Prolonged Exposure to Free Fatty Acids Has Cytostatic and Pro-Apoptotic Effects on Human Pancreatic Islets

Evidence that β-Cell Death Is Caspase Mediated, Partially Dependent on Ceramide Pathway, and Bcl-2 Regulated

Roberto Lupi,[1] Francesco Dotta,[2] Lorella Marselli,[1] Silvia Del Guerra,[1] Matilde Masini,[1] Carmela Santangelo,[2] Giovanni Patané,[3] Ugo Boggi,[4] Salvatore Piro,[3] Marcello Anello,[3] Ettore Bergamini,[1] Franco Mosca,[4] Umberto Di Mario,[2] Stefano Del Prato,[1] and Piero Marchetti[1]

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024

In an effort to better understand the phenomenon of lipotoxicity in human β-cells, we evaluated the effects of 48-h preculture with 1.0 or 2.0 mmol/l free fatty acid (FFA) (2:1 oleate to palmitate) on the function and survival of isolated human islets and investigated some of the possible mechanisms. Compared with control islets, triglyceride content was significantly increased and insulin content and glucose-stimulated insulin release were significantly reduced in islets precultured with increased FFA concentrations. These changes were accompanied by a significant reduction of glucose utilization and oxidation. By cell death detection techniques, it was observed that exposure to FFAs induced a significant increase of the amount of dead cells. Electron microscopy showed the involvement of β-cells, with morphological appearance compatible with the presence of apoptotic phenomena. FFA-induced islet cell death was blocked by inhibition of upstream caspases and partially prevented by inhibiton of ceramide synthesis or serine protease activity, whereas inhibition of nitric oxide synthesis had no effect. RT-PCR studies revealed no major change of iNOS and Bax mRNA expression and a marked decrease of Bcl-2 mRNA expression in the islets cultured with FFA. Thus, prolonged exposure to FFAs has cytostatic and pro-apoptotic effects on human pancreatic β-cells. The cytostatic action is likely to be due to the FFA-induced reduction of intraislet glucose metabolism, and the proapoptotic effects are mostly caspase mediated, partially dependent on ceramide pathway, and possibly Bcl-2 regulated. *Diabetes* 51:1437–1442, 2002

From the [1]Department of Endocrinology and Metabolism, University of Pisa, Pisa, Italy; the [2]Department of Clinical Sciences-Endocrinology, "La Sapienza" University, Rome, Italy; the [3]Department of Endocrinology, University of Catania, Catania, Italy; and the [4]Department of Oncology, University of Pisa, Pisa, Italy.

Address correspondence and reprint requests to Dr. Piero Marchetti, Department of Endocrinology and Metabolism, Metabolic Unit, Ospedale Cisanello, Via Paradisa 2, 56100 Pisa, Italy. E-mail: marchant@immr.med.unipi.it.

Received for publication 6 December 2000 and accepted in revised form 11 February 2002.

DCI, 3,4-dichlorolsocoumarin; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; HBSS, Hanks' balanced salt solution; KRB, Krebs-Ringer bicarbonate; n-NAME, *N*ω-nitro-L-arginine methyl ester; NF-κB, nuclear factor κB; NO, nitric oxide; OD, optical density; PPAR, peroxisome proliferator–activated receptor; TUNEL, transferase-mediated dUTP nick-end labeling; VAD-FMK, valine alanine aspartic acid-fluoromethylketone.

Under physiological conditions, free fatty acids (FFAs) sustain basal insulin secretion in the fasted state and potentiate hormone release acutely in the presence of glucose (1). However, evidence is growing that prolonged exposure to increased FFA concentrations has detrimental effects on pancreatic β-cells (1,2), a phenomenon that has been termed lipotoxicity. Culturing rodent islets in the presence of elevated FFA levels usually causes reduced insulin release in response to high glucose, suppression of proinsulin biosynthesis, and decreased insulin stores (1–3). Extensive studies in the obese Zucker diabetic fatty (ZDF) rat have shown that elevated circulating FFA concentrations in these animals contribute to cause impairment of β-cell function and loss of insulin-secreting cells by apoptosis, leading to the development of diabetes (4–6). These findings have renewed the interest on the concept that in genetically predisposed human subjects, prolonged exposure of β-cells to raised concentrations of circulating FFAs may contribute to qualitative and quantitative abnormalities of pancreatic islet function, leading to type 2 diabetes (7). However, studies with human islet cells are still scanty, and several issues remain to be clarified. Zhou and Grill (8) found that 0.125 mmol/l palmitate or oleate had a marked cytostatic effect on glucose-stimulated insulin release after 48 h incubation. More recently, it was shown that elevated fatty acids increased the proinsulin/insulin ratio (9). It is unknown whether prolonged exposure to FFAs affects the survival of isolated human islet cells and, if so, which pathways are involved.

In the present study, we evaluated the occurrence and some characteristics of lipotoxicity in isolated human islets cultured in the presence of increased FFA concentrations. We found that prolonged exposure to FFAs has cytostatic and pro-apoptotic effects on human pancreatic β-cells. The cytostatic action was accompanied by reduced intraislet glucose metabolism, and the pro-apoptotic effects were mostly, but not solely, caspase mediated, partially dependent on ceramide pathway, and possibly Bcl-2 regulated.

RESEARCH DESIGN AND METHODS

Islet preparation and incubation experiments. Pancreatic islets were prepared by collagenase digestion and density gradient purification (10,11) from 18 nonobese human multiorgan donors (10 men and 8 women, aged 63 ± 10 years, BMI 24 ± 2 kg/m^2; cause of death: 11 cerebral vascular event and 7 trauma). All protocols were approved by our local Ethics Committee.

The enzyme collagenase (Collagenase P; Roche Mannheim, Germany) was used for digestion of the pancreas. The pancreatic duct was cannulated, and the digestion solution (collagenase 1.5–2.0 mg/ml, dissolved in 300 ml Hanks' balanced salt solution [HBSS]) (Sigma Chemical, St. Louis, MO) was slowly injected to distend the tissue. The collagenase solution injected was about threefold in volume the weight of the pancreas. After distension, the gland was placed into a 500-ml glass beaker, and the digestion solution not used for distension was added into the beaker. This was loaded into a shaking water bath at 37°C, activated at 120 rpm. After 10 min, the pancreas was shaken with forceps for 60 s, and then the digestate was filtered through 300- and 90-μm mesh stainless steel filters in sequence. The solution that passed through the filters and the tissue entrapped on the 300-μm mesh filter were placed back into the water bath for further digestion. The tissue remaining on the 90-μm mesh filter was washed with HBSS and 10% bovine serum. The same procedures of filtration, washing, and settling in the HBSS solution were repeated each 8–10 min up to 40–50 min.

For the purification procedure, 3 ml of tissue was loaded into 250-ml plastic conicals and resuspended in 50 ml of 80% Histopaque 1.077 (Sigma) and 20% HBSS, topped with 40 ml HBSS. After centrifugation at 800*g* for 5 min at 4°C, the islets were recovered at the interface between the Histopaque and the HBSS layers. The islets were washed with HBSS by centrifugation at 800*g* for 2 min at 4°C, resuspended in M199 culture medium (supplemented with 10% serum and antibiotics), and cultured at 37°C in a CO_2 incubator.

At the end of the isolation procedure, the islets were resuspended in M199 culture medium, supplemented with 10% adult bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomicin, 50 μg/ml gentamicin, and 0.25 μg/ml amphotericin B) (10,11), and cultured at 37°C in a CO_2 incubator.

Within 5–7 days from isolation, aliquots of the islet preparations were placed into 60-mm Petri dishes and maintained in suspension culture for 48 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium consisted of the supplemented M199 medium, either with or without 1.0 or 2.0 mmol/l long-chain fatty acid mixture (2:1 oleate to palmitate) (Sigma Chemical), containing 2% human albumin and prepared as previously reported (2,4–6,8,9). At the end of the 48-h culture period, islet triglyceride concentration was measured by a commercial kit (GPO-Trinder; Sigma) after extraction (12,13).

Insulin secretion studies. At the end of the varying incubation conditions, insulin secretion was assessed as previously detailed (10,11). After a 45-min preincubation period at 3.3 mmol/l glucose, batches of ~50 islets of comparable size were kept at 37°C for 45 min in Krebs-Ringer bicarbonate (KRB) solution, 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. At the end of this challenge, the medium was completely removed and replaced with KRB containing 16.7 mmol/l glucose. After an additional 45-min incubation, the medium was removed and assayed. Islet insulin content was assessed after alcohol-acid extraction (10). Insulin concentrations in the various media were measured by immunoradiometric assay (Pantec Forniture Biomediche, Turin, Italy).

Glucose utilization and oxidation. After 48-h exposure to 2.0 mmol/l FFA, glucose utilization and oxidation were assessed based on methods previously developed and tested (13). The rate of glucose utilization was determined by measuring the formation of ^3H_2O from [5-^{3}H]glucose in the presence of either 3.3 or 16.7 mmol/l nonradioactive glucose. Glucose oxidation was measured by determining the formation of $^{14}CO_2$ from [U-^{14}C]glucose in the presence of either 3.3 or 16.7 mmol/l unlabelled glucose.

Evaluation of islet cell death/survival. Islet cell death/survival was assessed by the transferase-mediated dUTP nick-end labeling (TUNEL) technique (In Situ Cell Death Detection system; Roche) and the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) plus assay (Roche), both according to the procedures recommended by the manufacturer and applied to human islet experiments (14).

For the TUNEL technique, ~50 hand-picked islets were dissociated into single cells as previously described (11) and fixed on glass slides by paraformaldehyde. After inhibition of endogenous peroxidase and membrane permeabilization, islet cells were exposed to terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides for 60 min at 37°C. Then, the slides were treated with an anti-fluorescein antibody conjugated with horseradish peroxidase followed by exposure to 3,3′-diaminobenzidine substrate. The preparations were finally observed on a light microscope, and dead cells were counted based on nuclear staining.

For the ELISA method, aliquots of ~15 islets of comparable size were incubated for 30 min with a lysis buffer at room temperature and then centrifuged at 200*g* for 10 min at 4°C. Aliquots of the supernatant (20 μl) were placed into microtiter plate wells coated with streptavidin. A total of 80 μl of a mixture containing anti–histone-biotin antibody and anti–DNA-peroxidase antibody was then added, and incubation was allowed for 120 min at 37°C. Then, the preparations were washed and 100 μl of a solution containing ABTS (2,2′-azino-di[3-ethylbenzthiazalin-sulfonate]) (the substrate for peroxidase) was added. At the end of the 15-min incubation, absorbance of samples was read spectrophotometrically at 405 nm.

Electron microscopy studies were performed as previously described (14). Human islets were pelleted by centrifugation at 1,300*g* and fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4, for 1 h at 4°C. After rinsing in cacodylate buffer, islet pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature, dehydrated in a graded series of ethanol, briefly transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections (60- to 80-nm thick) were cut with a diamond knife, placed on formvar carbon–coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate.

Evaluation of the role of proteases and the ceramide/nitric oxide pathway. To assess whether the caspase (cysteine protease) system was involved, in some experiments hand-picked islets were cultured free-floating in the presence of FFAs, with or without the addition of 10 μmol/l valine alanine aspartic acid-fluoromethylketone (VAD-FMK) (Roche), a synthetic peptide inhibitor that functions upstream of the effector caspases (15). The possible role of serine proteases was also assessed by adding 100 μmol/l 3,4-dichlorolsocoumarin (DCI), an inhibitor of these proteases (16), to the FFA-containing medium. In addition, because ceramide has been reported as a possible inducer of FFA-mediated apoptosis, we tested whether myriocin, a ceramide synthesis inhibitor (17), could affect (used at 50 nmol/l) the phenomenon of lipotoxicity. Finally, *N*ω-nitro-L-arginine methyl ester (n-NAME), an inhibitor of nitric oxide (NO) synthesis (18), was tested (at 10 mmol/l) to evaluate the possible role of the NO pathway on FFA-induced damage. In these experiments, cell death was assessed by the ELISA technique described above.

RT-PCR experiments. RT-PCR studies were performed in three separate experiments in order to analyze mRNA expression of iNOS, Bax, and Bcl-2 (14). Total RNA was extracted from purified human pancreatic islets with Trizol (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions and quantitated by optical density (OD). First-strand cDNA synthesis was performed in a total volume of 20 μl, using 2 μg of each RNA sample primed with random examers with 200 units of Superscript II (Gibco-BRL); cDNA aliquots corresponding to 200 ng RNA were subsequently amplified in 100 μl reaction volume containing 20 pmol of upstream- and downstream-specific primers, 2.5 units of *Taq* DNA polymerase (Gibco-BRL), 200 μmol/l of each deoxynucleoside triphosphate, and 1.5 mmol/l $MgCl_2$. The specific primer for human iNOS amplified a 461-bp product (sense: 5′-TCC GAG GCA AAC AGC ACA TTC A-3′; antisense: 5′-GGG TTG GGG GTG TGG TGA TGT-3′). The human Bcl2 primer pair (5′-ACA ACA TCG CCC TGT GGA TGA C-3′ and 5′-ATA GCT GAT TCG ACG TTT TGC C-3′) and human Bax primer pair (5′-GGC CCA CCA GCT CTG AGC AGA-3′ and 5′-GCC ACG TGG GCG TCC CAA AGT-3′) generated a 408- and 477-bp product, respectively (14). Expression of β-actin as RNA control was analyzed using the following primers, generating a 354-bp product (5′-ACC AAC TGG GAG GAG ATG GAG-3′ and 5′-CGT GAG GAT CTT CAT GAG GTA AGT C-3′). Multiple exons spanning primers were used to avoid the detection of genomic DNA. All PCR products were electrophoresed on 1.2% Separide agarose gel and bands visualized by ethidium bromide staining. To confirm the identity of the bands, amplified products were sequenced with the Ampli-*Taq* cycle method using an automated sequencer (ABI373A; Perkin Elmer, Norwalk, CT).

Statistical analysis. Results are expressed as means ± SD. Statistical analysis was performed by the two-tailed Student's *t* test or ANOVA plus the Bonferroni correction.

RESULTS

Effects of FFAs on human islet function. Triglyceride content was measured in islets prepared from six separate pancreata, and it increased from 17.0 ± 2.4 ng/islet in control preparations to 27.4 ± 2.8 ng/islet after culture for 48 h in 2.0 mmol/l FFA medium ($P < 0.02$). Insulin content, as determined in islets from six separate pancreata, was 6.3 ± 2.1 ng/islet in control cells and 2.7 ± 0.5 ng/islet in the islets preexposed to 2.0 mmol/l FFA for 48 h ($n = 6$, $P < 0.02$).

Table 1 shows the glucose-stimulated insulin secretion

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024

TABLE 1
Insulin secretion in response to glucose from human islets cultured for 48 h with or without varying FFA mixture concentrations

	Insulin secretion (μU/ml)	
FFA (mmol/l)	3.3 mmol/l glucose*	16.7 mmol/l glucose*
0	169 ± 21	388 ± 76†
1.0	184 ± 23	297 ± 88†
2.0	153 ± 23‡	157 ± 51§

Data are means ± SD of 24 replicates from 8 pancreata. *$P < 0.01$ by ANOVA for the values in the column; †$P < 0.01$ vs. 3.3 mmol/l glucose by Student's t test; ‡$P < 0.05$ vs. 3.3 mmol/l glucose and 0 and 1.0 mmol/l FFA, by Bonferroni test; §$P < 0.05$ vs. 16.7 mmol/l glucose and 0 and 1.0 mmol/l FFA, by Bonferroni test.

TABLE 3
Amount of dead islet cells after 48-h incubation with control culture medium or medium containing 2.0 mmol/l FFA mixture

	Amount of dead cells	
	Controls	FFA exposed
TUNEL (%)	8.9 ± 2.0	24.0 ± 4.0*
ELISA (OD)	0.9 ± 0.1	2.9 ± 0.6*

Data are means ± SD. TUNEL experiments were from four separate pancreata; ELISA experiments were from nine separate pancreata. *$P < 0.01$ vs. controls.

results. Compared with control islets, basal (at 3.3 mmol/l glucose) insulin release tended to be higher after 48 h incubation with 1.0 mmol/l FFA and decreased significantly after preexposure to 2.0 mmol/l FFA. When challenged with 16.7 mmol/l glucose, control islets showed a significant, 2.3-fold, increase of insulin release, consistent with previous studies using islets cultured for a few days (10,14). Insulin release from 1.0 mmol/l FFA-exposed cells was ~25% lower than that from control islets, but it was still significantly higher than the secretion at 3.3 mmol/l glucose, showing at least partially maintained glucose responsivity. However, insulin release at 16.7 mmol/l glucose from islets preexposed to 2.0 mmol/l FFA mixture was markedly impaired, resulting in significantly lower levels than those observed with control and 1.0 mmol/l exposed islets.

As summarized in Table 2, in control islets, both glucose oxidation and utilization increased significantly at augmented glucose levels. In the islets cultured for 48 h with 2.0 mmol/l FFA, a significant decrease of the rate of glucose metabolism was observed in the presence of 16.7 mmol/l glucose, with a reduction in glucose oxidation of ~40% and a reduction in glucose utilization of ~50%.

Effects of FFAs on human islet death/survival. Cell death detection techniques revealed a significant increase (about threefold) of the amount of dead cells in islets preexposed for 48 h to 2.0 mmol/l FFA, as compared with control islets (Table 3). To assess whether β-cells were involved in this phenomenon, aliquots of control and 2.0 mmol/l FFA-exposed islets were sampled for electron microscopy analysis, and some of the findings are shown in Fig. 1*A–D*. No difference between control islets (Fig. 1*A*) and the islets incubated for 6 h with FFAs (not shown) was observed. After 24 h, islets exposed to FFAs showed changes of β-cell morphology suggestive of apoptotic phenomena, including autophagy (Fig. 1*B*) and chromatin clumping (Fig. 1*C*), and these features became more marked after 48 h (Fig. 1*D*).

The role of intracellular mediators of apoptosis was assessed in seven separate experiments. When VAD-FMK, an inhibitor of upstream caspases, was added to islets incubated in the presence of FFAs, a complete prevention of cell death was observed (OD in FFA-exposed islets from 3.0 ± 0.4 to 1.0 ± 0.3, $P < 0.05$ by the Bonferroni test) (Fig. 2). A partial but significant reduction of islet cell death was also obtained with the use of DCI, an inhibitor of serine proteases (OD 2.2 ± 0.4, $P < 0.05$ by the Bonferroni test), or myriocin, an inhibitor of ceramide synthesis (OD 2.1 ± 0.4, $P < 0.05$ by the Bonferroni correction) (Fig. 2). On the other hand, inhibition of NO synthesis by the use of n-NAME did not affect the rate of human islet cell death (OD 3.0 ± 0.3).

As shown in Fig. 3, RT-PCR studies revealed no major change of iNOS and Bax mRNA expression and revealed a marked decrease of Bcl-2 mRNA expression in the islets preexposed to 2.0 mmol/l FFA, as compared with control islets.

DISCUSSION

In the present report, the direct effects of long-term elevated oleate and palmitate concentrations on isolated human islets were studied at the functional and survival level. At the functional level, we found that these fatty acids caused a dose-dependent inhibition of glucose-stimulated insulin release (in agreement with previous data obtained by using either oleate or palmitate alone) (8). According to rodent results (13), this cytostatic action was accompanied by reduced islet glucose metabolism. This supports the hypothesis that the FFA-induced inhibition of glucose-stimulated insulin release may also be related, at least in part, to the glucose-FFA (Randle) cycle (19) in human islets. Based on this view, an enhanced availability of FFAs favors their oxidation, which leads to impaired glucose metabolism by substrate competition. Indeed, when FFA oxidation is reduced by using, for example, the biguanide metformin (13), an improvement of glucose

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024

TABLE 2
Glucose oxidation and utilization ($pmol^{-1} \cdot islet^{-1} \cdot 120\ min^{-1}$) in control islets and islets cultured for 48 h with 2.0 mmol/l FFA mixture

	Glucose oxidation		Glucose utilization	
	3.3 mmol/l glucose	16.7 mmol/l glucose	3.3 mmol/l glucose	16.7 mmol/l glucose
Controls	19.5 ± 8.7	41.9 ± 12.1*	39.5 ± 12.7	140.6 ± 45.8†
FFAs	16.4 ± 10.0	25.9 ± 6.1‡	27.5 ± 12.7	69.7 ± 24.8§

Data are means ± SD of four separate experiments. *$P = 0.02$ vs. controls, 3.3 mmol/l glucose; †$P < 0.01$ vs. controls, 3.3 mmol/l glucose; ‡$P = 0.05$ vs. controls, 16.7 mmol/l glucose; §$P = 0.02$ vs. FFA, 3.3 mmol/l glucose and $P < 0.05$ vs. controls, 16.7 mmol/l glucose.

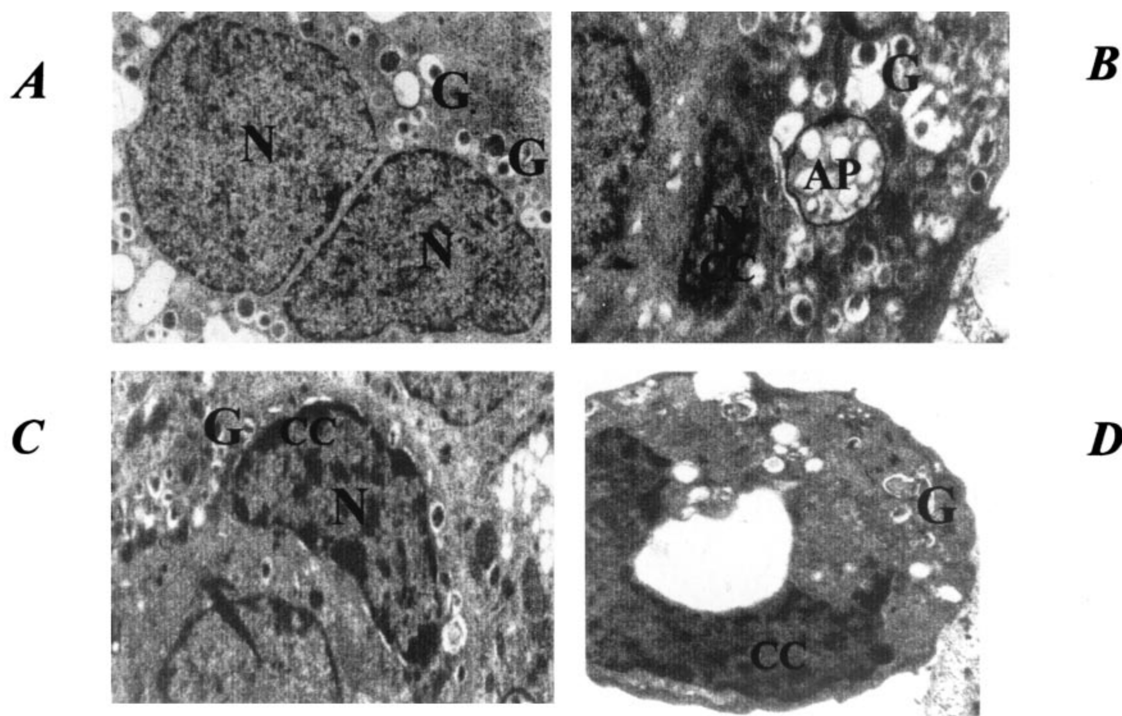

FIG. 1. Electron microscopy of human islet β-cells from control experiments (*A*) or after 24-h (*B* and *C*) or 48-h (*D*) exposure to FFA mixture. AP, areas of autophagy; CC, chromatin clumping; G, insulin granules; N, nucleus. Magnification ×10,000.

metabolism and insulin secretion is observed (13). Among the mechanisms through which FFAs may alter glucose metabolism, the reduced activity of pyruvate dehydrogenase due to increased FFA oxidation seems to play a major role (20). In our experiments, preexposure to FFAs induced a decrease not only of glucose oxidation but also of overall glucose utilization, and this latter was higher than that accounted for by oxidation, confirming previous data with rat islets (13). This suggests that other pathway(s) of intracellular glucose handling are likely to be affected by enhanced FFA availability. This issue will deserve specifically designed experiments to be addressed.

The role of other factors causing FFA-induced alterations of insulin release cannot be ruled out. In our experiments, human islet triglyceride content increased after FFA exposure, an effect also reported by other

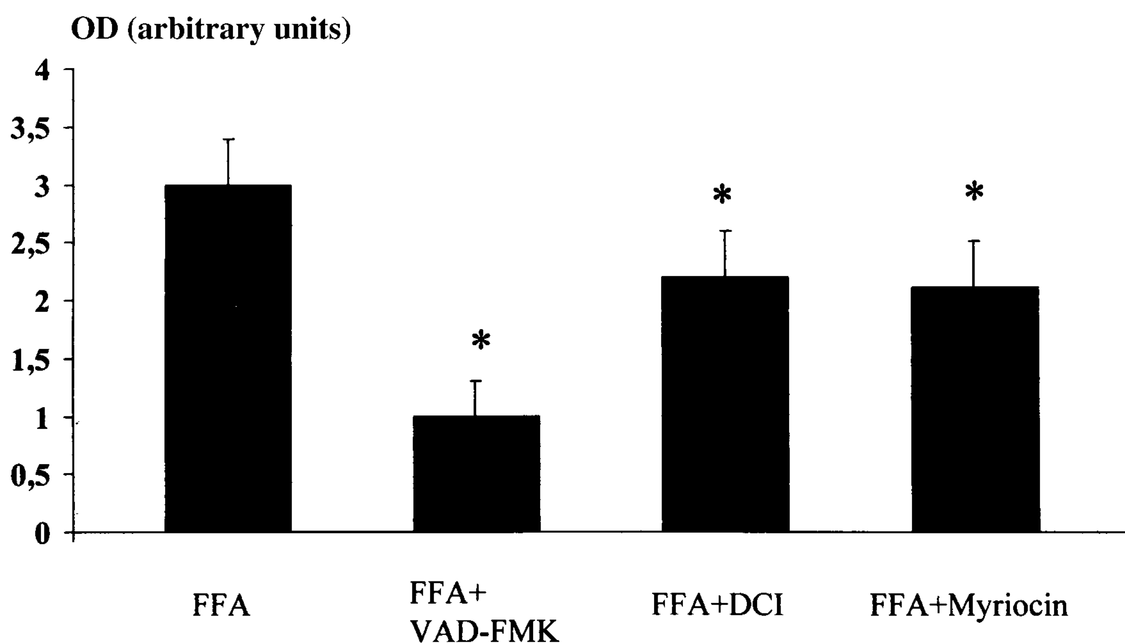

FIG. 2. Effect of caspase inhibition, serine protease inhibition, or ceramide synthesis inhibition on the survival of human islets exposed for 48 h to 2.0 mmol/l FFA mixture. FFA, islets exposed to FFAs only; FFA + VAD-FMK, islets exposed to FFAs plus upstream caspases inhibitor; FFA + DCI, islets exposed to FFAs plus an inhibitor of serine proteases; FFA + myriocin, islets exposed to FFAs plus an inhibitor of ceramide synthesis. Difference between groups was assessed by ANOVA ($P < 0.01$). *$P < 0.05$ vs. FFA by the Bonferroni test.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024

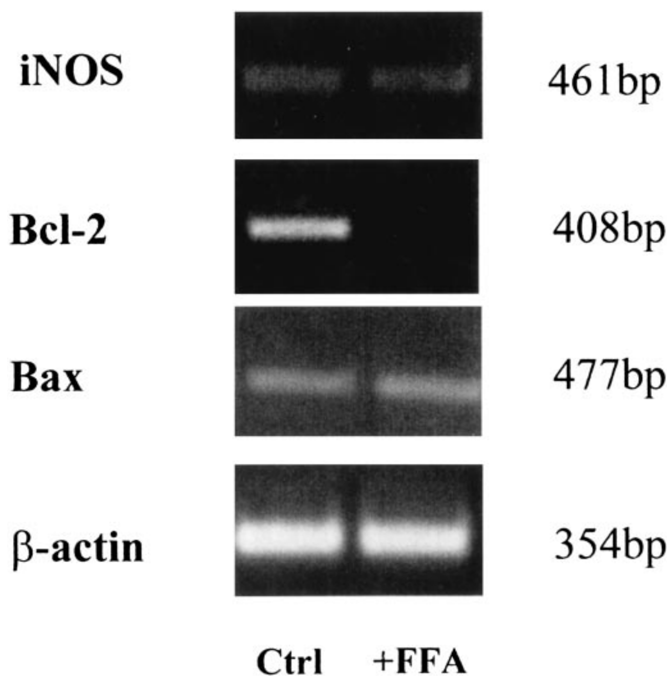

FIG. 3. iNOS, Bcl-2, and Bax mRNA expression in human islets either exposed or not exposed to 2.0 mmol/l FFA mixture for 48 h. PCR products (see text for details) were electrophoresed on Separide agarose gel, and bands were visualized by ethidium bromide staining.

studies of rat islets (21). However, the role of intracellular triglyceride accumulation on insulin secretion changes is questionable, and increasing evidence suggests a dissociation between triglyceride content and insulin secretory function (21). Alternatively, because insulin content decreased significantly in FFA-exposed islets, this could have led to decreased insulin secretion per se. However, preliminary data from our laboratory show that arginine- and sulfonylurea-induced insulin secretion is similar in control to that in FFA-cultured human islets (22), and this selective loss of glucose responsiveness, which was also observed in rat islets (23), implies a role of some alterations of glucose metabolism in the observed phenomena.

At the survival level, for the first time, we show that prolonged exposure to FFAs has a cytotoxic effect on human islets and that this citotoxicity is accompanied by morphological changes of human pancreatic β-cells typical of apoptosis. FFA-induced apoptosis of human islet cells is executed by the caspase system, as suggested by the finding that inhibition of these proteases blocks islet cell death. However, other proteases might be involved, as suggested by the fact that inhibition of serine proteases partially, but significantly, prevented islet cell apoptosis. This shows that FFAs can elicit the activation of a wide number of apoptosis effectors, including the classical caspase proteases (24) as well as the noncaspase proteases (25).

How these proteases can be activated is still unclear. In a model of diabetes-prone obese rats (the *fa/fa* ZDF rat), it has been shown that the lack of a functionally active leptin receptor leads to accumulation of lipids in the β-cells (26,27). This causes β-cell death through mechanisms that, though not fully elucidated, seem to involve increased ceramide production, activation of the transcriptor factor nuclear factor κB (NFκB), upregulation of iNOS, increased synthesis of NO, enhanced formation of NO-derived free radicals, DNA damage, and apoptosis (27). These effects could be prevented by overexpressing the leptin receptor in the *fa/fa* rat islets by adenoviral technology and exposing the modified islets to leptin (27). Our findings with human islets show that inhibition of ceramide formation prevents apoptosis only partially. In addition, and in agreement with a recent study with rat islets (22), we could not demonstrate any involvement of the iNOS/NO pathway. This suggests that ceramide formation may have a role in human islet lipotoxicity. If so, ceramide activation of NFκB (27) would cause upregulation of gene(s) other than the iNOS gene, which would not be surprising considering the pleiotropic effects of NFκB on gene regulation (28).

Additional pathways could be involved. For example, the peroxisome proliferator–activated receptors (PPARs), which are transcription factors belonging to the nuclear receptor superfamily (29), can be found in human islets (30). Binding of fatty acids with PPARs is able to elicit several changes in the expression of many proteins, including some caspases (31,32). Moreover, drugs able to interact with PPARs, such as glitazones (23,33), seem to protect β-cells from FFA-induced damage. This raises the possibility that the interaction of FFAs with PPARs can affect some key mechanisms of the human islet apoptotic pathways.

In rodent islets, FFA-induced apoptosis is characterized by a decrease of Bcl-2 mRNA expression (34). Bcl-2 is a member of the large family of apoptosis-regulator gene products that may either facilitate cell survival (Bcl-2, Bcl-XL, Bcl-w, and others) or promote cell death (Bax, Bak, Bad, and others), and the relative amount of these proteins is a regulatory rheostat that functions by selective protein-protein interaction (35,36). In the *fa/fa* ZDF rat, the reduction of Bcl2 induced by fatty acids has been linked to the action of ceramide through the effect of ceramide on tumor necrosis factor-α (34). Similar to rodent results, our data clearly show that apoptosis of human islet cells exposed to FFAs is accompanied by marked reduction of Bcl-2 mRNA, but our experiments do not indicate whether this is a direct or indirect effect. Interestingly, other human cells seem to have a similar behavior, as suggested by the recent finding that FFA-induced apoptosis in human granulosa cells is accompanied by downregulation of Bcl-2, without any clear relation to ceramide generation (37).

In conclusion, the present study shows that prolonged exposure to FFAs causes reduced glucose stimulated insulin secretion and apoptosis of human pancreatic β-cells. The cytostatic action was accompanied by reduced intraislet glucose metabolism, and the pro-apoptotic effects were mostly, but not solely, caspase mediated, partially dependent on ceramide pathway, and possibly Bcl-2 regulated.

In vivo studies have previously shown that a 24-h infusion of a triglyceride emulsion in healthy subjects was associated with inhibition of insulin release (38). In addition, reduction of plasma FFA levels in first-degree relatives of type 2 diabetic patients improved acute insulin secretion (39). On the other hand, Boden et al. (12) have reported that prolonged (48 h) fat infusion in healthy volunteers had no inhibitory effect on β-cells, and actually,

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024

it caused insulin hypersecretion during the first 24 h. The in vitro data of our study were obtained with FFA concentrations that have often been used in these kind of experiments and are close to those that can be found in humans during lipid challenge (4–6,12,13,38). Although a clear quantitative comparison of in vitro and in vivo FFA concentrations remains elusive, mainly due to the role that albumin and other proteins have in binding circulating fatty acids, the present results with human islets are consistent with the concept that raised FFA concentrations may contribute to causing and/or sustaining the abnormalities of pancreatic islet cell function and survival of type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the Italian Ministero Università e Ricerca Scientifica e Tecnologica (COFIN 2000), by a Telethon grant (E660), and by the Juvenile Diabetes Foundation International (1-1999-679).

REFERENCES

1. Mc Garry JD, Dobbins RL: Fatty acid, lipotoxicity and insulin secretion. *Diabetologia* 42:128–138, 1999
2. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acid inhibits glucose-induced insulin secretion and biosyntesis through a glucose fatty acid cycle. *J Clin Invest* 93:870–876, 1994
3. Bollheimer CL, Skelly RH, Chester MW, McGarry JD, Rhodes CJ: Chronic exposure to free fatty acid reduces pancreatic β cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J Clin Invest* 101:1094–1101, 1998
4. Milburn JL Jr, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, Beltran del Rio H, Newgard CB, Johnson JH, Unger RH: Pancreatic beta-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
5. Lee Y, Hirose H, Zhou YT, Esser V, McGarry JD, Unger RH: Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM. *Diabetes* 46:408–413, 1997
6. Shimabukuro M, Higa M, Zhou YT, Wang MY, Newgard CB, Unger RH: Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats: role of serine palmitoyltransferase overexpression. *J Biol Chem* 273:32487–32490, 1998
7. Unger RH: Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes* 44:863–870, 1995
8. Zhou YP, Grill V: Long-term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J Clin Endocrinol Metab* 80:1584–1590, 1995
9. Bjorklund A, Grill V: Enhancing effects of long-term elevated glucose and palmitate on stored and secreted proinsulin-to-insulin ratios in human pancreatic islets. *Diabetes* 48:1409–1414, 1999
10. Marchetti P, Dotta F, Ling Z, Lupi R, Del Guerra S, Santangelo C, Realacci M, Marselli L, Di Mario U, Navalesi R: The function of pancreatic islets isolated from type 1 diabetic patient. *Diabetes Care* 23:701–703, 2000
11. Antonelli A, Baj G, Marchetti P, Fallahi P, Surico N, Pupilli C, Malavasi F, Ferrannini E: Human anti-CD38 autoantibodies raise intracellular calcium and stimulate insulin release in human pancreatic islets. *Diabetes* 50:985–991, 2001
12. Boden G, Chen X, Rosner J, Barton M: Effects of a 48-h fat infusion on insulin secretion and glucose utilization. *Diabetes* 44:1239–1242, 1995
13. Patanè G, Piro S, Rabuazzo AM, Anello M, Vigneri R, Purrello F: Metformin restores insulin secretion altered by chronic exposure to free fatty acids or high glucose: a direct metformin effect on pancreatic beta-cells. *Diabetes* 49:735–740, 2000
14. Marselli L, Dotta F, Piro S, Santangelo C, Masini M, Lupi R, Realacci M, Del Guerra S, Mosca F, Boggi U, Purrello F, Navalesi R, Marchetti P: Th2 cytokines have a partial, direct protective effect on the function and survival of isolated human islets exposed to combined proinflammatory and Th1 cytokines. *J Clin Endocrinol Metab* 86:4974–4978, 2001
15. Chow SC, Weis M, Kass GE, Holstrom TH, Eriksson JE, Orrenius S: Involvement of multiple proteases during Fas-mediated apoptosis in T lymphocytes. *FEBS Lett* 364:134–138, 1995
16. Weaver VM, Lach B, Walker PR, Sikorska M: Role of proteolysis in apoptosis: involvement of serine protease in internucleosomal DNA fragmentation in immature thymocytes. *Biochem Cell Biol* 71:488–500, 1993
17. Miyake Y, Kozutsumi Y, Nakamura S, Fujita T, Kawasaki T: Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. *Biochem Biophys Res Commun* 21:396–403, 1995
18. Kiechle FL, Malinski T: Nitric oxide: biochemistry, pathophysiology and detection. *Am J Clin Pathol* 100:567–575, 1993
19. Randle PJ, Kerbey AL, Espinal J: Mechanism decreasing glucose oxidation in diabetes and starvation: role of lipid fuels hormones. *Diabete Metab Rev* 4:623–638, 1988
20. Zhou YP, Berggren PO, Grill VE: A fatty acid–induced decrease in pyruvate dehydrogenase activity is an important determinant of β-cell dysfunction in the obese diabetic *db/db* mouse. *Diabetes* 45:580–586, 1996
21. 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
22. Lupi R, Del Guerra S, Fierabracci V, Marselli L, Novelli M, Patanè G, Boggi U, Mosca F, Piro S, Masiello P, Del Prato S, Marchetti P: Lipotoxicity in human pancreatic islets and the effect of metformin. *Diabetologia* 44 (Suppl. 1):A140, 2001
23. Higa M, Zhou YT, Ravazzola M, Baetens D, Orci L, Unger RH: Troglitazone prevents mitochondrial alterations, beta-cell destruction and diabetes in obese prediabetic rats. *Proc Natl Acad Sci* 96:11513–11518, 1999
24. Cryns V, Yuan J: Proteases to die for. *Genes Dev* 12:1551–1570, 1998
25. Johnson DE: Noncaspase proteases in apoptosis. *Leukemia* 14:1695–1703, 2000
26. Zhou YT, Shimabukuro M, Lee Y, Koyama K, Higa M, Ferguson T, Unger RH: Enhanced de novo lipogenesis in the leptin-unresponsive pancreatic islets of prediabetic Zucker diabetic fatty rats: role in the pathogenesis of lipotoxic diabetes. *Diabetes* 47:1904–1908, 1998
27. Unger RH, Zhou Y-T: Lipotoxicity of beta-cells in obesity and other causes of fatty acid spillover. *Diabetes* 50 (Suppl. 1):S118–S121, 2001
28. Baldwin AS: Series introduction: the transcription factor NFκB and human disease. *J Clin Invest* 107:3–6, 2001
29. Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 20:649–688, 1999
30. Lupi R, Del Guerra S, Marselli L, Navalesi R, Marchetti P: Expression of peroxisome proliferator–activated mRNA in isolated human pancreatic islets (Abstract). *Diabetes* 49 (Suppl. 1):A248, 2000
31. Chattopadhyay N, Singh DP, Heese O, Godbole MM, Sinohara T, Black PM, Borwn EM: Expression of peroxisome proliferator-activated receptors (PPARS) in human astrocytic cells: PPARgamma agonists as inducers of apoptosis. *J Neurosci Res* 61:67–74, 2000
32. Chinetti G, Griglio S, Antonucci M, Torra IP, DeleriveP, Majed Z, Fruchart JC, Chamean J, Najeb J, Staels B: Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 273:25573–25580, 1998
33. Finegood DT, McArthur MD, Kojwang D, Thomas MJ, Topp BG, Leonard T, Buckingam RE: β-Cell mass dynamics in Zucker diabetic fatty rats: rosiglitazone prevents the rise in net cell death. *Diabetes* 50:1021–1029, 2001
34. Shimabukuro M, Wang MY, Zhou YT, Newgard CB, Unger RH: Protection against lipoapoptosis of β cells through leptin-dependent manteinance of Bcl-2 expression. *Proc Natl Acad Sci U S A* 95:9558–9561, 1998
35. Di Mario U, Dotta F: Beta-cell autoimmunity 1998. *Diabete Metab Rev* 14:1–2, 1998
36. Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G: Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* 16:2265–2282, 1998
37. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H: Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 142: 3590–3597
38. Paolisso G, Gambardella A, Amato L, Tortoriello R, D'Amore A, Varricchio M, D'Onofrio F: Opposite effects of short- and long-term fatty acid infusion on insulin secretion in healthy subjects. *Diabetologia* 38:1295–1299, 1995
39. Paolisso G, Tagliamonte MR, Rizzo MR, Gualdiero P, Saccomanno G, Gambardella A, Giugliano D, D'Onofrio F, Howard BV: Lowering fatty acids potentiates acute insulin response in first degree relatives of people with type 2 diabetes. *Diabetologia* 41:1127–1132, 1998

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/51/5/1437/658853/db0502001437.pdf by guest on 30 May 2024