Chronic Exposure to Free Fatty Acids or High Glucose Induces Apoptosis in Rat Pancreatic Islets: Possible Role of Oxidative Stress

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We investigated the effect of a chronic exposure to high levels of free fatty acid (FFA; 2 mmol/L oleate/palmitate 2:1) or glucose (16.7 mmol/L) on islet cell apoptosis. Apoptosis was detected using 4 different methods: (1) cell staining with annexin-V fluorescien isothiocyanate (FITC) conjugate and propidium iodide (PI); (2) quantification of cytoplasmatic DNA fragments by an enzyme-linked immunosorbent assay (ELISA); (3) assay of caspase 3 activity; and (4) TdT-mediated dUTP nick-end labeling (TUNEL). Islet cells were also costained with an anti-insulin antibody to identify apoptotic β cells. We also evaluated by reverse-transcriptase polymerase chain reaction (RT-PCR) the expression of *bax*, *bcl-2*, and *caspase 3*, genes involved in apoptosis. In islets cultured for 7 days in the presence of high FFA or for 3 days in the presence of high glucose levels, we observed: (1) a 2- to 3-fold increase of apoptotic cells conjugated with annexin-V FITC and PI; (2) a 4- to 6-fold increase of cytoplasmatic DNA fragments; (3) a 3- to 4-fold increase of caspase 3 activity; and (4) a significant increase of insulin positive apoptotic cells as detected with the TUNEL method. RT-PCR analysis indicated in islets exposed to high FFA or glucose levels an increase of *bax* (proapoptotic gene), a reduction of *bcl-2* (antiapoptotic gene), and a slight (although not significant) increase in caspase 3 expression. Western blot analysis also showed an increase of Bax protein levels in islets exposed to high FFA or glucose. The simultaneous presence of both metabolic abnormalities did not further increase the amount of apoptotic cells, although the time-course of the cellular damage induced by FFA was accelerated by the contemporary presence of high glucose. To elucidate the mechanism by which FFA and glucose may induce pancreatic β-cell damage, we examined whether nicotinamide prevents apoptosis in pancreatic islets cultured for 7 days with high FFA or for 3 days with high glucose. Nicotinamide was able to prevent β-cell damage by significantly reducing apoptosis in both experimental conditions. Also, the increase of Bax protein level was prevented by nicotinamide. These data indicate that chronic exposure to elevated FFA or glucose levels increases apoptosis in rat pancreatic islets and these cytotoxic effects could be mediated by oxidative stress. This may contribute to the β-cell failure that occurs in most in type 2 diabetic patients few years after clinical diabetes onset.

APOPTOSIS is an important biologic process that is involved in a variety of physiologic and pathologic conditions, including the regulation of the pancreatic β-cell mass.[1] A change of the balance between cell proliferation and growth and cell death may lead to impairment of glucose homeostasis. Beta-cell apoptosis might contribute to the autoimmune β-cell destruction that occurs in type 1 diabetes,[2] and to the reduction of the β-cell mass that is observed postpartum.[3]

Several data demonstrate that, at least in certain cases, β-cell mass may be reduced in type 2 diabetic patients compared with nondiabetic subjects.[4,5] In consideration of the fact that type 2 diabetic patients are often obese, and that obesity is characterized by an increase of the β-cell mass,[6,7] the modest reduction of the β-cell mass in these patients is even more relevant. The mechanism(s) underlying this defect is still unclear.[8] In particular, the specific role of genetic and environmental factors in determining the β-cell mass reduction in type 2 diabetic patients needs a better clarification.

Recent studies have demonstrated that the chronic exposure to high glucose or free fatty acids (FFA) levels may induce apoptosis of pancreatic β cells.[9,10] However, most of these studies were performed in animal strains with a genetic predisposition to diabetes, therefore making it difficult to differentiate between genetic and environmental factors. Moreover, the effect of the simultaneous presence of both metabolic abnormalities has never been directly investigated. We cultured normal Wistar rat pancreatic islets with high concentrations of either free fatty acids (FFA), or glucose, or both, to mimic in vitro the hyperglycemia and hypernefemia that may occur in vivo and to study the direct effects of these metabolic abnormalities on β-cell apoptosis. To investigate the possible role of oxidative stress, some experiments were performed with FFA or glucose in the presence of nicotinamide, a known antioxidant agent.

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MATERIALS AND METHODS

Materials

Crude collagenase was obtained from Boehringer Mannheim (Mannheim, Germany). RPMI 1640, heat-inactivated foetal calf serum (FCS), glutamine, and gentamycin were obtained from Gibco (Glasgow, UK). Oleate and palmitate sodium salt, and bovine serum albumin (BSA)-free FFA were from Sigma (St Louis, MO).

Islets Preparation and Culture Conditions

Pancreatic islets were isolated by the collagenase method from 200 to 250 g fed male Wistar rats, injected intraperitoneally with 0.2 mL of a 0.2% pilocarpine solution 2 hours before killing by decapitation. With this technique, 300 to 400 islets were isolated from each pancreas.[11] The whole procedure was completed within 120 minutes. After an overnight incubation, purified islets were cultured for up to 7 days in RPMI 1064 medium, containing 8.3 or 16.7 mmol/L glucose, 10% FCS, 2 mmol/L glutamine, 50 μg/mL gentamycin, 2% BSA (FFA-free)

either with or without 2 mmol/L long-chain fatty acid (oleate/palmitate 2:1), as described by Lee et al.[12] We first prepared a 22% fatty acid–free BSA solution in RPMI 1064 medium, and then mixed oleate and palmitate (2:1) to obtain a 22 mmol/L stock solution. The unbound FFA concentrations were 180.6 nmol/L and 20.3 nmol/L, respectively, for oleic and palmitic acids. Unbound concentration were calculated using the FFA-albumin association constants for the first 6 binding sites of albumin, as determined by Richieri et al[13] and reported by Cnop et al.[9] Medium was changed every 2 days. In some experiments, 5 mmol/L nicotinamide was simultaneously added with glucose or FFA.

Islet Dissociation

Dissociation of the islets was performed as previously described.[14] The islets were first dissociated in single cells by gently resuspending in a calcium-free Krebs-Ringer bicarbonate solution (KRB) with 1 mmol/L EGTA at 30°C. After 10 minutes, 16.5 μg/mL trypsin and 2 μg/mL DNAse (Sigma) were added, and the suspension incubated for 8 to 10 minutes at 30°C. Islet cell dissociation was monitored by observing a drop of the suspension at the microscope. When most cells were single or in small groups of 2 to 3 cells, the procedure was stopped by adding KRB buffer containing 1.67 mmol/L calcium, and 1% calf serum. At the end of the dissociation, cells were resuspended in RPMI 1064 medium containing glucose 8.3 mmol/L, washed with phosphate-buffered saline (PBS) and resuspended in the appropriate buffer for assay.

Evaluation of Apoptosis and Necrosis With Annexin-V Fluorescien Isothiocyanate Conjugate and Propidium Iodide

The percentage of apoptotic or necrotic cells was assessed by exposing the pancreatic cells to a solution containing a annexin-V fluorescien isothiocyanate (FITC) conjugate (Sigma) and the DNA binding dye propidium iodide (PI).[15] Annexin-V FITC is a 35.8-kd protein that has a strong natural affinity for phosphatidylserine, a membrane phospholipid that, soon after apoptosis initiates, translocates from the inner to the outer surface of the cell plasma membrane. Cells with preserved membranes are impermeable to PI which, on the contrary, enters in cells with a damaged membrane, and stains DNA red. At the end of the culture period, islets were dissociated in single cells (see above) and 10 μL of Annexin-V FITC (final concentration, 1 μL/mL) and 10 μL of PI were added. Cells were then placed on a glass slide, covered with a glass coverslip, and observed under a fluorescent microscope using a dual filter set for FITC and rhodamine. Double staining with annexin-V and PI identified apoptotic cells, whereas cells stained with PI only were identified as necrotic cells.

Measure of DNA Fragments

DNA fragments were quantified with the Cell Death Detection Enzyme-Linked Immunosorbent Assay (ELISA) Plus Kit from Roche (Mannheim, Germany). This assay is based on a quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes but not free histone or DNA that may generate during nonapoptotic cell death[16] in the cytoplasmic fraction of cell lysates. At the end of the culture period, groups of 50 islets were washed with PBS, lysed according to the manufacturer's protocol, centrifuged (200*g* × 10 minutes), placed in a streptavidin-coated microtiter plate, and incubated with a mixture of antihistone (biotin-labeled) and anti-DNA (conjugated with peroxidase) antibodies. After removal of the unbound antibodies by a washing step, the amount of nucleosomes was quantified photometrically by the peroxidase retained in the immunocomplex.

Detection of Apoptosis With TdT-Mediated dUTP Nick-End Labeling (TUNEL)

At the end of the culture period, islets were dissociated and dispersed in single cells and then attached on silanized glass slides with 4% paraformaldeyde in PBS, pH 7.4, for 10 minutes at room temperature. Staining was performed according to the manufacture's protocol, using the Apoptag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) to localize apoptotic nuclei in isolated islets. Briefly, after fixing, samples were incubated in 3% PBS for 5 minutes; hydrogen peroxide in PBS was used for quenching endogenous peroxidase. This protocol was aimed to label in situ the free 3'OH DNA termini with chemically labeled or unlabeled nucleotides. DNA fragments labeled with the digoxigenin-nucleotide were then allowed to bind an enzymatically conjugated antidigoxigenin antibody, which generates a permanent stain for apoptotic nuclei. The same slides were then incubated first with a mouse antihuman insulin monoclonal antibody (Sigma, 1:800 dilution) for 2 hours, and then with a rhodamine-conjugated antimouse IgG antibody (Sigma, 1:64 dilution) for 1 hour, to detect β cells.

Caspase 3 Activity

To measure caspase-3 (also known as CPC-32, yama, or apopain) activity, islets were lysed for 15 minutes in modified RIPA Buffer (50 mmol/L Tris-HCl pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EGTA) at 4°C and centrifuged at 14,000*g* at 4°C. The supernatant was then assayed for caspase-3 activity. Briefly, 10 μg of supernatant proteins was incubated in the presence of 7 μL (final concentration, 50 μmol/L) of caspase 3 fluorimetric substrate (Upstate Biotechnology, Waltham, MA). Relative fluorescence of the sample was determined at 380 nm excitation and 460 nm emission, after 0 and 5 minutes. Data are expressed as OD/10 μg of protein.

RT-PCR Analysis

RT-PCR analysis was performed as previously described.[17,18] Total RNA was extracted with Trizol (Life Technologies-Gibco, Glasgow, UK) from rat pancreatic islets cultured for 7 days in control medium or in medium with high levels of either FFA or glucose or both, by following the manufacturer's protocol. Purity of the RNA preparations and absence of contamination with DNA were confirmed by checking that the size of the amplified cDNAs was as expected and by performing PCR without the reverse transcription step. Reverse transcription of total RNA was performed for 1 hour at 42°C by using 2 μg of total RNA, 500 ng of oligo-dT primers (Roche Molecular Biochemicals, Mannheim, Germany), and 200 U of Superscript II Reverse Transcriptase in a total volume of 20 μL of 1x First Strand Buffer (Life Technologies-Gibco); different aliquots were then amplified with 1U of Taq polymerase (Applied Biosystem, Foster City, CA) to determine the range within which the amount of amplified cDNA increased linearly with that of the specific mRNA target of the reaction. Alternatively, total RNA from treated and controls pancreatic islets was used to perform RT-PCR with the Superscript One-Step RT-PCR System (Life Technologies-Gibco). Different aliquots of target mRNA were amplified to identify those conditions that allowed the reaction to proceed linearly. Primers were designed with the OMIGA program (Oxford Molecular, Oxford, UK) by using as template the published sequences of *Homo sapiens bcl-2* (GenBank accession no. M14745) and *bax-α* (GenBank accession no. NM_004324), and of *Rattus norvegicus caspase 3* (GenBank accession no. U49930) and *β-actin* (GenBank accession no. V01217). Primers sequences were: *bcl-2* forward 5'-TGCACCTGACGCCCTTCAC-3' and *bcl-2* reverse 5'AGACAG-CCAGGAGAAATCAAACAG3', which amplified a cDNA fragment of 293 bp; *bax-α* forward 5'ACCAAGAAGCTGAGCGAGTGTC3' and *bax-α* reverse 5'ACAAAGATGGTCACGGTCTGCC3', which

amplified a cDNA fragment of 368 bp; *caspase 3* forward 5'TTTTTGGAACGAACGGACC3' and *caspase 3* reverse 5'CCCTGACAGTTTTCTCATTTGG3', which amplified a cDNA fragment of 566 bp; *β-actin* forward 5'GCCAACCGTGAAAAGATGACC3' and *β-actin* reverse 5'TGCCGATAGTGATGACCTGACC3', which amplified a cDNA fragment of 415 bp.

By using these primers, PCR amplification of *bcl-2*, *bax-α*, caspase *3*, and *β-actin* cDNAs was performed with the following cycling parameters: for *bcl-2,* (1) 94°C for 5 minutes; (2) 27 cycles at 94°C for 1 minute, 60°C for 90 seconds, 72°C for 2 minutes; and (3) 72°C for 10 minutes; for *bax-α*, (1) 94°C for 5 minutes; (2) 22 cycles at 94°C for 1 minutes, 60°C for 90 seconds, 72°C for 2 minutes; and (3) 72°C for 10 minutes; for *caspase 3*, (1) 94°C for 5 minutes; (2) 25 cycles 94°C for 1 minute, 58°C for 90 seconds, 72°C for 2 minutes; and (3) 72°C for 10 minutes; for *β-actin*, (1) 94°C for 5 minutes; (2) 18 cycles at 94°C for 1 minute, 60°C for 90 seconds, 72°C for 2 minutes; and (3) 72°C for 10 minutes. Independently amplified *bcl-2*, *bax-α*, or *caspase 3* cDNAs from identical aliquots of the same RNA sample were loaded together with *β-actin* cDNA, amplified separately from another identical aliquot of the same sample, and analyzed through 1.5% agarose gel electrophoresis at 8 V/cm for 90 minutes; this procedure allowed for normalization of their amount. Densitometric analysis was performed with ImageMaster VDS by using TotalLab software (Amersham Pharmacia Biotech, Little Chalfont, UK).

Western Blot for Bax and Bcl-2 Measurement

Bax, Bcl-2, and β-actin protein levels were measured by Western blot analysis as previously described.[12] Briefly, at the end of the culture period, groups of 300 islets were washed twice in PBS and homogenized by sonication in sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) sample buffer. Islet proteins were denatured by boiling for 3 minutes and protein content was measured by the Bradford method, using BSA as standard. Equivalent amounts of proteins were used in the different experimental groups. Homogenates (25 μg of total cellular protein) were separated on 12% SDS-polyacrylamide gel (Mini-Protean, BioRad, Hercules, CA) and electrophoretically transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blotting efficiency as well as the position of protein standards were assessed by Ponceau staining. Membranes were then washed at room temperature with PBS containing 1% nonfat dried milk and 0.2% Tween 20 (2 changes within 30 minutes) and blocked with the same buffer containing 5% nonfat dried milk at room temperature for 1 hour. After blocking, the membranes were washed 2 times, as described above, and incubated with a mouse monoclonal anti–Bcl-2 (Trevigen, Gaithersburg, MD) at 1:1,000 dilution in blocking solution, at 4°C overnight, and mouse monoclonal anti-Bax (Trevigen) at 1:1,000 dilution in blocking solution, at 4°C overnight. As verified in each experiment, the total amount of protein load was similar in each lane for the different culture conditions; we reblotted the same membranes with antimonoclonal β-actin antibody (Sigma) at 1:3,000 dilution in blocking solution, at 4°C overnight. Membranes were then washed and blotted with a goat antimouse immunoglobulin peroxidase-linked whole antibody (Amersham Pharmacia Biotech) diluted 1:4,000 for 1 hour at room temperature. Peroxidase activity was detected using a ECL Kit (Amersham Pharmacia Biotech).

Statistical Analysis

Statistical analysis was assessed by Student's *t* test for unpaired comparison. Data are expressed as means ± SE.

RESULTS

High FFA Effects on Islet Cell Apoptosis

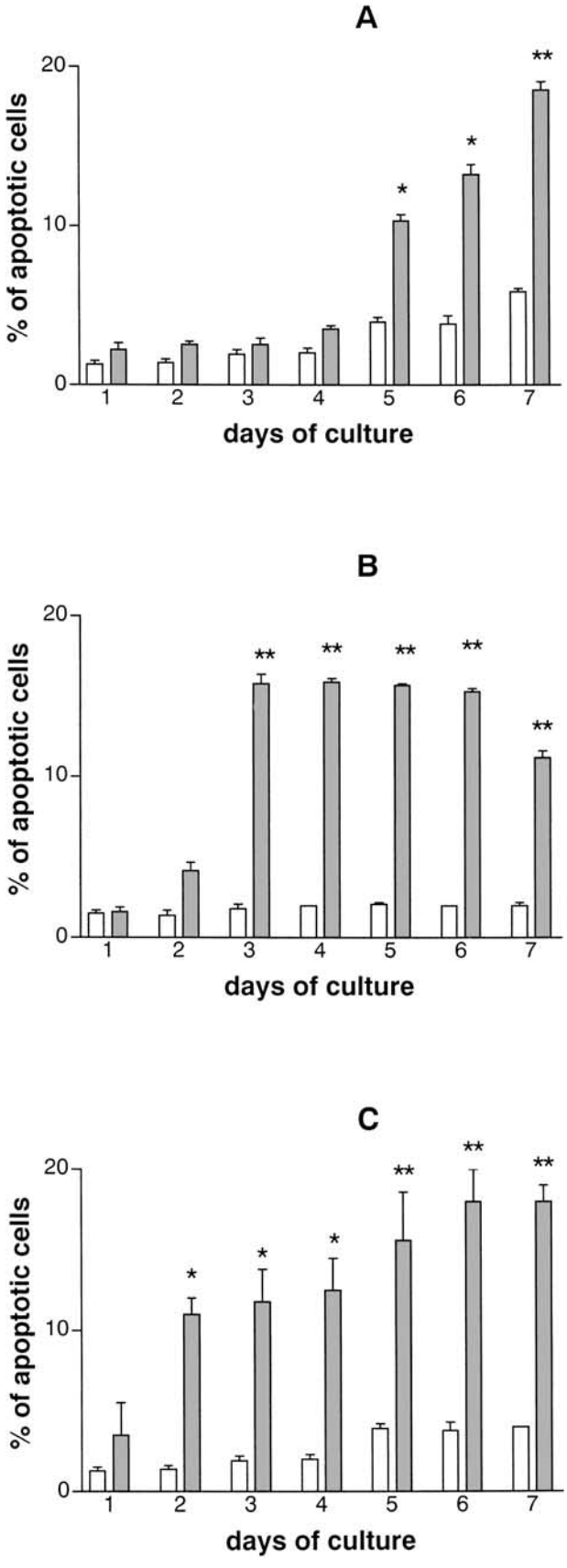
In islets cultured in the presence of high FFA, we measured apoptosis using different techniques and detected an increased amount of apoptotic cells by all methods. First, a significant increase of cells conjugated with both annexin-V FITC and PI was observed. This effect was present after 5 days of culture, and more evident after 7 days (Fig 1A). At that time apoptotic cells were 18.8% ± 4% in FFA-exposed islets, versus 5.6% ± 2% in control islets (mean ± SE; n = 5; $P < .001$). Necrotic cells, identified only by PI, were similar in the 2 groups (4.5% ± 0.2% and 4.2% ± 0.4%, respectively). DNA fragments, as measured by a specific ELISA assay after 7 days of culture, were significantly increased in islets cultured with high FFA in comparison to control islets (1.97 ± 0.6 *v* 0.49 ± 0.1 OD, mean ± SE; n = 5; $P < .001$). The TUNEL method confirmed the presence of an increased amount of apoptotic cells in islets cultured with high FFA (Fig 2A), and also demonstrated that apoptotic cells were β cells, as indicated by staining with an anti-insulin antibody (Fig 3).

Caspase 3 is an intracellular cystein protease that exists as a proenzyme and is activated during the cascade of events associated with apoptosis. The presence of caspase 3 in cells is required for the execution of apoptosis. Caspase 3 activity was greatly increased in islets exposed for 7 days to FFA in comparison to control islets (9.4 ± 1 *v* 2.1 ± 0.6 OD/10 μg protein; n = 4; $P < .001$).

RT-PCR analysis indicated a 37% ± 3% increase of *bax-α* (proapoptotic gene) expression and a 42.6% ± 2% reduction of *bcl-2* (antiapoptotic gene) expression in islets exposed to high FFA levels in respect to control islets (n = 3; $P < .05$) (Fig 4A and B). As a consequence, the *bcl-2*/*bax-α* ratio was clearly reduced in islets exposed to FFA. *Caspase 3* expression was slightly increased in islets exposed to FFA (+10% ± 2%; n = 3; not significant). To evaluate the effect of high FFA on protein levels, we measured Bax and Bcl-2 expression by Western blot analysis. In islets exposed to high FFA for 7 days, Bax protein content was significantly increased in respect to control islets (120 arbitrary densitometric units [ADU] *v* 100 ADU; $P < .001$; n = 4). Bcl-2 contents were similar in every condition (Fig 5A). Beta-actin content was comparable among the various groups, thus confirming that the total amount of protein was similar in each lane.

High Glucose Effects on Islet Cell Apoptosis

In islets cultured in the presence of 16.7 mmol/L of glucose, an increased amount of apoptotic cells was also detected by different methods. We found a significant increase of cells conjugated with both Annexin-V FITC and PI. This effect was present after 2 days of culture, and more evident after 3 days (Fig 1B), when apoptotic cells were 16.1% ± 1% in islets exposed to high glucose and 1.8% ± 2% in control islets (mean ± SE; n = 5; $P < .001$). Necrotic cells, identified only by PI, were similar in the 2 groups (3.5% ± 0.2% and 3.8% ± 0.4%, respectively). DNA fragments, as measured by a specific ELISA assay after 3 days of culture, were significantly increased in islets cultured with high glucose in comparison to control islets (1.38 ± 0.12 *v* 0.26 ± 0.02 OD; n = 5; $P < .001$).



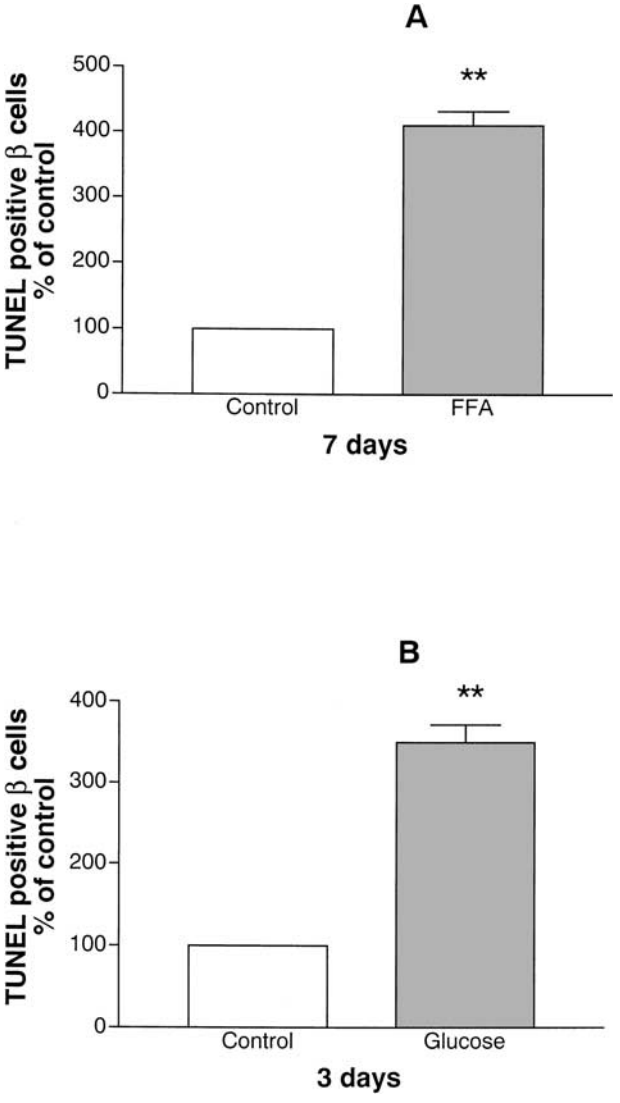


Fig 2. Fatty acids- and glucose-induced DNA fragmentation, as measured with TUNEL. (□) Control; (▩) FFA. Islets cultured in a medium containing (A) FFA 2 mmol/L for 7 days or (B) glucose 16.7 mmol/L for 3 days were assayed for TUNEL. Results are % of control, means ± SE, n = 5; **$P < .001$

The TUNEL method confirmed the increased amount of apoptotic cells in islets cultured with high glucose (Fig 2B), and also showed that apoptotic cells were β cells (data not shown). Caspase 3 activity was increased in islets exposed for 3 days to 16.7 mmol/L glucose in comparison to control islets (9.3 ± 1.9 *v* 2.8 ± 1.1 OD/10 μg protein; n = 4; $P < .001$).

Fig 1. Time-course of FFA and/or glucose effect on apoptosis in rat pancreatic islets. (□) Control; (▩) FFA. Islets were cultured up to 7 days in RPMI medium with or without (A) 2 mmol/L of oleate palmitate (2:1), (B) glucose 16.7 mmol/L, or (C) 2 mmol/L of oleate palmitate (2:1) plus glucose 16.7 mmol/L. Apoptotic cells were identified by the double staining with annexin-V FITC and PI. Results are means ± SE, n = 5; *$P < .05$, **$P < .001$

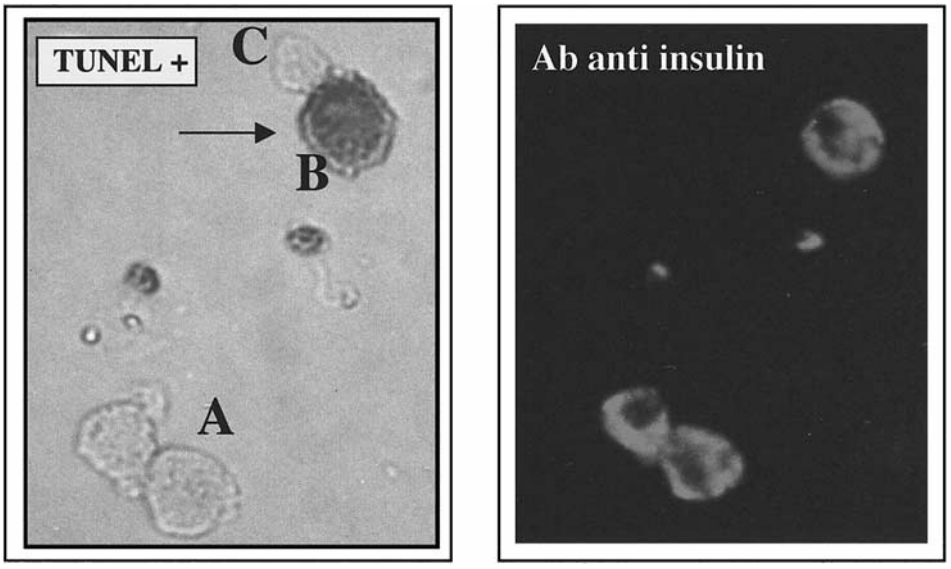


Fig 3. Staining of TUNEL-positive β cells. (Left) Cells from islets dissociation, stained with TUNEL. A and C, 3 nonapoptotic cells; B, an apoptotic cell. (Right) The same cells stained with a fluorescent anti-insulin antibody. Red cells are β cells. A, nonapoptotic β cell; B, apoptotic β cell; C, nonapoptotic non-β cell

RT-PCR analysis indicated a 29% ± 3% increase of *bax-α* expression and a 33% ± 4% reduction of *bcl-2* expression in islets exposed to high glucose levels (n = 3; $P < .05$). Caspase 3 expression was slightly increased in islets exposed to high glucose (+18% ± 3%; n = 3; not significant). Western blot analysis showed an increased of Bax content in respect to control islets (122 ADU *v* 100 ADU; n = 4; $P < .001$), and similar Bcl-2 content (Fig 5B).

High FFA and Glucose Effects on Islet Cell Apoptosis

Apoptotic cells were increased in islets cultured in the presence of both high FFA and glucose after 2 days of culture (as in islets cultured only with high glucose); the percentage of apoptotic cells further increased after 5 and 7 days (as in islets cultured only with high FFA) (Fig 1C). The extent of the cellular damage was similar in islets exposed to both nutrient abnormalities in respect to islets cultured with either high FFA or high glucose alone. After 7 days of culture, apoptotic cells stained with Annexin-V FITC and PI were 18.1% ± 3% in islets exposed to both high FFA and glucose, and 5.1% ± 1.8% in control islets (mean ± SE; n = 5; $P < .001$); DNA fragments were 1.80 ± 36 *v* 0.44 ± 0.1 OD (n = 5; $P < .0001$). RT-PCR analysis indicated a 32% ± 3% increase of *bax-α* expression and a 39% ± 3% reduction of *bcl-2* expression in respect to control islets. Caspase 3 expression was slightly increased in islets exposed to high glucose and FFA (+16% ± 2%; n = 3; not significant).

Effect of Nicotinamide on Islet Cell Apoptosis

In islets cultured in presence of both high FFA and nicotinamide, we detected a significantly decreased amount of apoptotic cells in respect to islets cultured only with high FFA (Fig 6A). After 7 days of culture, apoptotic cells stained with Annexin-V FITC and PI were 9.9% ± 0.7% in islets exposed to both high FFA and nicotinamide, and 25.4% ± 0.17% in islets cultured only with FFA (mean ± SE; n = 5; $P < .001$). Protein expression, measured by Western blot, showed that nicotinamide partially reversed the increase of Bax protein, measured by Western blot (112 ADU *v* 120 ADU in FFA groups; n = 4; $P < .05$). Bcl-2 content was similar to the control and high FFA groups (Fig 5A). Similar results were obtained in islets cultured in presence of high glucose and nicotinamide. After 3 days of culture, we found a significant decrease of cells conjugated with both Annexin-V FITC and PI (Fig 6B) also in islets cultured with high glucose and nicotinamide. We detected 6.7% ± 0.2% apoptotic cells in islets exposed to glucose plus nicotinamide, and 19.1% ± 0.3% in islets cultured with glucose only (mean ± SE; n = 5; $P < .001$). Necrotic cells, identified only by PI, were similar in the 2 groups (4.4% ± 0.4% and 4.1% ± 0.3%, respectively). Nicotinamide completely reverse the increased of Bax protein, measured by Western blot (98 ADU *v* 122 ADU in islets cultured only with glucose; n= 4; $P < .005$). Bcl-2 contents were similar to the control and high glucose groups (Fig 5B).

Similar results have been obtained with other antioxidants, and in particular with N-acetyl-cystein and with lipoic acid (data not shown).

DISCUSSION

We have demonstrated using several different methods that chronic exposure of normal rat pancreatic islets to high levels of either FFA or glucose or both may increase β-cell apoptosis. In islets exposed to these abnormal biochemical conditions, there was not only an increase in the amount of fragmented DNA (according to both the TUNEL method and a specific ELISA assay), but also the expression balance between *bax-α and bcl-2*, 2 genes of human apoptosis,[19] was clearly shifted in favor of the first, indicating the start of the apoptotic process. The simultaneous presence of both nutrient abnormalities did not further increase the number of apoptotic cells, but rather affected the time-course of the cellular damage, which was accelerated in respect to that induced by only high FFA. These findings suggest that both high FFA and glucose induce islet cell apoptosis by a similar mechanism. The similar changes

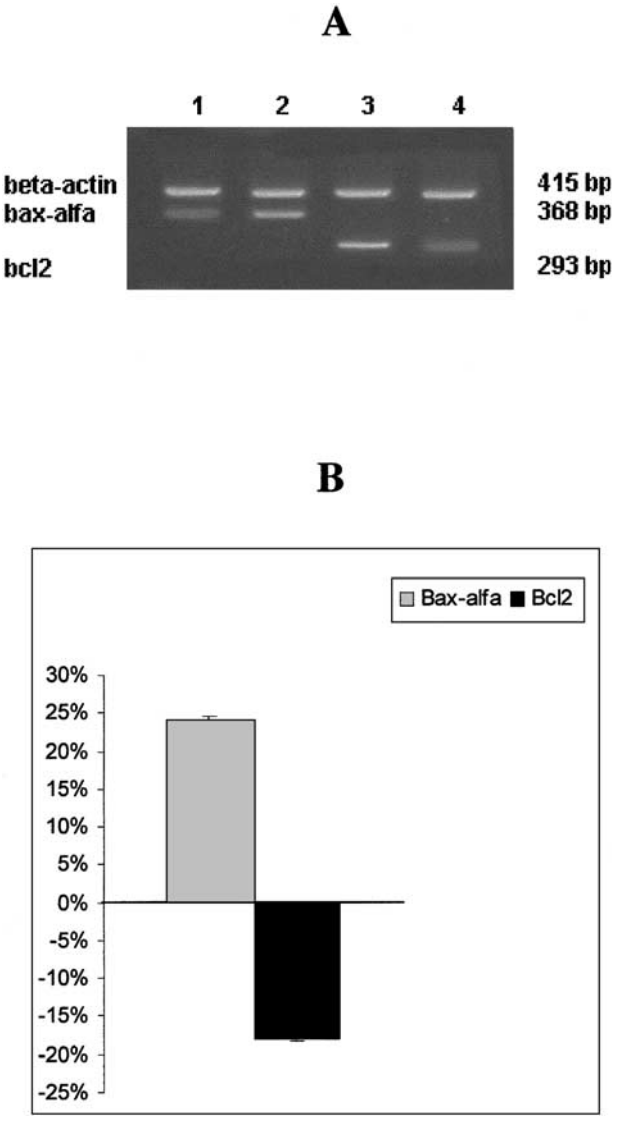


Fig 4. Variation of *bax-alfa* and *bcl-2* mRNA content in rat islet β cells cultured in the presence of high levels of FFA. (A) Agarose gel electrophoresis of amplified *β-actin* and *bax-alfa* and of *β-actin* and *bcl-2* cDNAs from rat pancreatic islets cultured in standard medium (lanes 1 and 3, respectively) or in medium containing high FFA (lanes 2 and 4, respectively). (B) Increase of *bax-alfa* mRNA levels and the decrease of *bcl-2* mRNA levels in rat islets exposed to high levels of FFA. Similar data have been obtained in islets cultured in the presence of high glucose.

observed in *bax* and *bcl-2* expression support this hypothesis. This effect was significantly prevented by the simultaneous addition of nicotinamide at the beginning of the culture period, suggesting a role of oxidative stress in β-cell apoptosis under our experimental conditions.

Nutrients as glucose and fatty acids have a dual effect on pancreatic β cells. After acute exposure both stimulate insulin secretion. In contrast, after long-term exposure, both impair β-cell function and may also affect β-cell survival.

Previous data have already shown that chronic exposure to high FFA or glucose levels may induce β-cell apoptosis.[20-25] The apoptotic effect of FFA was first demonstrated in Zucker Diabetic Fatty (ZDF) rats, an animal model of type 2 diabetes and obesity.[10,22] In these rats, an intracellular excess of fat might increase the synthesis of ceramide, a key component of a signal-transduction pathway leading to apoptosis. The role of ceramide in apoptosis is supported by the experimental demonstration that its addition causes DNA fragmentation, and that blockade of its synthesis with fumomisin-B prevents FFA-induced DNA fragmentation.[22] Recently, Maedler et al[23] showed in normal rat pancreatic islets that saturated but not monounsaturated fatty acids increase β-cell apoptosis. This effect was mimicked by a ceramide analog and blocked by a ceramide synthetase inhibitor, further supporting the role of ceramide in FFA-induced apoptosis.

The effect of high glucose is more controversial. Exposure of islets from diabetes-prone *Psammomys obesus* (an animal model of type 2 diabetes) to high glucose levels resulted in a dose-dependent increase in β-cell DNA fragmentation.[24] In contrast, islets from diabetes-resistant animals showed

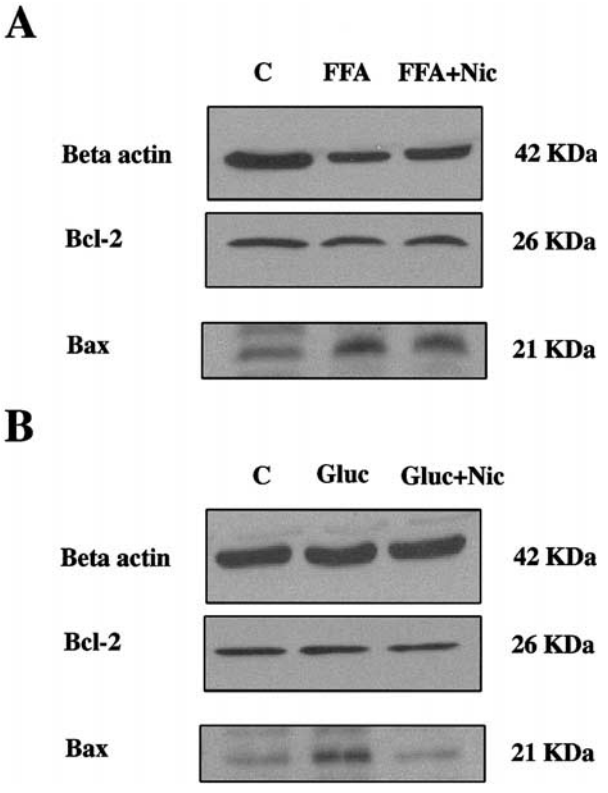


Fig 5. Western blot analysis for Bax, Bcl-2, and β-actin in islets exposed to high FFA or high glucose in the presence or in absence of nicotinamide. Bax and Bcl-2 protein levels measured by Western blot analysis. (A) Total homogenate of islets cultured for 7 days at 2.0 mmol/L FFA. A representative of 4 separate experiments is shown. In this experiment, 25 μg of proteins were resolved by electrophoresis on a 15% polyacrylamide gel. (B) Total homogenate of islets cultured for 3 days at 16.7 mmol/L glucose. A representative of 4 separate experiments is shown. In this experiment, 25 μg of proteins were resolved by electrophoresis on a 15% polyacrylamide gel. Beta-actin in A and B is the control gene.

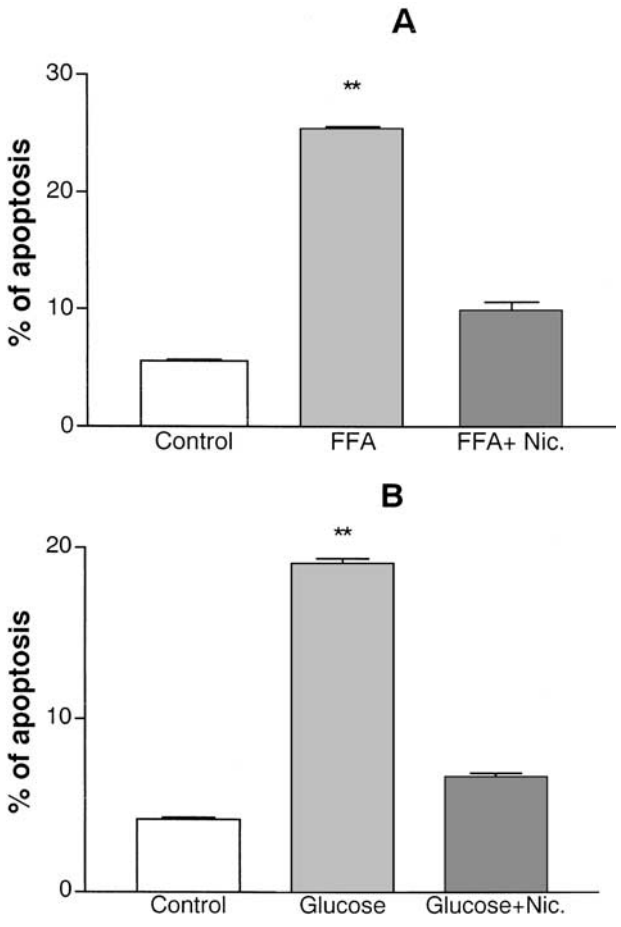


Fig 6. Effect of nicotinamide on FFA- or glucose-induced apoptosis in rat pancreatic islets. (□) Control; (▒) FFA; (■) FFA + nicotinamide. Islets were cultured for 7 days in RPMI medium with or without (A) 2 mmol/L of oleate palmitate (2:1) ± 5 mmol/L nicotinamide, and (B) for 3 days in RPMI medium with 8.5 or 16.7 mmol/L glucose ± 5 mmol/L nicotinamide. Apoptotic cells were identified by the double staining with annexin-V FITC and PI. Results are means ± SE, n = 5; **$P < .001$.

a reduced response. Efanova et al[20] showed that high glucose (17 to 27 mmol/L) might induce apoptosis of pancreatic β cells from *ob/ob* mice and normal Wistar rats in vitro. These data disagree with findings reported by Hoorens et al,[21] who found no significant difference in the rate of cell death when glucose concentration was increased from 10 to 20 mmol/L.

Because some of the previous data were in animal models with a genetic predisposition to the disease, the respective role of genetic and environmental factors is controversial. Our data, obtained in normal rat pancreatic islets, support the view that a genetic predisposition is not essential to observe the proapototic effect induced by high glucose or FFA, although it is conceivable that it may increase the individual susceptibility to these metabolic abnormalities. We have also obtained data (the first to our knowledge) on the effects of the simultaneous presence of both nutrient abnormalities, showing that the time-course of the cellular damage was accelerated in respect to that induced by only high FFA. Finally, our data give insight on the molecular effectors of FFA-/glucose-induced apoptosis. We observed a decrease of *bcl-2* and an increase of *bax* expression in islets cultured with high FFA or glucose levels. These proteins localize in mitochondria and belong to one of the most biologically relevant classes of apoptosis-regulatory gene products acting at the effector stage of apoptosis.[26] They control the permeability of the outer mitochondrial membranes by forming autonomous pores in the membrane or by opening a multiprotein complex, called the mitochondrial permeability transition core complex.[27] The ratio of death antagonists (ie, *bcl-2*) to agonists (ie, *bax*) determines whether a cell will respond to an apoptotic stimulus. Under our experimental conditions, this ratio was clearly altered, with a prevalence of the expression of a proapoptotic gene and protein. We also found an increase of caspase 3 activity in islets exposed to high glucose or FFA. Caspase 3 activation is a distal event in the apoptotic cascade, that ultimately stimulates other caspases and a variety of other cellular proteins, including caspase-dependent endonuclease, which is released from its inhibitor by caspase 3 and cuts DNA into oligonucleosomal fragments.

The mechanism by which high FFA and high glucose induces β-cell apoptosis is still unclear. The finding that nicotinamide prevented apoptosis of β cells cultured with high FFA and glucose seems to indicate that oxidative stress could be involved in the β-cell destruction.

Nicotinamide has been reported to protect β-cell function and survival both in vivo and in vitro.[28,29] Treatment with high doses of nicotinamide prevents or delays insulin-deficient diabetes in several animal model of type I diabetes[30,31] and protects islet cells against cytotoxic actions in vitro from damage induced by cytokines and streptozotocin.[28,32] Moreover, in the ZDF rat, nicotinamide was demonstrated to prevent NO-dependent β-cell apoptosis by decreasing expression of nitric oxide synthase.[30] In addiction, nicotinamide was shown to inhibit the oxidative damage induced by reactive oxygen species.[33] We suggest that nicotinamide might protect β cells from fuel damage by increasing the intracellular reducing equivalents, through an increased availability of NADH(P) produced or directly or with an inhibition of the PARP poly(ADP-ribose) polymerase.[34]

Our data disagree with findings reported by Cnop et al.[9] In their study, nicotinamide was unable to prevent the cytotoxic effect of palmitic or oleic acid on single β cells. These different results might be explained by the different experimental conditions (islets *v* single cells, length and type of culture, mixture of FFA).

In conclusion, our data show an increased amount of apoptotic cells in rat pancreatic islets chronically incubated with high FFA and/or glucose and this cellular damage might be mediated by oxidative stress. Since the altered metabolic environment of the pancreatic β cell may affect both cell function and survival, these effects may contribute to the β-cell failure observed in type 2 diabetic patients.

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