0.05$ relative to islets at 5.5 mmol/l glucose; **$P < 0.05$ between palmitic acid and fatty acid mixture at the same palmitic acid and glucose concentration; #$P < 0.05$ relative to islets at 33.3 mmol/l glucose.

cytosol, leading to β-cell apoptosis (15). In human islets, high concentrations of glucose induce β-cell apoptosis without changes in Bcl-2 expression and release of cytochrome c (10,11). However, the proapoptotic genes *Bad*, *Bid*, and *Bik* of the Bcl family are overexpressed in islets maintained in high glucose (11) and may sensitize β-cells to other proapoptotic stimuli. Therefore, we investigated the role of FFA and of high glucose concentrations alone or in combination on β-cell proliferation, apoptosis, and function in human islets. In addition, the involvement of the ceramide and of the apoptotic mitochondrial pathway was studied. To elucidate whether possible effects could be ascribed to the degree of saturation, we exposed β-cells to palmitic (C16:0), palmitoleic (16:1), and oleic (18:1) acid.

RESEARCH DESIGN AND METHODS

Islet isolation and culture. Islets were isolated from pancreases of nine organ donors at the Department of Surgery, University of Geneva Medical Center, as described previously (23). The islet purity was >95%, as judged by dithizone staining (when this degree of purity was not primarily achieved by routine isolation, islets were handpicked). The donors, aged 26–67 years, were heart-beating cadaver organ donors, and none had a previous history of diabetes or metabolic disorders. For long-term in vitro studies, the islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel), allowing the cells to attach to the dishes and spread, preserving their functional integrity (24,25). The contamination by ductal cells after 4 days in culture was of 5–15%, but almost all ductal cells were found in the periphery of the islets and did not co-localize with β-cells (12). Islets were cultured in CMRL 1066 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Gibco, Gaithersburg, MD), hereafter referred to as culture medium. Two days after plating, when most islets were attached and began to flatten, the medium was changed to culture medium containing 5.5 or 33.3 mmol/l glucose supplemented with or without fatty acids (Sigma Chemical, St. Louis, MO; palmitic acid [16:0], palmitoleic acid [16:1], oleic acid [18:1], or a mixture of fatty acids [16:0/16:1, 16:0/18:1]). Fatty acids were dissolved at 10 mmol/l in culture medium containing 11% fatty acid–free BSA (Sigma) under N_2-atmosphere, shaken overnight at 37°C, sonicated 15 min, and sterile-filtrated (stock solution) (15,26). For control experiments, BSA in the absence of fatty acids was prepared, as described above. The effective FFA concentration was determined after sterile filtration with a commercially available kit (Wako chemicals, Neuss, Germany). The calculated concentrations of non–albumin-bound FFA were derived from the molar ratio of total FFA (0.5 mmol/l) and albumin (0.15 mmol/l) using the stepwise equilibrium model (27). Unbound concentration of palmitic, palmitoleic, and oleic acids were of 0.832, 0.575, and 2.089 μmol/l, respectively, for a final concentration of 0.5 mmol/l FFA. In some experiments, islets were cultured with or without 15 μmol/l C_2-ceramide, 15 μmol/l C_2-Dihydroceramide (Biomol, Plymouth Meeting, PA), and 15 μmol/l fumonisin B1 (Sigma). All of them were first dissolved in prewarmed 37°C DMSO (Fluka, Buchs, Switzerland) at 5 mmol/l. For control experiments, islets were exposed to solvent alone (0.3% DMSO).

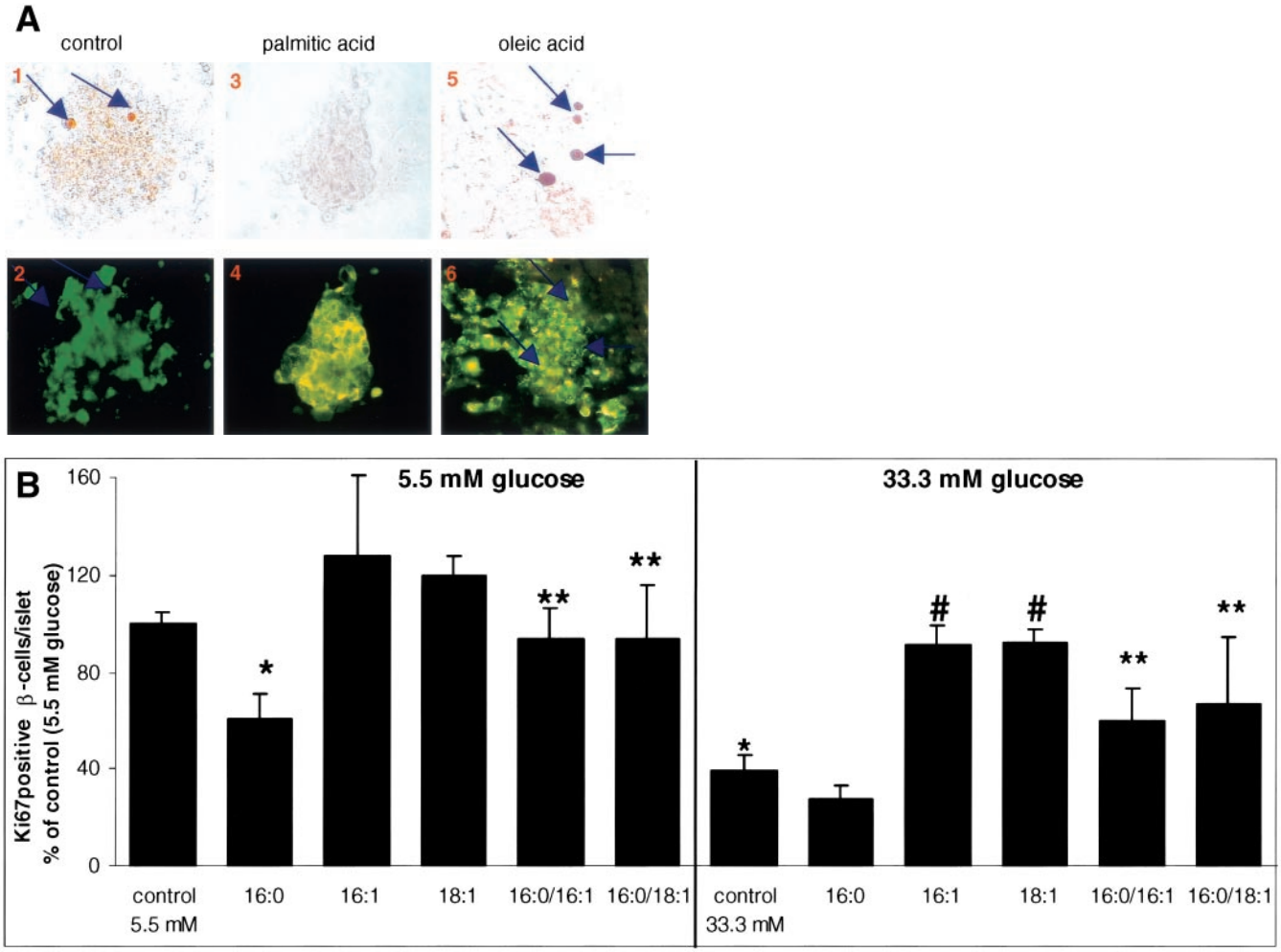

FIG. 2. Monounsaturated fatty acids protect from glucose- and palmitic acid–induced decrease in β-cell proliferation. *A*: Islets were plated on extracellular matrix–coated dishes and exposed for 4 days to media containing 5.5 mmol/l glucose alone (1, 2) and with 0.5 mmol/l palmitic (3, 4) or oleic acid (5, 6). Detection of β-cell proliferation with anti–Ki-67 (1, 3, 5) and with anti-insulin antibody (2, 4, 6). The arrows mark cells stained positive for Ki-67 and for insulin (light microscopy ×250). *B*: Relative number of Ki-67–positive β-cells per islets after 4-day culture in 5.5 or 33.3 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitic (16:0), palmitoleic (16:1), or oleic (18:1) acid or a mixture of palmitic and palmitoleic acid (16:0/16:1; 0.5 mmol/l each) or a mixture of palmitic and oleic acid (16:0/18:1; 0.5 mmol/l each). Results are means ± SE of the relative number Ki-67–positive β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value: 0.75 Ki-67–positive β-cells per islet, corresponding to 0.5% Ki-67–positive β-cells). The mean number of islets scored from each donor was 38 (range 22–62) for each treatment condition. Islets were isolated from six organ donors. *P < 0.05 relative to islets at 5.5 mmol/l glucose; **P < 0.05 between palmitic acid and fatty acid mixture at the same glucose concentration; #P < 0.05 relative to islets at 33.3 mmol/l glucose.

β-Cell replication. For β-cell proliferation studies, a monoclonal antibody against the human Ki-67 antigen was used (Zymed, San Francisco, CA) (3,28). After cultured islets were washed with PBS, they were fixed in 4% paraformaldehyde (30 min, room temperature) followed by permeabilization with 0.5% Triton X-100 (4 min, room temperature). Afterward, islets were incubated for 1 h at room temperature with monoclonal mouse anti–Ki-67 antibody diluted 1:10, followed by detection using a streptavidin-biotin-peroxidase complex (Histostain-Plus Kit, Zymed). Subsequently, islets were incubated for 30 min at 37°C with guinea pig anti-insulin antibody diluted 1:50 (Dako, Carpinteria, CA), followed by a 30-min incubation with a 1:20 dilution of fluorescein-conjugated rabbit anti-guinea pig antibody (Dako).

β-Cell apoptosis. The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (29). Islet cultures were fixed and permeabilized as described above, followed by the TUNEL assay, performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Boehringer Mannheim, Germany). The preparations were then rinsed with Tris-buffered saline and incubated (10 min, room temperature) with 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma). For staining of the activated caspase 3, after fixation and permeabilization, islets were incubated for 2 h at 37°C with a rabbit anti-cleaved caspase-3 antibody (1:50 dilution, D 175; Cell Signaling, Beverly, MA), followed by incubation (30 min, 37°C) with a Cy3-conjugated donkey anti-rabbit antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Thereafter, islets were incubated with a guinea pig anti-insulin antibody as above, followed by detection using the streptavidin-biotin-peroxidase complex (Zymed) or by a 30-min incubation with a 1:20 dilution of fluorescein-conjugated rabbit anti-guinea pig antibody (Dako).

The TUNEL assay detects DNA fragmentation associated with both apoptotic and necrotic cell death; therefore, islets were also treated with a fluorescent annexin V probe (Annexin-V-FLUOS staining kit, Boehringer Mannheim) according to the manufacturer's instructions. Double staining of cells with propidium iodide and annexin V enables the differentiation of apoptotic from necrotic cells.

Subcellular fractionation and Western blot analysis. For analysis of human islets subcellular fractions, groups of 200 islets were cultured in suspension in nonadherent plastic dishes. Mitochondrial and cytosolic (S100) fractions were prepared from islets suspended in 70 μl of ice-cold buffer containing 20 mmol/l HEPES-KOH (pH 7.5), 10 mmol/l KCl, 15 mmol/l $MgCl_2$, 1 mmol/l Na-EDTA, 1 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 250 mmol/l sucrose (21). Mechanical homogenization was achieved by repeated aspiration through a pipette. Unlysed cells and nuclei were pelleted by 10 min of centrifugation (750*g*, 4°C). The supernatant was centrifuged at 10,000*g* for 15 min at 4°C. The resulting pellet, representing the mitochondrial fraction, was then resuspended in 10 μl of the buffer described above. Finally, the supernatant was centrifuged at 100,000*g* for 1 h at 4°C. The supernatant from this final centrifugation represents the S-100 fraction (22). Both fractions were frozen at −80°C until used. Mitochondrial and cytosolic

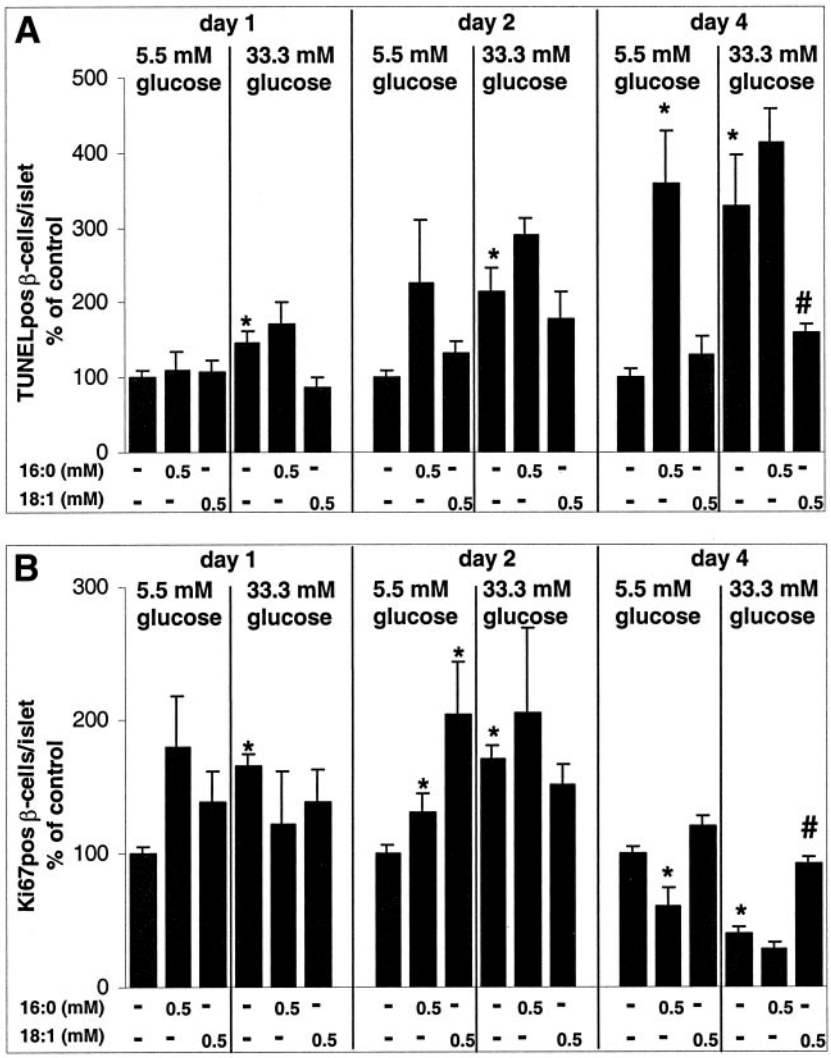

FIG. 3. Time course of the effect of 33.3 mmol/l glucose and 0.5 mmol/l palmitic and oleic acids on human β-cell apoptosis and proliferation. Relative number of TUNEL-positive (*A*) and Ki-67–positive (*B*) β-cells per islets after 1-, 2-, and 4-day culture in 5.5 or 33.3 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitic (16:0) or oleic (18:1) acid. Results are means ± SE of the relative number of TUNEL- or Ki-67–positive β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone. The mean number of islets scored from each donor was 31 (range 27–78) for each treatment condition. Islets were isolated from six organ donors. *$P < 0.05$ relative to islets at 5.5 mmol/l glucose; #$P < 0.05$ relative to islets at 33.3 mmol/l glucose.

fractions were diluted in sample buffer (187.5 mmol/l Tris-HCL [pH 6.8], 6% SDS, 30% glycerol, 150 mmol/l DTT, and 0.3% bromphenol blue) and then boiled for 5 min. Equivalent amounts from each treatment group in a relation of 3:1 for the cytosolic-to-mitochondrial fraction ratio were run on 15% SDS polyacrylamide gels. Proteins were electrically transferred to nitrocellulose filters and incubated with a mouse anti–cytochrome c monoclonal antibody (PharMingen, San Diego, CA) followed by incubation with horseradish-peroxidase–linked anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The emitted light was captured on X-ray film after Lumiglo reagent was added (Phototope-HRP Western blot detection Kit; Biolabs, Beverly, MA). As a marker, biotinylated protein molecular weight standard (Biolabs) was run in parallel. For Bcl-2 analysis, nitrocellulose filters were incubated with polyclonal rabbit anti-human Bcl-2 antibody (PharMingen) followed by incubation with horseradish-peroxidase–linked anti-rabbit IgG (Santa Cruz Biotechnology). Bands were analyzed by densitometric scanning using a Multianalyst (Bio Rad, Laboratories, Hercules, CA).

Insulin release and content. To determine acute insulin release in response to glucose stimulation, we washed islets in RPMI 1640/10% FCS medium containing 3.3 mmol/l glucose and preincubated them for 1 h in the same medium. The medium was then discarded and replaced with fresh medium containing 3.3 mmol/l glucose for 1 h for basal secretion, followed by an additional 1-h incubation in medium containing 16.7 mmol/l glucose. Thereafter, islets were extracted with 0.18 N HCl in 70% ethanol, and the acid-ethanol extracts were collected for determination of insulin content. Insulin was determined by a human insulin RIA kit (CIS Bio International, Gif-Sur-Yvette, France).

Cultures evaluation and statistical analysis. Cultures were evaluated in a randomized manner by a single investigator (K.M.), who was blinded to the treatment conditions. Care was taken to score islets of similar size. Some larger islets did not completely spread and were several cells thick. Such larger islets were excluded because a monolayer is a prerequisite for single-cell evaluation. Data were analyzed by Student's *t* test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

RESULTS

Monounsaturated fatty acids protect human β-cells from glucose- and palmitic acid–induced apoptosis and impaired β-cell proliferation. Exposure of human islets cultivated at 5.5 mmol/l glucose to 0.1, 0.25, and 0.5 mmol/l palmitic acid for 4 days induced a 2.12-, 2.51-, and 3.59-fold increase, respectively, of the number of TUNEL-positive β-cells (Fig. 1). Exposure to 33.3 mmol/l glucose induced a 3.3-fold increase of the number of TUNEL-positive β-cells relative to islets at 5.5 mmol/l glucose (Fig. 1*B*). Glucose (33.3 mmol/l) together with 0.1–0.5 mmol/l palmitic acid had no additive effect (Fig. 1*B*). In contrast, the monounsaturated palmitoleic acid (0.5 mmol/l) and oleic acid (0.1–0.5 mmol/l) did not induce β-cell DNA fragmentation (Fig. 1). When added together with the corresponding concentration of palmitic acid, 0.5 mmol/l palmitoleic acid and 0.25–0.5 mmol/l oleic acid reduced the palmitic acid effect on β-cell death (Fig. 1*B*). Furthermore, 0.5 mmol/l palmitoleic acid and 0.25–0.5 mmol/l oleic acid prevented the β-cells from 33.3 mmol/l glucose-induced apoptosis and limited the apoptotic effect of both,

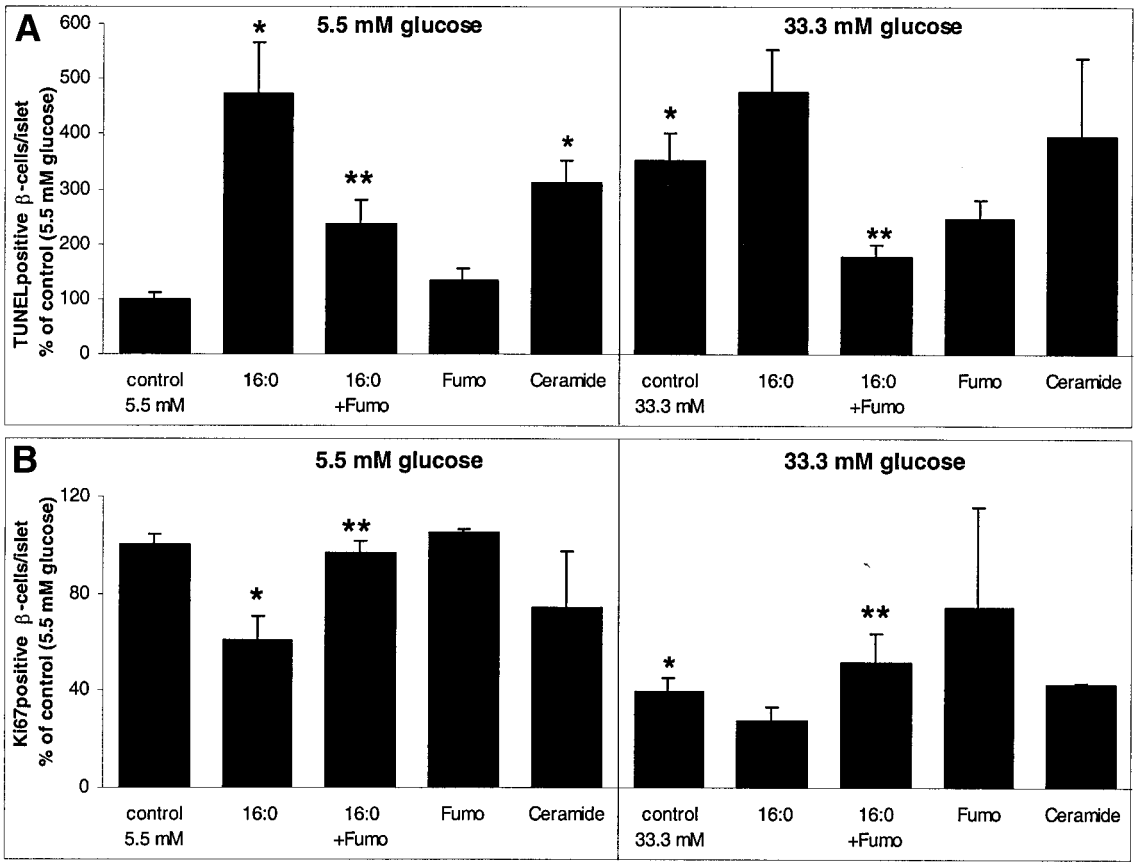

FIG. 4. Effect of blockade of ceramide synthesis by fumonisin B1 and of exogenous ceramide on β-cell DNA fragmentation (*A*) and proliferative activity (*B*). Islets were cultured for 4 days in 5.5 or 33.3 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitic acid (16:0), without or with 15 μmol/l fumonisin B1 (Fumo) or in the presence of 15 μmol/l C_2-ceramide. Results are means ± SE of the relative number of TUNEL-positive (*A*) and Ki-67–positive (*B*) β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; the results originate from the same experiments as in Figs. 1*B* and 2*B* and therefore have the same controls). *$P < 0.05$ relative to islets at 5.5 mmol/l glucose; **$P < 0.05$ relative to palmitic acid treated islets.

increased glucose concentrations and palmitic acid (Fig. 1*B*).

In parallel to the TUNEL assay, treated islets were incubated with annexin V and propidium iodide or stained with an anti-cleaved caspase 3 antibody to discriminate apoptotic from necrotic cells. Exposure of cultured islets to palmitic acid markedly increased the number of cells that exhibited phosphatidylserine molecules translocated to the outer leaflet of the plasma membrane, as revealed by annexin V binding (2.7 ± 0.26 Annexin-V-FLUOS–positive cells/islet in islets at 5.5 mmol/l glucose alone vs. 5.4 ± 0.31 in 0.5 mmol/l palmitic acid–treated islets; $P < 0.01$). A minority of these cells were necrotic (or late apoptotic), with plasma membranes permeable to the DNA-binding dye propidium iodide (0.73 ± 0.04 propidium iodide–positive cells/islet in 5.5 mmol/l glucose alone vs. 2.15 ± 0.41 in 0.5 mmol/l palmitic acid–treated islets; $P < 0.01$). Moreover, exposure of islets to increasing glucose concentrations (from 5.5 to 33.3 mmol/l) did not lead to increased propidium iodide uptake into the cultured cells (data not shown). Finally, triple immunostaining for DNA fragmentation, insulin, and cleaved caspase-3 demonstrated cleaved caspase-3 in most TUNEL-positive β-cells (data not shown). Therefore, DNA fragmentation as determined by the TUNEL assay mainly represents apoptotic cell death induced by palmitic acid and almost exclusively represents an apoptotic and not necrotic process of glucose-induced β-cell destruction.

Exposure of cultured human islets to 0.5 mmol/l palmitic acid and to 33.3 mmol/l glucose for 4 days decreased the number of proliferating (Ki-67–positive) β-cells by 40 and 60%, respectively, relative to islets at 5.5 mmol/l glucose alone (Fig. 2). Palmitoleic and oleic acids prevented the β-cells from high glucose– and palmitic acid–induced impairment of β-cell proliferation (Fig. 2*B*). Moreover, monounsaturated fatty acids partially restored β-cell proliferation in islets exposed to both increased glucose concentrations and palmitic acid (Fig. 2*B*).

The time-course effect of 0.5 mmol/l palmitic acid at 5.5 mmol/l glucose on β-cell DNA fragmentation became significant only after 4 days of exposure to palmitic acid (Fig. 3*A*). High glucose level induced a significant increase in β-cell apoptosis after 1 day and persisted throughout the study. Oleic acid (0.5 mmol/l) tended to protect the β-cells from glucose-induced apoptosis at each time point, although it reached statistic significance only after 4 days. As previously shown (10,12), short-time (1–2 days) exposure of cultured human islets to 33.3 mmol/l glucose increased the number of proliferating (Ki-67–positive) β-cells, whereas prolonged (4-day) exposure resulted in an inhibition of proliferation, relative to islets at 5.5 mmol/l glucose (Fig. 3*B*). It is interesting that the time course of

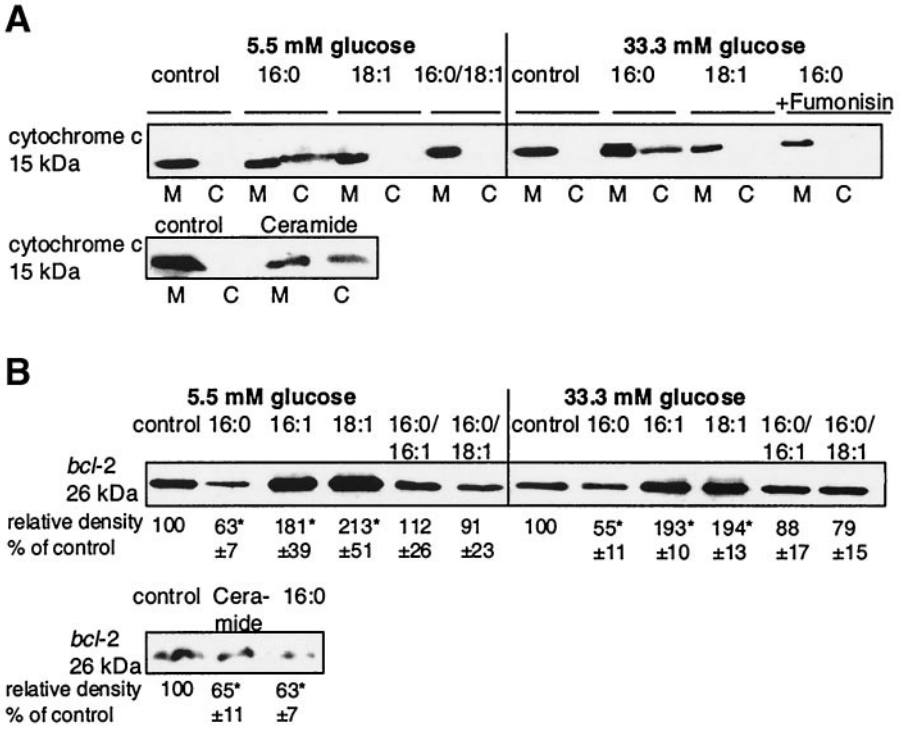

FIG. 5. Subcellular localization of cytochrome c and regulation of Bcl-2 in glucose-, fatty acid–, and ceramide-treated islets. Human islets were cultured at 5.5 and 33.3 mmol/l glucose for 42 h without and with 0.5 mmol/l palmitic (16:0), 0.5 mmol/l palmitoleic (16:1), or 0.5 mmol/l oleic acid (18:1), or a mixture of palmitic and palmitoleic acid (16:0/16:1; 0.5 mmol/l each) or a mixture of palmitic and oleic acid (16:0/18:1; 0.5 mmol/l each), or 0.5 mmol/l palmitic acid with 15 μmol/l fumonisin B1, or 15 μmol/l C_2-ceramide. Immunoblotting of cytochrome c (*A*) was performed on mitochondrial (M) and cytosolic (C) fractions of islets and immunoblotting of Bcl-2 (*B*) on whole islet lysate. One representative of three experiments from three donors is shown. In each experiment, cytochrome c was detectable only in the cytosolic fractions of islets treated with palmitic acid or ceramide alone. For Bcl-2 expression (*B*), immunoreactive bands were quantified by densitometric scanning for relative optical density normalized to control incubations. The results are given as mean ± SE. *$P < 0.05$ relative to controls at same glucose concentration.

the effect of palmitic acid on β-cell proliferation revealed a similar dual effect. Indeed, exposure of human islets to 0.5 mmol/l palmitic acid at 5.5 mmol/l glucose for 2 days induced an initial increase of proliferating β-cells, whereas longer exposure resulted in a marked inhibition of the β-cells' proliferative capacity. Similarly, exposure to 0.5 mmol/l oleic acid at 5.5 mmol/l glucose for 2 days induced an initial increase of proliferating β-cells. However, in contrast to palmitic acid and high glucose, prolonged exposure to oleic acid did not affect β-cell proliferation at 5.5 mmol/l glucose and restored β-cell proliferation at 33.3 mmol/l glucose.

Ceramide signaling in palmitic acid–induced β-cell apoptosis and inhibition of proliferation. In human islets cultured with 15 μmol/l C_2-ceramide in the presence of 5.5 mmol/l glucose, β-cell DNA fragmentation was increased 3.1-fold, whereas β-cell proliferation was not significantly decreased by C_2-ceramide (Fig. 4). C_2-ceramide had no additive effect on glucose-induced β-cell apoptosis and did not further impair proliferation in islets cultured in 33.3 mmol/l glucose (Fig. 4). Addition of 15 μmol/l of the ceramide synthase inhibitor fumonisin B1 in medium containing 5.5 or 33.3 mmol/l glucose and 0.5 mmol/l palmitic acid reduced the effect of palmitic acid on β-cell apoptosis and proliferation (Fig. 4). Fumonisin B1 had no significant protective effect on β-cell apoptosis and proliferation in islets exposed to 33.3 mmol/l glucose alone. As control, we used the metabolically inactive ceramide analogue C_2-dihydroceramide, which had no effect on the cells (data not shown).

Monounsaturated fatty acids protect human β-cells from palmitic acid–induced activation of the apoptotic mitochondrial pathway. Exposure of human islets to 33.3 mmol/l glucose and to oleic acid did not induce cytochrome c release from the mitochondria to the cytosol (Fig. 5*A*). In contrast, palmitic acid induced a release of cytochrome c from mitochondria to cytosol at 5.5 and 33.3 mmol/l glucose. The addition of oleic acid or of fumonisin B1 blocked palmitic acid–induced cytochrome c release.

Palmitoleic and oleic acids increased expression of the antiapoptotic mitochondrial Bcl-2 irrespective of the ambient glucose concentrations (Fig. 5*B*). However, palmitic acid decreased Bcl-2 expression; addition of palmitoleic or oleic acid to palmitic acid prevented this effect.

Exposure of human islets to 15 μmol/l C_2-ceramide at 5.5 mmol/l glucose induced a release of cytochrome c from mitochondria to cytosol and decreased Bcl-2 expression, mimicking the effect of palmitic acid on the mitochondria (Fig. 5).

Monounsaturated fatty acids improve impaired β-cell function as a result of "lipotoxicity" and "glucotoxicity." Chronic exposure of human islets to 33.3 mmol/l glucose or to 0.5 mmol/l palmitic acid for 4 days completely abolished acute glucose-stimulated insulin release (Fig. 6*A*). Addition of the monounsaturated palmitoleic or oleic acid partly restored such glucose stimulation. Insulin content of islets cultured at high glucose or with palmitic acid was lower compared with control (5.5 mmol/l glucose) and increased after addition of monounsaturated fatty acids (Fig. 6*B*). The deleterious effect of palmitic acid on glucose-stimulated insulin release was mimicked by ceramide and partly restored by coincubation with fumonisin B1 for 4 days (1.5 ± 0.20- and 2.4 ± 0.43-fold induction after an acute glucose challenge in 15 μmol/l C_2-ceramide–treated and in 0.5 mmol/l palmitic acid/15 μmol/l fumonisin B1–treated islets, respectively, versus 3.4 ± 0.59- and 1.5 ± 0.23-fold in 5.5 mmol/l glucose alone or with 0.5 mmol/l palmitic acid, respectively; $P < 0.05$). Similarly, exposure of human islets to ceramide for 4 days inhibited insulin content by 18.5 ± 5.6% relative to islets at 5.5 mmol/l glucose alone ($P < 0.05$), and addition of fumonisin B1 to palmitic acid completely blocked the

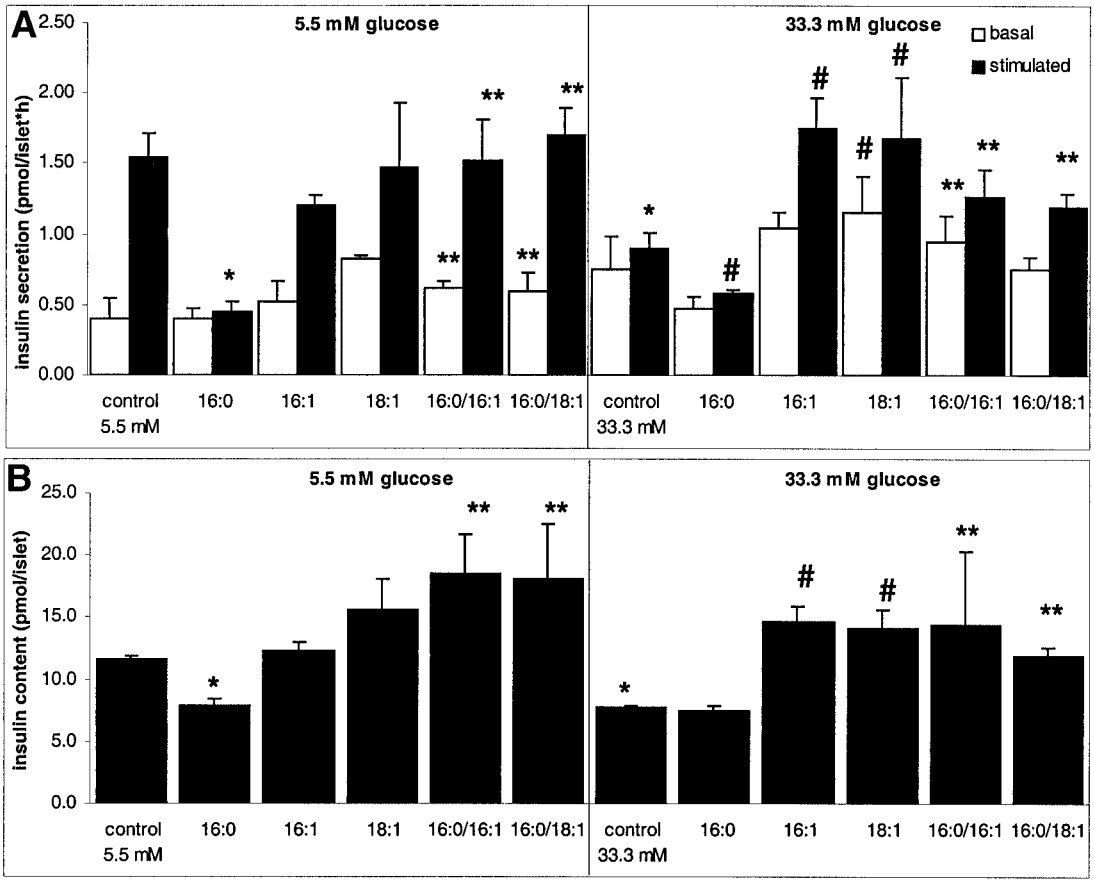

FIG. 6. Monounsaturated fatty acids restore glucose-stimulated insulin secretion in human islets exposed to high glucose or to palmitic acid. Islets were incubated in 5.5 and 33.3 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitic (16:0), palmitoleic (16:1), or oleic (18:1) acid or a mixture of saturated and unsaturated fatty acid (0.5 mmol/l each; 16:0/16:1, 16:0/18:1) for 4 days. *A*: Basal and stimulated insulin secretion denotes the amount secreted over 1-h incubation at 3.3 and 16.7 mmol/l glucose, respectively. *B*: Insulin content. Data are represented as mean of three experiments ± SE from three separate donors. In each experiment, the data were collected from three plates per treatment. *$P <$ 0.05 relative to islets at 5.5 mmol/l glucose; **$P < 0.05$ relative to palmitic acid treated islets at same glucose concentration; #$P < 0.05$ relative to islets at 33.3 mmol/l glucose.

palmitic acid–induced decrease in insulin content (data not shown).

DISCUSSION

This study examined the effects of elevated glucose and FFA levels, alone and in combination, on human β-cell turnover and function. The saturated palmitic acid and elevated glucose concentration reduced the proliferative capacity of β-cells and induced β-cell death. Conversely, the monounsaturated palmitoleic and oleic acids exhibited the opposite effects. They did not affect apoptosis but promoted β-cell proliferation and counteracted the toxic effects of palmitic acid and of high glucose. The beneficial effects of monounsaturated FFA were also reflected by improved parameters of β-cell function. Palmitoleic and oleic acids prevented the palmitic acid– and glucose-induced decrease in islet insulin content and impaired glucose-stimulated insulin secretion.

The distinct effects of the saturated palmitic acid and of the monounsaturated fatty acids on β-cell turnover and function are striking. The specific toxic effects of saturated fatty acids may relate to ceramide formation. Recent studies indicate that signal transduction through the ceramide pathway activates apoptosis in various cell types (18), including islets from the ZDF rats (9) and normal rats (15). Moreover, an increase in cellular levels of palmitic or stearic acid but not of palmitoleic acid is correlated with de novo synthesis of ceramide (30). In the present study, we extend these findings to human islets by the observation that the ceramide synthetase inhibitor fumonisin B1 blocked the deleterious effects of palmitic acid.

Ceramide induces cytochrome c release from isolated rat liver mitochondria (31). In our study, we could demonstrate that palmitic acid induces cytochrome c release also from mitochondria of human islets. In contrast, neither high glucose nor monounsaturated FFA induced cytochrome c release. Moreover, palmitoleic and oleic acids prevented the release of cytochrome c by palmitic acid. This is probably mediated by upregulation of Bcl-2, which inhibits the release of cytochrome c from mitochondria, thereby blocking the apoptotic process. In line with this proposition, it has been shown that in islets of the ZDF rat, Bcl-2 protein is 70% below control (32), and in the present study we observed that palmitoleic and oleic acids induced Bcl-2, whereas palmitic acid reduced Bcl-2 expression. Thus, our results attest that the mitochondrion is an important target for palmitic acid–induced apoptosis in human islets and that apoptosis can be prevented by monounsaturated FFA, possibly via induction of Bcl-2. An alternative explanation for the beneficial effect of monounsaturated fatty acids could be related to competition

between the fatty acids for transport into the cell and/or metabolism.

The susceptibility to glucose-induced β-cell apoptosis depends on the genetic background. In islets of the diabetes-prone *Psammomys obesus* (3) and of humans (10–13), an increase in glucose concentrations induce β-cell apoptosis. In contrast, in rodent islets, an increase in glucose concentration to 11 mmol/l promotes β-cell survival (3,14,15,33). When glucose concentrations are further increased, glucose proves to be pro- or antiapoptotic, depending on culture conditions. However, in human islets, monounsaturated FFA protected not only against the deleterious effects induced by palmitic acid, but also against those induced by glucose.

ACKNOWLEDGMENTS

This work was supported by a European Foundation for the Study of Diabetes/Johnson & Johnson Research Award and by the Swiss National Science Foundation Grants 3200-067049.01. M.Y.D. is supported by the Max Cloetta Foundation.

We thank G. Siegfried-Kellenberger for technical assistance.

REFERENCES

1. Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma A: Beta-cell adaptation and decompensation during the progression of diabetes. *Diabetes* 50 (Suppl. 1):S154–S159, 2001
2. Bonner-Weir S: Islet growth and development in the adult. *J Mol Endocrinol* 24:297–302, 2000
3. Donath MY, Gross DJ, Cerasi E, Kaiser N: Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. *Diabetes* 48:738–744, 1999
4. Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS: Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358–364, 1998
5. Zhu M, Noma Y, Mizuno A, Sano T, Shima K: Poor capacity for proliferation of pancreatic beta-cells in Otsuka-Long-Evans-Tokushima Fatty rat: a model of spontaneous NIDDM. *Diabetes* 45:941–946, 1996
6. Miralles F, Portha B: Early development of beta-cells is impaired in the GK rat model of type 2 diabetes. *Diabetes* 50 (Suppl. 1):S84–S88, 2001
7. Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patane G, Laybutt R, Bonner-Weir S, Weir GC: Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. *J Biol Chem* 274:14112–14121, 1999
8. Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas JC, Sgroi DC, Groff A, Ferran C, Bonner-Weir S, Sharma A, Weir GC: Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to beta-cell survival during chronic hyperglycemia. *Diabetes* 51:413–423, 2002
9. Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95:2498–2502, 1998
10. Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY: Glucose induces β-cell apoptosis via upregulation of the Fas-receptor in human islets. *Diabetes* 50:1683–1690, 2001
11. Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F, Marlier LN, Davalli AM, Carandente O, Pontiroli AE, Melino G, Marchetti P, Lauro R, Sesti G, Folli F: High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* 50:1290–1301, 2001
12. Maedler K, Fontana A, Ris F, Sergeev P, Toso C, Oberholzer J, Lehmann R, Bachmann F, Tasinato A, Spinas GA, Halban PA, Donath MY: FLIP switches Fas-mediated glucose signaling in human pancreatic β cells from apoptosis to cell replication. *Proc Natl Acad Sci U S A* 99:8236–8241, 2002
13. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta-cell production of interleukin-1β contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110:851–860, 2002
14. Efanova IB, Zaitsev SV, Zhivotovsky B, Kohler M, Efendic S, Orrenius S, Berggren PO: Glucose and tolbutamide induce apoptosis in pancreatic beta-cells: a process dependent on intracellular Ca2+ concentration. *J Biol Chem* 273:33501–33507, 1998
15. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY: Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 50:69–76, 2001
16. Bonner-Weir S, Deery D, Leahy JL, Weir GC: Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. *Diabetes* 38:49–53, 1989
17. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 50:1771–1777, 2001
18. Mathias S, Pena LA, Kolesnick RN: Signal transduction of stress via ceramide. *Biochem J* 335:465–480, 1998
19. Kroemer G, Reed JC: Mitochondrial control of cell death. *Nat Med* 6:513–519, 2000
20. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147–157, 1996
21. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275:1132–1136, 1997
22. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 275:1129–1132, 1997
23. Oberholzer J, Triponez F, Mage R, Andereggen E, Buhler L, Cretin N, Fournier B, Goumaz C, Lou J, Philippe J, Morel P: Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. *Transplantation* 69:1115–1123, 2000
24. Kaiser N, Corcos AP, Sarel I, Cerasi E: Monolayer culture of adult rat pancreatic islets on extracellular matrix: modulation of B-cell function by chronic exposure to high glucose. *Endocrinology* 129:2067–2076, 1991
25. Marshak S, Leibowitz G, Bertuzzi F, Socci C, Kaiser N, Gross DJ, Cerasi E, Melloul D: Impaired beta-cell functions induced by chronic exposure of cultured human pancreatic islets to high glucose. *Diabetes* 48:1230–1236, 1999
26. Dyntar D, Eppenberger-Eberhardt M, Maedler K, Pruschy M, Eppenberger HM, Spinas GA, Donath MY: Glucose and palmitic acid induce degeneration of myofibrils and modulate apoptosis in rat adult cardiomyocytes. *Diabetes* 50:2105–2113, 2001
27. Spector AA, Fletcher JE, Ashbrook JD: Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. *Biochemistry* 10:3226–3232, 1971
28. Bouwens L, Lu WG, De KR: Proliferation and differentiation in the human fetal endocrine pancreas. *Diabetologia* 40:398–404, 1997
29. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992
30. Paumen MB, Ishida Y, Muramatsu M, Yamamoto M, Honjo T: Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J Biol Chem* 272:3324–3399, 1997
31. Ghafourifar P, Klein SD, Schucht O, Schenk U, Pruschy M, Rocha S, Richter C: Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. *J Biol Chem* 274:6080–6084, 1999
32. Shimabukuro M, Wang MY, Zhou YT, Newgard CB, Unger RH: Protection against lipoapoptosis of beta cells through leptin-dependent maintenance of Bcl-2 expression. *Proc Natl Acad Sci U S A* 95:9558–9561, 1998
33. Hoorens A, Van de Casteele M, Kloppel G, Pipeleers D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568–1574, 1996