Inverse Relationship Between Cytotoxicity of Free Fatty Acids in Pancreatic Islet Cells and Cellular Triglyceride Accumulation

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Studies in Zucker diabetic fatty rats have led to the concept that chronically elevated free fatty acid (FFA) levels can cause apoptosis of triglyceride-laden pancreatic β-cells as a result of the formation of ceramides, which induce nitric oxide (NO)-dependent cell death. This "lipotoxicity" hypothesis could explain development of type 2 diabetes in obesity. The present study examines whether prolonged exposure to FFA affects survival of isolated normal rat β-cells and whether the outcome is related to the occurrence of triglyceride accumulation. A dose-dependent cytotoxicity was detected at 5–100 nmol/l of unbound oleate and palmitate, with necrosis occurring within 48 h and an additional apoptosis during the subsequent 6 days of culture. At equimolar concentrations, the cytotoxicity of palmitate was higher than that of oleate but lower than that of its nonmetabolized analog bromopalmitate. FFA cytotoxicity was not suppressed by etomoxir (an inhibitor of mitochondrial carnitine palmitoyltransferase I) or by antioxidants; it was not associated with inducible NO synthase expression or NO formation. An inverse correlation was observed between the percentage of dead β-cells on day 8 and their cellular triglyceride content on day 2. For equimolar concentrations of the tested FFA, oleate caused the lowest β-cell toxicity and the highest cytoplasmic triglyceride accumulation. On the other hand, oleate exerted the highest toxicity in islet non–β-cells, where no FFA-induced triglyceride accumulation was detected. In conditions without triglyceride accumulation, the lower FFA concentrations caused primarily apoptosis, both in islet β-cells and non–β-cells. It is concluded that FFAs can cause death of normal rat islet cells through an NO-independent mechanism. The ability of normal β-cells to form and accumulate cytoplasmic triglycerides might serve as a cytoprotective mechanism against FFA-induced apoptosis by preventing a cellular rise in toxic free fatty acyl moieties. It is conceivable that this potential is lost or insufficient in cells with a prolonged triglyceride accumulation as may occur in vivo. *Diabetes* 50:1771–1777, 2001

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Received for publication 5 October 2000 and accepted in revised form 1 May 2001.

FFA, free fatty acid; IL, interleukin; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PCR, polymerase chain reaction; RT, reverse transcriptase.

It is suspected that chronically elevated concentrations of free fatty acids (FFAs) contribute to the development of type 2 diabetes (1). They have been found to exert negative influences at the level of both insulin action and insulin release. FFAs can induce a state of insulin resistance (2–5) and impair pancreatic β-cell function (6–9). There is also evidence that FFAs may cause β-cell death, at least in islets of Zucker diabetic fatty rats. In this animal model of obesity-associated diabetes, the sustained increase in circulating FFAs is held responsible for a triglyceride accumulation in the islet cells and for elevated cellular free fatty acyl levels that are cytotoxic (10). In isolated islets from prediabetic Zucker diabetic fatty rats, FFAs were shown to increase ceramide formation, leading to expression of nitric oxide (NO) synthase and NO-dependent β-cell apoptosis (11). These processes have not yet been elucidated at the level of the β-cells; it is unknown whether they can occur in β-cells from normal rats. The mode of FFA-induced β-cell death should be further investigated, in particular, because NO has been identified as a potent cause of β-cell necrosis (12,13). The present study follows the survival of β-cells purified from normal Wistar rats during culture with increasing concentrations of palmitate or oleate. A cytotoxicity assay is used to quantify the number of necrotic or apoptotic cells (14); accumulation of cellular triglycerides is determined to detect its possible correlation with cellular susceptibility to death. Islet non–β-cells are examined in parallel to test the specificity of the observations in β-cells.

RESEARCH DESIGN AND METHODS

Purification and culture of rat β-cells and non–β-cells. Adult male Wistar rats were housed according to the guidelines of the Belgian Regulations for Animal Care. The protocol was approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel. Rats were sedated and killed with CO_2 followed by decapitation. Pancreatic islets were isolated by collagenase digestion and dissociated into single cells in calcium-free medium containing trypsin and DNase (15). Single β-cells (>90% pure) and non–β-cells (>75% α-cells) were purified by autofluorescence-activated sorting, using cellular light-scatter and flavin adenine dinucleotide–autofluorescence as discriminating parameters (15). Single-cell preparations were used in toxicity assays and were analyzed in parallel for inducible NO synthase (iNOS) expression and NO production.

For viability testing, cells were cultured in polylysine-coated microtiter plates in Ham's F10 medium containing 6 or 10 mmol/l glucose, 1% bovine serum albumin pretreated with charcoal (fraction V, radioimmunoassay grade; Sigma, St. Louis, MO), 2 mmol/l L-glutamine, 50 μmol/l 3-isobutyl-1-methylxanthine, 0.075 mg/ml penicillin, and 0.1 mg/ml streptomycin (16). For the islet non–β-cells, this medium was supplemented with 0.5 μg/ml growth hormone

(recombinant human Somatropin; Pharmacia & Upjohn, Stockholm, Sweden), which was found to improve the survival of α-cells during 10-day culture periods (13). Palmitate and oleate (sodium salt; Sigma) were added after solubilization in 95% ethanol and heated to 60°C (1:100 dilution in culture medium); control conditions contained a similar dilution of the solvent. After 48 h or 8 days of culture with or without these fatty acids, the percentage of living cells was counted after staining with neutral red (16). The mode of cell death was determined by a Hoechst 33342-propidium iodide fluorescent assay, which distinguishes the percentage of dead cells with or without a fragmented nucleus, thus quantifying the percentage of apoptotic and necrotic cells (14).

Calculation of unbound FFA concentrations. Unbound FFA concentrations were derived from the total FFA and albumin concentrations. A multiple stepwise equilibrium model (17), described in Eq. 1, takes into account the multiple FFA binding sites of the albumin molecule.

$$r = \frac{K_1 \cdot [fa] + 2 \cdot K_1K_2 \cdot [fa]^2 \ldots + n \cdot K_1 \cdot K_2 \ldots K_n \cdot K_n \cdot [fa]^n}{1 + K_1 \cdot [fa] + K_1 \cdot K_2 \cdot [fa]^2 + \ldots + K_1 \cdot K_2 \ldots K_n \cdot [fa]^n} \quad (Eq.\ 1)$$

where r equals molar ratio FFA/albumin; [fa] equals unbound FFA concentration; and K_i equals FFA-albumin association constant for binding site i.

Using the FFA-albumin association constants for the first six binding sites of albumin, as determined by Richieri et al. (18), we numerically solved Eq. 1 for our experimental conditions (see RESULTS).

Determination of nitrite formation and iNOS expression. Single β-cells and non–β-cells, as well as intact isolated islets, were cultured in multi-well plates (100,000 cells per condition) in medium containing 6 mmol/l glucose and 1% albumin. After a 24-h culture with or without oleate or palmitate, medium was collected for nitrite determination using the Griess reagent (19). As a positive control for iNOS expression and NO production, β-cells were exposed in parallel to the cytokine interleukin (IL)-1β (30 U/ml). FFA-supplemented media were used as blanks for each condition because addition of FFAs to medium resulted in a considerable increase in background levels.

After exposure to FFAs, the cells were harvested from the culture wells for reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis of iNOS expression. RNA was extracted using Dynabeads (DYNAL, Oslo, Norway) and reverse transcribed with the GeneAmp RNA PCR Kit (Perkin-Elmer, Norwalk, CT). iNOS cDNA was amplified in a 34-cycle PCR as described previously (20). There were 32 cycles used for the housekeeping gene GAPDH. This number of cycles was selected to allow linear amplification of the cDNAs under study. Reaction products were separated in an agarose gel electrophoresis, and intensity of ethidium bromide staining was quantified by Biomax 1D Image Analysis Software (Kodak) and expressed in pixel intensities. The intensity of iNOS expression was corrected with that of GAPDH.

Analysis of cellular triglyceride content. After 48 h of culture, aggregates of islet β-cells or islet non–β-cells were collected for determination of their triglyceride content. After three washes and sampling for DNA assay and electron microscopy, cells were stored at −20°C in phosphate-buffered saline containing 20 mmol/l EDTA and 100 μl/dl Triton X-100. Frozen samples were sonicated in 1 ml chloroform/methanol mixture (2:1 by volume) and centrifuged so that the chloroform layer could be removed and the upper phase re-extracted. Pooled chloroform/methanol extracts were dried at 40°C under nitrogen and solubilized in isopropanol (HPLC grade; Sigma) for triglyceride measurement using the Sigma Triglyceride (GPO-Trinder) kit.

Samples for electron microscopy were fixed in cacodylate-buffered glutaraldehyde (4.5%, pH 7.3), postfixed in osmium tetroxide (1%), and embedded in Spurr's resin. Ultrathin sections were stained with uranylacetate and lead citrate and examined for lipid accumulations on a Zeiss EM 109 electron microscope.

Data analysis. The cytotoxicity of FFAs was calculated from the percentage of dead (neutral red [NRneg]) cells counted in the FFA-containing condition x and in the corresponding control c (21):

$$\text{Cytotoxicity } x = \frac{\%\text{NRneg}(x - \%\text{NRneg}(c)}{100\% - \%\text{NRneg}(c)} \times 100\% \quad (Eq.\ 2)$$

For the Hoechst 33342-propidium iodide assay, the cytotoxicity index for necrosis was calculated against the percentage of necrotic cells in the control condition:

$$\text{Necrosis index } x \frac{\%\text{ necrosis } (x - \%\text{ necrosis } (c)}{100 - \%\text{ necrosis } (c)} \times 100\% \quad (Eq.\ 3)$$

The apoptosis index was similarly calculated.

Results are presented as means ± SE. Single comparisons were performed by the Student's paired t test. For multiple comparisons, data were analyzed by analysis of variance, followed by group comparisons using Student's paired or unpaired t test, as indicated, with correction of the P values for multiple comparisons by the Bonferroni method (22).

TABLE 1
Cytotoxic effect of oleate and palmitate in cultured rat β-cell preparations

	Exposure time (mmol/l)	Cytotoxicity	
		2 days	8 days
Oleate	0.125	<5	7 ± 1*
	0.250	7 ± 2*	13 ± 2†
	0.500	9 ± 2*	25 ± 2†
Palmitate	0.125	5 ± 1*	15 ± 2†
	0.250	12 ± 2†	43 ± 3†
	0.500	54 ± 5†	94 ± 2†
Oleate	0.125	<5	<5‡§
Palmitate	0.125		
Oleate	0.250	7 ± 1‖	13 ± 3¶
Palmitate	0.250		
Bromopalmitate	0.125	5 ± 1#	37 ± 3†
	0.250	13 ± 2†	77 ± 1†
	0.500	75 ± 6†	100 ± 0†

Data are means ± SE for six to nine experiments. FFA-induced toxicity after 2 and 8 days of culture at 10 mmol/l glucose and 1% bovine serum albumin. Cytotoxicity of single fatty acids was assessed by comparison with control condition (no fatty acids added): *$P < 0.01$; †$P < 0.001$; ‡$P < 0.01$ vs. condition without palmitate; §$P < 0.01$; ‖$P < 0.05$; ¶$P < 0.001$ vs. condition without oleate; #$P < 0.05$. (analysis of variance, followed by paired t test with Bonferroni correction).

RESULTS

Effects of oleic and palmitic acid on survival of cultured β-cells. Culture of rat β-cells in the presence of oleic or palmitic acid resulted in time-dependent cell death. Oleic acid (0.5 mmol/l) exerted a minor toxicity after 48 h (<10%, $P < 0.01$ vs. control) but killed 25% of the cells after 8 days ($P < 0.001$). Palmitic acid (0.5 mmol/l) killed 54% after 48 h ($P < 0.001$) and virtually all cells after 8 days ($P < 0.001$) (Table 1).

The cytotoxicity of both fatty acids was lower when higher albumin concentrations were used in the culture medium. It was therefore expressed as a function of the molar ratio of FFA over albumin. FFA toxicity increased with this molar ratio (Fig. 1*A*). Up to a ratio of 3.5, the toxicity of oleate was minimal (<10%) after 48 h, but it was clearly detectable after 8 days and increased with the FFA/albumin molar ratio. The toxicity of palmitate was also ratio dependent but was more marked at both time points. At ratios >5, virtually all cells were killed after 48 h; in this condition, however, the unbound fatty acid concentrations exceeded the threshold for their solubility and are known to precipitate (23,24), which made us exclude these data from further analysis.

The free oleic and palmitic acid concentrations were calculated by the stepwise equilibrium model, taking into account their respective binding affinities for albumin (see RESEARCH DESIGN AND METHODS). In the presence of 1% albumin, the unbound FFA concentrations were 6, 13, and 47 nmol/l after addition of 0.125, 0.250, and 0.500 mmol/l oleate, respectively, and 5, 9, and 27 nmol/l after addition of 0.125, 0.250, and 0.500 mmol/l palmitate, respectively. The unbound FFA concentrations were plotted against their cytotoxic effects, leaving out the concentrations

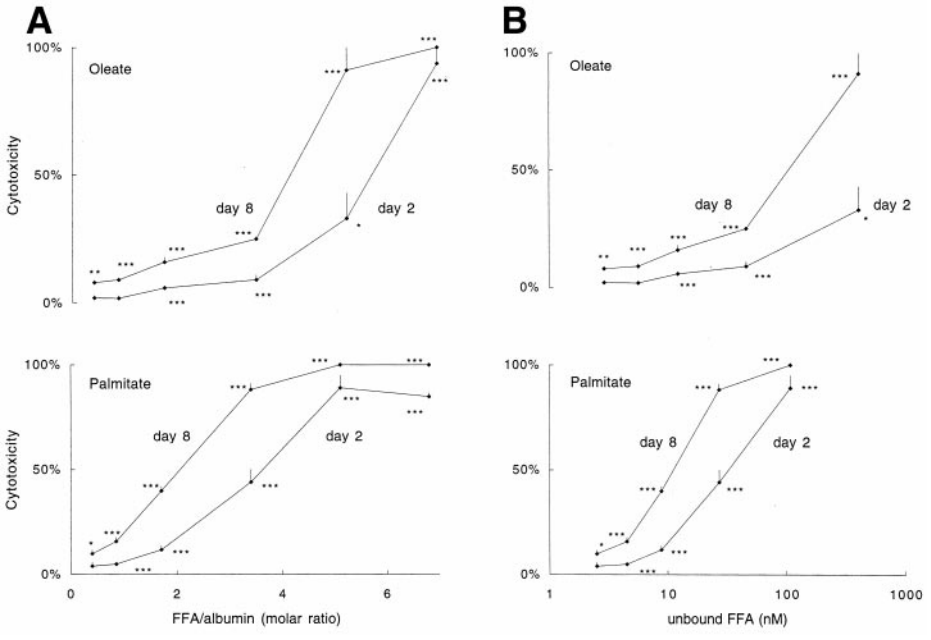

FIG. 1. FFA-induced β-cell toxicity. The FFA-induced β-cell toxicity as a function of (*A*) the molar ratio of FFA over albumin and (*B*) the calculated unbound concentrations of oleate and palmitate, excluding values above the critical micellar concentration, is shown. Data represent means ± SE for cytotoxicity after 2 and 8 days of culture in medium with 10 mmol/l glucose and 0.5, 1, or 2% bovine serum albumin. Statistical significance of differences was calculated by analysis of variance, followed by Student's paired *t* test with the Bonferroni correction: values at day 2 are compared with control without fatty acid added, control at day 8 with control at day 2, and other values at day 8 with control at day 8. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

known to exceed FFA solubility (oleate >6,000 nmol/l, palmitate >4,000 nmol/l) (23). At equimolar unbound concentrations (5–400 nmol/l), palmitic acid was more toxic than oleate (Fig. 1*B*). The β-cell toxicity of oleate affected only a minority of the cells (7–35% at day 8), whereas that of palmitate reached 50% after 48 h and affected nearly all β-cells after 8 days (Fig. 1*B*).

Addition of oleate to the palmitate condition did not further increase cytotoxicity. On the contrary, it significantly reduced palmitate toxicity: β-cell survival in the presence of palmitate plus oleate (both at either 0.25 or 0.125 mmol/l) was significantly better at day 8 than with palmitate alone (Table 1).

Mechanisms involved in FFA-induced β-cell death. To identify the possible mechanisms mediating FFA-induced β-cell death, we measured the cytotoxicity of oleic and palmitic acid in culture conditions that were previously found to be cytoprotective against other toxic agents (12,21,25). These studies were conducted with 0.5 mmol/l oleate or palmitate in the presence of 1% albumin, which represents calculated free concentrations of 47 nmol/l for oleic acid and 27 nmol/l for palmitic acid. Cytotoxicity after 8 days of exposure was 25 ± 2% for oleate and 93 ± 3% for palmitate ($n = 7$). These percentages were not reduced ($P > 0.05$) by the addition of 5 mmol/l nicotinamide (36 ± 7 and 90 ± 5%, $n = 3$), 100 μg/ml superoxide dismutase (22 ± 6 and 79 ± 19%, $n = 6$), or an antioxidant mixture containing vitamin A (1 μmol/l), vitamin C (10 μg/ml), vitamin E acetate (0.5 μmol/l), dithiothreitol (10 μmol/l), and glutathione (1 μmol/l) (18 ± 5 and 97 ± 2%, $n = 4$). The toxicity of both fatty acids was similar at 6 or 20 mmol/l glucose. Addition of etomoxir (1–5 μmol/l), an inhibitor of mitochondrial carnitine palmitoyltransferase I (26), did not increase FFA-induced cell death (data not shown), suggesting that the FFA toxicity does not depend on mitochondrial oxidation of FFA. This notion is supported by the finding that bromopalmitate, a nonmetabolizable analog of palmitate, is β-cytotoxic (Table 1).

It has been previously reported that fatty acids are toxic to islets through the induction of iNOS and the production of NO (11). We measured nitrites in the culture medium and found that both fatty acids markedly interfered with the nitrite assay. After subtraction of the high medium

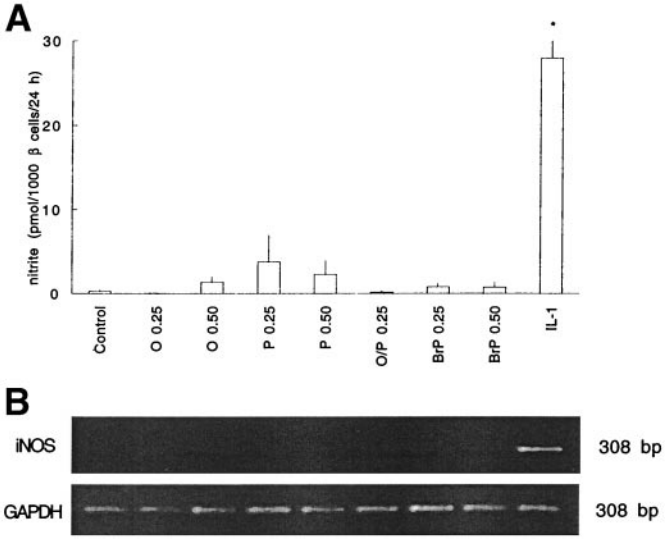

FIG. 2. Failure of FFAs to induce iNOS expression and nitrite production in β-cells. *A*: The 24-h nitrite production by β-cells exposed to FFAs (0.25 or 0.50 mmol/l) or IL-1β (30 U/ml). BrP, bromopalmitate; O, oleate; O/P, oleate plus palmitate (each 0.25 mmol/l); P, palmitate. Data represent means ± SE for five to nine experiments. **P* < 0.001 vs. control (paired *t* test). *B*: RT-PCR analysis of iNOS expression by β-cells after 24 h of exposure to FFAs or IL-1β (30 U/ml). GAPDH cDNA is present in all lanes. One representative experiment of five similar experiments is shown.

TABLE 2
Fatty acid induction of necrosis and apoptosis in β-cells

		Cytotoxicity			
		2 days		8 days	
	Exposure time (mmol/l)	Necrosis	Apoptosis	Necrosis	Apoptosis
Oleate	0.125	<5	<5	<5	7 ± 2
	0.250	<5	<5	10 ± 2*	11 ± 2†
	0.500	9 ± 2*	<5	14 ± 2†	25 ± 4†
Palmitate	0.125	<5	<5	7 ± 2‡	<5
	0.250	9 ± 2*	<5	9 ± 2‡	7 ± 2
	0.500	33 ± 5†	7 ± 2*	33 ± 5†	26 ± 3†
Bromopalmitate	0.125	<5	12 ± 2†	<5	52 ± 4†
	0.250	<5	20 ± 3*	<5	66 ± 3†
	0.500	35 ± 5*	27 ± 4*	52 ± 6†	24 ± 4*

Data are means ± SE for n experiments. Apoptosis and necrosis index for FFAs after 2 (n = 5–8) and 8 (n = 5–13) days of culture of β-cells in Ham's F10 medium with 6 mmol/l glucose and 1% bovine serum albumin. Statistical significance (FFA conditions vs. control) was calculated by analysis of variance, followed by paired t test with the Bonferroni correction. *$P < 0.01$; †$P < 0.001$; ‡$P < 0.05$.

background, no FFA-induced increase in cellular NO production was noticed during the 24-h culture with 0.25 or 0.5 mmol/l oleate, palmitate, or bromopalmitate ($P > 0.05$) (Fig. 2*A*). On the other hand, addition of the cytokine IL-1β (30 U/ml) induced a high NO production ($P < 0.001$ vs. control) (Fig. 2*A*). When expression of iNOS mRNA was analyzed by RT-PCR, no induction was observed after 24 h of exposure to oleate, palmitate, or bromopalmitate (0.25 or 0.5 mmol/l), whereas a clear induction occurred after IL-1β treatment for 24 h (Fig. 2*B*). Similar negative data were obtained after a 48-h FFA exposure or when islet non–β-cells or whole cultured islets were exposed to FFAs ($n = 3$).

Distinction between necrosis and apoptosis in FFA-induced cell death. A fluorescence cytotoxicity assay using a combination of Hoechst 33342 and propidium iodide examined whether FFA-induced β-cell injury resulted in necrosis and/or apoptosis (14). Both oleate and palmitate caused necrosis and apoptosis (Table 2). Their necrotic effect was completed within 2 days (no further increase in percent necrosis after 8 days compared with 2 days, $P > 0.05$); at equimolar concentrations (0.5 mmol/l), palmitate caused more necrotic cells than oleate ($P < 0.01$) (Table 2). Their apoptotic effect became clear only after 8 days of exposure and was comparable at equimolar concentrations (Table 2). Bromopalmitate also induced necrosis and apoptosis, but its necrotic effect was only observed at 0.5 mmol/l; at fourfold lower concentrations, this compound caused apoptosis in 52% of the β-cells (Table 2).

When islet non–β-cells were examined in parallel, virtually no necrosis was noticed after 2 or 8 days of exposure to either oleate and palmitate (Table 3). On the other hand, both fatty acids caused apoptosis in islet non–β-cells, affecting comparable percentages of cells (~30% after 8 days), as in β-cell preparations. Likewise, bromopalmitate primarily induced apoptosis in islet non–β-cells (Table 3).

Ultrastructural changes in FFA-exposed β-cells and non–β-cells. We performed an electron microscopic analysis of both β-cells and non–β-cells that were cultured with FFA. After 48 h of exposure to oleate, numerous β-cells exhibited gray cytoplasmic lipid droplets (Fig. 3*A*). These droplets occurred only sporadically in islet non–β-cells. Exposure of β-cells to palmitate alone resulted in spindle-shaped cytoplasmic lipid accumulations (Fig. 3*B*). When β-cells were cultured with a mixture of oleate and palmitate (0.125 mmol/l each), both spherical and spindle-shaped forms were noticed. Cytoplasmic lipid accumulation was never observed in the bromopalmitate condition, which contained β-cells exhibiting ultrastructural features

TABLE 3
FFA-induced apoptosis and necrosis in islet non–β-cells

		Cytotoxicity			
		2 days		8 days	
	Exposure time (mmol/l)	Necrosis	Apoptosis	Necrosis	Apoptosis
Oleate	0.125	<5	5 ± 1*	<5	24 ± 3*
	0.250	<5	8 ± 2†	<5	31 ± 5*
	0.500	<5	10 ± 3†	<5	27 ± 4*
Palmitate	0.125	<5	<5	<5	6 ± 4
	0.250	<5	<5	<5	17 ± 5
	0.500	5 ± 3	12 ± 2*	5 ± 2	35 ± 7*
Bromopalmitate	0.125	<5	10 ± 5	<5	51 ± 5‡
	0.250	<5	13 ± 4†	<5	56 ± 6‡
	0.500	6 ± 3	22 ± 4*	14 ± 5	39 ± 8*

Data are means ± SE for five experiments. Apoptosis and necrosis indexes after a 2- and 8-day FFA exposure of single non–β-cells in culture medium containing 6 mmol/l glucose, 1% bovine serum albumin, and 0.5 μg/ml growth hormone are shown. Statistical significance (FFA conditions vs. control) was calculated by analysis of variance A, followed by paired t test with the Bonferroni correction. *$P < 0.01$; †$P < 0.05$; ‡$P < 0.001$.

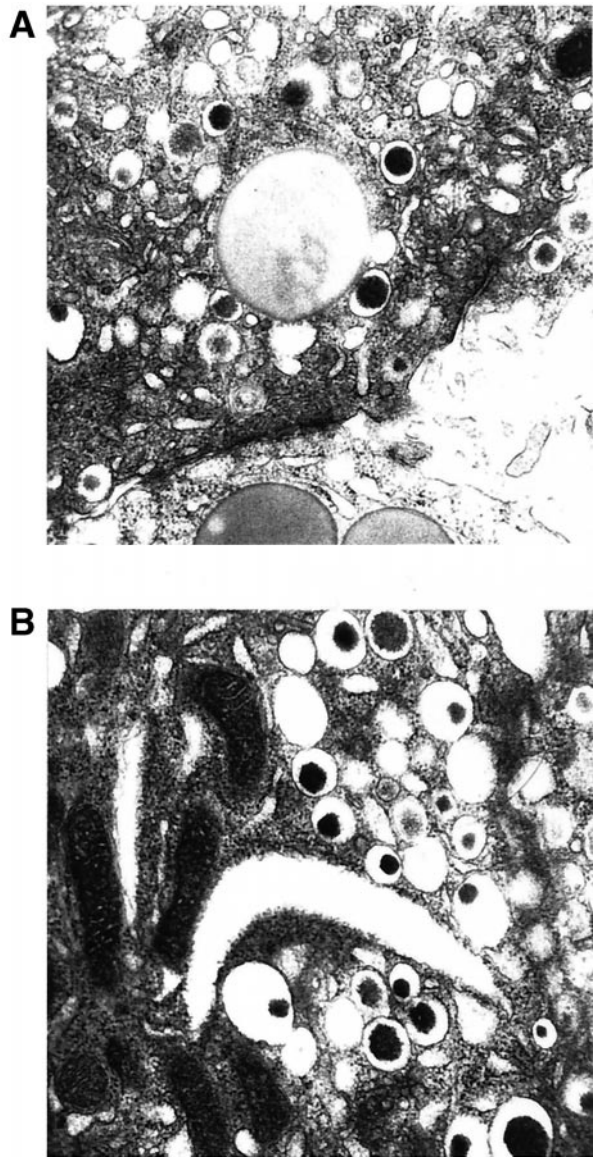

FIG. 3. Triglyceride accumulation in β-cells. Electron micrographs of β-cells after 2 days of culture with (*A*) 0.25 mmol/l oleate–1% bovine serum albumin (the cells contain gray cytoplasmic lipid droplets [magnification 20,000×]) and (*B*) 0.25 mmol/l palmitate–1% bovine serum albumin (the cells present electron-lucent spindle-shaped cytoplasmic structures [magnification 30,000×]) are shown.

of apoptosis, such as condensed and marginated chromatin and fragmentation of the nucleus, condensation of the cytoplasm, and blebbing of the plasma membrane.

Triglyceride content of FFA-exposed β-cells and non–β-cells. Purified β-cells and non–β-cells were cultured in the presence of 0.250 mmol/l FFA for 48 h and assayed for their triglyceride content. Addition of oleate, palmitate, or both resulted in an accumulation of triglycerides in β-cells (Table 4) but not in islet non–β-cells. With bromopalmitate, no triglyceride accumulation was measured. Oleic acid (13 nmol/l unbound) induced a larger increase in β-cell triglyceride content (plus 490 pg/ng DNA) than palmitic acid (9 nmol/l unbound) (plus 220 pg triglyceride/ng DNA) ($P < 0.05$). This 48-h triglyceride accumulation correlated inversely to the FFA-induced β-cell toxicity, as measured after 8 days of culture (Fig. 4).

TABLE 4
Triglyceride content of islet β-cells and non–β-cells after 48 h of culture

Addition	Cellular triglyceride content (pg/ng DNA)	
	β-Cells	Non–β-Cells
Control	162 ± 30	173 ± 50
0.25 mmol/l oleate	652 ± 82*	258 ± 24†
0.25 mmol/l palmitate	389 ± 38*	259 ± 51
0.125 mmol/l oleate 0.125 mmol/l palmitate	686 ± 135‡	261 ± 106
0.25 mmol/l bromopalmitate	251 ± 112	235 ± 115

Data are means ± SE for five β-cell and six (non–β-cell) independent experiments. Statistical significance of differences between FFA condition and control was calculated by analysis of variance, followed by paired t test with the Bonferroni correction: *$P < 0.005$, ‡$P < 0.05$. Statistical significance of differences between islet β-cells and islet endocrine non–β-cells for identical conditions was calculated by analysis of variance, followed by unpaired t test with the Bonferroni correction: †$P < 0.001$.

DISCUSSION

Chronically elevated fatty acid levels can impair the function of pancreatic β-cells (6–9). In Zucker rats, they have been proposed to induce apoptosis of β-cells (11). Their cytotoxic effect was attributed to an increased lipogenesis in the β-cells, which would then result in formation of ceramides and, subsequently, in induction of nitric oxide synthase followed by NO-mediated apoptosis (10,11,27). Addition of FFAs to islets isolated from Wistar rats also induced iNOS expression and NO formation (27), suggesting that they may also cause death of β-cells from Wistar islets. In the present study, we demonstrate that the FFAs oleate and palmitate can kill normal Wistar rat β-cells in vitro, but the underlying mechanisms seem different from those proposed for the Zucker rat islets (27,28). Previous articles from our laboratory have validated the purified

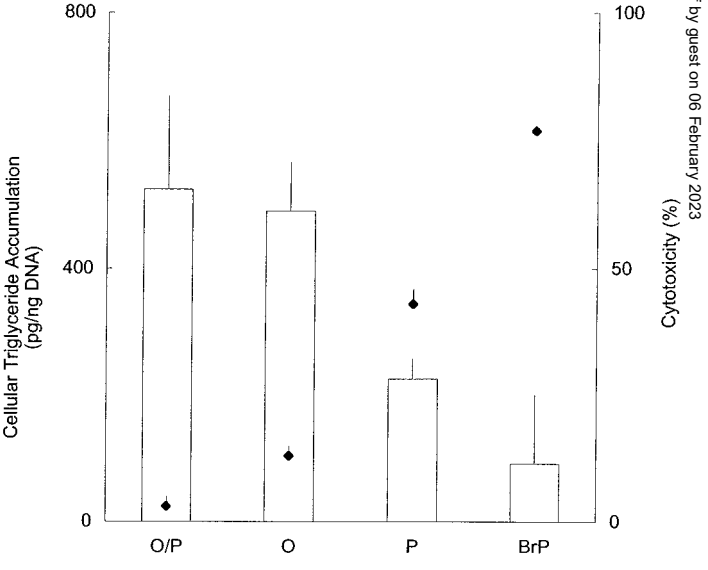

FIG. 4. Relationship between FFA cytotoxicity and triglyceride accumulation. Inverse correlation between the cytotoxicity measured after 8 days and the cellular triglyceride accumulation (□) measured after 2 days culture of β-cells with 0.25 mmol/l FFA is shown. BrP, bromopalmitate; O, oleate; O/P, oleate plus palmitate (each 0.125 mmol/l); P, palmitate. Triglyceride accumulation is calculated as the difference of the values measured in the FFA condition and those in the control.

β-cell preparations as models for studying the regulation of cell survival and function (12–16,21); they have also illustrated the pitfalls in using isolated islets for cytotoxicity experiments (13).

The toxicity of oleate and palmitate for β-cells from normal Wistar rats depended on the level of their unbound fraction rather than on their total concentration. An increase in total FFAs did not lead to a higher toxicity if the ratio of total FFAs over albumin remained constant and, hence, unbound FFA levels. Ratios >5 should not be included because they represent conditions in which unbound FFAs precipitate (24); this physical process may influence the interpretation of data at a high concentration ratio of FFAs over albumin (9,10,27,29–33). We have restricted our further experiments on conditions with a ratio of ≥3.5. This lower range covered unbound FFA levels that were also measured in humans (18) and that were cytotoxic to a proportion of isolated rat β-cells. Similar concentrations have been previously found to kill other rodent cell types in vitro (34,35). At equimolar concentrations, palmitate was more toxic than oleate; furthermore, palmitate became less toxic when oleate was simultaneously present—a phenomenon that was also observed in isolated cardiomyocytes (35). Thus, the fact that FFAs can kill different cell types may be less relevant than the potential to protect cells against FFA-induced death.

The β-cell toxicity of oleate and palmitate resulted in both necrosis and apoptosis. As in other β-cell toxic conditions (12–14), necrotic cells appeared rapidly (within 2 days), whereas apoptotic cells were only noticed after longer exposure periods. FFA-induced β-cell death was not prevented by inhibitors of fatty acid oxidation, such as etomoxir. The nonmetabolized FFA bromopalmitate was clearly cytotoxic; its unbound concentration could not be calculated because its association constants for albumin binding have not been determined but are considered to be comparable to those of palmitate (36,37). In its lower toxicity range, the bromopalmitate analog caused only apoptosis of the β-cells. In islet non–β-cells, both metabolized and nonmetabolized FFA induced only apoptosis. The action mechanism of the nonmetabolized palmitate analog may thus be representative for the apoptotic effect of FFAs in islet cells. After cellular uptake, this compound is converted to its acyl-CoA form, which will block entry of fatty acyl-CoA into the mitochondria (38,39) and compete for binding sites on intracellular proteins (40). The resulting increase in cytoplasmic fatty acyl-CoA moieties may stimulate ceramide synthesis and result in activation of the enzymatic cascade that leads to apoptosis, as previously described in a hematopoietic cell line (41). In this view, inhibition of mitochondrial FFA oxidation by etomoxir could be expected to increase the cytotoxicity of oleate or palmitate; however, etomoxir also decreases cellular FFA uptake (42), which may explain why such an increase was not observed in the present experiments.

The FFA-induced toxicity was not counteracted by increasing the glucose concentration (and hence mitochondrial activities) or by supplements of anti-oxidative compounds and free radical scavengers. This suggests that oxidative stress is not the main mechanism of FFA-induced cell death. The absence of any FFA-induced NO production or iNOS expression in the currently studied islet cell preparations excludes NO as the mediator for the observed cytotoxicity. This contrasts with the previous report on FFA-induced NO production in islets from both normal Wistar and Zucker rats (27) but is consistent with the findings of Carlsson et al. (43).

The apoptotic effect of bromopalmitate was not preceded by a cellular triglyceride accumulation, as judged from electron micrographs and from assays in cellular extracts. This is at variance with the concept that FFA-induced apoptosis of β-cells is initiated by lipogenesis and triglyceride accumulation, as suggested in the Zucker rat (10,11). On the contrary, FFA conditions that increased triglyceride content in our normal rat β-cell preparations did result in lower percentages of dead cells. An inverse relationship was found between the cellular triglyceride content after 2 days of FFA exposure and the percentage of dead cells after 8 days. These triglycerides accumulated in the cytoplasm, forming spherical droplets when produced from oleate and crescent-like particles when composed of saturated fatty acids, such as palmitate. No triglyceride formation was noticed in islet non–β-cells exposed to either metabolized or nonmetabolized fatty acids. This correlated with a higher susceptibility of non–β-cells compared with β-cells for the cytotoxic effect of FFA levels that induced triglyceride formation in β-cells. It has been previously reported that FFA-induced accumulation of triglycerides may influence β-cell functions (44), but this effect seemed reversible and thus not cytodestructive. In view of our observations, formation of triglycerides in β-cells might be seen as a cytoprotective mechanism that captures toxic-free acyl moieties. It is so far unknown whether human β-cells form cytoplasmic triglycerides, whether this protects them against FFA-induced damage, and whether they need this mechanism in vivo. As for all in vitro studies, experiments on isolated islets and β-cells are not necessarily relevant for processes that occur in vivo, such as in the Zucker diabetic rat, where islet cells are exposed for much longer periods to elevated FFA levels and lipogenetic pathways.

In conclusion, FFAs can be cytotoxic for normal islet β-cells as well as for normal islet non–β-cells, leading to cell death by both necrosis and apoptosis in β-cells and mostly by apoptosis in non–β-cells. Both cell types were equally sensitive to apoptosis induction by the nonmetabolized bromopalmitate analog. The ability of β-cells to oxidize FFAs (45) and to incorporate them into cytoplasmic triglycerides (present data) might serve as protection against their apoptotic effect. This protection might become inadequate when excessive levels of unbound FFAs destabilize membranes and cause rapid necrosis.

ACKNOWLEDGMENTS

This study was supported by grants from the European Community (BMH-CT95-1561), the Juvenile Diabetes Foundation International (JDF 995004), the Belgian Fonds voor Wetenschappelijk Onderzoek (F.W.O.G.0039.96 and G.0376.97), and the services of the Prime Minister (Interuniversity Attraction Pole P4/21). M.C. is Aspirant of the Fund for Scientific Research-Flanders (F.W.O.).

We thank the staff of the Diabetes Research Center for preparing rat islet cells and Geert Stangé, Eveline Verheu-

gen, and Ruth Leeman for excellent technical assistance. Etomoxir was a gift from Dr. Valdemar E. Grill, Trondheim University, Norway.

REFERENCES

1. Paolisso G, Tataranni PA, Foley JE, Bogardus C, Howard BV, Ravussin E: A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. *Diabetologia* 38:1213–1217, 1995
2. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* i:785–789, 1963
3. Lillioja S, Bogardus C, Mott D, Kennedy LA, Knowler WC, Howard BV: Relationship between insulin-mediated glucose disposal and lipid metabolism in man. *J Clin Invest* 75:1106–1115, 1985
4. Ferrannini E, Barrett EJ, Bevilacqua S, De Fronzo RA: Effect of fatty acids on glucose production and utilization in man. *J Clin Invest* 72:1737–1747, 1983
5. Waldhausl WK, Roden M: The effects of free fatty acids on glucose transport and phosphorylation in human skeletal muscle. *Curr Opin Endocrinol Diabetes* 7:211–216, 2000
6. Bollheimer LC, 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
7. Zhou Y, 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* 93:870–876, 1994
8. Zhou Y, Grill VE: Long term exposure to fatty acids and ketones inhibits β-cell functions in human pancreatic islets of Langerhans. *J Clin Endocrinol Metab* 80:1584–1590, 1995
9. Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH: β-Cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-β-cell relationships. *Proc Natl Acad Sci U S A* 91:10878–10882, 1994
10. Lee Y, Hirose H, Zhou Y, Esser V, McGarry JD, Unger RH: Increased lipogenic capacity of the islets of obese rats. *Diabetes* 46:408–413, 1997
11. Shimabukuro M, Zhou Y, 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
12. Hoorens A, Pipeleers DG: Nicotinamide protects human beta cells against chemically-induced necrosis, but not against cytokine-induced apoptosis. *Diabetologia* 42:55–59, 1999
13. Hoorens A, Stangé G, Pavlovic D, Pipeleers D: Distinction between interleukin-1-induced necrosis and apoptosis of islet cells. *Diabetes* 50: 551–557, 2001
14. Hoorens A, Van de Casteele M, Klöppel G, Pipeleers DG: Glucose promotes survival of rat pancreatic β cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568–1574, 1996
15. Pipeleers DG, in't Veld PA, Van De Winkel M, Maes E, Schuit FC, Gepts W: A new in vitro model for the study of pancreatic α and β cells. *Endocrinology* 117:806–816, 1985
16. Ling Z, Hannaert JC, Pipeleers DG: Effect of nutrients, hormones and serum on survival of rat islet beta cells in culture. *Diabetologia* 37:15–21, 1994
17. Spector AA, Fletcher JE, Ashbrook JD: Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. *Biochemistry* 10:3229–3232, 1971
18. Richieri GV, Anel A, Kleinfeld AM: Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry* 32:7574–7580, 1993
19. Green LC, Wagner DA, Goglowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal Biochem* 126:131–138, 1982
20. Flodström M, Chen MC, Smismans A, Schuit F, Pipeleers DG, Eizirik DL: Interleukin 1β increases arginine accumulation and activates the citrulline-NO cycle in rat pancreatic β cells. *Cytokine* 11:400–407, 1999
21. Pipeleers DG, Van De Winkel M: Pancreatic β cells possess defense mechanisms against cell-specific toxicity. *Proc Natl Acad Sci U S A* 83:5267–5271, 1986
22. Wallenstein S, Zucker CL, Fleiss JL: Some statistical methods useful in circulation research. *Circ Res* 47:1–9, 1980
23. Richieri GV, Ogata RT, Kleinfeld AM: A fluorescently labeled intestinal fatty acid binding protein. *J Biol Chem* 267:23495–23501, 1992
24. Cistola DP, Hamilton JA, Jackson D, Small DM: Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule. *Biochemistry* 27:1881–1888, 1988
25. Stocker R, Bowry VW: Tocopherol-mediated peroxidation of lipoprotein lipids and its inhibition by co-antioxidants. In *Handbook of Antioxidants.* Cadenas E, Packer L, Eds. New York, Marcel Dekker, 1996, p. 27–41
26. Chen S, Ogawa A, Ohneda M, Unger RH, Foster DW, McGarry JD: More direct evidence for a malonyl-CoA-carnitine palmitoyltransferase I interaction as a key event in pancreatic β-cell signaling. *Diabetes* 43:878–883, 1994
27. Shimabukuro M, Ohneda M, Lee Y, Unger RH: Role of nitric oxide in obesity-induced β cell disease. *J Clin Invest* 100:290–295, 1997
28. Unger RH, Zhou Y, Orci L: Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proc Natl Acad Sci U S A* 96:2327–2332, 1999
29. Milburn JL, Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, Unger RH: Pancreatic β-cells in obesity. *J Biol Chem* 270:1295–1299, 1995
30. Hirose H, Lee YH, Inman LR, Nagasawa Y, Johnson JH, Unger RH: Defective fatty acid-mediated β-cell compensation in Zucker diabetic fatty rats. *J Biol Chem* 271:5633–5637, 1996
31. Prentki M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Corkey BE: Malonyl-CoA and long-chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J Biol Chem* 267:5802–5810, 1992
32. Liu YQ, Tornheim K, Leahy JL: Fatty acid-induced β cell hypersensitivity to glucose. *J Clin Invest* 101:1870–1875, 1998
33. Warnotte C, Gilon P, Nenquin M, Henquin JC: Mechanisms of the stimulation of insulin release by saturated fatty acids. *Diabetes* 43:703–711, 1994
34. Gordon GB: Saturated free fatty acid toxicity. *Exp Mol Pathol* 27:262–276, 1977
35. de Vries JE, Vork MM, Roemen THM, de Jong YF, Cleutjens JPM, 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
36. Grimaldi PA, Knobel SM, Whitesell RR, Abumrad NA: Induction of aP2 gene expression by nonmetabolized long-chain fatty acids. *Proc Natl Acad Sci U S A* 89:10930–10934, 1992
37. Oakes ND, Kjellstedt A, Forsberg GB, Clementz T, Camejo G, Furler SM, Kraegen EW, Olwegard-Halvarsson M, Jenkins AB, Ljung B: Development and initial evaluation of a novel method for assessing tissue-specific plasma free fatty acid utilization in vivo using (R)-2-bromopalmitate tracer. *J Lipid Res* 40:1155–1169, 1999
38. Chase JFA, Tubbs PK: Specific inhibition of mitochondrial fatty acid oxidation by 2-bromopalmitate and its coenzyme A and carnitine esters. *Biochem J* 129:55–65, 1972
39. Declercq PE, Falck JR, Kuwajima M, Tyminski H, Foster DW, McGarry JD: Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. *J Biol Chem* 262:9812–9821, 1987
40. Luxon BA: Inhibition of binding to fatty acid binding protein reduces the intracellular transport of fatty acids. *Am J Physiol* 271:G113–G120, 1996
41. 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
42. Luiken JJFP, van Nieuwenhoven FA, America G, van der Vusse GJ, Glatz JFC: Uptake and metabolism of palmitate by isolated cardiac myocytes from adult rats: involvement of sarcolemmal proteins. *J Lipid Res* 38:745–758, 1997
43. Carlsson C, Borg LAH, Welsh N: Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140:3422–3428, 1999
44. Zhou Y, Ling Z, Grill VE: Inhibitory effects of fatty acids on glucose-regulated β-cell function: association with increased islet triglyceride stores and altered effect of fatty acid oxidation on glucose metabolism. *Metabolism* 45:981–986, 1996
45. Berne C: The metabolism of lipids in mouse pancreatic islets: the oxidation of fatty acids and ketone bodies. *Biochem J* 152:661–666, 1975