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Free Fatty Acids and Cytokines Induce Pancreatic β-Cell Apoptosis by Different Mechanisms: Role of Nuclear Factor-κB and Endoplasmic Reticulum Stress

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Apoptosis is probably the main form of β-cell death in both type 1 diabetes mellitus (T1DM) and T2DM. In T1DM, cytokines contribute to β-cell destruction through nuclear factor-κB (NF-κB) activation. Previous studies suggested that in T2DM high glucose and free fatty acids (FFAs) are β-cell toxic also via NF-κB activation. The aims of this study were to clarify whether common mechanisms are involved in FFA- and cytokine-induced β-cell apoptosis and determine whether TNFα, an adipocyte-derived cytokine, potentiates FFA toxicity through enhanced NF-κB activation. Apoptosis was induced in insulinoma (INS)-1E cells, rat islets, and fluorescence-activated cell sorting-purified β-cells by oleate, palmitate, and/or cytokines (IL-1β, interferon-γ, TNFα). Palmitate and IL-1β induced a similar percentage of apoptosis in INS-1E cells, whereas oleate was less toxic. TNFα did not potentiate FFA toxicity in primary β-cells. The NF-κB-dependent genes inducible nitric oxide synthase and monocyte chemoattractant protein-1 were induced by IL-1β but not by FFAs. Cytokines activated NF-κB in INS-1E and β-cells, but FFAs did not. Moreover, FFAs did not enhance NF-κB activation by TNFα. Palmitate and oleate induced C/EBP homologous protein, activating transcription factor-4, and immunoglobulin heavy chain binding protein mRNAs, X-box binding protein-1 alternative splicing, and activation of the activating transcription factor-6 promoter in INS-1E cells, suggesting that FFAs trigger an endoplasmic reticulum (ER) stress response. We conclude that apoptosis is the main mode of FFA- and cytokine-induced β-cell death but the mechanisms involved are different. Whereas cytokines induce NF-κB activation and ER stress (secondary to nitric oxide formation), FFAs activate an ER stress response via an NF-κB- and nitric oxide-independent mechanism. Our results argue against a unifying hypothesis for the mechanisms of β-cell death in T1DM and T2DM. (*Endocrinology* 145: 5087–5096, 2004)

HYPERCALORIC DIETS CONTAINING large amounts of fat, also called the Western diet, contribute to a major extent to the increasing prevalence of obesity and type 2 diabetes mellitus (T2DM) (1). T2DM is characterized by peripheral insulin resistance, pancreatic β-cell dysfunction, and decreased β-cell mass (2–5) associated with increased rates of β-cell apoptosis (5). Elevated levels of circulating free fatty acids (FFAs) contribute to the pathogenesis of T2DM (6–9). High concentrations of FFAs lead to both impairment of insulin action (2, 10) and β-cell dysfunction (9, 11–13). Moreover, FFAs have been shown to cause β-cell death, mainly by apoptosis (14, 15). Of note, increased adiposity is associated not only with increased FFA release but also with adipocyte secretion of a variety of cytokines and cytokine-like adipokines, such as TNFα, IL-6, leptin, resistin, and adiponectin. TNFα has direct cytotoxic effects on pancreatic β-cells, especially in combination with other cytokines (16, 17).

In type 1 diabetes mellitus (T1DM), the autoimmune assault on pancreatic β-cells leads to progressive β-cell loss. Macrophages and T cells, attracted to the islets, secrete soluble mediators such as oxygen free radicals, nitric oxide (NO), and the cytokines IL-1β, interferon (IFN)-γ and TNFα. Increasing evidence suggests that these mediators induce apoptosis, the main mode of β-cell death in the development of T1DM (18).

β-Cell apoptosis may thus be a common feature of T1DM and T2DM, but the mechanisms leading to initiation of the cell death program remain to be clarified. The transcription factor nuclear factor-κB (NF-κB) plays a crucial role in cytokine- and double-stranded RNA + cytokine-induced apoptosis (19–21). Cytokine-induced activation of NF-κB leads to: 1) loss of differentiated β-cell functions by down-regulation of Isl-1 and pancreatic duodenal homeobox-1 (Pdx-1) (22, 23), 2) up-regulation of inducible nitric oxide synthase (iNOS) (24) and excessive NO production, 3) up-regulation of chemokines such as monocyte chemoattractant protein-1 (MCP-1) (25, 26), and 4) down-regulation of the Ca^{2+} pump sarcoendoplasmic reticulum Ca^{2+} ATPase type 2b (SERCA-2b) (22, 23). Decreased SERCA-2b expression leads to endoplasmic reticulum (ER) calcium depletion and ER stress (23, 27, 28) (Cardozo, A. K., F. Ortis, Y.-M. Feng, J. Rasschaert, F.

Abbreviations: ATF, Activating transcription factor; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; FFA, free fatty acid; GADD, growth arrest and DNA damage; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO 342, Hoechst 33342; IFN, interferon; iNOS, inducible nitric oxide synthase; INS, insulinoma; MCP, monocyte chemoattractant protein; NF-κB, nuclear factor-κB; NO, nitric oxide; Pdx-1, pancreatic duodenal homeobox-1; PI, propidium iodide; SERCA-2b, sarcoendoplasmic reticulum Ca^{2+} ATPase type 2b; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; UCP, uncoupling protein-2; XBP-1, X-box binding protein-1.

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Van Eylen, J. Storling, T. Mandrup-Poulsen, A. Herchuelz, and D. L. Eizirik, submitted for publication). The cellular response to ER stress is mediated by several ER transmembrane proteins including the kinase, inositol requiring and ER-to-nucleus signaling kinase 1, PKR-like ER kinase (PERK), activating transcription factor (ATF)-6, and X-box binding protein-1 (XBP-1) (29). The activation of the transcription factor XBP-1 through its alternative splicing by IRE1 is an important indicator of ER stress (30), and XBP-1 alternative splicing is detected in both thapsigargin- and cytokine-treated β-cells (Cardozo, A. K., F. Ortis, Y.-M. Feng, J. Rasschaert, F. Van Eylen, J. Storling, T. Mandrup-Poulsen, A. Herchuelz, and D. L. Eizirik, submitted for publication). Thapsigargin is a well-known inducer of ER stress, previously shown to cause apoptosis of insulin-producing cells (31, 32). β-Cell exposure to IL-1β + IFNγ or thapsigargin also triggers expression of the mRNA for growth arrest and DNA damage (GADD) 153, also known as C/EBP homologous protein (CHOP) (GADD153/CHOP) (22, 23) (Cardozo, A. K., F. Ortis, Y.-M. Feng, J. Rasschaert, F. Van Eylen, J. Storling, T. Mandrup-Poulsen, A. Herchuelz, and D. L. Eizirik, submitted for publication), a transcription factor involved in ER stress-induced apoptosis (33).

The mechanisms involved in FFA-induced β-cell apoptosis in T2DM remain to be clarified. We have previously shown that FFA cytotoxicity is inversely related with cytoplasmic triglyceride accumulation (15). This suggests that cytoplasmic accumulation of fatty acyl-CoA is directly β-cell toxic, whereas their esterification probably functions as a protective mechanism (15). Increased β-cell FFA levels might lead to *de novo* ceramide formation (34, 35) and mitochondrial cytochrome C release (14, 36). Other proposed mechanisms for FFA-induced β-cell death are the activation of protein kinase C (37), inhibition of protein kinase B activity (38), and activation of calpain-10 (39). Moreover, microarray studies of global gene expression in FFA-treated β-cells (40–42) indicate similarities between several of the genes induced by FFAs and those observed downstream of cytokine-induced NF-κB, including ornithine decarboxylase, b-2 microglobulin, DNA-binding protein A, and MCP-1 (22, 26). In line with these observations, a recent study reported that palmitate induces a pronounced (>10-fold) NF-κB activation in insulinoma (INS)-1 cells, comparable with the activation observed with TNFα (43). Based on these and additional observations obtained in islets cultured at high glucose concentrations, it has been proposed that prolonged exposure to excessive concentrations of nutrients results in a proinflammatory β-cell response, contributing to β-cell damage and death in T2DM (35, 44–46). A unifying hypothesis has also been suggested for the mechanisms of nutrient- and cytokine-induced β-cell death in T1DM and T2DM, in which activation of NF-κB is a common and crucial step for both proapoptotic stimuli (45, 46).

Against this background, the aim of this study was to clarify whether common mechanisms are indeed involved in FFA- and cytokine-induced β-cell apoptosis. The questions we asked were: 1) is FFA-induced apoptosis associated with the activation of the transcription factor NF-κB and the induction of its downstream genes iNOS and MCP-1, as is the case for cytokines; 2) does TNFα potentiate the toxic effects of FFAs via a potentiation of NF-κB activation; and 3) do these FFA-mediated effects result in the induction of an ER stress response in β-cells? To address these questions, we exposed the insulinoma cell line INS-1E, rat islets, and fluorescence-activated cell sorting (FACS)-purified β-cells to oleate or palmitate, alone or in combination with TNFα. For comparative purposes, these cells were also exposed to IL-1β or, in some experiments, IL-1β + IFNγ or thapsigargin.

Materials and Methods

Culture of primary rat islets, FACS-purified β-cells, and insulin-producing INS-1E cells

Male Wistar rats were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Rat islets were isolated by collagenase digestion followed by hand picking under a stereomicroscope. Whole islets were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% BSA (fraction V, Sigma, Bornem, Belgium) treated with charcoal, 100 U/ml penicillin, and 100 μg/ml streptomycin. For β-cell isolation, islets were dispersed and β-cells purified by autofluorescence-activated cell sorting (FACS; FACStar, Becton Dickinson and Co., Sunnyvale, CA) (47). The preparations used in the present experiments contained 95 ± 1% β-cells (n = 5). Purified β-cells were precultured for 16 h in Ham's F-10 medium supplemented with 10 mM glucose, 2 mM glutamine, 5% fetal bovine serum, and 1% BSA (48, 49). During FFA, cytokine, or thapsigargin exposure, cells were maintained under the same culture conditions but in the absence of serum. The insulin-producing INS-1E cells (a kind gift from Prof. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) were cultured in RPMI 1640 (with Glutamax-1) supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 1% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (50) and always used between passages 65 and 70. Islets, FACS-purified β-cells and INS-1E cells were exposed to the following cytokines: IL-1β (30 or 50 U/ml; kindly provided by Dr. C. W. Reynolds, National Cancer Institute, Bethesda, MD); recombinant rat IFNγ (100 U/ml; R&D Systems, Oxon, UK); rat TNFα (1000 U/ml; Innogenetics, Gent, Belgium); or the FFAs oleate and palmitate. Oleate and palmitate (sodium salt, Sigma) were solubilized in 90% ethanol, heated to 60 C, and used in a 1:100 dilution in culture medium as previously described (15, 51). The control condition contained a similar dilution of ethanol, which by itself does not affect any of the variables evaluated in the present study (data not shown). The FFAs and cytokine concentrations were selected based on our previous time course and dose-response studies and the calculation of unbound FFA concentrations derived from the total FFA and albumin concentrations (15, 18, 51, 52).

Determination of cell viability

The percentage of viable, apoptotic, and necrotic cells was determined after 24–72 h exposure to FFAs or cytokines. For this purpose, islets, FACS-purified β-cells, and INS-1E cells were incubated for 15 min with the DNA binding dyes propidium iodide (PI, 10 μg/ml, Sigma) and Hoechst 33342 (HO 342, 20 μg/ml, Sigma) (15, 52, 53). The cells were examined by inverted microscopy with UV excitation at 340–380 nm. Viable cells were identified by their intact nuclei with blue fluorescence (HO 342), necrotic cells by their intact nuclei with yellow-red fluorescence (HO 342 + PI), and apoptotic cells by their fragmented nuclei, exhibiting either a blue (HO 342; early apoptosis) or yellow-red fluorescence (HO 342 + PI; late apoptosis) (53). This fluorescence assay is quantitative for single β-cells and has been validated by systematic comparison with electron microscopy (53). This method has previously been used to evaluate apoptosis/necrosis in rat (15, 21, 53, 54) (and present data), mouse (55), and human (56) β-cells and INS-1 cells (52).

By the use of FACS-purified β-cells (95% purity) in some of our present experiments, we avoided the problem of apoptosis/necrosis detection in non-β-cells, inherent to studies performed in whole islets. In each experimental condition a minimum of 500 cells was counted by at least two observers, one of them blinded for the sample identity. Of note, exact quantification of cell death in whole islets is difficult due to

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the superposition between cells. The data obtained in whole islets thus provide an approximate percentage of dead cells (54, 55). There was a more than 90% agreement between observers in the different assessments of viability, confirming the reproducibility of the method. Findings obtained using this apoptosis assay were corroborated by a second method, namely the cell death detection ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany). This method detects enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells, indicating apoptosis-associated DNA degradation. The method was validated for detection of apoptosis in our experimental conditions by systematic comparison with the HO 342 + PI method (described above) in purified β-cells exposed either to a proapoptotic stimulus (the ER stress inducer thapsigargin; 1 μM) or a pronecrotic stimulus (the mitochondrial poison antimycin A, 0.5 μg/ml) (data not shown). We found that the cell death detection ELISA^{PLUS} provides reliable and reproducible data in single cells and cell monolayers (β-cells or INS-1 cells) but not in whole islets, probably due to the islet dispersion procedure.

Analysis of mRNA expression by real-time PCR

Poly(A)$^+$ RNA was isolated from islets and INS-1E cells, using oligo(dT)25-coated polystyrene Dynabeads (Dynal, Oslo, Norway) and reverse transcribed as previously described (25). The real-time PCR amplification reaction was done in 20 μl containing 3 mM $MgCl_2$, 0.5 μM forward and reverse primers, 2 μl FastStart SYBR Green mix (Roche Diagnostics), and 2 μl cDNA. Standards for each gene were prepared using appropriate primers in a conventional PCR and purified for subsequent analyses. PCR products were quantified fluorometrically using SYBR Green, and their concentration was calculated as copies per microliter by comparison with a standard curve (57). The expression level of the gene of interest was corrected for that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (57). The mRNAs analyzed were: iNOS, MCP-1, Pdx-1, GADD153/CHOP, BiP, ATF-4, and uncoupling protein-2 (UCP-2). The specific primers used and their respective PCR fragment lengths were as follows: GAPDH, forward, 5′-AGTTCAACGGCACAGTCAAG-3′, reverse, 5′-TACTCAGCACCAGCATCACC-3′ (118 bp); iNOS, forward, 5′-GGGAGCCAGAGCAGTACAAG-3′, reverse, 5′-GGCTGGACTTCTCACTCTGC-3′ (138 bp); MCP-1, forward, 5′-CTTCTGGGCCTGTTGTTCA-3′, reverse, 5′-CCAGCCGACTCATTGGGATCA-3′ (127 bp); Pdx-1, forward, 5′-GGTATAGCCAGCGAGATGCT-3′, reverse, 5′-TCAGTTGGGAGCCTGATTCT-3′ (153 bp); GADD153, forward, 5′-CCAGCAGAGGTCACAAGCAC-3′, reverse, 5′-CGCACTGACCACTCTGTTTC-3′ (125 bp); UCP-2, forward, 5′-CGAAATGCCATTGTCAACTG-3′, reverse, 5′-CAAGGGAGGTCGTCTGTCAT-3′ (100 bp); ATF-4, forward, 5′-GTTGGTCAGTGCCTCAGACA-3′, reverse, 5′-CATTCGAAACAGAGCATCGA-3′ (109 bp); and BiP, forward, 5′-CCACCAGGATGCAGACATTG-3′, reverse, 5′-AGGGCCTCCACTTCCATAGA-3′ (100 bp).

Analysis of XBP-1 activation and ATF-6 promoter activity

Alternative splicing of XBP-1 mRNA during ER stress leads to the loss of a Pst1 restriction site (58, 59), and the activation of this transcription factor can be evaluated by restriction analysis after PCR amplification. The XBP-1 cDNA fragment (601 bp) encompassing the region of restriction site was amplified by conventional PCR using previously described primers (58). The purified PCR product was incubated with the Pst1 restriction enzyme for 5 h at 37 C, followed by separation of the restriction digests on a 2% agarose gel with ethidium bromide. The gels were photographed under UV transillumination using a Digital Science DC 290 camera (Kodak, Rochester, NY) and quantified by densitometry. The amount of 601-bp material (indicative of XBP-1 activation and thus of ER stress) was expressed as a percent of the total amount of amplified material, considering the sum of the two bands in each lane as 100%.

For the determination of ATF-6 promoter activation, another indicator of ER stress, a reporter plasmid containing the luciferase gene under the control of five ATF-6 binding sites (60) was kindly provided by Prof. Prywes (Columbia University, New York, NY). Of note, it has been reported that this construct might also be activated by XBP-1 under some experimental conditions (61). INS-1E cells were cotransfected with luciferase test plasmids and pRL-CMV (Promega, Madison, WI) by lipofection with Lipofectamine 2000 (Invitrogen, Baesley, Scotland) (24), and luciferase activities were assayed with the dual-luciferase reporter assay system (Promega) as previously described (24, 26). Test values were corrected for the luciferase value of the internal control plasmid pRL-CMV.

EMSA and immunofluorescence of NF-κB activation

Nuclear extracts were obtained from INS-1E cells as described (62–64). Extracted nuclear protein (4 μg) was preincubated for 10 min at 4 C with 1 μg of poly(dIdC) in 20 μl medium containing 10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol before addition of the radiolabeled probe (40,000 cpm) for the NF-κB consensus sequence 5′-AGCTTCAGAGGGGACTTTCCGAGA (64). Specificity of protein-oligo binding was tested by addition of 50 molar excess of cold oligonucleotide. After 20 min incubation at 4 C, the samples were electrophoresed on 5% polyacrylamide gels in 25 mM Tris, 25 mM boric acid, and 0.5 mM EDTA.

NF-κB nuclear translocation was also determined, as previously described (20, 65) by immunofluorescent staining using an anti-p65 antibody (sc-372x; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5000 dilution. The percentage of cells with nuclear staining (indicating NF-κB activation) was determined in at least 200 cells by two observers, one of them blinded for sample identity.

Statistical analysis

Data are presented as means ± SE. Comparisons *vs.* the control conditions were performed by the Student's paired *t* test or ANOVA followed by paired *t* test with the Bonferroni correction for multiple comparisons. $P \leq 0.05$ was considered statistically significant.

Results and Discussion

We have previously shown that FFAs induce pancreatic β-cell death (15). It was confirmed in the present study that palmitate and, to a lesser extent oleate, cause apoptosis in the well-differentiated insulin-producing INS-1E cells. Thus, exposure of INS-1E cells to oleate or palmitate leads to a time-dependent increase in cell death by apoptosis (Fig. 1). In the presence of 0.25 mM palmitate, apoptosis was detected from 24 h on. Palmitate induced more apoptosis than a similar concentration (0.5 mM) of oleate, and it was as cytotoxic to INS-1E cells as IL-1β (30 U/ml). The percentage of necrotic cells did not change significantly after FFA treatment (data

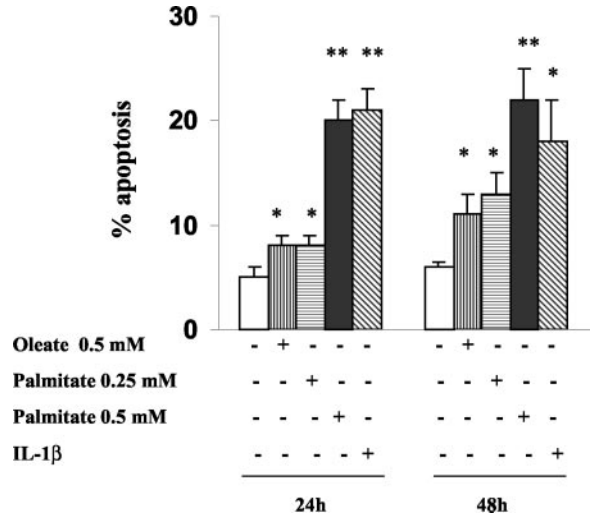

FIG. 1. FFAs and IL-1β induce apoptosis in INS-1E cells. INS-1E cells were cultured for 24 and 48 h in the presence or absence of oleate, palmitate, or IL-1β (30 U/ml) in medium containing 1% BSA. The results are the means ± SE of five independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

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not shown). Exposure of rat islets to palmitate decreased islet cell viability only after 72 h (Fig. 2), whereas increased cell death in the presence of IL-1β was already detected at 24 h (Fig. 2).

We next evaluated whether TNFα potentiates the deleterious effect of FFAs on INS-1E and FACS-purified β-cell viability. Confirming the findings shown in Figure 1, treatment of INS-1E cells for 24–48 h with oleate or palmitate induced apoptosis. TNFα alone induced a minor increase in apoptosis rates, which did not reach statistical significance (Fig. 3A). The combination of palmitate with TNFα induced a mild increase in cell death after 24 h, compared with palmitate alone, whereas there was a TNFα-induced increase in oleate-triggered apoptosis after 48 h only (Fig. 3A). Oleate and palmitate induced apoptosis in FACS-purified β-cells, but this effect was not augmented by TNFα (Fig. 3B). The observation of FFA-induced apoptosis in pure β-cell preparations (Fig. 3B) (15) and clonal insulin-producing cells (Fig. 3A) strongly suggests that the deleterious effects of FFAs in whole islets (Fig. 2) occur at least in part at the β-cell level. FFA-induced β-cell apoptosis was confirmed by the cell death detection ELISA^{PLUS}. After treatment of purified β-cells with oleate or palmitate (same experimental conditions as in Fig. 3B), there was a respective 2.4 ± 0.4- and 4.1 ± 0.6-fold increase in apoptosis, compared with control (n = 4; $P < 0.05$), and similar results were obtained in INS-1E cells. In primary β-cells, there was again no potentiating effect by TNFα (2.7 ± 0.7- and 3.1 ± 0.8-fold increase in apoptosis for oleate + TNFα and palmitate + TNFα, NS *vs.* FFAs alone), whereas in INS-1E cells, TNFα tended to increase oleate-mediated cell death by 50% at 48 h (data not shown).

The presently described lack of a potentiating effect of TNFα on FFA-induced primary β-cell death, and the presence of only a minor additive effect on INS-1E cells at some time points, contrasts with previous observations showing that oleate increases by more than 2-fold IL-1β toxicity in INS-1E cells (66). Both IL-1β and TNFα induce NF-κB expression and NF-κB-dependent genes in β-cells, but the effects of TNFα are less pronounced than the effects of IL-1β (63, 67). Moreover, and as shown in the present study (Figs. 1 and 3), IL-1β alone, but not TNFα, induces INS-1E cell death. This might explain these somewhat divergent observations.

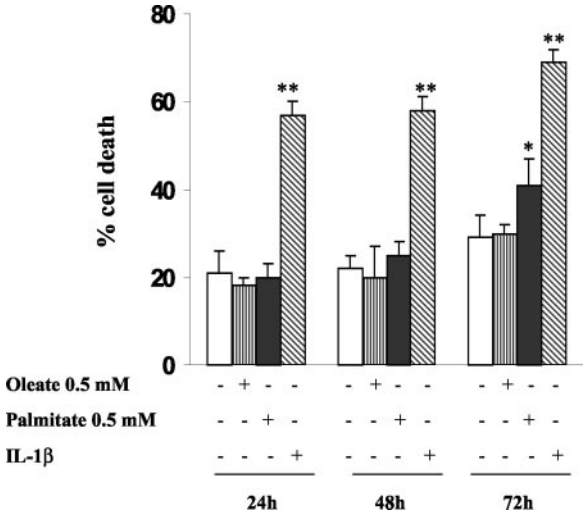

FIG. 2. FFAs and IL-1β are cytotoxic to rat islets. Rat islets were cultured for 24, 48, or 72 h in the presence of oleate (0.5 mM), palmitate (0.5 mM), or IL-1β (50 U/ml). The results are the means ± SE of four independent experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* control.

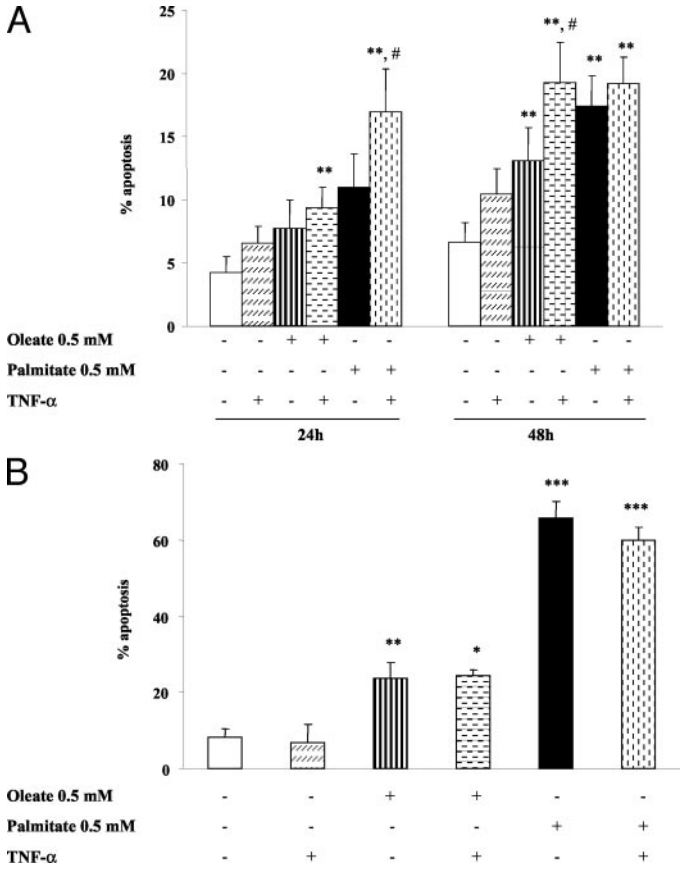

FIG. 3. Effects of TNFα and FFAs on apoptosis in INS-1E and primary β-cells. INS-1E cells (A) were cultured for 24 and 48 h and FACS-purified β-cells (B) for 72 h in the presence or absence of oleate (0.5 mM), palmitate (0.5 mM), or TNFα (1000 U/ml) alone or in combination. The results are the means ± SE of seven (INS-1E cells) or five to six (β-cells) independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control; and #, $P < 0.05$ *vs.* FFAs alone.

NF-κB activation plays a crucial role for both IL-1β + IFNγ- or IFNγ + double-stranded RNA-induced β-cell death (18–21). This, and the fact that microarray analysis of cytokine- or FFA-treated β-cells indicated some similarities in the pattern of gene expression (22, 23, 26, 40–42), prompted us to assess the role of NF-κB for FFA-induced β-cell gene expression and apoptosis. In a first series of experiments, we studied two genes previously shown to be regulated by NF-κB in β-cells, namely iNOS and MCP-1 (24, 26). Because the deleterious effects of FFAs were detected earlier in INS-1E cells, compared with whole islets (see Figs. 1 and 2), we selected 6 h as the first time point for mRNA determination in INS-1E cells, whereas 24 h was selected for rat islets. iNOS gene expression was not induced after exposure of INS-1E cells to oleate or palmitate, whereas a clear induction was observed with the positive control IL-1β (Fig. 4A). Similarly, rat islets exposed for 24 h to 0.5 mM oleate or palmitate did not increase iNOS expression, whereas IL-1β induced

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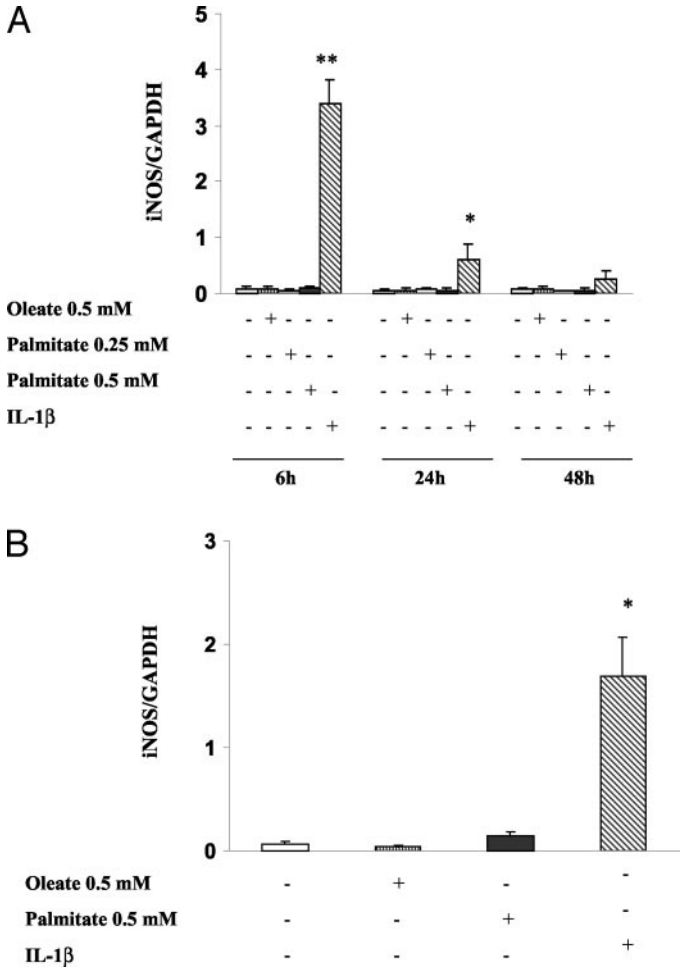

FIG. 4. FFAs do not induce iNOS mRNA expression. iNOS mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression of the housekeeping gene GAPDH. A, INS-1E cells were cultured for 6–48 h with oleate, palmitate, or IL-1β (30 U/ml) as a positive control. Data represent means ± SE of five independent experiments. B, Primary rat islets were cultured for 24 h with oleate, palmitate, or IL-1β (50 U/ml). Results represent means ± SE of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

iNOS mRNA (Fig. 4B). In line with these results, there was no increased accumulation of nitrite in the culture medium of FFA-treated cells, whereas IL-1β induced a marked increase in nitrite production (data not shown).

The observed lack of FFA-induced iNOS expression in INS-1E cells and whole islets is in agreement with our previous observations in FACS-purified primary β-cells (15) and the observations by other groups in both whole islets and insulin-producing cells (36, 68), all showing absence of FFA-induced iNOS expression. They contrast, however, with the findings by Shimabukuro *et al.* (44), suggesting that FFAs induce NO production in both Wistar and ZDF rat islets. Of note, we did observe a marked interference of FFAs with the nitrite assay (15) (Cnop, M., R. Leeman, and D. L. Eizirik, unpublished data), a phenomenon not reported by Shimabukuro *et al.* (44) and that might help to explain their findings of high nitrite accumulation in the medium of FFA-exposed cells.

Oleate and palmitate also failed to modify MCP-1 mRNA expression. Thus, there was no induction of MCP-1 mRNA in INS-1E cells (Fig. 5A) or in rat islets (Fig. 5B) after 6–48 h exposure to FFAs. IL-1β treatment, on the other hand, led to a clear induction of MCP-1 expression in both cell types, in good agreement with previous observations (25, 26, 52).

We have previously reported that IL-1β inhibits Pdx-1 expression in β-cells, an effect mediated via NF-κB activation and NO production (22), and we next evaluated the effects of FFAs or IL-1β on Pdx-1 mRNA expression. In line with our previous findings (22), there was a decrease in Pdx-1 expression in both INS-1 cells and pancreatic islets after 24 h treatment with IL-1β (Fig. 6). On the other hand, neither oleate nor palmitate decreased Pdx-1 expression (Fig. 6).

To confirm the biological effects of FFAs on mRNA expression, we determined the expression of mRNA for UCP-2 in FFA- or cytokine-treated INS-1 cells. Induction of UCP-2 is a well-known effect of FFAs on β-cells (69, 70). After 24 h, oleate and palmitate induced, respectively, a 45 and 36%

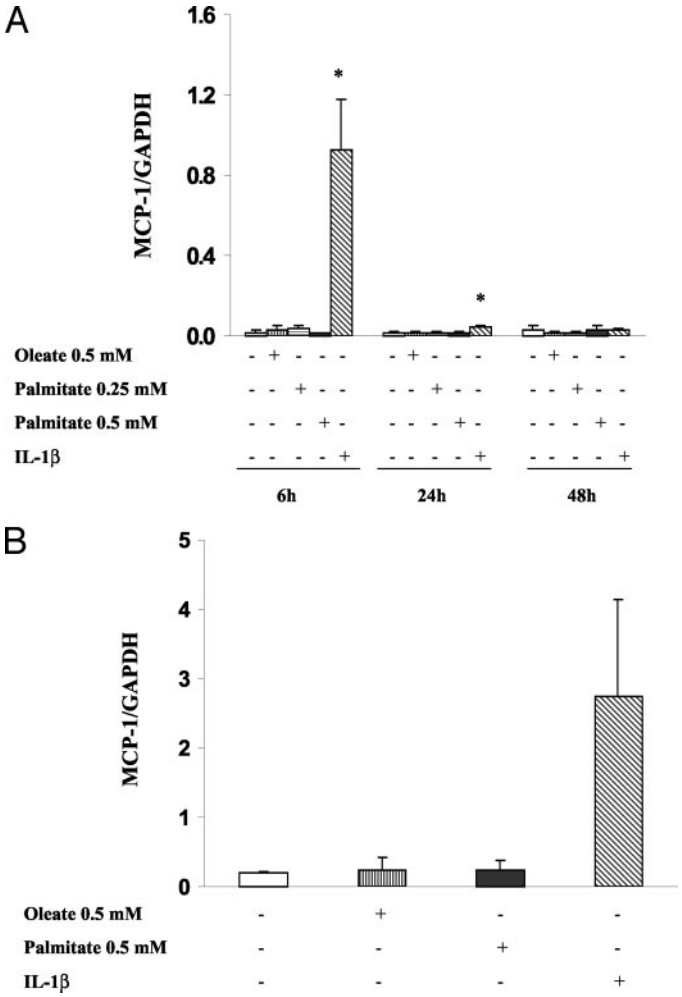

FIG. 5. FFAs do not induce MCP-1 mRNA expression. MCP-1 mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression of the housekeeping gene GAPDH. A, INS-1E cells cultured for 6–48 h in the presence or absence of oleate, palmitate, or IL-1β (30 U/ml) (n = 5). B, Primary rat islets exposed for 24 h to oleate, palmitate, or IL-1β (50 U/ml). Data represent means ± SE of three independent experiments. *, $P < 0.05$ *vs.* control.

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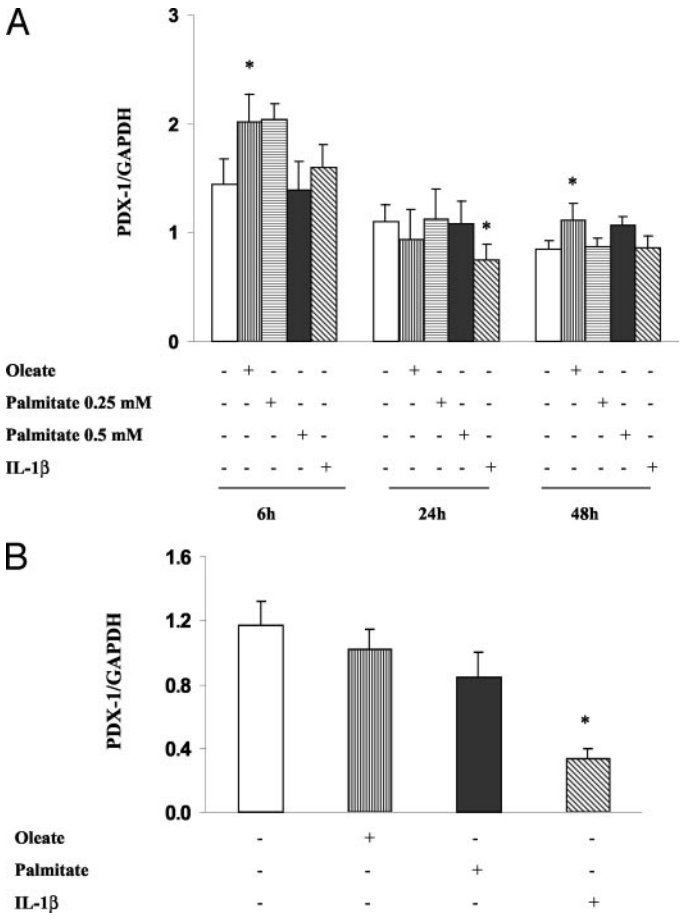

FIG. 6. Effects of FFAs on Pdx-1 mRNA expression. Pdx-1 mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression of the housekeeping gene GAPDH. A, INS-1E cells were cultured for 6–48 h in the presence or absence of oleate, palmitate, or IL-1β (30 U/ml). Data represent means ± SE of five independent experiments. B, Primary rat islets were exposed for 24 h to oleate, palmitate, or IL-1β (50 U/ml). Results represent means ± SE of three independent experiments. *, $P < 0.05$ *vs.* control.

increase in UCP-2 expression in INS-1E cells ($P < 0.05$ *vs.* control; n = 5), whereas IL-1β did not modify UCP-2 mRNA expression.

The data presented above indicate that FFAs do not induce the expression of NF-κB-dependent genes in INS-1E cells or pancreatic islets. The fact that palmitate and oleate, under similar experimental conditions, induced both UCP-2 mRNA expression and apoptosis in these cells confirms that the FFAs presently used are biologically active. Moreover, the observation that IL-1β affects expression of iNOS, MCP-1, and Pdx-1, as previously described (22–26), confirms that the primers and RT-PCR conditions used for the detection of these mRNAs were adequate. Thus, lack of FFA-induced iNOS or MCP-1 expression is a true biological phenomenon and not an artifact induced by inadequate experimental conditions.

We next performed EMSA and immunofluorescence to determine the nuclear translocation of activated NF-κB and thus directly assess a putative involvement of NF-κB on the effects of FFA. Neither oleate nor palmitate induced NF-κB activation in INS-1E cells examined after 30 min, 4 h, or 12 h of FFA exposure (Fig. 7, lanes 2 and 3). In contrast, specific DNA binding by nuclear NF-κB was observed at all time points studied in INS-1E cells exposed to TNFα (used as a positive control for NF-κB activation) (Fig. 7, lane 4). The specificity of DNA binding was confirmed by competition between labeled and cold target sequence in 50-fold molar excess in cells treated with TNFα for 30 min (Fig. 7, lane 7) and by supershift after incubation with antibodies directed against either the NF-κB p50 or p65 subunits (data not shown). The time course observed for TNFα-induced NF-κB activation, namely early (30 min) activation followed by progressive decrease after 4–12 h, is consistent with our previous observations (62, 63). The addition of FFAs to TNFα did not affect the magnitude or time course of NF-κB activation (Fig. 7, lanes 5 and 6), arguing against a synergistic effect between FFAs and TNFα on NF-κB activation. These observations were confirmed by immunostaining for subcellular localization of p65 (20), an NF-κB subunit, in primary β-cells (Fig. 8) and INS-1E cells (data not shown). A 30-min exposure to IL-1β or TNFα induced NF-κB nuclear migration in nearly 90% of the β-cells, whereas no activation of NF-κB was observed with oleate or palmitate at 30 min or at 12 h (Fig. 8). Moreover, and in line with the gel shift results (Fig. 7), the FFAs did not modify TNFα-induced NF-κB activation after 30 min or 12 h. Similar results were obtained in INS-1E cells after 30 min, 4 h, or 12 h of exposure to FFA and/or TNFα (data not shown). These observations, using two complementary methods to determine NF-κB activation and taken together with the mRNA expression data (see above), make it unlikely that NF-κB activation plays a role in FFA-induced β-cell dysfunction and death.

In the next series of experiments, we assessed whether

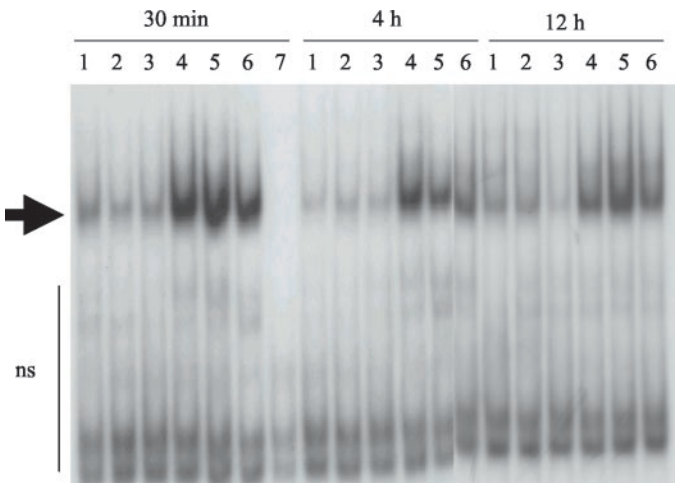

FIG. 7. TNFα but not FFAs induces NF-κB nuclear translocation and DNA binding in INS-1E cells, as measured by EMSA. Nuclear protein extracts were prepared from control INS-1E cells (lane 1) and cells exposed to 0.5 mM oleate (lane 2), 0.5 mM palmitate (lane 3), 1000 U/ml TNFα alone (lane 4), or in combination with oleate (lane 5) or palmitate (lane 6) for 30 min, 4 h, and 12 h. Nuclear extracts were incubated with labeled oligoprobe for the NF-κB consensus sequence. Specificity of nuclear binding was determined by addition of excess cold oligonucleotide (lane 7) to nuclear extract from cells treated with TNFα for 30 min. *Arrow*, NF-κB-specific DNA-protein binding; ns, nonspecific complexes. The experiment shown is representative of two similar experiments.

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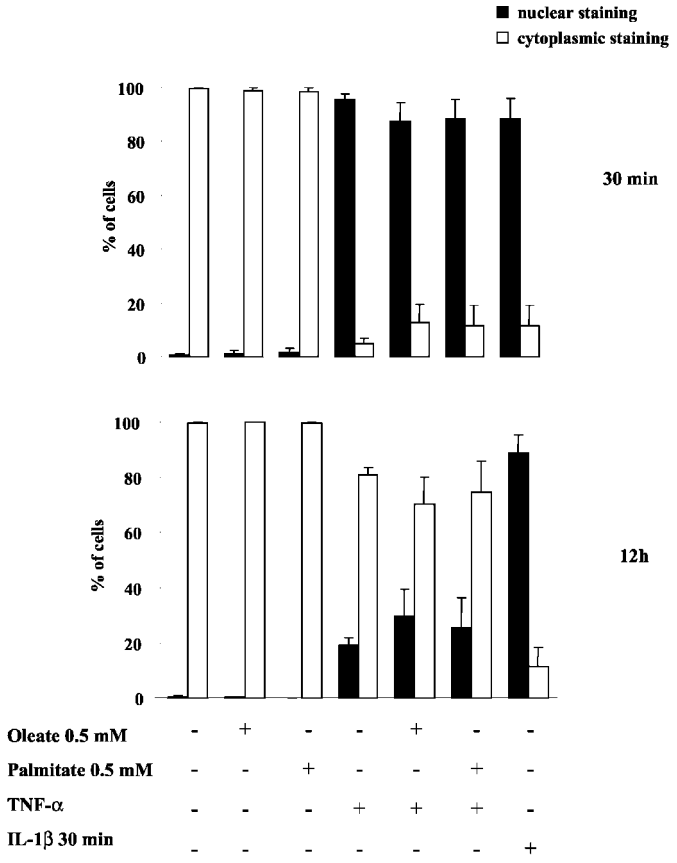

FIG. 8. TNFα but not FFAs induces NF-κB nuclear translocation and DNA binding in primary β-cells, as measured by immunofluorescence. FACS-purified β-cells were exposed to 0.5 mM oleate, 0.5 mM palmitate, or 1000 U/ml TNFα alone or in combination with oleate or palmitate for 30 min or 12 h. As an additional positive control, cells were also exposed to IL-1β (30 U/ml) during the last 30 min of culture. Fixed cells were immunostained for p65, an NF-κB subunit, and nuclear (indicating NF-κB activation) or cytoplasmic subcellular NF-κB localization was quantified. The results are means ± SE of three independent experiments.

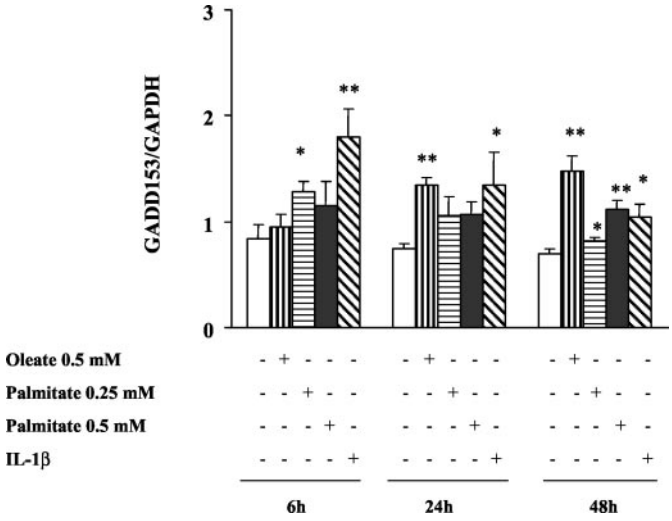

FIG. 9. FFAs induce GADD153/CHOP expression in INS-1E cells. GADD153/CHOP mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression level of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–48 h in the presence of oleate, palmitate, or IL-1β (30 U/ml). The results represent means ± SE of five independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

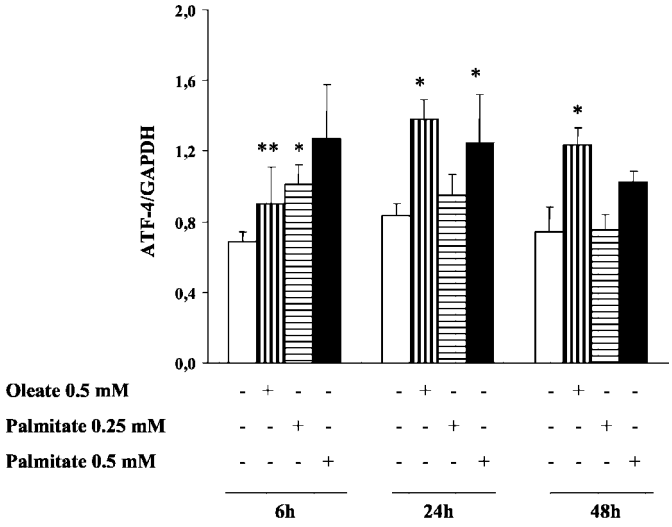

FIG. 10. FFAs induce ATF-4 expression in INS-1E cells. ATF-4 mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression level of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–48 h in the presence of oleate or palmitate. The results represent means ± SE of five independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

FFAs induce an ER stress response in INS-1E cells. For this purpose, we examined the activation of the ER stress-induced effectors GADD153/CHOP, XBP-1, BiP, ATF-4, and ATF-6 (71). GADD153/CHOP mRNA expression was examined in INS-1E cells treated with oleate, palmitate, or IL-1β. Oleate induced a 2-fold increase of GADD153/CHOP expression at 24 and 48 h (Fig. 9), whereas palmitate effects were most marked after 48 h (Fig. 9). GADD153/CHOP induction by IL-1β was already present after 6 h (Fig. 9). This effect of IL-1β was potentiated by the addition of IFNγ, leading to a 4- to 5-fold increase in GADD153/CHOP, compared with control cells (data not shown).

ER stress also activates PKR-like ER kinase (PERK), leading to increased expression of the transcription factor ATF-4 (30, 72). ATF-4 contributes to induce CHOP mRNA expression (73), and in line with this, we observed a 1.5- to 2.0-fold increase in ATF-4 mRNA expression in INS-1E cells exposed for 24 or 48 h to oleate or 0.5 mM palmitate (Fig. 10). In view of the induction of the downstream ER stress effectors GADD153/CHOP and ATF-4, we examined whether other specific ER stress mediators such as XBP-1, ATF-6, and BiP were also activated in β-cells by FFAs.

ER stress leads to activation of the transcription factor XBP-1 by alternative splicing, resulting in the removal of a Pst1 restriction site (29, 30, 71). XBP-1 activation was thus examined in INS-1E cells exposed to FFAs or cytokines (IL-1β + IFNγ) by its PCR amplification followed by incubation with Pst1. In the control condition, most of the XBP-1 PCR product was cut by the Pst1 restriction enzyme into two fragments of 300 bp, corresponding to native form of XBP-1, and only 23 ± 2% and 27 ± 1% of the total were detected as

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the 601-bp form at 24 and 48 h, respectively (n = 3). In INS-1E cells exposed to FFAs, a larger proportion of the XBP-1 PCR product kept its full 601-bp length (for 0.5 mM oleate 50 ± 5% at 24 h and 42 ± 4% at 48 h, n = 3, $P < 0.05$ *vs.* control; for 0.5 mM palmitate 56 ± 3% at 24 h and 51 ± 6% at 48 h, n = 3, $P < 0.05$ *vs.* control), indicating partial XBP-1 activation (Fig. 11) and presence of ER stress. The combination of cytokines (IL-1β + IFNγ) as well as thapsigargin, which blocks SERCA-2b (31), was used as positive controls for an ER stress response. These compounds also induced a clear XBP-1 activation (for cytokines 50 ± 2% at 24 h and 50 ± 3% at 48 h, n = 2; for thapsigargin 84 ± 2% at 24 h and 82 ± 1% at 48 h, n = 2), more pronounced in the case of thapsigargin than the activation induced by FFA (Fig. 11).

During the ER stress response, ATF-6 is cleaved from the ER membrane and translocates to the nucleus (30, 60, 61, 71). To determine whether FFAs induce activation of ATF-6 in β-cells, we used a construct containing the luciferase reporter gene downstream of five ATF-6 binding sites (5 × ATF-6). Both oleate, palmitate (0.5 mM) and the positive control thapsigargin activated the ATF-6 luciferase construct in INS-1E cells (Fig. 12). The ER chaperone BiP is a known target of ATF-6 (74), and, in agreement with the ATF-6 promoter data, INS-1E cells exposed for 24 or 48 h to FFA had a 2-fold increase in BiP mRNA expression (Fig. 13).

These observations, together with previous ultrastructural studies indicating that the ER is dilated in FFA-exposed β-cells (75), suggest that a 24- to 48-h exposure to FFAs induces ER stress in β-cells. Palmitate induced 2-fold more apoptosis than an equimolar concentration of oleate, but both FFAs induced a similar degree of ER stress as judged by the diverse parameters evaluated in this study. Thus, it seems unlikely that FFA-induced ER stress is the sole determinant of FFA-mediated β-cell death. It is, however, conceivable that a nonlethal level of ER stress induced by FFA sensitizes the β-cells to additional proapoptotic stimuli that might be present in early T2DM.

It has been previously shown that NO production, followed by SERCA2b inhibition, is the main mechanism by which cytokines cause ER stress in β-cells (22, 27, 28) (Cardozo, A. K., F. Ortis, Y.-M. Feng, J. Rasschaert, F. Van Eylen, J. Storling, T. Mandrup-Poulsen, A. Herchuelz, and D. L. Eizirik, submitted for publication). As discussed above, FFAs induce neither iNOS expression nor NO production in β-cells, suggesting that FFAs induce ER stress by a different mechanism. Oleate and palmitate have been shown to affect Ca^{2+} homeostasis in the ER in other cell types (76–78), which might impair the processing and folding of proteins (30, 58) and contribute to ER stress. Additional studies are required to clarify the mechanisms involved in FFA-induced ER stress.

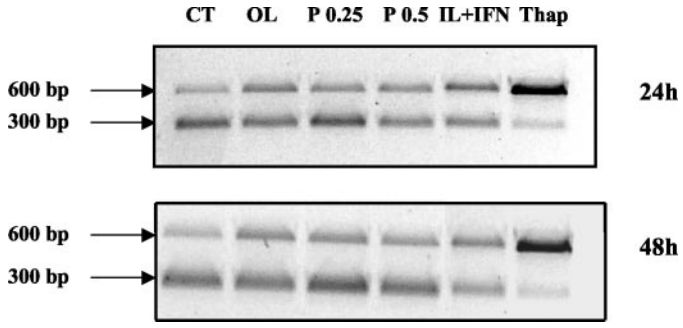

FIG. 11. Exposure of INS-1E cells to FFAs and cytokines leads to XBP-1 alternative splicing. INS-1E cells were exposed for 24 and 48 h to oleate (OL, 0.5 mM), palmitate (P, 0.25 mM and 0.5 mM), cytokines (IL-1β, 30 U/ml + IFNγ 100 U/ml), or thapsigargin (Thap, 1 μM). After mRNA extraction and reverse transcription, XBP-1 cDNA was amplified by PCR. This was followed by incubation with Pst1, which cuts the PCR products derived from nonprocessed XBP-1 into two fragments of 300 bp each (*lower arrow*). On the other hand, PCR products obtained from cells with activated XBP-1 (indicating ER stress) lack the restriction site and remain intact (*upper arrow*). One representative experiment for three similar experiments is shown; mean OD measurements for the three experiments are indicated in the text.

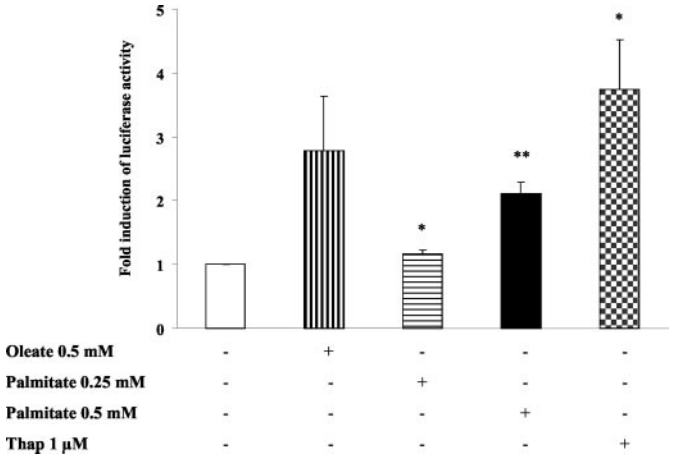

FIG. 12. FFAs activate the ATF-6 reporter gene in INS-1E cells. INS-1E cells were cotransfected with the 5 × ATF-6 site luciferase reporter gene (p5xATF6GL3) and the internal control pRL-CMV, encoding *Renilla* luciferase. After overnight transfection, the cells were exposed for 24 h to oleate, palmitate, or thapsigargin (Thap, 1 μM) and assayed for firefly and *Renilla* luciferase activities. The results were normalized for *Renilla* luciferase activity. The results are means ± SE of five independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

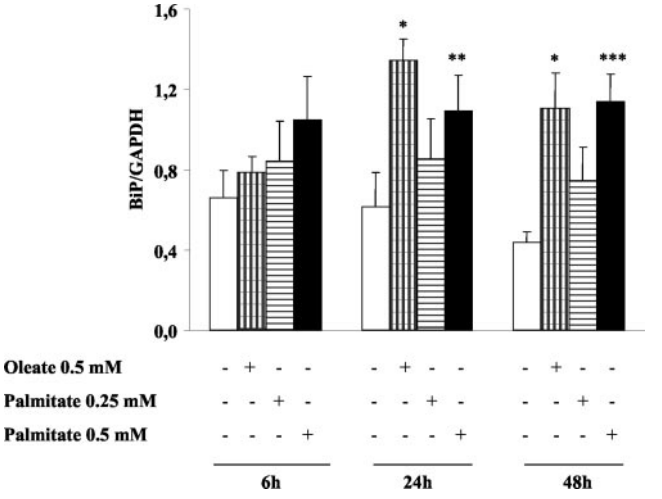

FIG. 13. FFAs induce BiP expression in INS-1E cells. BiP mRNA expression was analyzed by real-time PCR, and the data were normalized for the expression level of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–48 h in the presence of oleate or palmitate. The results represent means ± SE of five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

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Previous studies have reported similarities between nutrient- and cytokine-induced β-cell apoptosis (35, 44–46), and the hypothesis of a common cell death mechanism involving activation of NF-κB has been proposed (43, 45, 46). Against this background, we presently compared the mechanisms involved in FFA-induced β-cell death with the mechanisms of β-cell death induced by IL-1β and, in some experiments, IL-1β + IFNγ. The main conclusions of this study are the following: 1) cytokines, but not FFAs, induce expression of the NF-κB-dependent genes iNOS and MCP-1; 2) cytokines, but not FFAs, induce NF-κB activation; 3) FFAs do not potentiate TNFα-induced NF-κB activation; and 4) both FFAs and cytokines induce ER stress in INS-1E cells, but ER stress seems to be triggered by different mechanisms. These observations indicate that FFAs and cytokines lead to β-cell death by fundamentally distinct mechanisms, namely an NF-κB-dependent mechanism that culminates in caspase-3 activation in the case of cytokines (18) and an NF-κB-independent mechanism in the case of FFAs. FFA-induced apoptosis may be mediated by activation of caspases via other pathways (34), but recent evidence indicates that FFA-induced β-cell death may be related to calpain-10 activation and does not necessarily require caspase-3 (39). As a whole, the present findings argue against a unifying hypothesis for the mechanisms of β-cell death in T1DM and T2DM. Moreover, and if the present *in vitro* observations are indeed representative of the clinical situation, the findings suggest that different approaches should be pursued when attempting to prevent β-cell death in T1DM and T2DM.

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