Cytokine-Induced β-Cell Death Is Independent of Endoplasmic Reticulum Stress Signaling

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OBJECTIVE—Cytokines contribute to β-cell destruction in type 1 diabetes. Endoplasmic reticulum (ER) stress–mediated apoptosis has been proposed as a mechanism for β-cell death. We tested whether ER stress was necessary for cytokine-induced β-cell death and also whether ER stress gene activation was present in β-cells of the NOD mouse model of type 1 diabetes.

RESEARCH DESIGN AND METHODS—INS-1 β-cells or rat islets were treated with the chemical chaperone phenyl butyric acid (PBA) and exposed or not to interleukin (IL)-1β and γ-interferon (IFN-γ). Small interfering RNA (siRNA) was used to silence C/EBP homologous protein (CHOP) expression in INS-1 β-cells. Additionally, the role of ER stress in lipid-induced cell death was assessed.

RESULTS—Cytokines and palmitate triggered ER stress in β-cells as evidenced by increased phosphorylation of PKR-like ER kinase (PERK), eukaryotic initiation factor (EIF)2α, and Jun NH_2-terminal kinase (JNK) and increased expression of activating transcription factor (ATF)4 and CHOP. PBA treatment attenuated ER stress, but JNK phosphorylation was reduced only in response to palmitate, not in response to cytokines. PBA had no effect on cytokine-induced cell death but was associated with protection against palmitate-induced cell death. Similarly, siRNA-mediated reduction in CHOP expression protected against palmitate- but not against cytokine-induced cell death. In NOD islets, mRNA levels of several ER stress genes were reduced (ATF4, BiP [binding protein], GRP94 [glucose regulated protein 94], p58, and XBP-1 [X-box binding protein 1] splicing) or unchanged (CHOP and Edem1 [ER degradation enhancer, mannosidase α–like 1]).

CONCLUSIONS—While both cytokines and palmitate can induce ER stress, our results suggest that, in contrast to lipoapoptosis, the PERK-ATF4-CHOP ER stress–signaling pathway is not necessary for cytokine-induced β-cell death. *Diabetes* **57:3034–3044, 2008**

Type 1 diabetes is mediated by an autoimmune and inflammatory process ultimately leading to pancreatic β-cell death. Cytokines, such as interleukin (IL)-1β and γ-interferon (IFN-γ), are mediators of β-cell death, possibly via the generation of nitric oxide (NO) (1–5). However, the mechanisms by which β-cells are destroyed remain to be clarified.

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Recent studies have proposed that cytokine-induced β-cell death involves endoplasmic reticulum (ER) stress (6–8). The ER stress response is an evolutionarily conserved, adaptive program triggered by accumulation of unfolded protein in the lumen of the ER (9,10). Upon initiation of ER stress, cells activate intracellular signaling pathways that transmit information from the ER to the cytoplasm and nucleus, known as the unfolded protein response (UPR). The UPR includes transient attenuation of de novo protein synthesis that limits protein load on the ER. This is followed by transcriptional activation of genes encoding ER chaperones to increase protein folding capacity in the ER and proteins involved in ER-associated degradation to eliminate misfolded proteins by the ubiquitin-proteasome system. When functions of the ER are severely or irreversibly impaired, the UPR activates apoptosis. Signaling from the ER is initiated by ER-localized stress sensors PKR-like ER kinase (PERK), activating transcription factor (ATF)6, and inositol-requiring (IRE)1, which remain inactive under nonstress conditions due to association of their ER luminal domains with binding protein (BiP) (11). Upon accumulation of unfolded protein, BiP dissociates from the sensor proteins, leading to their activation. PERK activation by phosphorylation leads to phosphorylation of the eukaryotic initiation factor (EIF)2α, which in turn leads to translational attenuation and induction of ATF4 (12). ER stress can lead to apoptosis by various pathways, including via ATF4 transcriptional activation of the gene for C/EBP homologous protein (CHOP) (13–15) and activation of Jun NH_2-terminal kinase (JNK) and caspase-12 (9,10).

The importance of ER stress signaling in regulating β-cell function and survival was first demonstrated in *PERK*-deficient mice (16) and in mice with a mutation in the EIF2α phosphorylation site (Ser51Ala) (17,18). Other experiments in the Akita mouse have shown that β-cell apoptosis and diabetes could be delayed by inhibiting CHOP induction (14). That ER stress is at least partially required for lipoapoptosis in β-cells was suggested by the recent finding that MIN6 cells overexpressing BiP are partially protected against apoptosis induced by the saturated fatty acid palmitate (19). Furthermore, increased ER stress gene expression has been observed in islets of *db/db* mice (19) and humans with type 2 diabetes (19,20). The relevance of ER stress signaling to β-cell destruction in type 1 diabetes was suggested in studies showing that treatment of β-cells with cytokines or NO leads to increased expression of ATF4 and CHOP (6–8) and that pancreatic islets depleted in CHOP are partially protected against NO-induced cell death (6). These correlations led us to investigate the role of ER stress in cytokine-induced β-cell death and the development of type 1 diabetes. For comparison, we also assessed the role of ER stress in lipid-induced β-cell death.

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RESEARCH DESIGN AND METHODS

Cell culture. INS-1 cells were passaged in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 0.2 mmol/l glutamine, 10 mmol/l HEPES, 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mmol/l sodium pyruvate, and 50 μmol/l 2-mercaptoethanol. MIN6 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 25 mmol/l glucose, 10 mmol/l HEPES, 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at either 1–1.5 $\times$ 10^5 in 0.5 ml per well in a 24-well plate or at 5 $\times$ 10^5 in 2 ml per well in a 6-well plate. Cells were pretreated with the chemical chaperones phenyl butyric acid (PBA) (2.5 mmol/l; Sigma, St. Louis, MO) or trimethylamine *N*-oxide (TMAO) (100 mmol/l) for 24 h. Cells were then treated with PBA or TMAO in combination with 50 units/ml IL-1β and 100 units/ml IFN-γ (R&D Systems, Minneapolis, MN) or 0.4 mmol/l palmitate coupled to 0.92% BSA (palmitate to BSA molar ratio 3:1) (Sigma). Cell death was measured with an ELISA kit (Cell Death Detection ELISA; Roche Diagnostics, Castle Hill, Australia) (21). NO was measured in culture media using Griess reagent.

Rat islet isolation and culture. Islets were isolated from male Wistar rats (175–200 g) by pancreatic digestion with liberase RI (Roche Diagnostics). Islets were further separated with a Ficoll-Paque PLUS gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden) and handpicked under a stereomicroscope. Procedures were approved by the Garvan Institute/St.Vincent's Hospital Animal Experimentation Ethics Committee following guidelines issued by the National Health and Medical Research Council of Australia. Islets were cultured for 3–4 days at 37°C in RPMI-1640 medium supplemented with 0.2 mmol/l glutamine, 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Islets with central necrosis were discarded, and those of similar size were then pretreated with 2.5 mmol/l PBA for 24 h. Islets were then treated for 16 h with 2.5 mmol/l PBA in combination with 50 units/ml IL-1β and 100 units/ml IFN-γ. Cell death was assessed as above, and RNA was extracted for RT-PCR analysis.

Plasmid construction and transfection. Rat *BIP* cDNA was amplified by PCR, cloned into the Gateway donor vector pDONR 221, and then subcloned into the Gateway expression vector pcDNA-DEST40 (Invitrogen). Two days before transfection, INS-1 cells were seeded at 3 $\times$ 10^5 cells per well in a 12-well plate. *BIP*-DEST40 or control pmaxGFP (green fluorescent protein) was transfected into INS-1 cells using Lipofectamine 2000 (Invitrogen) with >80% transfection efficiency. Twenty four hours later, cells were treated with cytokines (50 units/ml IL-1β and 100 units/ml IFN-γ).

CHOP (DDIT3) ON-TARGET*plus* SMARTpool siRNA or negative control nontargeting siRNA were transfected into INS-1 cells using DharmaFECT Transfection Reagent (Dharmacon, Lafayette, CO). Forty eight hours later, cells were treated with cytokines (50 units/ml IL-1β and 100 units/ml IFN-γ) or 0.4 mmol/l palmitate coupled to 0.92% BSA.

Wild-type CHOP and a dominant-negative mutant CHOP construct lacking the basic DNA-binding region (CHOPΔBR) were gifts from Prof. Nicholas Hoogenraad (22). Wild-type CHOP, CHOPΔBR, or pmaxGFP were electroporated into MIN6 cells by nucleofection (AMAXA Biosystems, Cologne, Germany), according to the manufacturers' instructions, with >70% transfection efficiency. Cells were seeded at 5 $\times$ 10^5 cells in 1 ml of DMEM per well in a 12-well plate. After 24 h, cells were treated with cytokines (50 units/ml IL-1β, 100 units/ml IFN-γ, and 100 units/ml tumor necrosis factor [TNF]-α; R&D Systems, Minneapolis, MN).

Western blotting. Total protein levels were measured (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA), and 20–50 μg protein per lane was separated on NuPage SDS-PAGE gels (Invitrogen) and transferred to polyvinylidine difluoride membrane. Membranes were incubated in primary antibodies for either 1–2 h at room temperature or overnight at 4°C. The following antibodies were used (1:1,000 dilution unless otherwise indicated): CHOP (sc-575), total EIF2α (sc-11386), ATF4 (sc-200), and inducible NO synthase (iNOS) (NOS2, N-20, sc-651, 1:400) (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-PERK (Thr980, 16F8, 3179), phospho-EIF2α (Ser51, 9721), phospho-JNK (Thr183/Tyr185, 9251), total JNK (9252), and cleaved caspase-3 (Asp175, 9664, 1:500) (Cell Signaling Technology, Danvers, MA); and BiP (generously provided by Dr. Linda Hendershot, St. Jude Children's Research Hospital, Memphis, TN). Equal loading of protein between lanes was confirmed by subsequent β-actin immunoblots (β-actin 1:5,000; Sigma). After incubation with horseradish peroxidase–conjugated goat anti-mouse or donkey anti-rabbit (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA) antibody for 1 h at room temperature, immunodetection was performed by chemiluminescence (PerkinElmer, Wellesley, MA; or Millipore, Billerica, MA).

RNA analysis. Total RNA was extracted from cells or islets, and real-time PCR was performed using a LightCycler (Roche Diagnostics) (23). Oligonucleotide primer sequences (Supplementary Table 1 [available in an online appendix at http://dx.doi.org/10.2337/db07-1802]) were designed with MacVector software (Oxford Molecular Ltd.). The values obtained for each specific product was normalized to the control gene (cyclophilin A) and expressed as a percent of the value in control extracts. *Xbp1* cDNA was amplified by PCR and digested with *Pst*I, which cuts unprocessed *Xbp1* cDNA into fragments. Processed (activated) *Xbp1* cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) *Xbp1* was quantified by densitometry. The value obtained for processed *Xbp1* was expressed as a ratio of the total (processed + unprocessed) *Xbp1* mRNA levels for each sample. These ratios are expressed as a percentage of the ratio in control INS-1 cells.

NOD mice. Nonobese diabetic (NOD/LtJ) and C57BL/6J mice were obtained from ARC (Perth, WA, Australia). Islets were isolated from 13- to 18-week-old mice as described above. RNA extraction was performed immediately following islet collection.

Statistical analysis. All results are presented as means ± SEM. Statistical analyses were performed using Student's *t* test or one-way ANOVA.

RESULTS

PBA attenuates cytokine-induced ER stress in INS-1 cells. We first tested whether PBA was capable of attenuating ER stress induced by cytokines in INS-1 cells. PBA acts as a chemical chaperone to reduce the load of unfolded proteins in the ER by improving folding capacity and trafficking of mutant proteins out of the ER (24). In control (non–PBA-treated) INS-1 cells, IL-1β + IFN-γ treatment induced ER stress after both 6 and 24 h, as evidenced by increased phosphorylation of PERK (Fig. 1*A* and *B*) and EIF2α (Fig. 1*A* and *C*) and increased protein expression (Fig. 1*A*, *D*, and *E*) and mRNA levels (Fig. 1*F* and *G*) of ATF4 and CHOP. In PBA-treated INS-1 cells, ER stress due to IL-1β + IFN-γ was attenuated, as indicated by reduced PERK (Fig. 1*A* and *B*) and EIF2α phosphorylation (Fig. 1*A* and *C*) and reduced protein expression (Fig. 1*A*, *D*, and *E*) and mRNA levels (Fig. 1*F* and *G*) of ATF4 and CHOP.

PBA does not protect against cytokine-induced cell death in INS-1 cells. We tested the sensitivity of PBA-treated INS-1 cells to cytokine-mediated cytotoxicity. In control (non–PBA-treated) INS-1 cells, IL-1β + IFN-γ treatment led to a significant increase in cell death after both 6 and 24 h (Fig. 1*H*). However, PBA treatment did not protect INS-1 cells from cytokine-induced cell death; IL-1β + IFN-γ–induced cell death was similar in control and PBA-treated cells after both 6 and 24 h (Fig. 1*H*). Thus, PBA-treated cells retain their sensitivity to IL-1β + IFN-γ cytotoxicity despite the attenuation of ER stress, suggesting that ER stress induced by cytokines is not required for β-cell death.

PBA protects against palmitate-induced cell death and ER stress in INS-1 cells. We also tested the sensitivity of PBA-treated INS-1 cells to lipotoxicity induced by the saturated fatty acid palmitate. In control INS-1 cells, treatment with 0.4 mmol/l palmitate for 6 h led to a significant increase in cell death (Fig. 2*F*). Palmitate treatment of control INS-1 cells was also associated with induction of ER stress, as indicated by increased phosphorylation of PERK (Fig. 2*A* and *B*) and EIF2α (Fig. 2*A* and *C*) and increased expression of ATF4 (Fig. 2*A* and *D*) and CHOP (Fig. 2*A* and *E*). In PBA-treated INS-1 cells, palmitate-induced ER stress was attenuated, as indicated by reduced PERK (Fig. 2*A* and *B*) and EIF2α (Fig. 2*A* and *C*) phosphorylation and decreased expression of ATF4 (Fig. 2*A* and *D*) and CHOP (Fig. 2*A* and *E*). Strikingly, this was associated with a complete block of palmitate-induced cell death in PBA-treated INS-1 cells (Fig. 2*F*). Thus, PBA-treated cells acquired selective resistance to the toxic effects of palmitate. These data suggest that,

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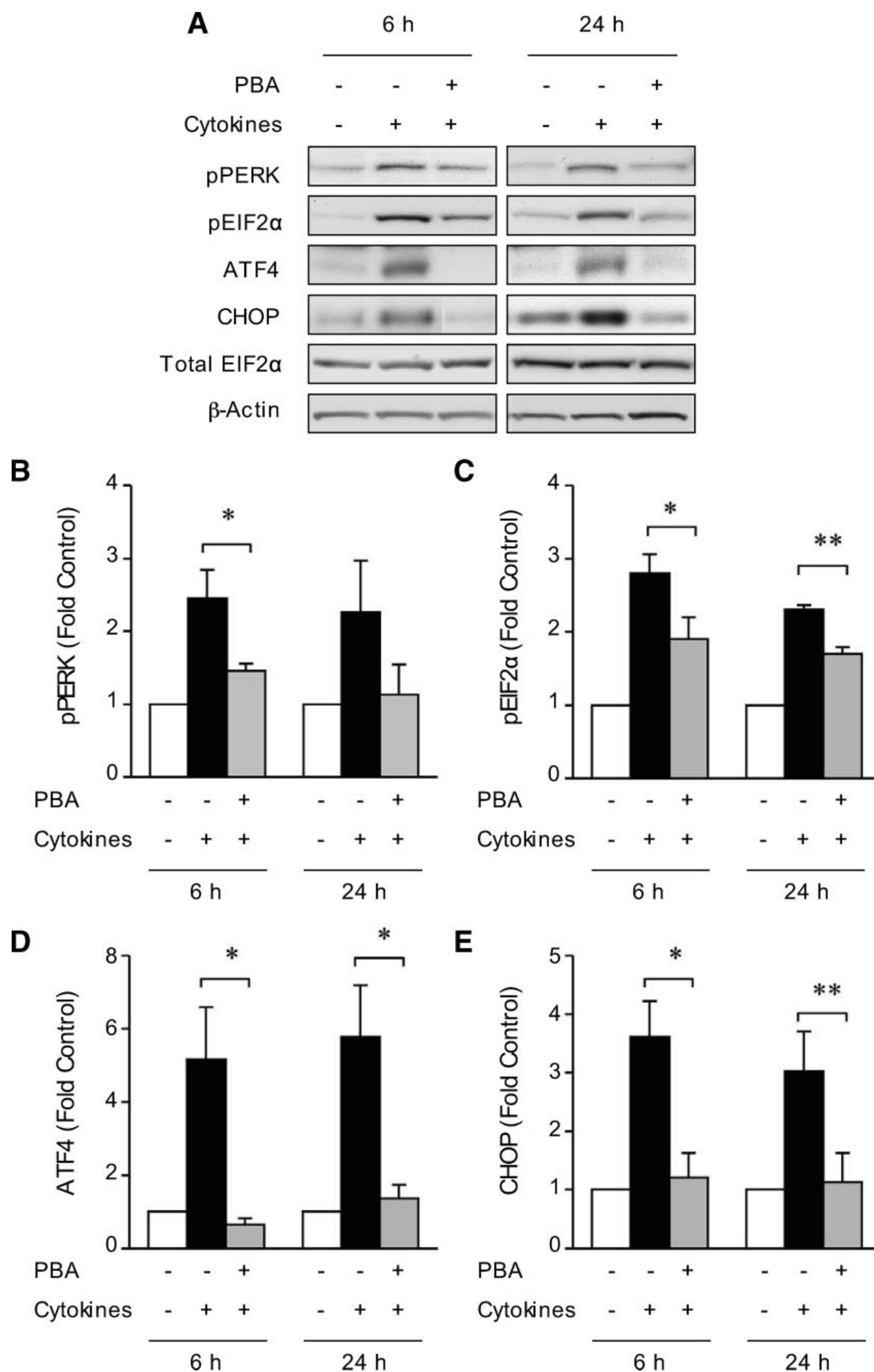

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unlike cytokines, ER stress is required for lipid-induced cell death in β-cells.

We further assessed the role of ER stress in β-cell death using another chemical chaperone, trimethylamine oxide (TMAO). Treatment of INS-1 cells with TMAO reduced ER stress, as indicated by decreased

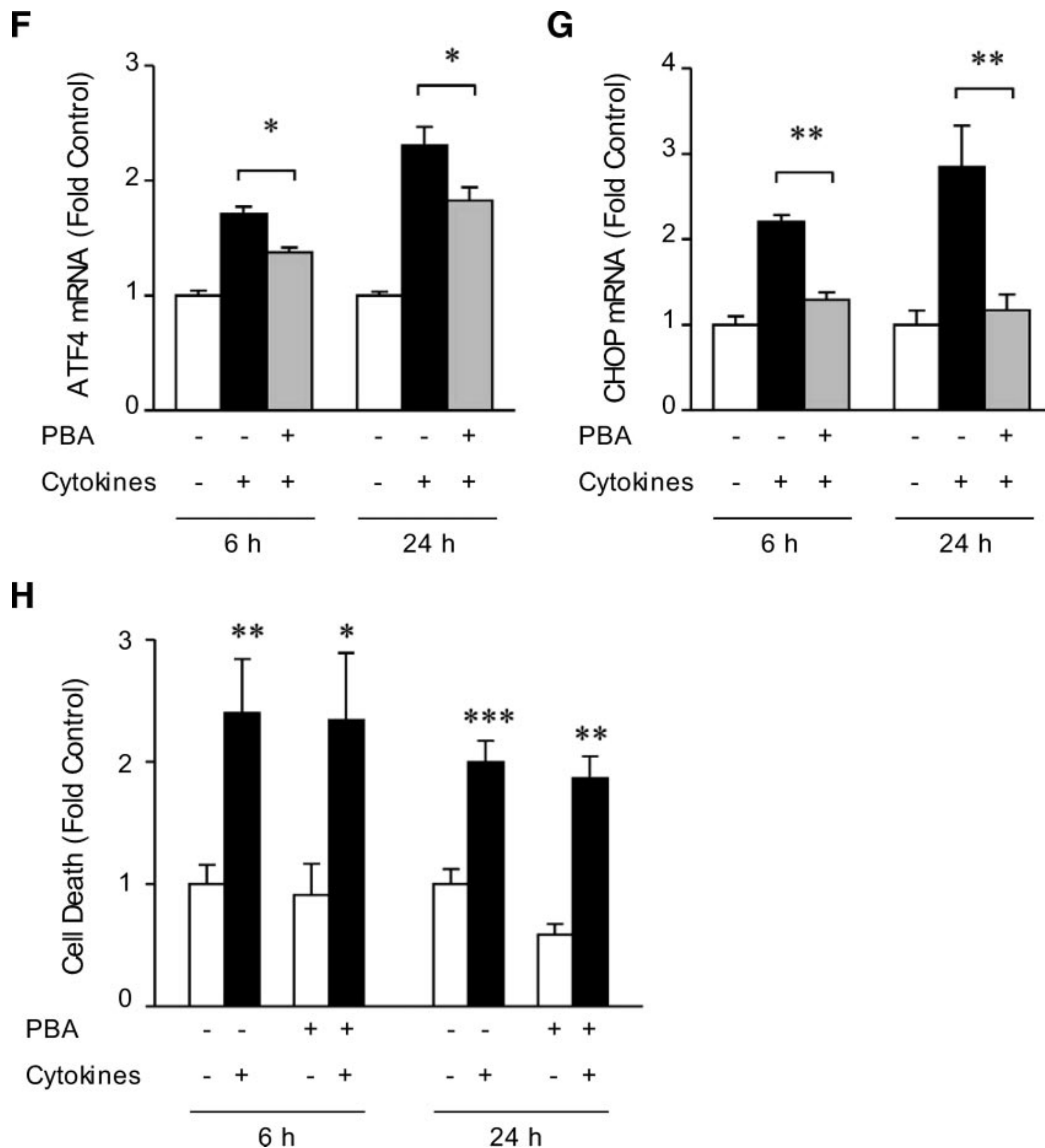

FIG. 1. The chemical chaperone PBA reduces cytokine activation of genes involved in ER stress in INS-1 cells but does not protect against cytokine-induced cell death. INS-1 cells grown in RPMI-1640 were pretreated in the absence or presence of PBA (2.5 mmol/l) for 24 h and then cultured in combination with IL-1β (50 units/ml) and IFN-γ (100 units/ml) for 6 or 24 h as indicated. *A*: Western blot analysis comparing changes in PERK and EIF2α phosphorylation (p) and expression of ATF4 and CHOP. Total EIF2α and β-actin protein served as loading controls. pPERK (*B*), pEIF2α (*C*), ATF4 (*D*), and CHOP (*E*) bands were quantified by densitometry and are expressed as fold change compared with control. Results are means ± SEM determined from three to four experiments. *$P < 0.05$, **$P < 0.01$ for PBA + cytokine-treated versus control cytokine-treated INS-1 cells at the same time point. ATF4 (*F*) and CHOP (*G*) mRNA levels. Total RNA was extracted and analyzed by real-time RT-PCR. Results are means ± SEM determined from three experiments performed in triplicate or quadruplicate and are expressed as fold-change of mRNA levels in control INS-1 cells. *$P < 0.05$, **$P < 0.01$ for PBA + cytokine-treated versus control cytokine-treated INS-1 cells at the same time point. *H*: Cell death was measured using a cell death detection ELISA. Results are means ± SEM determined from three experiments performed in triplicate and are expressed as fold change compared with control untreated INS-1 cells at each time point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control untreated INS-1 cells at the same time point.

ATF4 and CHOP protein expression in palmitate- (Supplementary Fig. 1*A* and *B*) and cytokine-treated (Supplementary Fig. 2*A* and *B*) INS-1 cells. This was associated with reduced cell death in palmitate-treated cells (Supplementary Fig. 1*C*), whereas cytokine-induced cell death was not affected (Supplementary Fig. 2*C*). Thus, the data using TMAO support the suggestion that ER stress is required for lipid- but not for cytokine-induced cell death. We also examined cleaved caspase-3 expression under these experimental conditions. Cleaved caspase-3 expression was induced in both palmitate- (Supplementary Fig. 3*A*) and cytokine-treated (Supplementary Fig. 3*B*) INS-1 cells. In PBA- and TMAO-treated INS-1 cells, cleaved caspase-3 expression was reduced in response to palmitate (Supplementary Fig. 3*A*) and not in response cytokines (Supplementary Fig. 3*B*).

PBA treatment does not lower cytokine-induced iNOS expression or NO production in INS-1 cells. To check that the ability of PBA to attenuate cytokine-induced ER stress was not due to lower NO production, we measured iNOS expression and NO production after cytokine exposure in control and PBA-treated β-cells. Treatment of INS-1 cells with IL-1β + IFN-γ led to an increase in iNOS expression (Fig. 3*B*) and stimulated production of NO (Fig. 3*A*). With PBA treatment, cytokine-induced iNOS expression and NO production were not

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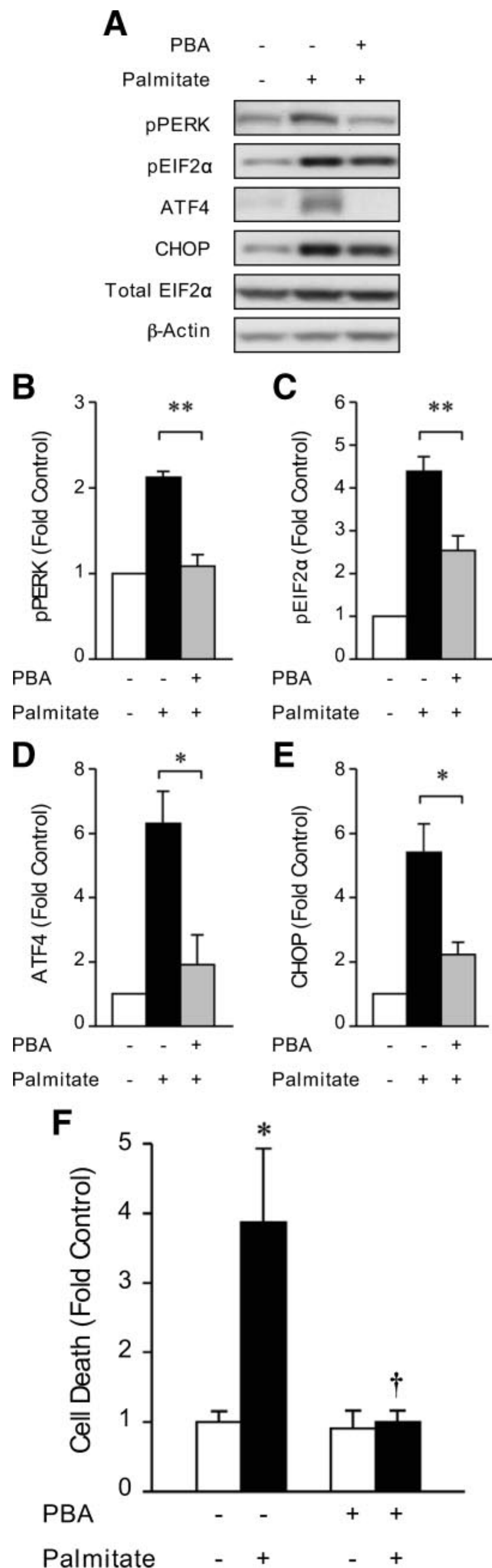

FIG. 2. PBA treatment protects against palmitate-induced ER stress and cell death in INS-1 cells. INS-1 cells grown in RPMI-1640 were pretreated in the absence or presence of PBA (2.5 mmol/l) for 24 h and then cultured in combination with 0.4 mmol/l palmitate coupled to 0.92% BSA for 6 h as indicated. *A*: Western blot analysis comparing changes in PERK and EIF2α phosphorylation (p) and expression of ATF4 and CHOP. Total EIF2α and β-actin protein served as loading controls. pPERK (*B*), pEIF2α (*C*), ATF4 (*D*), and CHOP (*E*) bands were quantified by densitometry and are expressed as fold change compared with control. Results are means ± SEM determined from three to four experiments. *$P < 0.05$, **$P < 0.01$ for PBA + palmitate-treated versus control palmitate-treated INS-1 cells. *F*: Cell death was measured using a cell death detection ELISA. Results are means ± SEM determined from three experiments performed in triplicate and are expressed as fold change compared with control. *$P < 0.05$ for control palmitate-treated versus control untreated INS-1 cells, †$P < 0.05$ for PBA + palmitate-treated versus control palmitate-treated INS-1 cells.

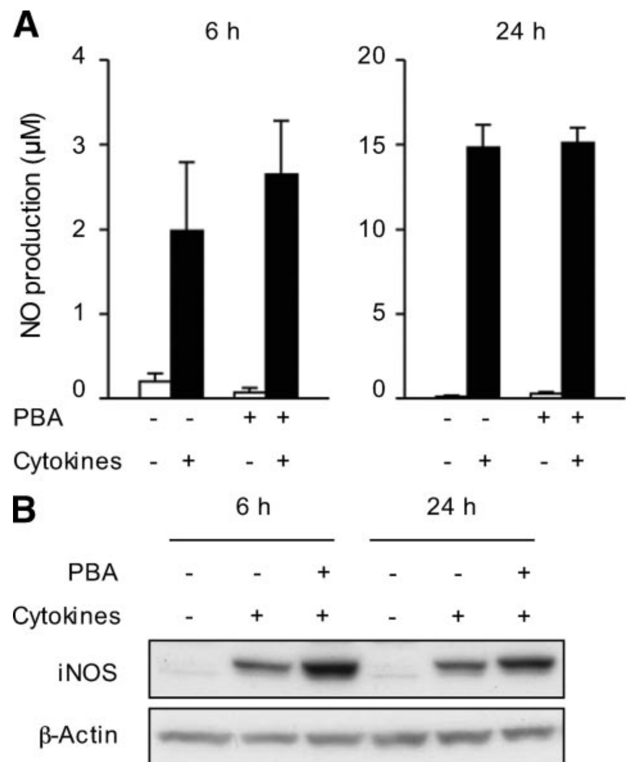

FIG. 3. PBA treatment does not lower cytokine-induced iNOS expression or NO production in INS-1 cells. INS-1 cells were treated with PBA (2.5 mmol/l) for 24 h and then cultured in combination with IL-1β (50 units/ml) and IFN-γ (100 units/ml) for 6 or 24 h as indicated. *A*: Medium was taken to determine levels of NO production by the Griess reaction. Results are means ± SEM. $n = 5$ in each group. *B*: Western blot analysis comparing changes in iNOS expression. Representative images are shown from two experiments.

inhibited (Fig. 3*A* and *B*). When INS-1 cells were treated with palmitate, we found no induction of iNOS expression and no stimulation of NO production (data not shown).

PBA treatment lowers palmitate-induced XBP1 splicing, whereas cytokines do not activate XBP1 in INS-1 cells. We next examined ER stress signaling downstream of IRE1 by assessing XBP1 splicing. Palmitate treatment led to an increase in the spliced form of XBP1 mRNA in INS-1 cells (Fig. 4*A*), indicating activation. In contrast, cytokine treatment was associated with reduced XBP1 splicing in INS-1 cells (Fig. 4*B*), suggesting reduced signaling via this arm of the ER stress response. In PBA-treated cells, palmitate activation of XBP1 was attenuated, as indicated by reduced mRNA levels of the spliced form (Fig. 4*A*).

PBA lowers JNK phosphorylation in response to palmitate but not in response to cytokines. We next examined JNK phosphorylation (activation), which can also be stimulated by ER stress downstream of IRE1 (25). Increased phosphorylation of JNK1 (46 kDa) and JNK2 (54 kDa) was observed in both palmitate- (Fig. 5*A–C*) and cytokine-treated (Fig. 5*D–F*) INS-1 cells. PBA treatment

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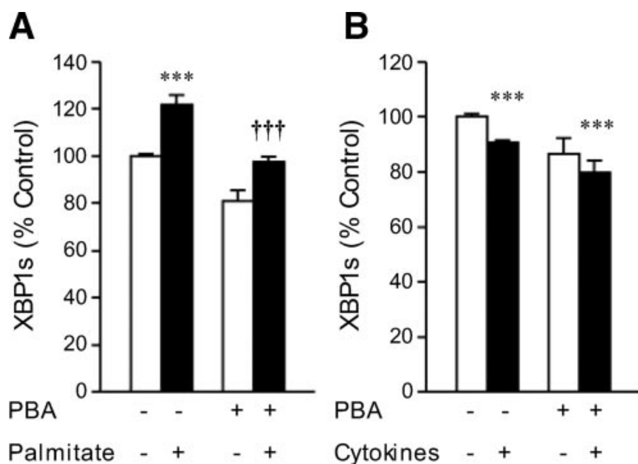

FIG. 4. Palmitate activates, whereas cytokines reduce, XBP1 splicing in INS-1 cells. PBA treatment lowers palmitate-induced XBP1 splicing. INS-1 cells were pretreated in the absence or presence of PBA (2.5 mmol/l) for 24 h and then cultured for 6 h in combination with the absence (□) or presence (■) of either 0.4 mmol/l palmitate coupled to 0.92% BSA (*A*), or IL-1β (50 units/ml) and IFN-γ (100 units/ml) (*B*). Total RNA was extracted and reverse transcribed. *Xbp1* cDNA was amplified by PCR and digested with *Pst*I, which cuts unprocessed *Xbp1* cDNA into fragments. Processed (activated) *Xbp1* cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) *Xbp1* was quantified by densitometry. The value obtained for processed *Xbp1* was expressed as a ratio of the total (processed + unprocessed) *Xbp1* mRNA levels for each sample. These ratios are expressed as a percentage of the ratio in control INS-1 cells. Results are means ± SEM determined from three experiments performed in triplicate or quadruplicate. *A*: ***$P < 0.001$ versus control untreated INS-1 cells, †††$P < 0.001$ for PBA + palmitate-treated versus control palmitate-treated INS-1 cells. *B*: ***$P < 0.001$ versus control untreated INS-1 cells.

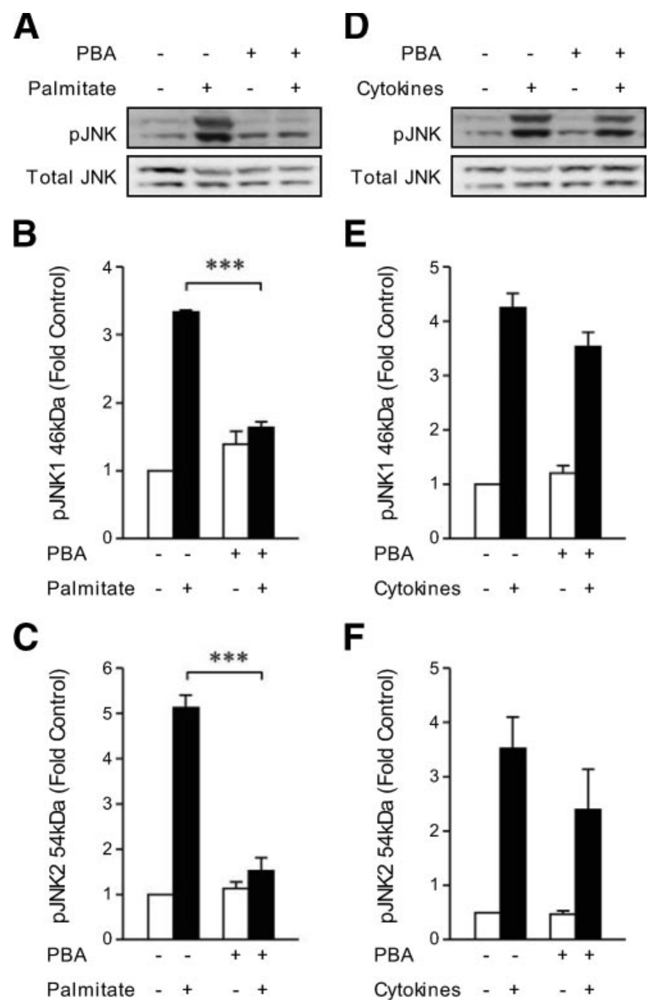

FIG. 5. PBA treatment reduces JNK phosphorylation in response to palmitate but not in response to cytokines. INS-1 cells were pretreated in the absence or presence of PBA (2.5 mmol/l) for 24 h and then cultured for 6 h in combination with the absence or presence of 0.4 mmol/l palmitate coupled to 0.92% BSA (*A–C*) or IL-1β (50 units/ml) and IFN-γ (100 units/ml) (*D–F*). *A* and *D*: Western blot analysis comparing changes in JNK phosphorylation (p). Total JNK protein served as loading control. pJNK1 (46 kDa) (*B* and *E*) and pJNK2 (54 kDa) (*C* and *F*) bands were quantified by densitometry and are expressed as fold change compared with control. Results are means ± SEM determined from three experiments. ***$P < 0.001$ for PBA + palmitate-treated versus control palmitate-treated INS-1 cells.

lowered JNK1/2 phosphorylation in response to palmitate (Fig. 5*A–C*), suggesting that ER stress is required for palmitate-induced JNK activation. In contrast, cytokine-induced JNK1/2 phosphorylation was not significantly altered by PBA treatment (Fig. 5*D–F*), suggesting that ER stress is not required for cytokine-induced JNK activation. Thus, ER stress-independent activation of JNK may provide a mechanism for the inability of PBA to protect against cytokine-induced cell death.

PBA does not protect against cytokine-induced cell death in primary islets. We next tested in rat islets whether attenuation of ER stress with PBA altered the sensitivity of primary β-cells to cytokine-induced cell death. In control rat islets, IL-1β + IFN-γ treatment for 16 h led to a significant increase in cell death (Fig. 6*A*), as well as increased ATF4 and CHOP mRNA levels (Fig. 6*B*), suggestive of UPR activation. In PBA-treated islets, exposure to cytokines led to significantly reduced ATF4 and CHOP mRNA levels compared with cytokine-treated control islets (Fig. 6*B*). However, in PBA-treated islets, cytokine-induced cell death was similar to that in control islets (Fig. 6*A*). Thus, the PBA-mediated reduction in UPR activation had no effect on cytokine-induced cell death in primary islets. These data are in accordance with the results obtained in INS-1 cells and confirm the suggestion that ER stress is not required for cytokine-induced β-cell death in islets.

BiP overexpression does not protect INS-1 cells from cytokine-induced cell death. The overexpression of the ER chaperone BiP attenuates ER stress in β-cells (19), as it does in other cell types (11,13,26). BiP acts as an ER resident molecular chaperone by enhancing protein folding and maintaining PERK, ATF6, and IRE1 in their inactive states (11). We previously demonstrated that lipid-induced cell death was significantly reduced in BiP-overexpressing β-cells (19). Here, we tested whether BiP overexpression influenced cytokine-induced toxicity in β-cells. INS-1 cells were transfected with an expression vector encoding BiP or GFP (Fig. 7*A*) and then cultured in the absence or presence of cytokines. Cell death induced by IL-1β + IFN-γ was similar in GFP- and BiP-overexpressing INS-1 cells (Fig. 7*B*), despite lowered CHOP expression in BiP-overexpressing cells (Fig. 7*C*). Similarly, in studies with MIN6 cells, overexpressing murine BiP had no influence on cytokine-induced cell death (data not shown). These data indicate that BiP-overexpressing β-cells retain their sensitivity to the toxic effects of cytokines.

siRNA-mediated silencing of CHOP expression in β-cells protects against palmitate- but not against cytokine-induced cell death. In a previous study (6), a modest reduction in cell death induced by cytokines was observed in islets from CHOP-deficient mice. To assess the

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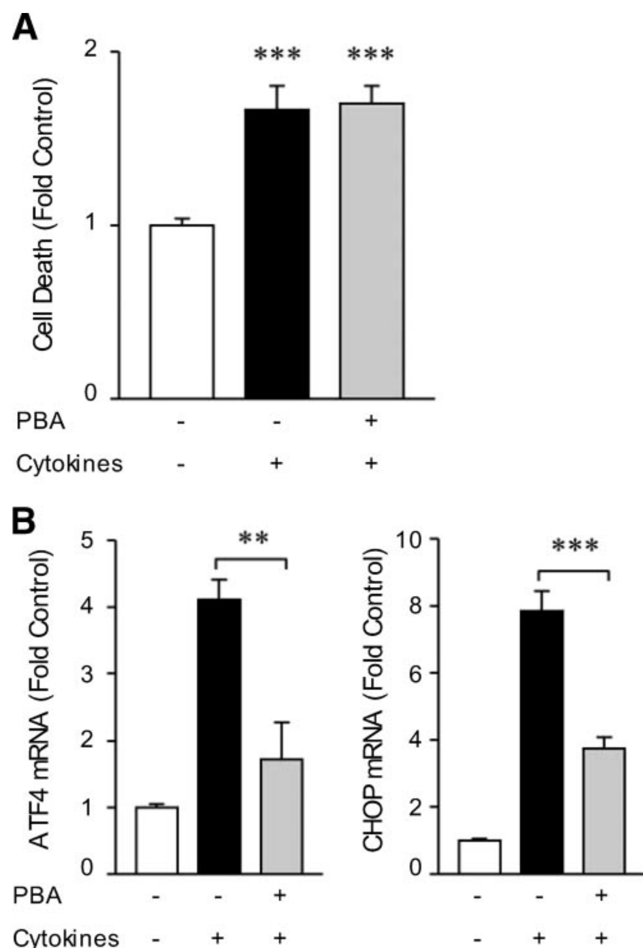

FIG. 6. Attenuation of ER stress with PBA does not protect against cytokine-induced cell death in primary rat islets. Isolated rat islets were pretreated in the absence or presence of PBA (2.5 mmol/l) for 24 h and then cultured in combination with the IL-1β (50 units/ml) and IFN-γ (100 units/ml) for 16 h. *A*: Cell death was measured using a cell death detection ELISA. Results are means ± SEM determined from three experiments performed in triplicate and are expressed as fold change compared with control untreated islets. ***P < 0.001 versus control untreated islets. *B*: Total RNA was extracted from islets and ATF4 and CHOP mRNA levels analyzed by real-time RT-PCR. Results are means ± SEM determined from four experiments performed in duplicate and are expressed as fold change of mRNA levels in control untreated islets. **P < 0.01, ***P < 0.001 for PBA + cytokine-treated versus control cytokine-treated islets.

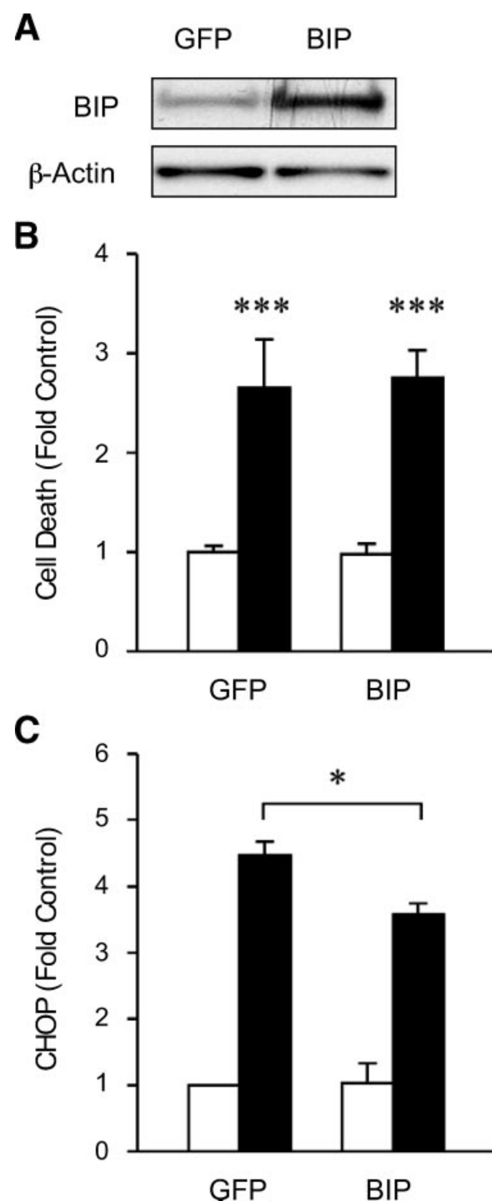

FIG. 7. BiP overexpression does not protect INS-1 cells from cytokine-induced cell death. INS-1 cells were transfected with an expression vector encoding for *BIP* (pBIP-DEST40) or GFP (pmaxGFP) by lipofectamine. *A*: Western blot analysis of BiP in GFP- and BiP-overexpressing INS-1 cells. β-Actin served as a loading control. *B*: Effect of BiP overexpression on cytokine-induced cell death in INS-1 cells. BiP- and GFP-overexpressing INS-1 cells were cultured in the absence (□) or presence (■) of IL-1β (50 units/ml) and IFN-γ (100 units/ml). Cell death was measured using a cell death detection ELISA. Results are means ± SEM determined from three experiments performed in triplicate. ***P < 0.001 versus untreated GFP-overexpressing cells. *C*: Effect of BiP overexpression on cytokine-induced CHOP expression in INS-1 cells. Western blots analyzing CHOP protein were quantified by densitometry and are expressed as fold change compared with untreated GFP-overexpressing cells. Results are means ± SEM determined from three experiments. *P < 0.05 for cytokine-treated BiP-overexpressing cells versus cytokine-treated GFP-overexpressing cells.

role of CHOP as a potential mediator of β-cell death, INS-1 cells were transfected with CHOP siRNA or control siRNA. CHOP siRNA transfection led to reduced CHOP expression in INS-1 cells exposed to palmitate (Fig. 8*A* and *B*) or cytokines (Fig. 8*E* and *F*) compared with control siRNA-transfected cells. This resulted in protection against palmitate-induced cell death (Fig. 8*D*) and reduced cleaved caspase-3 expression in response to palmitate (Fig. 8*A* and *C*). In contrast, siRNA-mediated reduction of CHOP expression had no effect on cell death (Fig. 8*H*) or cleaved caspase-3 expression (Fig. 8*E* and *G*) induced by cytokines. This evidence suggests that CHOP expression is necessary for lipid- but not for cytokine-induced β-cell death.

To further assess the role of CHOP as a potential mediator of cytokine-induced β-cell death, MIN6 cells were transfected with CHOP, CHOPΔBR (dominant-negative mutant), and GFP constructs (Supplementary Fig. 4*A*). By immunostaining, we confirmed that overexpressed CHOP was localized in the nucleus of transfected cells (not shown). Furthermore, induction of CHOP target genes Gadd34 and Car6 in CHOP-overexpressing cells was blocked in cells cotransfected with CHOP and CHOPΔBR (Supplementary Fig. 4*B*). In tissues other than β-cells, overexpression of CHOP is sufficient to induce cell death (15,27–30). Here, under basal conditions, the rate of cell death in CHOP-overexpressing MIN6 cells was not different compared with control GFP-expressing MIN6 cells

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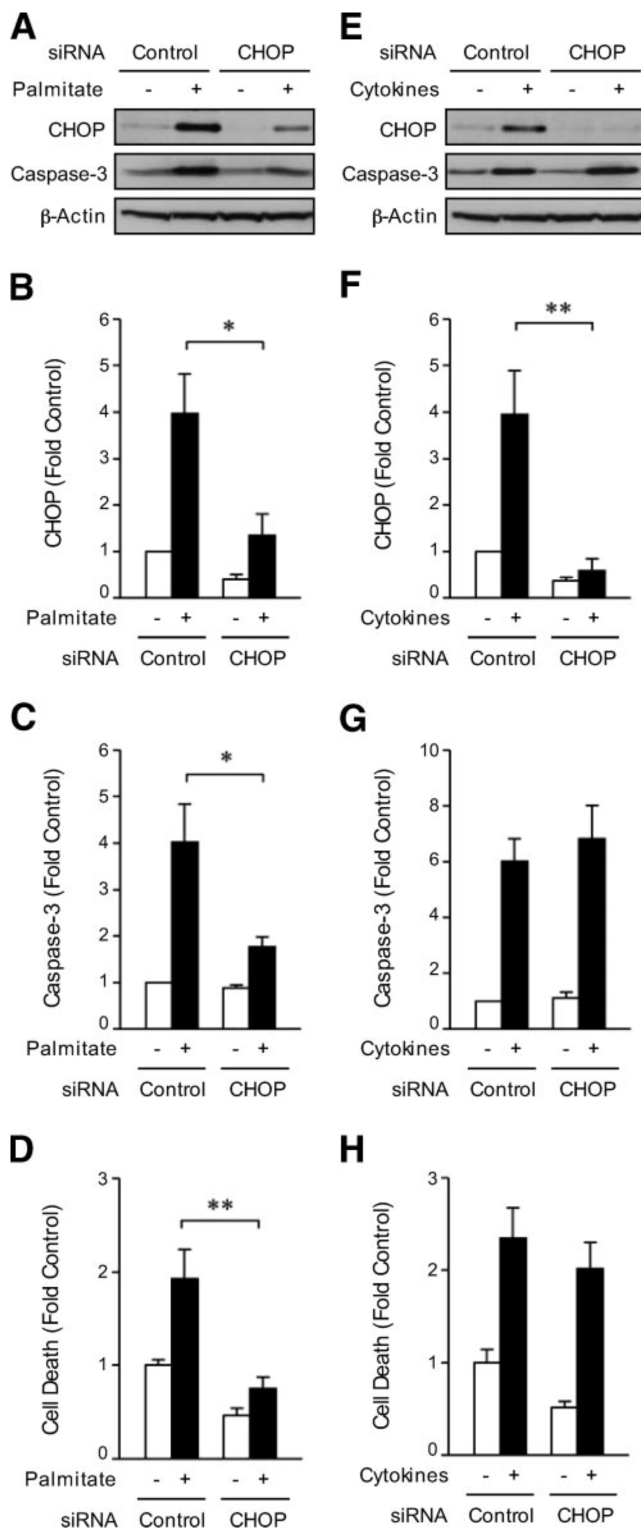

FIG. 8. siRNA-mediated silencing of CHOP expression in INS-1 cells protects against palmitate- but not against cytokine-induced cell death. INS-1 cells were transfected with CHOP ON-TARGET*plus* SMARTpool siRNA or negative control nontargeting siRNA using DharmaFECT Transfection Reagent and treated with 0.4 mmol/l palmitate coupled to 0.92% BSA (*A–D*) or IL-1β (50 units/ml) and IFN-γ (100 units/ml) (*E–H*). *A* and *E*: Western blot analysis of CHOP and cleaved caspase-3. β-Actin served as a loading control. CHOP (*B* and *F*) and cleaved caspase-3 bands (*C* and *G*) were quantified by densitometry and are expressed as fold change compared with untreated control siRNA. Results are means ± SEM, n = 5–6 in each group. *P < 0.05, **P < 0.01 versus treated control siRNA. *D* and *H*: Cell death was measured using a cell death detection ELISA. Results are means ± SEM, n = 6–7 in each group. **P < 0.01 versus palmitate-treated control siRNA.

TABLE 1
Changes to mRNA levels of genes involved in ER stress in islets isolated from pre-diabetic and diabetic NOD mice

	Pre-diabetic		Diabetic	
	C57BL/6J	NOD	C57BL/6J	NOD
ATF4	100 ± 2	82 ± 10	100 ± 13	62 ± 2*
BiP	100 ± 3	84 ± 5*	100 ± 2	87 ± 1**
GRP94	100 ± 2	82 ± 4**	100 ± 9	72 ± 4*
CHOP	100 ± 4	115 ± 17	100 ± 13	124 ± 8
Edem1	100 ± 2	90 ± 3	100 ± 19	90 ± 2
p58	100 ± 4	78 ± 5*	100 ± 10	59 ± 2**†
XBP1s	100 ± 3	77 ± 8*	100 ± 4	69 ± 2***
Fas	100 ± 6	180 ± 29*	100 ± 4	931 ± 93***††
MCP-1	100 ± 6	372 ± 50***	100 ± 18	5437 ± 284***††

Data are means ± SEM and are expressed as a percent of mRNA levels in islets isolated from control C57BL/6J mice at the same time. n = 3–5 independent determinations per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for pre-diabetic and diabetic NOD mice versus control C57BL/6J mice at the same time point. †$P < 0.05$, ††$P < 0.001$ for diabetic NOD mice versus pre-diabetic NOD mice.

(Supplementary Fig. 4*C*). This indicates that increased expression of CHOP alone is not sufficient to trigger MIN6 β-cell death. Furthermore, after cytokine (IL-1β + IFN-γ + TNF-α) exposure, the induction in cell death did not differ in GFP- and CHOP-overexpressing cells (Supplementary Fig. 4*C*). In CHOPΔBR-overexpressing MIN6 cells, the basal and cytokine-stimulated rate of cell death was similar to that in control GFP-expressing cells (Supplementary Fig. 4*C*). Thus, the overexpression or inhibition of CHOP in MIN6 cells does not influence cell viability under basal conditions or after exposure to cytokines. Similar results were found in INS-1 cells transfected with GFP, CHOP, and CHOPΔBR and subsequently treated with IL-1β + IFN-γ (data not shown).

Absence of ER stress gene activation in islets from NOD mice. Finally, we assessed whether ER stress was present in islets of NOD mice. mRNA levels were compared in islets isolated from non–diabetes-prone C57BL/6 mice and pre-diabetic and diabetic NOD mice. Blood glucose levels were 5.3 ± 0.4 mmol/l in pre-diabetic NOD mice and 13.4 ± 1.7 mmol/l in diabetic NOD mice. Using real-time RT-PCR, we determined that the expression of several ER stress genes were either downregulated or unchanged in NOD islets compared with control C57BL/6 islets. Islet mRNA levels were reduced for BiP, GRP94, and spliced XBP1 in pre-diabetic and diabetic NOD mice and for ATF4 in diabetic NOD mice (Table 1). p58 mRNA levels were reduced in islets of pre-diabetic NOD mice and were further reduced in islets of diabetic NOD mice ($P < 0.05$). CHOP and Edem1 mRNA levels were unchanged in islets of NOD mice both before and after the onset of diabetes compared with their control C57BL/6 mice (Table 1). In contrast, Fas and monocyte chemoattractant protein-1 mRNA levels were significantly increased in islets of pre-diabetic and diabetic NOD mice, indicative of the autoimmune and inflammatory insult. Thus, in contrast to *db/db* mice (19,31) and humans with type 2 diabetes (19,20), there was no increase in ER stress gene expression in islets of NOD mice.

DISCUSSION

The mechanisms by which cytokines stimulate pancreatic β-cell death have been the subject of much attention because of their potential relevance to type 1 diabetes

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(1–5). Previous studies have proposed a role of ER stress in cytokine-mediated cytotoxicity in β-cells (6–8). Here, we tested whether ER stress makes a necessary contribution to cytokine-induced β-cell death and whether it occurs in islets of the premier animal model of type 1 diabetes, the NOD mouse. Consistent with previous studies (6–8), we show that cytokines activate the UPR in clonal β-cells and cultured rodent islets. For the first time, we show that cytokines lead to increased phosphorylation of PERK and thus activation of this ER stress sensor pathway. This is accompanied by increased phosphorylation of EIF2α and transcriptional activation of ATF4 and CHOP. Importantly, we show that this activation of the UPR is not necessary for the associated β-cell death. Chemical and molecular approaches to increase the chaperone capacity of the ER failed to confer protection to the toxic effects of cytokines, suggesting an ER stress–independent mechanism of cell death. Furthermore, inhibition of CHOP had no influence over cytokine-mediated β-cell death. The relevance of this to type 1 diabetes is demonstrated by the fact that expressions of ER stress–responsive proteins are either reduced or not changed, rather than increased, in islets of NOD mice.

Importantly, our studies strengthen the case for ER stress, providing a mechanism for lipotoxicity and the reduced β-cell mass that characterizes type 2 diabetes (5,32). This is based on the observations that lipotoxicity was significantly reduced by CHOP knock down and by the treatments of PBA and TMAO, chemical chaperones that enhance ER functional capacity. This is consistent with our previous studies indicating that BiP overexpression partially protects against the effects of lipid overexposure (19). With ER stress potentially also providing a link between obesity and insulin resistance in liver and fat, our studies, together with others (8,19,20,24,33,34), suggest that ER stress may act as a common mechanism for β-cell failure and defective insulin signaling. The findings highlight the potential for increasing chaperone capacity of the ER as a therapeutic approach for type 2 diabetes treatment by preventing β-cell lipotoxicity and peripheral insulin resistance.

There are several differences in cytokine and palmitate signaling that may contribute to the divergent β-cell responses to ER stress (5). Whereas cytokines induce nuclear factor-κB activation, NO production, downregulation of the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b (SERC2b), and ER Ca^{2+} depletion (7,8), palmitate induces ER stress via nuclear factor-κB–, NO–, and ER Ca^{2+}–independent mechanisms (8,34). Furthermore, unlike palmitate (8,19), cytokines fail to activate ATF6 in β-cells (7), and, indeed, the presence of IFN-γ within the inflammatory milieu may actually lower the expression of ATF6/XBP1-targeted genes (chaperones) and thus ER stress defenses (35). We confirmed this in our own studies, finding slightly reduced BiP and p58 mRNA levels in 24-h IL-1β + IFN-γ–treated INS-1 cells (data not shown). There are also subtle differences in the way palmitate and cytokines regulate CHOP transcription (36). Moreover, prolonged phosphorylation of EIF2α leads to marked potentiation of the deleterious effects of fatty acids but not cytokines (37). Other studies have demonstrated that UPR activation may in fact inhibit cytokine signaling (38). Recently, cytokine-induced cell death and UPR activation were dissociated on the basis that IL-1 failed to stimulate caspase-3 activity (39).

Interestingly, BiP overexpression in NIT-1 insulinoma cells was shown to lower cytokine-mediated cytotoxicity (40), in opposition to our findings in INS-1 and MIN6 cells. However, it is important to note that in the studies with NIT-1 cells, BiP overexpression through unknown mechanisms led to lowered cytokine-induced NO production and increased superoxide dismutase activity (40). Thus, the mechanism by which BiP overexpression protects NIT-1 cells may be secondary to effects of lowering NO and/or increasing capacity of the cell to cope with reactive oxygen species rather than to attenuation of ER stress. In contrast, neither BiP overexpression (data not shown) nor PBA treatment (Fig. 3) lowered cytokine-induced NO production or iNOS expression in our studies.

In the present study, we provide evidence of differential signaling mechanisms for the activation of JNK by palmitate and cytokines. The ability of palmitate to increase XBP1 splicing and of PBA to lower palmitate-induced JNK1/2 phosphorylation is consistent with the possibility that ER stress is necessary for palmitate-induced JNK activation. On the other hand, the failure of cytokines to activate XBP1 and of PBA to reverse cytokine-induced JNK1/2 phosphorylation suggests that cytokine-mediated activation of JNK occurs independently of ER stress. Several previous studies have highlighted the importance of JNK protein kinases in cytokine- (41,42) and palmitate-induced (43) cell death and in T-cell–mediated killing of β-cells in NOD mice (44).

Our data suggesting a CHOP-independent mechanism of cytokine-induced β-cell death are at variance with prior studies by Oyadomari et al. (6) that apportioned CHOP with a modest contribution. This apparent discrepancy might relate to important differences in experimental design. Given that β-cells appear especially sensitive to disruption of ER stress signaling (16,17,45), it is possible that developmental changes and alterations in cell differentiation in CHOP (−/−) mice (15,46,47) may influence outcomes from experiments using their islets ex vivo. In contrast, our experiments were performed in otherwise normal β-cells without influence of potential changes in cell development or differentiation, with similar results found in pure β-cell populations of INS-1 and MIN6 cells.

We conclude that although both cytokines and palmitate induce ER stress in cultured β-cells, UPR activation is selectively necessary for lipotoxicity and not for cytokine-induced β-cell death. Furthermore, we did not find evidence of ER stress in islets of NOD mice, and it was not found in islets of humans with type 1 diabetes either (20). On the other hand, islets from type 2 diabetic patients may be susceptible to ER stress induction after metabolic challenge (48), thus underlining the potential importance of ER stress and UPR activation in the development of type 2 diabetes (19,20). These data support the view that different mechanisms are responsible for β-cell death in type 1 and type 2 diabetes.

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