

Distinct Effects of Saturated and Monounsaturated Fatty Acids on β -Cell Turnover and Function

K. Maedler, G.A. Spinas, D. Dyntar, W. Moritz, N. Kaiser, and Marc Y. Donath

Glucotoxicity and lipotoxicity contribute to the impaired β -cell function observed in type 2 diabetes. Here we examine the effect of saturated and unsaturated fatty acids at different glucose concentrations on β -cell proliferation and apoptosis. Adult rat pancreatic islets were cultured onto plates coated with extracellular matrix derived from bovine corneal endothelial cells. Exposure of islets to saturated fatty acid (0.5 mmol/l palmitic acid) in medium containing 5.5, 11.1, or 33.3 mmol/l glucose for 4 days resulted in a five- to ninefold increase of β -cell DNA fragmentation. In contrast, monounsaturated palmitoleic acid alone (0.5 mmol/l) or in combination with palmitic acid (0.25 or 0.5 mmol/l each) did not affect DNA fragmentation. Increasing concentrations of glucose promoted β -cell proliferation that was dramatically reduced by palmitic acid. Palmitoleic acid enhanced the proliferation activity in medium containing 5.5 mmol/l glucose but had no additional effect at higher glucose concentrations (11.1 and 33.3 mmol/l). The cell-permeable ceramide analog C_2 -ceramide mimicked both the palmitic acid-induced β -cell apoptosis and decrease in proliferation. Moreover, the ceramide synthetase inhibitor fumonisin B1 blocked the deleterious effects of palmitic acid on β -cell viability. Additionally, palmitic acid but not palmitoleic acid decreased the expression of the mitochondrial adenine nucleotide translocator and induced release of cytochrome c from the mitochondria into the cytosol. Finally, palmitoleic acid improved β -cell-secretory function that was reduced by palmitic acid. Taken together, these results suggest that the lipotoxic effect of the saturated palmitic acid involves an increased apoptosis rate coupled with reduced proliferation capacity of β -cells and impaired insulin secretion. The deleterious effect of palmitate on β -cell turnover is mediated via formation of ceramide and activation of the apoptotic mitochondrial pathway. In contrast, the monounsaturated palmitoleic acid does not affect β -cell apoptosis, yet it promotes β -cell proliferation at low glucose concentrations, counteracting the negative effects of palmitic acid as well as improving β -cell function. *Diabetes* 50:69–76, 2001

From the Division of Endocrinology and Diabetes (K.M., G.A.S., D.D., W.M., M.Y.D.), University Hospital, Zurich, Switzerland; and the Department of Endocrinology and Metabolism (N.K.), Hebrew University–Hadassah Medical Center, Jerusalem, Israel.

Address correspondence and reprint requests to Marc 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 14 July 2000 and accepted in revised form 2 October 2000.

ANT, adenine nucleotide translocator; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

At the initial stages of type 2 diabetes, individuals lose the ability to produce sufficient quantities of insulin to maintain normoglycemia in the face of insulin resistance (1). The capacity to produce insulin is determined by the total β -cell number and β -cell functional activity. The β -cell mass is capable of long-term adaptation by increasing the β -cell number through hyperplasia and neogenesis (2,3). However, β -cell expansion can be offset by concomitant apoptosis (4). The failure of these long-term feedback adaptations in genetically susceptible individuals with concurrent insulin resistance may result in type 2 diabetes (3,5). The etiology and mechanisms leading to apoptosis of β -cells together with an inadequately low proliferation rate has not been completely elucidated. Previously, we analyzed β -cell turnover in pancreata of *Psammomys obesus*, a rodent with a natural tendency to diet-induced type 2–like diabetes (6). Elevated glucose concentrations directly induced β -cell apoptosis in cultured islets from diabetes-prone *P. obesus*, but not in islets from diabetes-resistant rats (6,7). Glucose-induced β -cell proliferation was observed in both rat and *P. obesus* islets; however, *P. obesus* showed only a limited capacity of β -cell proliferation in response to elevated glucose concentrations. Based on these observations, we suggested the existence of a novel process of glucotoxicity, in which chronic hyperglycemia is linked to a progressive loss of β -cell mass, when genetic susceptibility to diabetes exists.

Apart from hyperglycemia, plasma long-chain free fatty acid levels are often increased in states of insulin resistance, further impairing β -cell-secretory function (8,9). In an adipogenic model of diabetes, the Zucker diabetic fatty rat, fatty acid-induced β -cell apoptosis was observed (5). However, it is not known whether islets of these diabetic models behave differently from those of normal rats. Furthermore, the effect of fatty acids on β -cell proliferation has been investigated only at high fatty acid concentrations (2 mmol/l) in rat islets maintained in suspension for 7 days (10).

Because ceramide is synthesized from long-chain fatty acids, it has been postulated that free fatty acid-induced cell death is mediated via formation of ceramide. Ceramide serves as a second messenger for cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis (11). Shimabukuro et al. (5,12) showed that islets from Zucker diabetic fatty rats manifested elevated ceramide levels. In response to a challenge with fatty acids, these islets displayed increased incorporation of fatty acids into ceramide, accompanied by apoptosis. Fumonisin B1, a specific inhibitor of ceramide synthase activity, blocked both ceramide generation and apoptosis, indicating that ceramide

generation was necessary for the apoptotic response. However, the possible role of ceramide signaling on β -cell proliferation has not been addressed in these studies.

A critical event leading 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 (13,14). The loss of barrier function of both mitochondrial membranes is controlled, at least in part, by the permeability transition pore complex, a polyprotein complex that includes the most abundant protein of the inner mitochondrial membranes, the adenine nucleotide translocator (ANT) (15). ANT catalyses the exchange of ADP and ATP across the inner mitochondrial membrane. Recent studies indicate that proapoptotic proteins, such as Bax or Bak, interact with ANT to facilitate membrane permeabilization (16,17). Others have shown that agents that inhibit ANT activity induce mitochondrial swelling (18). It has also been postulated that mitochondrial swelling and rupture of the outer membrane may explain how cytochrome *c* is released (19). Palmitoyl-CoA esters are natural ligands for ANT (20); however, whether fatty acids affect ANT expression and induce release of cytochrome *c* from mitochondria of β -cells is unknown.

Therefore, we investigated the role of fatty acids at different ambient glucose concentrations on β -cell proliferation, apoptosis, and function in adult rat islets. Pancreatic islets were cultured onto plates coated with extracellular matrix derived from bovine corneal endothelial cells, allowing *in situ* identification of β -cell apoptosis and proliferation. In addition, the possible 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 at an identical carbon chain length, β -cells were exposed to palmitic (C16:0) and palmitoleic (16:1) acid alone and in combination.

RESEARCH DESIGN AND METHODS

Islet isolation. Male Sprague-Dawley rats (200–220 g) were anesthetized by ether, and islets isolated from the pancreata using an adaptation for rat islets of the method of Gotoh et al. (21). Briefly, after cannulation of the common bile duct and instillation of 10 ml cold Hank's balanced salt solution (Gibco, Gaithersburg, MD) containing 0.17 mg/ml Liberase (Boehringer Mannheim, Mannheim, Germany), 0.1 mg/ml DNase I (Boehringer Mannheim), and 25 mmol/l HEPES, each pancreas was removed and digested in 5 ml of the above described Liberase solution for 35 min at 37°C in a shaking water bath. This was followed by dilution and washing with Hank's balanced salt solution containing 0.1 mg/ml DNase I, 25 mmol/l HEPES, and 10% fetal calf serum (Gibco). Islets from the crude pancreas digest were purified by centrifugation through a discontinuous Histopaque 1077 (Sigma Chemical, St. Louis, MO) gradient, washed with RPMI-1640 medium (Gibco), and dispersed into culture dishes.

Islet culture. Details of the procedure have been described previously (22,23). Islets pooled from four rats were suspended in 7 ml RPMI-1640 medium (11.1 mmol/l glucose) containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 250 ng/ml amphotericin B, 40 μ g/ml gentamicin, and 10% fetal calf serum (Gibco). Of the islet suspension, 200 μ l (30–50 islets) were added to 2 ml of the above described medium and cultured onto 35-mm plates coated with extracellular matrix derived from bovine corneal endothelial cells (23) (Novamed, Jerusalem, Israel). Two days after plating, when most islets were attached and began to flatten, the culture medium was changed to RPMI containing 5.5, 11.1, or 33.3 mmol/l glucose supplemented with bovine serum albumin (BSA) alone or with fatty acids (0.5 mmol/l palmitic acid, 0.5 mmol/l palmitoleic acid, or a mixture of 0.25 mmol/l or 0.5 mmol/l each). Fatty acids (Sigma) were dissolved at 10 mmol/l in RPMI-1640 medium containing 11% fatty acid-free BSA (Sigma) under an N_2 -atmosphere, shaken overnight at 37°C, sonicated 15 min, and filtrated under sterile conditions (stock solution). For control incubations, 11% BSA was prepared, as described above. Before use, the effective free fatty acids concentrations were controlled with a commercially available

kit (Wako, Neuss, Germany). In some experiments, islets were cultured with 15 μ mol/l C_2 -ceramide (Biomol, Plymouth Meeting, PA) or 15 μ mol/l fumonisin B1 (Sigma); both were first dissolved in prewarmed 37°C DMSO (Fluka, Buchs, Switzerland) at 5 mmol/l.

β -cell replication. For β -cell proliferation studies, a monoclonal antibody to Ki-67 was used (MIB-5; Dianova, Hamburg, Germany). Ki-67 is a nuclear antigen expressed by proliferating cells that is used as a marker for late G1, S, G2, and M phases of the cell cycle (24). After washing with phosphate-buffered saline (PBS), cultured islets were fixed in 4% paraformaldehyde (30 min at room temperature) followed by permeabilization with 0.5% Triton X-100 (4 min at room temperature). Afterwards, 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, San Francisco, CA). 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 10-min incubation with a 1:10 dilution of fluorescein-conjugated rabbit anti-guinea pig antibody (Dako, Glostrup, Denmark).

Detection of apoptotic β -cells. The free 3'-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (25). After islet cultures were fixed and permeabilized as described above, the TUNEL assay was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Boehringer Mannheim). The preparations were then rinsed with Tris-buffered saline and incubated (10 min at room temperature) with a 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma). Thereafter, islets were incubated with a guinea pig anti-insulin antibody as described above and detection was performed using the streptavidin-biotin-peroxidase complex (Zymed).

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 cells from necrotic cells.

After staining for proliferation or apoptosis, islets were embedded in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) and analyzed by light and fluorescent microscopy (microscope Axiolab; Zeiss, Jena, Germany).

Cytochrome *c* immunofluorescence staining and confocal microscopy. Islet cultures were fixed and permeabilized as described above, incubated for 2 h at room temperature with mouse anti-cytochrome *c* monoclonal antibody that was diluted 1:50 (PharMingen), and then incubated for 1 h with a 1:50 dilution of fluorescein-conjugated goat anti-mouse antibody (Jackson Immuno Research Lab, West Grove, PA) and embedded in Dako fluorescent mounting medium. Images were produced by a confocal laser scanning microscope (Zeiss Axiophot fluorescence microscope) with a Zeiss Neofluar \times 40/1.3 objective lens connected to a Bio-Rad MRC-600 confocal scanner (Bio-Rad, Lasership, Oxfordshire, U.K.) and a Silicon Graphics Personal Iris 4D/25 Work-Station (Silicon Graphics, Mountain View, CA).

Subcellular fractionation. For analysis of rat islet's subcellular fractions, islets were cultured in suspension in RPMI-1640 medium containing 11.1 mmol/l glucose, as described above. One day after isolation, the medium was changed, and groups of 200 islets were incubated for 6 or 20 h in medium containing 11.1 mmol/l glucose with 0.5 mmol/l palmitate, 0.5 mmol/l palmitoleic acid, or solvent. At the end of the incubations, islets were washed in PBS, and mitochondrial and cytosolic (S100) fractions were prepared from islets resuspended in 70 μ l 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 (26). Mechanical homogenization was achieved by repeated aspiration through a pipette. Unlysed cells and nuclei were pelleted by a 10-min centrifugation (750g for 4°C). The supernatant was centrifuged at 10 000g for 15 min at 4°C. This pellet representing the mitochondrial fraction was then resuspended in 10 μ l of the above-described buffer. Finally, the supernatant was centrifuged at 100,000g for 1 h at 4°C. The supernatant from this final centrifugation represents the S-100 fraction (27). Both fractions were frozen at -80°C until use.

Western blot analysis. Mitochondrial and cytosolic fractions were diluted 1:3 in sample buffer containing 187.5 mmol/l Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 150 mmol/l dithiothreitol, and 0.3% Bromphenol blue and were boiled for 5 min. Equivalent amounts of each treatment group at a ratio of 5:3 cytosolic to mitochondrial fraction 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) (1 μ g/ml for 1 h at room temperature), followed by incubation with horseradish peroxidase-linked anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (1 h at room temperature). After adding Lumiglo reagent (Phototope-HRP

Western Blot Detection Kit; Biolabs, Beverly, MA), the emitted light was captured on X-ray film. As a marker, a biotinylated protein molecular weight standard (Biolabs) was run in parallel according to the manufacturer's instructions.

For ANT analysis, the nitrocellulose membrane was stripped for 30 min at 50°C in 40 ml of a watery solution containing 280 μ l β -mercaptoethanol, 5 ml 0.5 mol/l Tris-HCl, pH 6.8, and 10% SDS, then washed for 1 h in Tris-buffered saline containing 0.1% Tween-20, incubated with an anti-ANT antibody (provided by T. Wallimann, Institute of Cell Biology, Swiss Federal Institute of Technology, Zurich, Switzerland [28]) and then incubated with horseradish peroxidase-linked anti-rabbit IgG (Santa Cruz) (1:5,000 for 1 h at room temperature) and detected as described above.

Insulin release and content. Chronic insulin release was evaluated in the culture medium collected before the termination of each experiment. To determine acute insulin release in response to glucose stimulation, islets were washed in RPMI-1640 medium containing 3.3 mmol/l glucose and were preincubated 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. Incubates were collected and frozen for insulin assays. Thereafter, islets were washed with PBS and extracted with 0.18N HCl in 70% ethanol for 24 h at 4°C; the acid-ethanol extracts were collected and frozen for determination of insulin content.

Insulin was determined by a human insulin radiomunoassay kit (CIS Bio International, Gif-Sur-Yvette, France) with rat insulin as the standard. Cross-reactivity of the anti-human insulin antibody used with rat insulin is 89.5%.

Statistical analysis. Data are presented as the means \pm SE and were analyzed by Student's *t* test. $P < 0.05$ was considered significant. 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.

RESULTS

Modulation of β -cell proliferation and apoptosis after exposure to free fatty acids and glucose. Exposure of adult rat islets in long-term culture to palmitic acid for 4 days resulted in an increased number of β -cells with TUNEL-positive nuclei (Fig. 1A and B). The increase was 5.2-, 8.9-, and 5.5-fold at 5.5, 11.1, and 33.3 mmol/l glucose, respectively, compared with islets exposed to identical glucose concentrations and solvent (albumin) (Fig. 3A). In contrast, the monounsaturated palmitoleic acid did not induce DNA fragmentation and, when present with palmitic acid (0.25 mmol/l and 0.5 mmol/l each), its effect on β -cell death was inhibited. Baseline β -cell death in the absence of fatty acids was minimal at 11.1 mmol/l glucose and increased 3.6- and 3.0-fold at 5.5 and 33.3 mmol/l glucose, respectively.

In parallel with the TUNEL assay, treated islets were incubated with annexin V and propidium iodide to discriminate apoptotic from necrotic cells (Fig. 1C–F). Exposure of cultured islets to palmitic acid markedly increased the number of cells exhibiting phosphatidylserine molecules translocated to the outer leaflet of the plasma membrane, as revealed by annexin V binding (1.96 ± 0.36 Annexin-V-FLUOS-positive cells/islet in control [11.1 mmol/l glucose] vs. 6.78 ± 1.05 in 0.5 mmol/l palmitic acid-treated islets, $P < 0.01$). Most of these cells had intact plasma membranes, impermeable to the DNA-binding dye propidium iodide (0.8 ± 0.01 propidium iodide-positive cells/islet in control vs. 2.14 ± 0.26 in 0.5 mmol/l palmitic acid-treated islets, $P < 0.01$). Therefore, the palmitic acid-induced DNA fragmentation, as determined by the TUNEL assay, mainly represents apoptotic cell death.

Exposure to high concentrations of glucose for 4 days resulted in increased proliferation of β -cells by 1.9- and 1.8-fold at 11.1 and 33.3 mmol/l glucose, respectively, compared with islets at 5.5 mmol/l glucose (Figs. 2 and 3B). In contrast, addition of palmitic acid resulted in a similar decrease in β -cell proliferation at all glucose concentrations (45, 33, and 46% decreases at 5.5, 11.1, and 33.3 mmol/l glucose, respectively,

compared with islets incubated in identical glucose concentrations and solvent) (Fig. 3B). On the other hand, palmitoleic acid augmented β -cell proliferation by 65% at 5.5 mmol/l glucose, whereas at higher glucose concentrations, when the maximal proliferative activity was achieved with glucose alone, no additional effect was obtained. Treatment of the cells with a mixture of palmitoleic and palmitic acid had no influence on β -cell proliferation (Fig. 3B).

Ceramide signaling in palmitic acid induced changes in β -cell apoptosis and proliferation. In pancreatic islets cultured with 15 μ mol/l C_2 -ceramide in the presence of 11.1 mmol/l glucose, β -cell DNA fragmentation was increased 2.6-fold, whereas β -cell proliferation was decreased by 71.1%, showing the same trend as that observed in palmitic acid-treated islets (Fig. 4). Addition of 15 μ mol/l of the ceramide synthase inhibitor fumonisin B1 in cultured medium containing 11.1 mmol/l glucose and 0.5 mmol/l palmitic acid, reduced the effect of palmitate on β -cell apoptosis and had a tendency to reverse its antiproliferative effect (Fig. 4).

Palmitic acid-induced decrease in ANT expression and cytochrome c release. Exposure of pancreatic islets to palmitic acid, compared with palmitoleic acid and solvent-treated islets, induced a time-dependent decrease of mitochondrial ANT expression (Fig. 5). The decrease was already detectable after 6 h of treatment, and after 20 h, ANT in the mitochondrial fraction of palmitic acid-treated islets was almost undetectable. At the same time, cytochrome c of palmitic acid-treated islets was shifted from the mitochondrial to the cytosolic fraction, whereas most cytochrome c in palmitoleic acid or control islets was confined to the mitochondria (Figs. 5 and 6). No ANT was detectable in any cytosolic fraction, thereby confirming the integrity of the mitochondrial and cytosolic preparations.

Insulin release and content. Exposure of rat islets to 0.5 mmol/l palmitoleic acid for 4 days increased islet insulin content and chronic insulin secretion as compared to control and palmitic acid treated islets (Fig. 7A and B). Incubation with 0.5 mmol/l palmitic acid decreased islet insulin content; addition of 0.5 mmol/l palmitoleic to 0.5 mmol/l palmitic acid prevented this effect. An acute glucose challenge of palmitoleic acid-treated cells induced a higher insulin release rate compared with control, whereas treatment with palmitic acid abolished the insulin response to glucose (Fig. 7C).

DISCUSSION

This study shows that the saturated palmitic acid reduces the proliferative capacity of β -cells and induces β -cell death mainly by apoptosis. Conversely, palmitoleic acid, a monounsaturated fatty acid with identical carbon chain length, exhibits the opposite effects: it does not affect apoptosis, but promotes β -cell proliferation and counteracts the toxic effects of palmitic acid. The cell-permeable ceramide analog C_2 -ceramide mimics the palmitic acid-induced changes in the cell cycle, which were blocked by the ceramide synthetase inhibitor fumonisin B1. Thus, formation of ceramide is required to mediate the palmitic acid effects on β -cell turnover. Additionally, palmitic acid but not palmitoleic acid decreases the expression of ANT. This is accompanied by increased translocation of cytochrome c from the mitochondria to the cytosol. The overall beneficial effect of palmitoleic acid is also reflected by improved parameters of β -cell function: palmitoleic acid increased islet insulin content as well as chronic and glucose-stimulated insulin secretion.

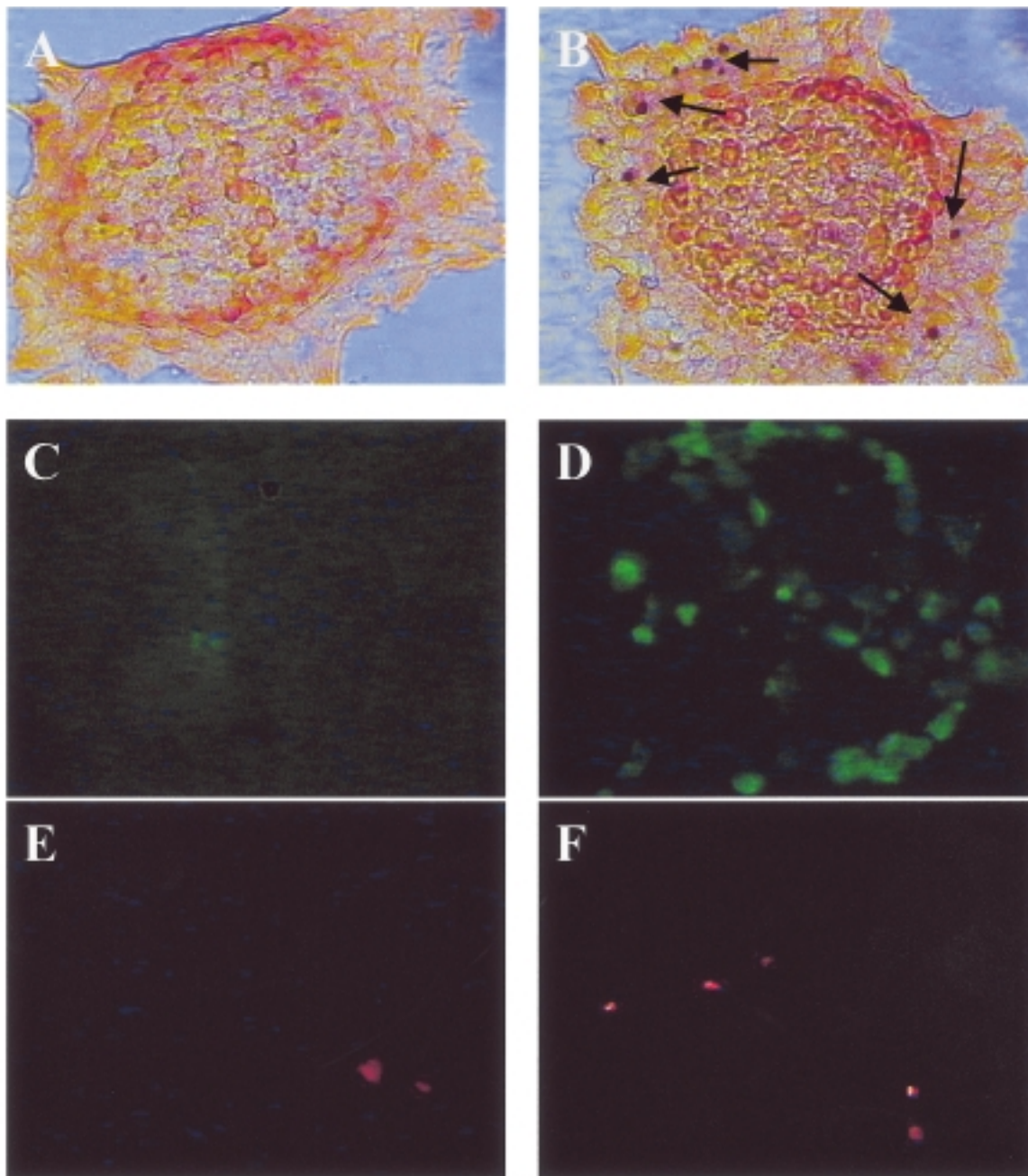


FIG. 1. Characterization of the effect of palmitic acid on β -cell death by double staining with the TUNEL assay (alkaline phosphatase) and anti-insulin antibody (peroxidase) (*A* and *B*) and by double fluorescent staining with Annexin-V-FLUOS (green) (*C* and *D*) and propidium iodide (red) (*E* and *F*). Islets were plated on extracellular matrix-coated dishes and exposed for 4 days to media containing 11.1 mmol/l glucose alone (*A*, *C*, and *E*) and with 0.5 mmol/l palmitic acid (*B*, *D*, and *F*). The arrows indicate nuclei stained positive for the TUNEL reaction (light and fluorescence microscopy $\times 400$).

Furthermore, it prevented the palmitic acid-induced decrease in islet insulin content and impaired glucose-stimulated insulin secretion.

The distinct effects of the saturated palmitic acid and the monounsaturated palmitoleic acid on β -cell turnover and function are striking. The fact that fatty acids longer than 15 carbons may be harmful to various cell types has been previously reported (29–35). At the same concentrations, chronic exposure to saturated fatty acids of different length have similar effects. Likewise, monounsaturated fatty acids C16:1 and C18:1 induced similar effects (29–35). However, saturated fatty acids

were associated with different effects than unsaturated fatty acids. A putative explanation for this difference is based on the relative high melting point of saturated fatty acids (63°C for palmitic acid) compared with unsaturated fatty acids (0.5°C for palmitoleic acid). Consequently, triacylglycerols synthesized from saturated fatty chains are insoluble at 37°C. This observation has led to the hypothesis that, immediately after their formation, saturated triacylglycerol molecules precipitate at the site of synthesis (i. e., the sarcoplasmic reticulum) (36,37). These precipitates are thought to hamper sarcoplasmic reticulum function. However, it remains to be established

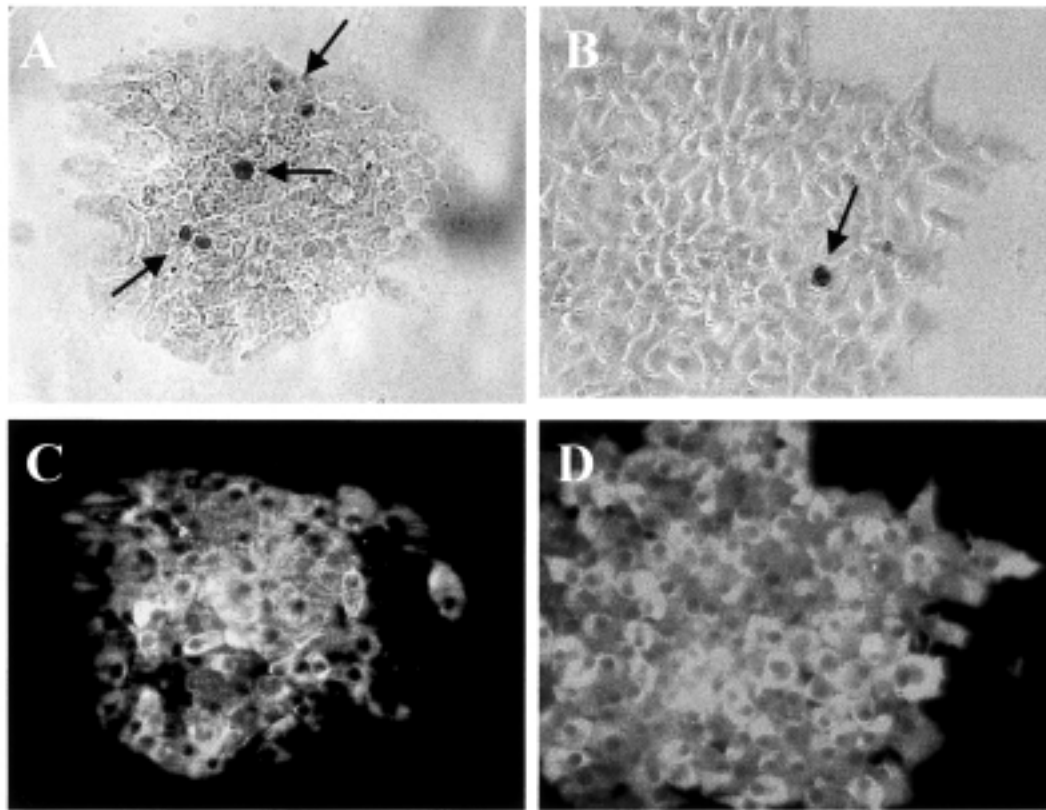


FIG. 2. Double immunostaining for the Ki-67 nuclear antigen (peroxidase) (A and B) and insulin (fluorescein) (C and D). Islets plated on extracellular matrix-coated dishes were exposed for 4 days to media containing 11.1 mmol/l glucose alone (A and C) or including 0.5 mmol/l palmitic acid (B and D). The arrows indicate nuclei stained positive for Ki-67 (light and fluorescence microscopy $\times 400$).

whether this process is linked to apoptosis. A second explanation may be related to changes in membrane fluidity. Indeed, enrichment of phospholipids by saturated fatty acids lowers membrane fluidity, which severely hinders membrane function

(38,39). Moreover, it has been shown in a neuronal cell line that an increase of saturated fatty acids in the phospholipid pool forms an essential part of the apoptotic process (40). Furthermore, alterations in the composition of membrane phospholipids

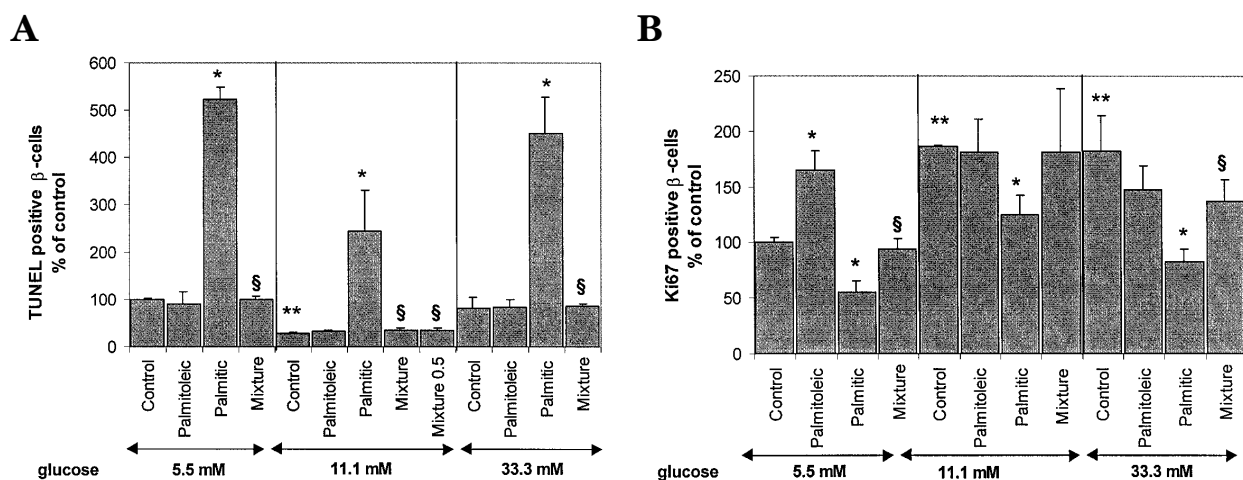


FIG. 3. Fatty acids and glucose-induced β -cell DNA fragmentation and proliferative activity. Islets were cultured for 4 days in the absence or presence of different fatty acids (0.5 mmol/l) or a mixture of palmitoleic and palmitic acid (0.25 mmol/l [mixture] or 0.5 mmol/l [mixture 0.5] each) in 5.5, 11.1, and 33.3 mmol/l glucose. Results are means \pm SE of the relative number of TUNEL⁺ (A) and Ki-67⁺ β -cells (B) per islet, normalized to control incubations at 5.5 mmol/l glucose alone (100%) (in absolute value: 0.82 TUNEL⁺ β -cells per islet and 4.06 Ki-67⁺ β -cells per islet at 5.5 mmol/l glucose alone). The mean number of islets scored for DNA fragmentation was 40, 68, and 49 and the mean number for proliferative activity by anti-Ki-67 staining was 78, 69, and 63 in media containing 5.5, 11.1, and 33.3 mmol/l glucose, respectively. Islets were isolated from 40 rats. * $P < 0.01$ between control and palmitic or palmitoleic acid at the same glucose concentration; § $P < 0.05$ between palmitic acid and fatty acid mixture at the same glucose concentration; ** $P < 0.01$ relative to islets at 5.5 mmol/l glucose.

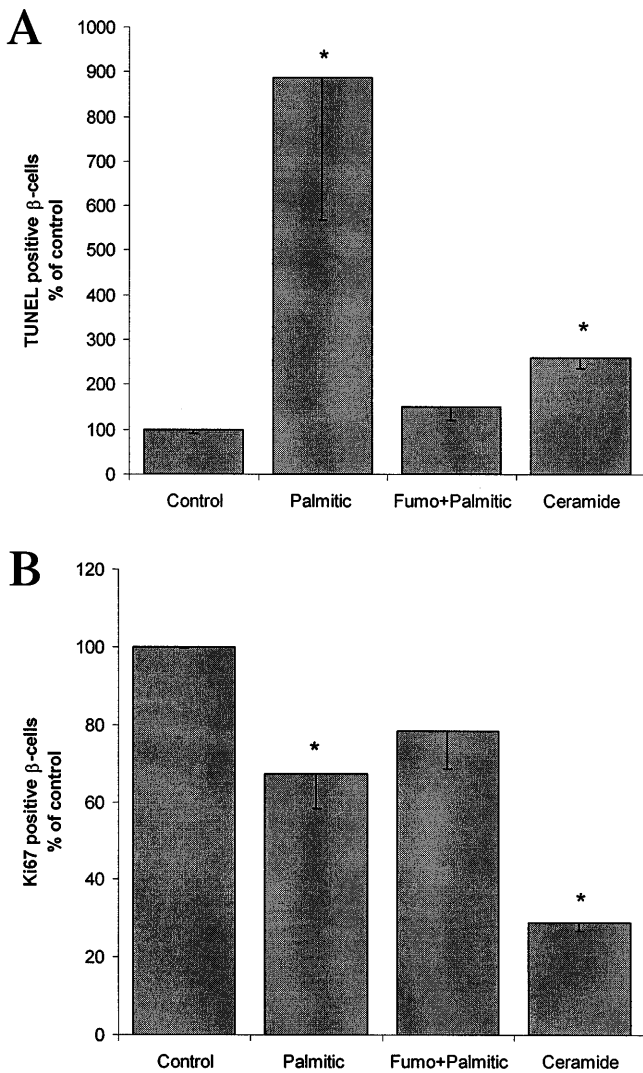


FIG. 4. Effects of blockade of ceramide synthesis by fumonisin B1 and of exogenous ceramide on β -cell DNA fragmentation and proliferative activity. Islets were cultured for 4 days in 11.1 mmol/l glucose alone (control) or in the presence of 0.5 mmol/l palmitic acids with or without 15 μ mol/l fumonisin B1 (Fumo) or in the presence of 15 μ mol/l C_2 -ceramide. Results are means \pm SE of the relative number of TUNEL⁺ (A) and Ki-67⁺ β -cells (B) per islet normalized to the solvent-treated control (100%) (in absolute value: 0.61 TUNEL⁺ β -cells per islet and 7.32 Ki-67⁺ β -cells per islet at 11.1 mmol/l glucose alone). The mean number of islets scored for DNA fragmentation and for proliferative activity by anti-Ki-67 staining was 85 and 71, respectively. Islets were isolated from 16 rats. * $P < 0.01$ relative to solvent-treated controls.

may act as a trigger for apoptosis, as demonstrated in a promyelocytic cell line, by inhibition of the remodeling of long-chain unsaturated fatty acids between the phospholipids of cells (41). Finally, 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 (11), including islets from the Zucker diabetic fatty rats (5). Moreover, an increase in cellular levels of palmitic or stearic acid but not in levels of palmitoleic acid is correlated with de novo synthesis of ceramide (42). In line with these findings, the present study shows that the ceramide synthetase inhibitor fumonisin B1 blocked the deleterious effects of palmitic acid.

Palmitic acid has a powerful acute insulinotropic effect (32). However, exposure to palmitic acid for 4 days did not increase total insulin secretion. Possibly, the acute insulinotropic effect gradually disappears in conjunction with increased β -cell apoptosis, leading to an overall unchanged insulin release.

The enhancement of TUNEL⁺ β -cells by ceramide is modest compared with the effect of palmitic acid. The difference is probably not due to reduced cell permeability of C_2 -ceramide, because it has a dramatic effect on β -cell proliferation, stronger than the palmitic acid effect. The time course of the C_2 -ceramide effect possibly differs from the palmitic acid effect, with an earlier increase in apoptosis that leads to a faster decrease in proliferation.

Glucose-induced apoptosis was observed in β -cells of *ob/ob* mice and of Wistar rats maintained in medium containing 10% fetal calf serum (43). In contrast, high glucose concentrations promoted survival of purified β -cells from adult Wistar rats cultured in serum-free conditions (7). Islets from the diabetes-prone *P. obesus*, which were cultured on extracellular matrix-coated plates in the presence of serum, responded to elevated glucose levels by increasing the rate of β -cell apoptosis, whereas no significant change was observed in similarly treated islets of adult Sprague-Dawley rats (6). In the present study, elevated glucose concentrations had only a marginal effect on β -cells apoptosis, whereas exposure to palmitic acid resulted in a substantial increase in the number of β -cells with TUNEL⁺ nuclei independent of the medium glucose levels.

The disruption of mitochondrial ATP/ADP exchange is among the earliest identified events that may initiate apoptosis (44). ANT catalyses the exchange of ADP and ATP across the inner mitochondrial membrane. Here we demonstrate that palmitic acid decreases the expression of ANT. The loss of ANT activity induces mitochondrial swelling (18), as well as rupture of the outer membrane leading to cytochrome c release and apoptosis (19), as observed in the present study. The mechanism leading to decreased ANT expression by palmitic acid is unknown. Yet, irrespective of the mechanism

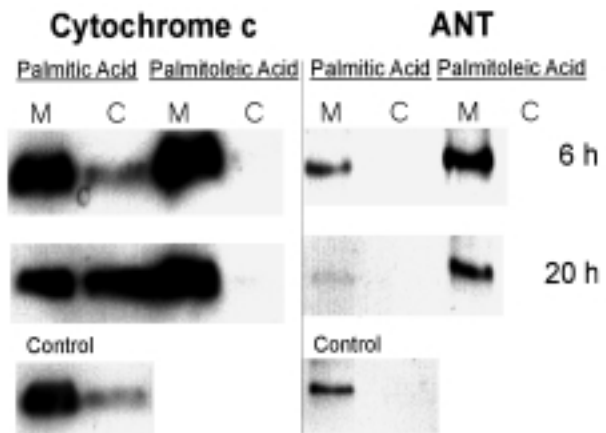


FIG. 5. Subcellular localization of ANT and cytochrome c in fatty acid-treated islets. Immunoblotting of ANT (molecular weight 30 kDa) and cytochrome c (molecular weight 15 kDa) was performed on mitochondrial (M) and cytosolic (C) fractions of islets cultured at 11.1 mmol/l glucose for 6 or 20 h with 0.5 mmol/l palmitic acid or 0.5 mmol/l palmitoleic acid. Control islets were analyzed after 20 h of incubation. Both antibodies were blotted on the same membrane after stripping. One representative of three experiments is shown.

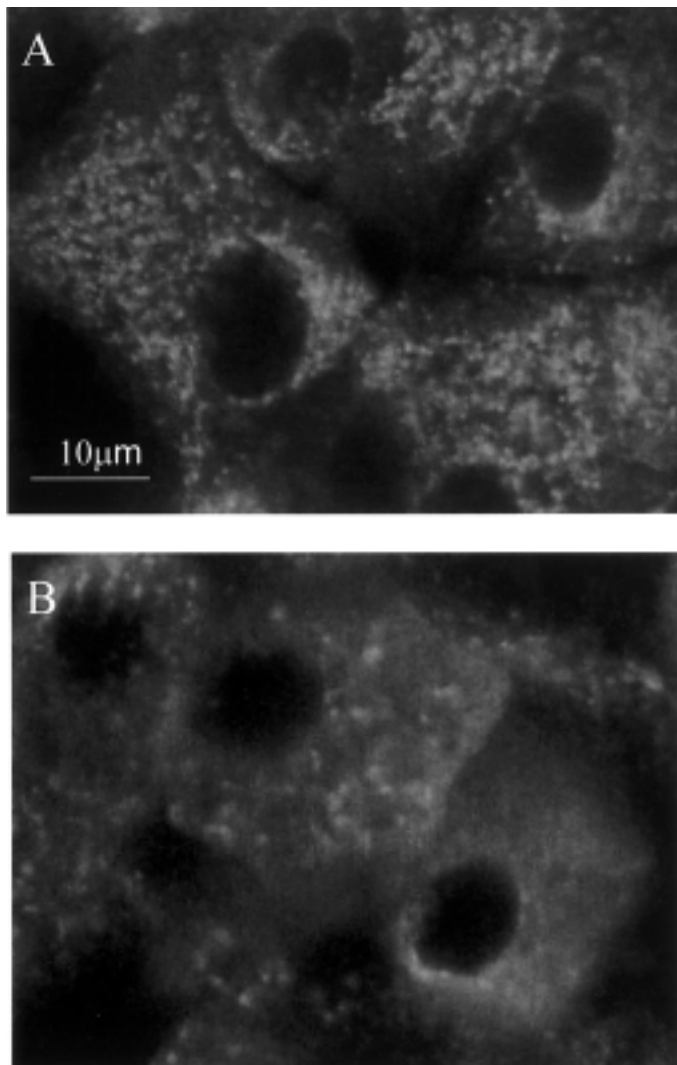


FIG. 6. Confocal images showing cytochrome c diffusely localized to the cytoplasm but not significantly to the mitochondria in palmitic acid-treated islets. Islets were plated on extracellular matrix-coated dishes and were exposed for 4 days to media containing 11.1 mmol/l glucose alone (A) and with 0.5 mmol/l palmitic acid (B) and stained with anti-cytochrome c antibody.

involved, our results attest that the mitochondrion is an important target for palmitic acid-induced apoptosis in β -cells from adult rat islets.

Although various factors including glucose, fatty acids, and amino acids have been shown to govern β -cell proliferation (2,6,10,45), to our knowledge, this study is the first to demonstrate the distinct role of saturated and monounsaturated fatty acids in β -cell replication. Whereas palmitoleic acid was shown to stimulate β -cell proliferation at normoglycemic glucose concentrations, palmitic acid exhibited an inhibitory effect independent of a medium glucose level. With the induction of apoptosis, the deleterious effects of palmitic acid could lead to a reduction in β -cell mass, an important determinant of β -cell functional activity.

ACKNOWLEDGMENTS

This work was supported by grants from the Swiss National Science Foundation (no. 3200-057595.99 to M.Y.D.) and the

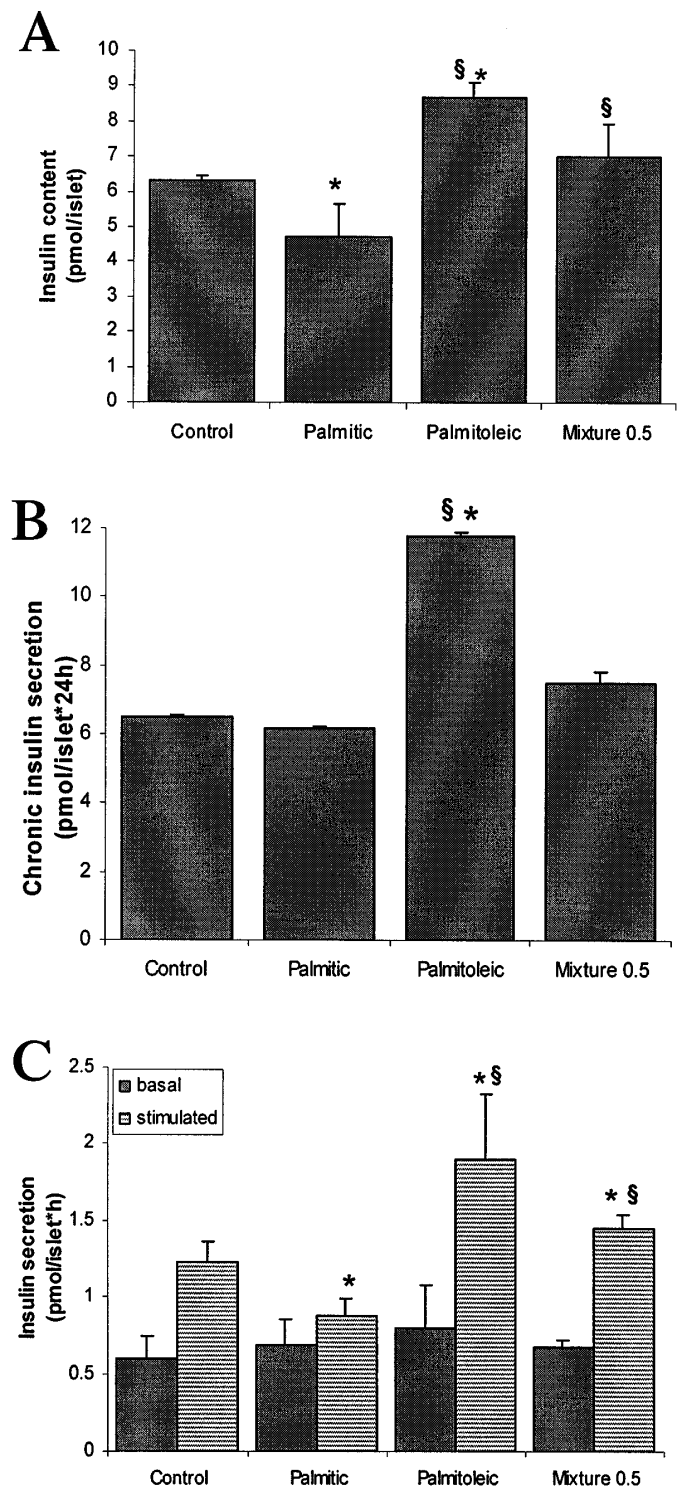


FIG. 7. Effect of prolonged exposure of cultured islets to fatty acids on islet insulin content (A), on chronic insulin release into the culture medium (B), and on basal and glucose-stimulated insulin secretion (C). Islets were incubated in 11.1 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitic or palmitoleic acid or a mixture of both (0.5 mmol/l each) for 4 days. Chronic insulin secretion represents the amount secreted into the culture medium during the experiment. Basal and stimulated insulin secretion denotes the amount secreted over 1 h incubation at 3.3 and 16.7 mmol/l glucose, respectively. Each bar represents the mean of 4 separate experiments \pm SE. In each experiment, the data were collected from 4 plates per treatment. * $P < 0.05$ relative to solvent-treated controls; § $P < 0.05$ for the difference between palmitic acid and palmitoleic acid or fatty acid mixture.

Israel Science Foundation (no. 251/97 to N.K.). M.Y.D. is supported by the Max Cloetta Foundation.

We wish to acknowledge the skillful assistance of Gretha Siegfried-Kellenberger and Heidi Seiler.

REFERENCES

- Kloppel G, Lohr M, Habich K, Oberholzer M, Heitz PU: Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Surv Synth Pathol Res* 4:110–125, 1985
- Bonner-Weir S: Islet growth and development in the adult. *J Mol Endocrinol* 24:297–302, 2000
- Polonsky KS, Sturis J, Bell GI: Seminars in Medicine of the Beth Israel Hospital, Boston: non-insulin-dependent diabetes mellitus: a genetically programmed failure of the β -cell to compensate for insulin resistance. *N Engl J Med* 334:777–783, 1998
- Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS: Role of apoptosis in failure of β -cell mass compensation for insulin resistance and β -cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358–364, 1998
- Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced β -cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95:2498–2502, 1998
- Donath MY, Gross DJ, Cerasi E, Kaiser N: Hyperglycemia-induced β -cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes. *Diabetes* 48:738–744, 1999
- Horens A, Van de Castele 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
- Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD: Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* 37:1020–1024, 1988
- Walker KZ, O'Dea K, Johnson L, Sinclair AJ, Piers LS, Nicholson GC, Muir JG: Body fat distribution and non-insulin-dependent diabetes: comparison of a fiber-rich, high-carbohydrate, low-fat (23%) diet and a 35% fat diet high in monounsaturated fat. *Am J Clin Nutr* 63:254–260, 1996
- Milburn JL Jr, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, Unger RH: Pancreatic β -cells in obesity: evidence for induction of functional, morphologic, and metabolic abnormalities by increased long-chain fatty acids. *J Biol Chem* 270:1295–1299, 1995
- Mathias S, Pena LA, Kolesnick RN: Signal transduction of stress via ceramide. *Biochem J* 335:465–480, 1998
- Shimabukuro M, Higa M, Zhou YT, Wang MY, Newgard CB, Unger RH: Lipoapoptosis in β -cells of obese prediabetic *fafa* rats: role of serine palmitoyltransferase overexpression. *J Biol Chem* 273:32487–32490, 1998
- Kroemer G, Reed JC: Mitochondrial control of cell death. *Nat Med* 6:513–519, 2000
- 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
- Klingenberg M: Dialectics in carrier research: the ADP/ATP carrier and the uncoupling protein. *J Bioenerg Biomembr* 25:447–457, 1993
- Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G: Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 281:2027–2031, 1998
- Shimizu S, Narita M, Tsujimoto Y: Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399:483–487, 1999
- Stoner CD, Sirak HD: Adenine nucleotide-induced contraction on the inner mitochondrial membrane. II. Effect of bongkrekic acid. *J Cell Biol* 56:65–73, 1973
- Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB: Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 91:627–637, 1997
- Woldegiorgis G, Yousufzai SY, Shrago E: Studies on the interaction of palmitoyl coenzyme A with the adenine nucleotide translocase. *J Biol Chem* 257:14783–14787, 1982
- Gotoh M, Maki T, Kiyozumi T, Satomi S, Monaco AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437–438, 1985
- Gross DJ, Leibowitz G, Cerasi E, Kaiser N: Increased susceptibility of islets from diabetes-prone *Psammomys obesus* to the deleterious effects of chronic glucose exposure. *Endocrinology* 137:5610–5615, 1996
- 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, 1988
- Bouwens L, Lu WG, De KR: Proliferation and differentiation in the human fetal endocrine pancreas. *Diabetologia* 40:398–404, 1996
- 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
- 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
- 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
- Rojo M, Wallimann T: The mitochondrial ATP/ADP carrier: interaction with detergents and purification by a novel procedure. *Biochim Biophys Acta* 1187:360–367, 1994
- Buttke TM: Inhibition of lymphocyte proliferation by free fatty acids. I. Differential effects on mouse B and T lymphocytes. *Immunology* 53:507–514, 1984
- de Vries JE, Vork MM, Roemen TH, de Jong YF, Cleutjens JP, van der Vusse GJ, van Bilsen M: Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J Lipid Res* 38:1384–1394, 1997
- Doi O, Doi F, Schroeder F, Alberts AW, Vagelos PR: Manipulation of fatty acid composition of membrane phospholipid and its effects on cell growth in mouse LM cells. *Biochim Biophys Acta* 509:239–250, 1978
- McGarry JD, Dobbins RL: Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* 42:128–138, 1999
- Roche E, Buteau J, Aniento I, Reig JA, Soria B, Prentki M: Palmitate and oleate induce the immediate-early response genes *c-fos* and *nur-77* in the pancreatic β -cell line INS-1. *Diabetes* 48:2007–2014, 1999
- Zhang CL, Lyngmo V, Nordoy A: The effects of saturated fatty acids on endothelial cells. *Thromb Res* 65:65–75, 1992
- Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest* 93:870–887, 1994
- Gordon GB: Saturated free fatty acid toxicity. III. Potentiation by chlorophenoxyisobutyrate (clofibrate) in strain L cells. *Res Commun Chem Pathol Pharmacol* 20:79–99, 1978
- Hawley HP, Gordon GB: The effects of long-chain free fatty acids on human neutrophil function and structure. *Lab Invest* 34:216–222, 1976
- Innis SM, Clandinin MT: Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid. *Biochem J* 198:167–175, 1981
- Stubbs CD, Smith AD: Essential fatty acids in membrane: physical properties and function. *Biochem Soc Trans* 18:779–781, 1990
- Singh JK, Dasgupta A, Adavey T, Shahmehdi SA, Hammond D, Banerjee P: Apoptosis is associated with an increase in saturated fatty acid containing phospholipids in the neuronal cell line, HN2-5 [published erratum appears in *Biochim Biophys Acta* 1346:205–206, 1997]. *Biochim Biophys Acta* 1304:171–178, 1996
- Surette ME, Winkler JD, Fonteh AN, Chilton FH: Relationship between arachidonate-phospholipid remodeling and apoptosis. *Biochemistry* 35:9187–9196, 1996
- 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* 272:3324–3329, 1997
- Efanova IB, Zaitsev SV, Zhivotovsky B, Kohler M, Efendic S, Orrenius S, Berggren PO: Glucose and tolbutamide induce apoptosis in pancreatic β -cells: a process dependent on intracellular Ca^{2+} concentration. *J Biol Chem* 273:33501–33507, 1998
- Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB: Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell* 3:159–167, 1999
- Sjoholm A: Diabetes mellitus and impaired pancreatic β -cell proliferation. *J Intern Med* 239:211–220, 1996