Mild Endoplasmic Reticulum Stress Augments the Proinflammatory Effect of IL-1β in Pancreatic Rat β-Cells via the IRE1α/XBP1s Pathway

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The prevalence of obesity and type 1 diabetes in children is increasing worldwide. Insulin resistance and augmented circulating free fatty acids associated with obesity may cause pancreatic β-cell endoplasmic reticulum (ER) stress. We tested the hypothesis that mild ER stress predisposes β-cells to an exacerbated inflammatory response when exposed to IL-1β or TNF-α, cytokines that contribute to the pathogenesis of type 1 diabetes. INS-1E cells or primary rat β-cells were exposed to a low dose of the ER stressor cyclopiazonic acid (CPA) or free fatty acids, followed by low-dose IL-1β or TNF-α. ER stress signaling was inhibited by small interfering RNA. Cells were evaluated for proinflammatory gene expression by RT-PCR and ELISA, gene reporter activity, p65 activation by immunofluorescence, and apoptosis. CPA pretreatment enhanced IL-1β-induced, but not TNF-α-induced, expression of chemokine (C-C motif) ligand 2, chemokine (C-X-C motif) ligand 1, inducible nitric oxide synthase, and Fas via augmented nuclear factor-κB (NF-κB) activation. X-box binding protein 1 (XBP1) and inositol-requiring enzyme 1, but not CCAAT/enhancer binding protein homologous protein, knockdown prevented the CPA-induced exacerbation of NF-κB-dependent genes and decreased IL-1β-induced NF-κB promoter activity. XBP1 modulated NF-κB activity via forkhead box O1 inhibition. In conclusion, rat β-cells facing mild ER stress are sensitized to IL-1β, generating a more intense and protracted inflammatory response through inositol-requiring enzyme 1/XBP1 activation. These observations link β-cell ER stress to the triggering of exacerbated local inflammation. (Endocrinology 153: 3017–3028, 2012)

Type 1 diabetes (T1D) is a chronic autoimmune disease triggered by the interaction between genetic and environmental factors (1–3). The prevalence of T1D is increasing at an alarming rate, with a particularly marked increase in countries such as Poland, Romania, and the Czech Republic. It is expected that new cases of T1D in young European children will double between 2005 and 2020 (4). This seems to be a global phenomenon, with an increase in new cases of T1D also observed in the United States, Australia, New Zealand, and Canada (5–8). This fast increase of T1D incidence suggests the effect of environmental rather than genetic factors (4).

Modern Western society has easy access to high-fat and high-caloric food. This, coupled to lack of physical activity, favors the development of obesity in children and adults (9). Obesity is usually associated with type 2 diabetes (9), but there is also a correlation between body mass index in children and risk for T1D (10, 11).

Obesity increases the demand for insulin secretion, secondary to peripheral insulin resistance (9). In parallel, high

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concentrations of circulating free fatty acids (FFA), present in obese insulin-resistant patients, impair β-cell function and survival (12). Increased FFA, coupled to the augmented demand of insulin synthesis, pose a heavy demand on the β-cell endoplasmic reticulum (ER), the organelle where synthesis and folding of insulin takes place. This may lead to ER stress and its consequent adaptive response known as the unfolded protein response (UPR) (12). The overall goals of the UPR are to reduce the arrival of newly synthesized proteins in the ER, to increase the folding capacity of the organelle, and to facilitate the extrusion of misfolded proteins. In case of excessive and/or prolonged ER stress, the UPR triggers the mitochondrial pathway of apoptosis in β-cells (12, 13). The three main branches of the UPR are regulated by membrane-associated ER proteins, namely activating transcription factor-6, RNA-activated protein kinase-like eukaryotic initiation factor 2α kinase, and inositol-requiring enzyme 1α (IRE1α) (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org) (9, 12, 13).

Recent studies have shown that the UPR can modulate the inflammatory response (14, 15). Thus, ER stress enhances interferon-β production by macrophages after engagement of toll-like receptors (16), and macrophages facing ongoing ER stress are hyperresponsive to toll-like receptor stimulation, leading to sustained production of proinflammatory cytokines and chemokines (17). In line with these observations, ER stress enhances polyIC (a byproduct of viral infection)-induced innate immunoresponse in dendritic cells (18).

Because of the strong inflammation present in pancreatic islets during early T1D (insulitis) (1), the potential connection between ER stress and inflammation is of particular interest, because it could provide a mechanistic link between the growing incidence of obesity and T1D in young individuals.

The inflammatory mediators involved in insulitis are produced by the infiltrating immune cells and by the β-cells themselves (1). Previous findings by us (1, 19) and others (20) suggest that the transcription factor nuclear factor κB (NF-κB) has a central role in the regulation of β-cell inflammatory responses, including synthesis of several chemokines and cytokines (21, 22). NF-κB is a family of proteins that are usually associated and retained in the cytosol by the inhibitory protein inhibitor κB (IκB) (23). Upon stimulation with proinflammatory cytokines or lipopolysaccharide, a cascade of intracellular events leads to the activation of the IκB kinase complex that phosphorylates and targets IκB to proteasome degradation. NF-κB then translocates into the nucleus and modulates the expression of proinflammatory genes (23).

Against this background, we have presently studied whether mild ER stress modulates the proinflammatory responses of pancreatic β-cells exposed to low doses of the proinflammatory cytokines IL-1β or TNF-α. We observed that preconditioning β-cells with the ER stressor cyclopiazonic acid (CPA) or FFA boosted IL-1β but not TNF-α-mediated NF-κB activation, leading to increased expression of NF-κB target genes involved in the inflammatory response, e.g. inducible nitric oxide synthase (iNOS), Fas, chemokine (C-C motif) ligand 2 (CCL2), and chemokine (C-X-C motif) ligand 1 (CXCL1). X-box binding protein 1

**FIG. 1.** The combination of low-dose CPA and IL-1β, but not TNF-α, enhances NF-κB activity. A, INS-1E cells were transfected with the NF-κB reporter and a pRL-CMV plasmid used as internal control. Forty-eight hours after transfection, cells were pretreated with DMSO (white bars) or CPA (6.25 µM, gray bars) for 6 h and then exposed to cytokines (750 U/ml TNF-α or 0.5 U/ml IL-1β) for 6 h or left untreated (control condition). Luciferase was assayed, and the values obtained corrected by the internal control. Results are the mean ± SEM of five independent experiments; #, P < 0.05 and ###, P < 0.001 vs. DMSO (control) and ***, P < 0.001 as indicated; ANOVA. AB, Arbitrary unit. B, The same experimental design with CPA or DMSO pretreatment and combination with cytokines (as in A) was used to evaluate mRNA expression of Fas, CCL2, CXCL1, IκBα, and iNOS by real-time RT-PCR. Values were corrected by the housekeeping gene GAPDH. Results are the mean ± SEM of four to five independent experiments; #, P < 0.05; ##, P < 0.01; and ###, P < 0.001 vs. DMSO; ***, P < 0.001 as indicated, ANOVA.
(XBP1) and IRE1α, but not CCAAT/enhancer binding protein homologous protein (CHOP) knockdown (KD), prevented this exacerbation. XBP1 KD increased forkhead box O1 (FoxO1) protein expression, and FoxO1 KD further increased NF-κB activation and the expression of its target genes. These observations indicate that ER stress potentiates NF-κB activation in β-cells via the IRE1α/XBP1 branch of the ER stress response, providing evidence for cross talk between a specific branch of the UPR and β-cell inflammatory responses.

Materials and Methods

Culture of INS-1E cells and fluorescence-activated cell sorting (FACS)-purified rat β-cells

The rat insulinoma cell line INS-1E [kindly provided by C. Wollheim (Centre Medical Universitaire, Geneva, Switzerland) and used between passages 60 and 71] was cultured in RPMI 1640 GlutaMAX-I, containing 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 1 mM Na-pyruvate, and 50 μM 2-mercaptoethanol (24).

Pancreatic islets were isolated from male Wistar rats (Charles River Laboratories, Brussels, Belgium), treated following the guidelines of the Belgian Regulations for Animal Care and with approval from the local Ethical Committee. Rat islets were dispersed and β-cells purified by FACS (FACSAria, BD Bioscience, San Jose, CA) (25, 26). The preparations used contained 93 ± 2% (n = 9) β-cells. β-Cells were cultured in Ham’s F-10 medium containing 10 mM glucose, 2 mM glutamine, 50 μM 3-isobutyl-L-methylxanthine, 0.5% fatty acid-free BSA (Roche, Indianapolis, IN), 5% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The same medium but without FBS was used during the treatments described below.

Cell treatment, NO, CXCL1, and CCL2 measurements

Cytokine concentrations were selected based on dose-response studies with recombinant murine TNF-α (range of 50–1000 U/ml; Innogenetics, Ghent, Belgium) or recombinant human IL-1β (range of 0.005–10 U/ml; R&D Systems, Abingdon, UK), aiming to find the lowest dose that induced consistent NF-κB stimulation. These are arbitrary concentrations, because there are no available data on the actual cytokine concentrations present in the islets during the early- or preinsulitis period. INS-1E cells and rat primary β-cells were pretreated with 6.25
μM CPA (Sigma-Aldrich, Steinheim, Germany), a concentration previously shown by us in dose-response studies to induce mild ER stress (27). CPA was dissolved in dimethyl sulfoxide (DMSO); cells were cultured with CPA or DMSO (0.03%) alone for 6 h and then exposed to IL-1β (0.5 U/ml) or TNF-α (750 U/ml). Alternatively, INS-1E cells were preincubated with IL-1β (0.5 U/ml) for 6 h and then treated with CPA (6.25 μM) or DMSO, or exposed to CPA and IL-β together for 12 h. We also used 0.06 μM thapsigargin (THP) (Sigma, St. Louis, MO) to induce ER stress (28). For FFA exposure, INS-1E cells were cultured in medium containing 0.85% FFA-free albumin (Fraction V; Roche) and 1% FBS. Oleate and palmitate (Sigma-Aldrich) were dissolved in 90% ethanol (29–32) and used in a 1:1 ratio at a final concentration of 0.6 mM. This FFA mixture induces mild ER stress without triggering β-cell apoptosis (29). After 24 h of exposure to the FFA mixture or 1.2% ethanol, INS-1E cells were treated with 0.5 U/ml IL-1β for 6 h. The same experimental design was used to study olate (0.3 mM) or palmitate (0.3 mM) preconditioning followed by IL-1β treatment. For the experiments with recombinant FasL, INS-1E cells were pretreated with 6.25 μM CPA for 6 h, treated with IL-1β for 6 h, and then exposed to recombinant human soluble FasL (0.1 μg/ml; Alexis, Lülfingen, Switzerland) and enhancer (1 μg/ml; Alexis) for 18 h. The selected concentrations for FasL and the enhancer induce apoptosis in rat β-cells (33). The cytokines and FasL were added to CPA/DMSO or FFA/ethanol-containing medium. In some experiments, the culture medium was collected for nitrite (a surrogate measure of NO production) determination (34), and for CXCL1 (R&D Systems) and CCL2 measurement by ELISA (Invitrogen, Paisley, UK). Apoptosis was determined using the nuclear dyes Hoechst 33342/prodium iodide (25, 35, 36).

mRNA extraction and real-time RT-PCR

mRNA was extracted from INS-1E cells and rat primary β-cells and reverse transcribed as described (25). Expression of target genes was determined by real-time RT-PCR using SYBR Green (25, 37) and comparison with a standard curve (38). Expression values were corrected by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized by the highest value of each experiment considered as 1. GAPDH expression is not modified under the present experimental conditions (21, 28). Primer sequences are described in Supplemental Table 1. XBP1 spliced (XBP1s) expression was evaluated by real-time RT-PCR as previously described (39, 40).

RNA interference

Specific small interfering RNA (siRNA) were used for the silencing of CHOP, IRE1α, XBP1, and FoxO1 (Supplemental Table 2). We have previously shown (40) and confirmed here that similar biological effects are observed after knocking down XBP1 with another siRNA. Allstars Negative Control siRNA (QIAGEN, Venlo, The Netherlands) was used as negative control. β-Cells were transfected overnight with 30 nM of siRNA mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) (41). The negative control siRNA (siControl) does not affect β-cell gene expression or insulin release, compared with nontransfected cells (41, 42). Cells transfected with siRNA were used for experiments 24–48 h after transfection.

Promoter reporter assay

INS-1E cells were transfected using Lipofectamine 2000 (Invitrogen). The constructs used were the pRL-CMV (Promega, Madison, WI), as an internal control, and the pNF-κB-Luciferase (BD Biosciences, Palo Alto, CA), the pNOS-1002 luc (35, 43) or the pFas-811 luc (44). Forty-eight hours after transfection, cells were treated with TNF-α or...
IL-1β for 6 h. Alternatively, cells were exposed to CPA or THP or to the combination of oleate plus palmitate for 6 or 24 h, and then IL-1β was added for 12 or 16 h. Luciferase activities were studied with the Dual-Luciferase Reporter Assay System (Promega) (35). The values obtained were corrected for the luciferase values of the internal control plasmid pRL-CMV and normalized by the highest value of each experiment considered as 1.

**Immunofluorescence and Western blotting**

INS-1E cells were cultured in polylysine-coated glass slides (BD Biosciences). Cells were fixed with 4% paraformaldehyde and permeabilized with 70% acetone-30% methanol. Slides were incubated for 1 h with goat serum, followed by overnight incubation at 4°C with rabbit anti-p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described (42). p65+ nuclei were evaluated as the hallmark of NF-κB activation (36). Results are from at least 500 cells per experimental condition and expressed in percentage of positive cells/total cells.

For Western blotting, cells were treated as described (45). Primary antibodies against FoxO1 (Cell Signaling, Danvers, MA) and α-tubulin (Sigma), as control for protein loading, were used. Horseradish peroxidase-conjugated donkey antirabbit or antimouse IgG was used as secondary antibody (Lucron Bioproducts, De Pinte, Belgium). Immunoreactive bands were identified by enhanced chemiluminescence (Pierce) or the ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis**

Data are expressed as mean ± SEM. Comparisons were performed by paired t-test or by ANOVA followed by Student’s t test with Bonferroni correction, as indicated. A P value of less than 0.05 was considered as significant.

**Results**

**Low doses of cytokines induce NF-κB activation and p65 nuclear translocation**

A dose response with TNF-α and IL-1β was done to determine the threshold for NF-κB activation (Supplemental Fig. 2A), based on which the concentrations of 750 U/ml TNF-α and 0.5 U/ml IL-1β were chosen for subsequent experiments. The promoter reporter studies (Supplemental Fig. 2A) were confirmed by immunofluorescence for the nuclear translocation of p65, the main cytokine-induced NF-κB family member in β-cells (35). The selected doses were compared with higher cytokine concentrations used in previous studies (22, 35). Both TNF-α at 750 U/ml and IL-1β at 0.5 U/ml induced clear NF-κB translocation to the nucleus (Supplemental Fig. 2B), although with somewhat lower intensity than 1000 U/ml TNF-α and 10 U/ml IL-1β.

**Pretreatment with CPA sensitizes INS-1E cells and primary β-cells to IL-1β-induced NF-κB activation and expression of proinflammatory genes**

The sarcoplasmic/ER Ca2+-ATPase blocker CPA was used to induce ER stress in β-cells. The low dose of CPA (6.25 μM) selected induces UPR markers but has limited impact on cell viability (27). Both TNF-α and IL-1β activated the NF-κB reporter (Fig. 1A). CPA induced a mild increase in reporter activity and potentiated IL-1β-induced, but not TNF-α-induced, NF-κB activation (Fig. 1A). Preexposure with IL-1β followed by CPA treatment (Supplemental Fig. 3A) or a coadministration of CPA plus IL-1β (Supplemental Fig. 3B) also enhanced NF-κB activation. We next quantified the mRNA expression of five well-known proinflammatory genes (22, 35).

**Fig. 4.** CPA enhances IL-1β-induced iNOS, CCL2, CXCL1, and Fas mRNA expression and NO production in FACS-purified primary β-cells. A, Rat β-cells were pretreated with CPA (6.25 μM) or DMSO for 6 h and then exposed or not to IL-1β (0.5 U/ml) for the time points indicated on the x-axis. Cells were collected, mRNA expression was assayed for iNOS, CCL2, Fas, and CXCL1 and corrected for the housekeeping gene GAPDH. Results are the mean ± SEM of five independent experiments; *, P < 0.05; **, P < 0.01; and ###, P < 0.001 vs. DMSO; *, P < 0.05 and **, P < 0.01 as indicated, ANOVA. B, The same protocol as in A was used, but β-cells were exposed to IL-1β or not for 24 h. The medium was collected and assayed for nitrite. Results are the mean ± SEM of four independent experiments; **, P < 0.01 as indicated, paired t test.
NF-κB target genes in β-cells, i.e., iNOS (43), CCL2 (46), CXCL1 (22), Fas (44), and IkBα (35). In keeping with the reporter assay (Fig. 1A), CPA pretreatment synergized with IL-1β but not with TNF-α (Fig. 1B). This cross talk between CPA-induced ER stress and IL-1β-induced NF-κB activation resulted in a marked increase in iNOS, CCL2, CXCL1, and Fas mRNA expression, whereas no change was observed for IkBα, which inhibits NF-κB activation (Fig. 1B). The expression of another gene implicated in the negative feedback regulation of the NF-κB pathway, namely A20, was not potentiated by CPA plus IL-1β compared with IL-1β alone (data not shown). Because CPA did not augment expression of TNF-α-induced NF-κB-related genes, subsequent experiments were performed using the combination CPA-IL-1β.

CPA induced expression of UPR genes downstream of the three main UPR pathways, namely CHOP, BiP, and XBP1s (Supplemental Fig. 4). Expression of these genes was only mildly (in the case of CHOP) or not modified by the subsequent addition of IL-1β or TNF-α (Supplemental Fig. 4). A time-course analysis (Fig. 2A) confirmed that CPA pretreatment favors a higher IL-1β-induced expression of cytokines and chemokines at 6 h and leads to prolonged expression of CCL2, iNOS, CXCL1, and IkBα up to 12–24 h. Use of reporter constructs containing the rat iNOS promoter region from nucleotide −1002 to +132 (piNOS-1002luc) (43) or six NF-κB binding sites coupled to luciferase (35) showed a basal CPA-induced promoter activity and 2-fold augmentation of the stimulatory effect of IL-1β (Fig. 2B), confirming regulation at the transcriptional level.

We next evaluated whether other chemical or physiological ER stressors, e.g., THP and the equimolar combination of oleate and palmitate, or the two FFA alone (29), also favored IL-1β-induced NF-κB activation. Oleate plus palmitate induces a mild ER stress without β-cell apoptosis (29), a finding confirmed in the present series of experiments (see below). Pretreatment with THP, oleate plus palmitate, or oleate and palmitate separately increased by 2-fold IL-1β-induced NF-κB activation, confirming the observation with CPA (Fig. 3, A and B, and Supplemental
Fig. 5A, respectively). Furthermore, the FFA markedly amplified IL-1β-induced iNOS, CCL2, and CXCL1 expression (Fig. 3C). Oleate and palmitate alone increased IL-1β-induced activation and augmented expression of some NF-κB-regulated genes (Supplemental Fig. 5B) but to a lesser extent than the two FFA together (Fig. 3C). We confirmed that the presently used concentrations of FFA induced the ER stress markers CHOP, BiP, and XBP1s (Supplemental Fig. 6). CPA-induced exacerbation of the proinflammatory effects of IL-1β was also observed in FACS-purified primary β-cells (Fig. 4A). Thus, preculture with CPA augmented IL-1β-induced CCL2, CXCL1, and iNOS expression. There was also a significantly increased NO production, measured as medium nitrite accumulation, indicating that iNOS mRNA induction by CPA is functional (Fig. 4B). This was further evaluated in INS-1E cells by measuring NO and the chemokines CXCL1 and CCL2 (Supplemental Fig. 7, A–C). CPA increased by 2-fold the IL-1β effect, confirming the mRNA observations. The CPA plus IL-1β-induced increase in Fas mRNA expression in INS-1E cells (Fig. 1B) was paralleled by a nearly 2-fold increase in apoptosis, compared with IL-1β or CPA alone, when β-cells were exposed to recombinant FasL (Supplemental Fig. 7D), indicating that the overexpressed Fas is functional.

**XBP1s, but not CHOP, mediates the exacerbation of the NF-κB-mediated proinflammatory response**

The above-described experiments identified a cross talk between ER stress and NF-κB pathways in β-cells. We next examined which of the ER stress-related signals mediate this cross talk.

Previous reports showed that the ER stress-induced transcription factor CHOP modulates cytokine expression (47). CPA, and especially CPA plus IL-1β, induced CHOP expression in INS-1E cells (Supplemental Fig. 4A). This induction was largely prevented by a specific siCHOP (Supplemental Fig. 8), but the KD failed to prevent CPA plus IL-1β-induced expression of iNOS, CCL2, CXCL1, and Fas. Induction of these genes by CPA plus IL-1β was identical when comparing cells transfected with siControl or siCHOP (Supplemental Fig. 8), indicating that CHOP up-regulation does not mediate the pro-inflammatory effects of CPA-induced ER stress.

Another branch of the ER stress response that has been implicated in the regulation of inflammatory cytokines/chemokines (16) and in the activation of innate immunity (17, 48) is the IRE1α/XBP1 pathway. CPA activated this pathway, as evidenced by XBP1 splicing (Supplemental Fig. 4C). We next transfected INS-1E cells with a siControl or a previously validated siRNA against XBP1 (siXBP1) (40). siXBP1, but not siControl, decreased basal and CPA-induced XBP1s mRNA expression by more than 70% (Fig. 5A). siXBP1 prevented CPA plus IL-1β-mediated enhancement of iNOS, CCL2, CXCL1, and Fas expression in INS-1E cells (Fig. 5A). These results were confirmed in INS-1E cells by the use of a second and previously validated siRNA (40) that targets a different region of XBP1 (Supplemental Fig. 9). To confirm the results obtained at the mRNA level, we examined the promoter activity of iNOS (see above) and Fas promoter reporters, both containing NF-κB-responsive elements (44). XBP1 KD prevented CPA plus IL-1β-
induced exacerbation of both promoter activities (Fig. 5B). To evaluate whether XBP1 directly modulates NF-κB activation, we performed a reporter assay with the previously described NF-κB construct (35) and immunofluorescent staining to identify p65 translocation into the nucleus. siXBP1, but not siControl, blocked the exacerbation of NF-κB activation, as evaluated by the NF-κB reporter (Fig. 5B, bottom panel) and nuclear translocation into the nucleus, as shown by a representative picture (Fig. 5C) and quantification of p65 nuclei (Fig. 5D).

Activation of XBP1 requires splicing by IRE1 (12). As expected, KD of IRE1 decreased both IRE1 and XBP1s expression (Fig. 6). Similar to siXBP1 (Fig. 5A), siIRE1 prevented CPA plus IL-1β-induced expression of iNOS, CCL2, CXCL1, and Fas (Fig. 6).

XBP1s-mediated FoxO1 inhibition potentiates IL-1β-induced NF-κB activation

The above-described experiments suggest that ER stress cross talks with the NF-κB pathway via XBP1s. Previous findings in mouse embryonic fibroblasts indicate that XBP1s directly binds to FoxO1 and targets it for proteasome degradation (49). Because FoxO1 may inhibit NF-κB activation (50), we next studied FoxO1 expression in INS-1E cells transfected with XBP1 or siControl and then treated as in Fig. 5A (Supplemental Fig. 10). XBP1 KD alone increased by 2-fold FoxO1 protein expression (Supplemental Fig. 10A) without changing its mRNA expression (Supplemental Fig. 10B). We next investigated whether FoxO1 has a role in the NF-κB-mediated proinflammatory response of β-cells exposed to CPA plus IL-1β. FoxO1 KD sensitized the cells to the IL-1β treatment, inducing an activation of the NF-κB reporter comparable with the one observed after CPA plus IL-1β in the presence of the siControl (Fig. 7A). Pretreatment with CPA further increased NF-κB activity when FoxO1 was silenced. siFoxO1 decreased FoxO1 mRNA expression by more than 60% (Fig. 7B). FoxO1 KD further increased iNOS, CCL2, and Fas mRNA expression when cells were treated with CPA plus IL-1β, confirming its role as a negative regulator of the NF-κB pathway in β-cells.

These converging findings suggest that XBP1s augments the activation of the NF-κB pathway and consequent increase in the expression of proinflammatory genes at least in part via FoxO1 inhibition in pancreatic β-cells.

Discussion

In the present study, we evaluated the pancreatic β-cell response to low-dose proinflammatory cytokines after preconditioning with mild ER stress. ER stress enhances
the proinflammatory effect of IL-1β but not TNF-α. This effect is mediated by the IRE1α/XBP1s branch of the UPR via modulation of FoxO1 and the key proinflammatory transcription factor NF-κB.

It is thought that in T1D, a succession of environmental factors hits the β-cells of genetically susceptible individuals, causing local pancreatic islet inflammation and eventually apoptosis (1, 2, 7). The exact chronology and nature of events taking place in the period preceding clinical diagnosis of T1D is not known. The slow progression of the disease suggests that the triggers and/or boosters are probably mild and subclinical events, generating a chronic proinflammatory and hostile environment for pancreatic β-cells (1–3). The ER stress and the NF-κB pathways have been suggested to act alone (12, 36) or in combination (14, 15) in the pathogenesis of T1D and other autoimmune diseases. Macrophages and dendritic cells preconditioned with mild ER stress hyperrespond to inflammatory signals (51), exacerbating the expression of genes and proteins involved in the innate immunoresponse (16, 17). On the other hand, a low-grade ER stress may evoke a physiological oxidative (or other) stress response in some cell types that pre-adapts them to cope with a later oxidative stress, ER stress or inflammatory assault (52). Therefore, it could be that the observed inflammatory response is part of an adaptive response to the low-grade chronic ER stress.

CPA was used in the present study to induce a mild and “pure” ER stress based on previous findings from our group (27). The observations made with CPA were confirmed by β-cell exposure to the pathophysiologically relevant stimulus of oleate and/or palmitate (12). The present results indicate that sensitizing β-cells with ER stress inflates their response to a subsequent mild proinflammatory cytokine exposure. Indeed, iNOS, Fas, CCL2, and CXCL1 expression were enhanced and prolonged over time in INS-1E cells and primary β-cells treated with IL-1β. Increased expression of iNOS and Fas sensitizes β-cells to apoptosis (53), whereas the chemokines CCL2 and CXCL1 contribute to the attraction of mononuclear cells involved in the build up of insulitis (1). Interestingly, there was no increase in the expression of genes involved in the negative feedback regulation of NF-κB, namely IkBα and A20. IkBα and A20 are activated at early time points after cytokine exposure, whereas other genes, such as the chemokines CCL2 and CCL5, are up-regulated with higher doses and at later time points (54–56). Thus, our results indicate that CPA plus IL-1β treatment preferentially increases the expression of “late genes” involved in the proinflammatory response rather than the “early genes” contributing for the negative feedback loop. The mechanisms involved in these differential effects of ER stress remain to be clarified, but the net result will be an augmented and prolonged proinflammatory signal.

There was no enhancement in NF-κB activity or its downstream genes in β-cells treated with CPA and TNF-α, suggesting that the proinflammatory effects of ER stress in β-cells are context dependent. According to our previous results in β-cells, TNF-α induces NF-κB but to a lesser extent than IL-1β (35). In the present experiments, this difference was not an issue, because the cytokine concentrations were selected to allow similar activation of NF-κB; this required much higher concentrations of TNF-α (750 vs. 0.5 U/ml IL-1β). Because ER stress inhibits protein translation (12), an explanation for the weaker induction of the NF-κB pathway by TNF-α could be a decrease in the expression of TNF-receptor 1. We did not observe, however, any inhibitory effect of CPA on the TNF-receptor 1 protein expression (data not shown). Thus, the observed difference between the impact of the UPR on TNF-α- and IL-1β-induced NF-κB activation must lay in other intracellular events that remain to be clarified.

Previous findings in other cell types suggest that the synergistic effect of ER stress and proinflammatory me-

![Proposed model for NF-κB potentiation by ER stress in β-cells.](https://academic.oup.com/endo/article-abstract/153/7/3017/2423616/endo.endojournals.org)
diators is at least in part dependent on the splicing of XBP1 by IRE1α (16–18). The outcome of this cross talk is a maximization of the innate immunoresponse to pathogens, including enhanced TNF-α, IL-6, and interferon-β expression (16–18). Interestingly, the IRE1α/XBP1 pathway is activated by obesity (57), an environmental factor potentially related to T1D (10, 58). Here, we have shown that knocking down IRE1α or XBP1s in β-cells prevents the boost in the production of chemokines (CCL2 and CXCL1) and other proinflammatory agents induced by ER stress. Studies in macrophages indicate that XBP1s directly binds to the promoter region of some immune-related genes, enhancing their expression (16, 17). Moreover, XBP1s inhibits FoxO1 (49), and FoxO1 negatively regulates the NF-κB pathway (50). Our findings suggest that the ER stress-induced exacerbation of the proinflammatory response is mediated at least in part by XBP1s inhibition of the NF-κB negative regulator FoxO1. Indeed, FoxO1 silencing further increases NF-κB activation after CPA plus IL-1β. Of note, Martinez et al. (59) found that FoxO1 contributes to fatty acid-induced clonal mouse β-cell apoptosis, suggesting that FoxO1 may modulate different aspects of the response of β-cells to ER stress depending on species and experimental context.

We propose in Fig. 8 an overview of our main findings. Metabolic stimuli, such as obesity and insulin resistance, induce chronic ER stress in β-cells, with the consequent activation of XBP1s through the IRE1α branch of the UPR (12, 57). This preexistent ER stress, even if mild, sensitizes β-cells to low concentrations of IL-1β, which may be locally released by macrophages due to viral infection or exposure to other “danger signals” (1). This will cause a more intense and protracted local production of chemokines and other inflammatory mediators via the transcription factor NF-κB. The intersection between the UPR and NF-κB pathways may play a relevant role in the early stages of the insulitis, aggravating and prolonging this inflammation in obese and insulin-resistant children and accelerating the development of T1D.

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