The Role of Nitric Oxide and the Unfolded Protein Response in Cytokine-Induced β-Cell Death

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OBJECTIVE—The unfolded protein response (UPR) is a conserved cellular response designed to alleviate damage and promote survival of cells experiencing stress; however, prolonged UPR activation can result in apoptotic cell death. The UPR, activated by cytokine-induced nitric oxide (NO) production, has been proposed to mediate β-cell death in response to cytokines. In this study, the role of UPR activation in cytokine-induced β-cell death was examined.

RESEARCH DESIGN AND METHODS—The effects of cytokine treatment of rat and human islets and RINm5F cells on UPR activation, NO production, and cell viability were examined using molecular and biochemical methodologies.

RESULTS—UPR activation correlates with β-cell death in interleukin (IL)-1–treated rat islets. NO mediates both cytokine-induced UPR activation and β-cell death as NO synthase inhibitors attenuate each of these IL-1–stimulated events. Importantly, cytokines and tunicamycin, a classical UPR activator, induce β-cell death by different mechanisms. Cell death in response to the classical UPR activator is associated with a 2.5-fold increase in caspase-3 activity, while IL-1 fails to stimulate caspase-3 activity. In addition, cell death is enhanced by ~35% in tunicamycin-treated cells expressing an S51A eIF2α mutant that cannot be phosphorylated or in cells lacking PERK (protein kinase regulated by RNA/endoplasmic reticulum–like kinase). In contrast, neither the absence of PERK nor the expression of the S51A eIF2α mutant affects the levels of cytokine-induced death.

CONCLUSIONS—While cytokine-induced β-cell death temporally correlates with UPR activation, the lack of caspase activity and the ability of NO to attenuate caspase activity suggest that prolonged UPR activation does not mediate cytokine-induced β-cell death. ***Diabetes*** **57:124–132, 2008**

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Received for publication 11 July 2007 and accepted in revised form 2 October 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 10 October 2007. DOI: 10.2337/db07-0944.

ATF, activating transcription factor; CHOP, C/EBP homologous protein; DEA-NO, (Z)-1(*N*,*N*-diethylamino) diazen-1-ium-1,2-diolate; ER, endoplasmic reticulum; HMGB1, high-mobility group box 1 protein; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IRE, inositol-requiring enzyme; MEF, mouse embryonic fibroblast; NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; PERK, protein kinase regulated by RNA/ER–like kinase; PGJ_2, prostaglandin J_2; UPR, unfolded protein response; XBP, X-box binding protein.

Type 1 diabetes is an autoimmune disease characterized by an inflammatory reaction in and around pancreatic islets of Langerhans that results in the selective destruction of insulin-secreting β-cells (1). Cytokines, released during insulitis, have been shown to inhibit β-cell function and to induce islet destruction (2,3). The mechanism by which cytokines impair β-cell function includes the expression of the inducible isoform of nitric oxide (NO) synthase (iNOS), resulting in the production of high levels of NO (4–8). Interleukin (IL)-1 alone is sufficient to stimulate iNOS expression in rat islets, while IL-1 plus interferon (IFN)-γ are the minimal combination of cytokines required to stimulate iNOS expression and NO production in mouse and human islets (2,9,10). In response to IL-1 or combinations of IL-1 and IFN-γ, the insulin-secreting β-cell is the primary islet source of iNOS and NO (8,11). NO exerts its inhibitory effects on β-cell function by targeting iron-sulfur–containing enzymes, such as aconitase of the Krebs cycle and the electron transport chain, resulting in the inhibition of glucose oxidation to CO_2 (12) and reductions in cellular levels of ATP (9,13). It is this reduction in ATP that is responsible for the inhibitory actions of cytokines on insulin secretion, as glucose-induced insulin secretion requires the ATP-dependent inhibition of K^+ channel activity, allowing for depolarization and Ca^{2+}-dependent exocytosis (14,15).

Although it has been well established that NO is the primary mediator of the inhibitory actions of cytokines on β-cell function, the mechanisms responsible for the loss of β-cell viability in response to cytokines have not been fully identified. There have been many reports suggesting that cytokines stimulate β-cell apoptosis (16,17) and that NO may or may not participate in this process. Factors contributing to β-cell death include the type of cytokines present (IL-1 alone vs. IL-1 plus IFN-γ), the length of cytokine exposure (24–48 h vs. 7 days), and the source of islets (where rat is thought to be more sensitive to NO than mouse and human islets [16]). More recently, biochemical and molecular studies have been used to support a NO-dependent necrotic mechanism of β-cell death (rat and human) in response to cytokines, such as IL-1 or combinations of IL-1 and IFN-γ (18,19). While the debate regarding the type of cell death induced by cytokines and the role of NO in this process will continue, it is clear that cytokines kill β-cells.

Recent studies have proposed that cytokine-induced β-cell death is due to the induction of endoplasmic reticulum (ER) stress and prolonged unfolded protein response (UPR) activation (20,21). The UPR, a conserved cellular response designed to protect cells from stress, is activated by protein overload in the ER, nutrient deprivation, meta-

bolic changes, viral infection, and the generation of free radicals. There are also known chemical activators of the UPR such as the N-linked glycosylation inhibitor tunicamycin, the SERCA (sarcoplasmic ER calcium inhibitor) thapsigargin, and the thiol-reducing reagent dithiothreitol (12). Once activated, de novo protein synthesis is attenuated by phosphorylation of the translational initiation factor eukaryotic initiation factor-2α, while the transcription of genes involved in resolving the stress is induced (12). The major transducers of the UPR include *1*) protein kinase regulated by RNA/ER–like kinase (PERK, PEK, EIF2AK3), an eIF2α kinase that attenuates global protein synthesis concurrent with preferential translation of activating transcription factor (ATF)-4 mRNA encoding a key UPR transcription activator (12,22); *2*) ATF-6, which enhances the expression of chaperones that assist in protein folding; and *3*) inositol-requiring enzyme (IRE)1α, which splices X-box binding protein (XBP)1 mRNA, leading to expression of an active version of the XBP1 transcriptional activator that participates in the expression of components of ER-associated degradation designed to relieve protein overload (12,22). Once activated, the UPR attempts to remedy the cellular stress; however, if the insult is overwhelming or persists for an extensive time, apoptotic cascades may be initiated (12,22,23). β-Cells are highly sensitive to ER stress and prolonged UPR activation. Early studies showed that UPR activators such as thapsigargin stimulate β-cell death by apoptosis (24). The importance of translational control in regulating β-cell viability was first identified using PERK knockout mice (25,26). In these mice, both the exocrine and endocrine pancreas developed normally; however, within 21 days after birth, there is increased β-cell death resulting in diabetes. In addition, mice expressing a homozygous mutation to alanine at S51, the eIF2α phosphorylation site, died shortly after birth because of hypoglycemia (23). Thus, the ability to regulate translation via eIF2α is required for β-cell survival. Recently, the IRE1α signaling cascade has been shown to regulate insulin biosynthesis in response to glucose, further emphasizing the importance of the UPR in β-cell survival (27).

While the UPR is required for normal development and physiological function of β-cells, much less is known about the role of this pathway in the survival of β-cells undergoing oxidative damage following cytokine treatment. A number of recent studies have provided a clear association of cytokine-induced UPR activation with the inhibitory actions of cytokines on β-cell function (20,21). Cytokines have been shown to stimulate eIF2α phosphorylation, C/EBP homologous protein (CHOP) expression, and IRE1α activation in rat islets and insulinoma cell lines in a time-dependent manner that correlates with the production of NO, and importantly, all of these events are prevented by inhibitors of NOS (20,21). In addition, cytokine-induced β-cell death is modestly attenuated in islets isolated from Chop-deficient mice compared with wild-type controls (28). Since there is a strong correlation between β-cell production of NO and UPR activation and since prolonged UPR activation is associated with β-cell death by apoptosis, the potential role of UPR activation in cytokine-induced β-cell death has been examined.

RESEARCH DESIGN AND METHODS

Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). RINm5F (rat insulinoma) cells were obtained from Washington University Tissue Culture Support Center (St. Louis, MO). Human islets were obtained from the Islet Cell Resource at Washington University (St. Louis, MO) and University of Miami (Miami, FL). RPMI-1640, CMRL-1066 tissue culture medium, L-glutamine, penicillin, and streptomycin were from GIBCO-BRL (Grand Island, NY). PERK$^{-/-}$, A/A, wild-type, and IRE1α$^{-/-}$ mouse embryonic fibroblasts (MEFs) have previously been described (29,30). Empty pETFVA vector and pETFVA-eIF2α (S51A) were gifts from Dr. Randal Kaufman (University of Michigan), and pcDNA3-PERK (K621M) has been previously described (31). FCS was obtained from Hyclone Laboratories (Logan, UT). Human recombinant IL-1β and human and mouse recombinant IFN-γ were obtained from PeproTech (Rocky Hill, NJ). Prostaglandin J_2 (PGJ_2) was from Cayman Chemical (Ann Arbor, MI). Enhanced chemiluminescence was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Neutral red, camptothecin, and tunicamycin were obtained from Sigma (St. Louis, MO). Caspase-3 fluorometric assay was from R&D Systems (Minneapolis, MN). N^G-monomethyl-L-arginine (NMMA) and (Z)-1(*N*,*N*-diethylamino) diazen-1-ium-1,2-diolate (DEA-NO) were purchased from Axxora (San Diego, CA). Rabbit anti–phospho-eIF2α and rabbit anti-eIF2α were from Stressgen (San Diego, CA). Rabbit anti–caspase-3 and rabbit anti-active caspase-3 were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-CHOP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti–high-mobility group box 1 protein (HMGB1) was from BD Pharmingen (San Diego, CA). Horseradish peroxidase–conjugated donkey anti-rabbit and donkey anti-mouse antisera were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents were obtained from commercially available sources.

Islet isolation and cell culture. Islets were isolated from male Sprague-Dawley rats (250–300 g) by collagenase digestion as previously described (32). Islets were cultured overnight in complete CMRL-1066 (containing 2 mmol/l L-glutamine, 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37°C under an atmosphere of 95% air and 5% CO_2 before experimentation. RINm5F cells and MEFs were removed from growth flasks by treatment with 0.05% trypsin and 0.02% EDTA for 5 min at 37°C, washed two times with RPMI-1640 or Dulbecco's modified Eagle's medium, respectively, and plated at the indicated densities.

RINm5F cells were transiently transfected using the Amaxa Nucleofector electroporator (Amaxa Biosystems, Gaithersburg, MD) with 2 μg pEGFP (enhanced green fluorescent protein) and pETFVA-eIF2α (S51A) or pcDNA3-PERK (K621M) as previously described (33). Using this method, we routinely obtain a transfection efficiency of 50–60%, as determined by EGFP expression.

Western blot analysis. Western blot analysis of RINm5F cells and islet lysates were performed using semidry transfer conditions as previously described (34). All primary antibodies were diluted 1:1,000. Horseradish peroxidase–conjugated donkey anti-rabbit and donkey anti-mouse secondary antibodies were used at 1:7,000 and 1:5,000, respectively. Antigens were detected by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham Pharmacia Biotech, Piscataway, NJ).

Nitrite determination. Nitrite production was determined by adding 50 μl of the Greiss reagent to 50 μl culture supernatant (35). The absorbance was measured at 540 nm, and nitrite concentrations were calculated from a sodium nitrite standard curve.

Xbp1 splicing assay. Total RNA was isolated from rat islets or RINm5F cells using the RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed using oligo(dT) and the reverse transcriptase Superscript Preamplification System (Invitrogen, Carlsbad, CA), according to the manufacturer's specifications. A fragment of Xbp1 was then amplified using the following primers: 5′-AAACAGAGTAGCAGCGCAGACTGC-3′ (forward) and 5′-GGATC TCTAAAACTAGAGGCTTGGTG-3′ (reverse) (36). PCR products were digested with *Pst*I for 1h at 37°C, and samples were analyzed on a 2% agarose gel containing ethidium bromide. IRE1α catalyzes the splicing of a 26-nucleotide fragment from the Xbp1 mRNA transcript that contains a *Pst*I digestion site. Therefore, when IRE1α is active, Xbp1 is spliced, resulting in a fragment of ~600 bp, while unspliced Xbp1 appears as two fragments of ~300 bp due to the presence of the *Pst*I restriction site. GAPDH was amplified using previously described primers (37) to serve as a control for cDNA amounts.

Cell viability. Cell viability was determined using the neutral red assay as previously described (19,38). Following treatment, RINm5F cells (1.0×10^5 cells/200 μl RPMI-1640) were washed and then incubated in 200 μl fresh media containing neutral red (50 μg/ml) for 2 additional hours at 37°C. Following this incubation, the excess neutral red was discarded by washing and the cells were fixed with 1% formaldehyde-1% $CaCl_2$. The neutral red was extracted from the cells using 100 μl 50% ethanol/1% acetic acid lysing solution. The amount of neutral red in the lysate was measured at a wavelength of 540 nm. The percentage of dead cells was determined by comparing neutral red uptake of treated samples with uptake by the unstimulated control, where neutral red uptake by the unstimulated control cells is set at 100% viable (or the absence of cell death). For a limited number of

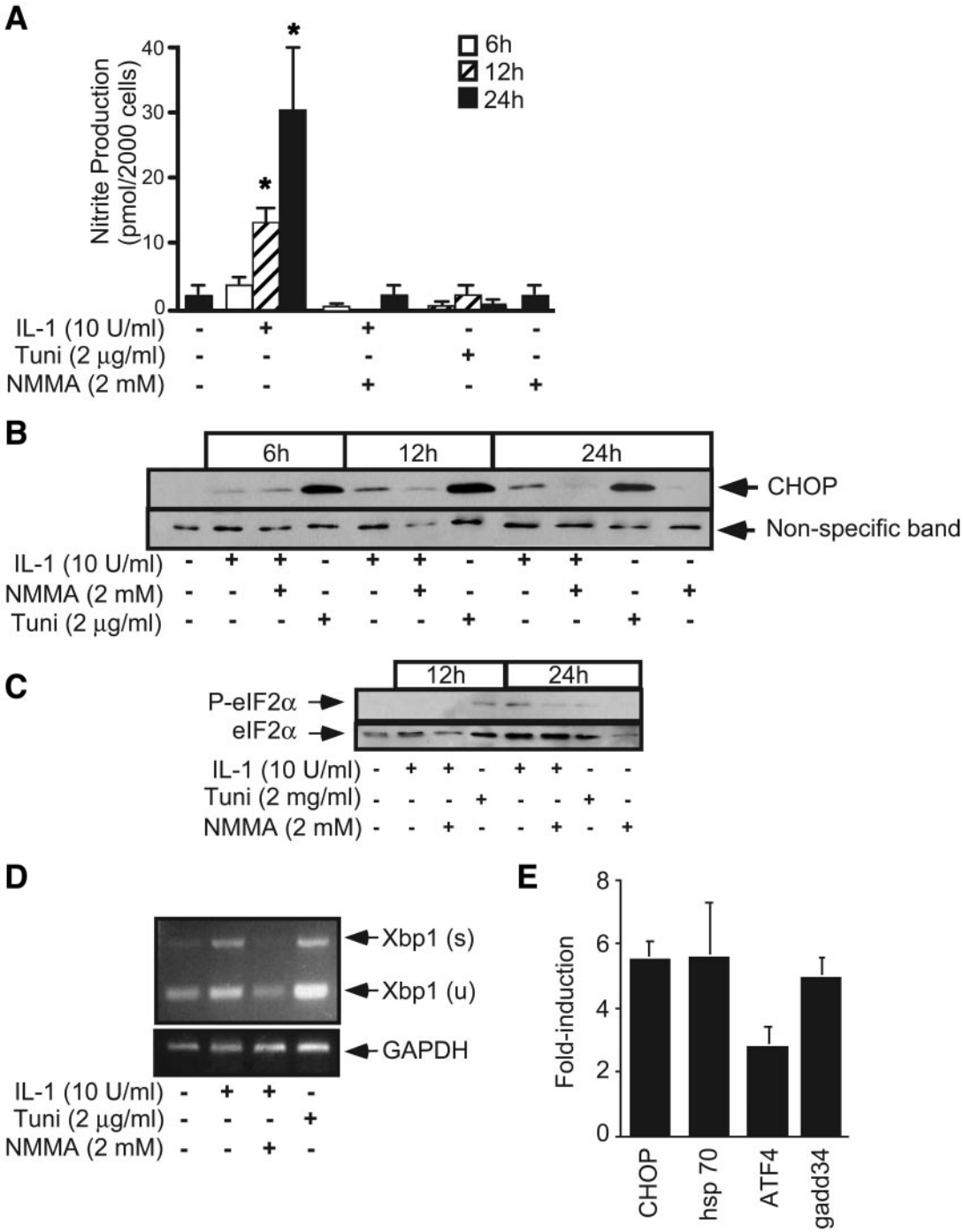

FIG. 1. IL-1 induces UPR activation in β-cells in an NO-dependent manner. RINm5F cells (2.0 × 10^5 cells/400 μl RPMI-1640) were treated for 6, 12, or 24 h with IL-1 (10 units/ml), NMMA (2 mmol/l), and tunicamycin (2 μg/ml), as indicated. The supernatants were removed for nitrite determination (*A*), and CHOP expression was examined by Western blot analysis of the cell lysate (*B*). After incubating for 12 or 24 h with IL-1, NMMA, and tunicamycin, rat islets (150 islets/400 μl cCMRL) were isolated and eIF2α phosphorylation and total eIF2α levels determined by Western blot analysis (*C*). IRE1α activity was assayed by examining Xbp1 splicing. Total RNA was isolated from rat islets (150 islets/400 μl cCMRL) treated for 24 h with the indicated concentrations of IL-1, NMMA, or tunicamycin (Tuni). Xbp1 mRNA was amplified via RT-PCR and digested with *Pst*I, resulting in detection of the spliced Xbp1(s) and unspliced XBP1(u). GAPDH mRNA accumulation was used as an internal loading control (*D*). CHOP, hsp 70, ATF-4, and gadd34 mRNA accumulation in human islets treated for 3 h with 0.5 mmol/l DEA-NO was examined by Affymextrix Genechip analysis (*E*). Results are means ± SE of 3–4 individual experiments (*A* and *E*) or representative of three independent experiments (*B* and *C*).

experiments, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used as an index of cell viability (19).

Caspase-3 activity. Following treatment, RINm5F cells (2.0 × 10^5 cells/400 μl RPMI-1640) or isolated rat islets (150 islets/400 μl CMRL-1066) were isolated, washed, and then lysed in 55 μl lysis buffer. Caspase-3 activity was determined using 50 μl lysate according to the manufacturer's specifications (Caspase-3 Fluorometric Assay Kit; R&D Systems). The relative fluorescence units were normalized to total protein content of sample determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Treated samples were compared with unstimulated controls, and results are reported as fold increase over control.

mRNA expression profiling. The effects of NO on gene expression were evaluated by Affymetrix gene chip analysis. In brief, human islets (1,000/condtion) were treated for 3 h with or without NO donor DEA-NO (0.5

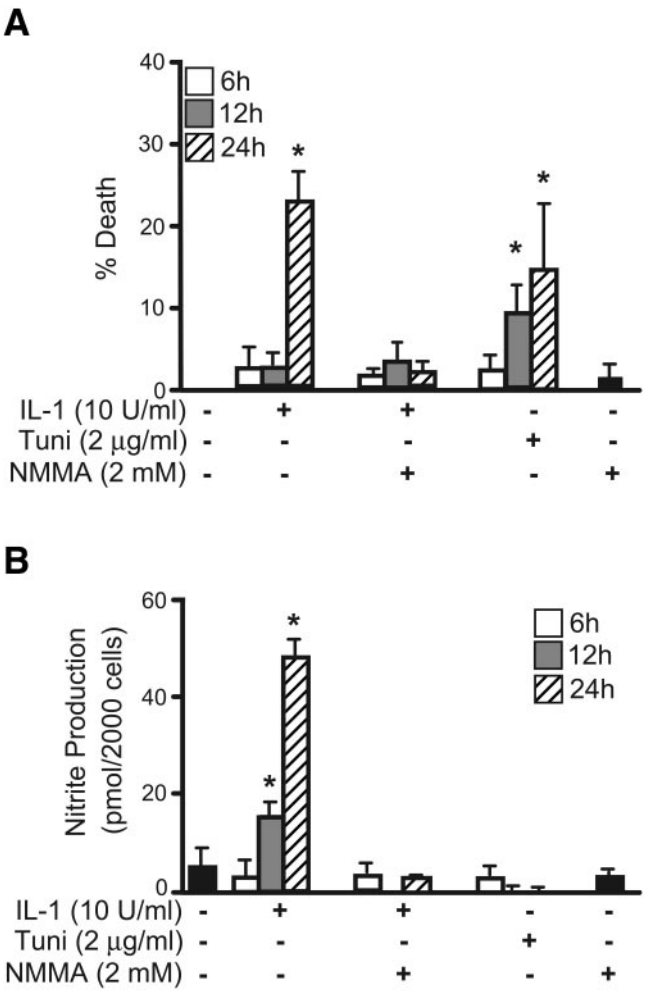

FIG. 2. IL-1 induces RINm5F cell death. After incubating for 6, 12, or 24 h with the indicated concentrations of IL-1, NMMA, and tunicamycin (Tuni), RINm5F cell (1.0×10^5 cells/400 μl RPMI-1640) viability was determined using the neutral red uptake assay (*A*). The culture supernatants were harvested for determination of nitrite production (*B*). Results are means ± SE of at least three independent experiments.

mmol/l). The islets were harvested by centrifugation and total RNA extracted using Trizol (as outlined by supplier, Gibco/BRL). The total RNA was further purified using the RNeasy Kit (Qiagen) and then used for expression profiling with Affymetrix U133 gene chips at the Washington University Gene Sequencing Facility (St. Louis, MO). Changes in mRNA levels, as determined by gene chip analysis, are the average of four experiments from four independent human islet preparations.

Statistics. Statistical comparisons were made between groups using one-way ANOVA. Significant differences between groups, treated versus unstimulated controls ($P < 0.05$, unless otherwise indicated), were determined by Newman-Keuls post hoc analysis.

RESULTS

NO activates the UPR in cytokine-treated β-cells. The UPR is a survival pathway that is activated in response to cellular stress. When cells are unable to sufficiently respond to and overcome the stress, a programmed death pathway leads to cellular demise. NO, the primary mediator of cytokine-induced β-cell damage and death (18,19,39,40), has been shown to activate the UPR in a number of cell types, including β-cells (20,21,41,42). Consistent with these previous studies, treatment of RINm5F cells (Fig. 1*A*) or rat islets (data not shown, reference 43) with IL-1 results in the time-dependent production of NO that is first apparent after a 12-h incubation, with an ~10-fold increase in nitrite formation after a 24-h incubation. Consistent with the time-dependent production of NO, IL-1 stimulates CHOP expression (Fig. 1*B*) in RINm5F cells and eIF2α phosphorylation in rat islets (Fig. 1*C*) that is first apparent after a 12-h incubation. Importantly, the NOS inhibitor NMMA, which prevents IL-1–induced NO production, also attenuates CHOP expression and eIF2α phosphorylation in response to IL-1. When active, IRE1α catalyzes the processing of a 26-nucleotide fragment (containing a *Pst*I restriction enzyme site) from Xbp-1 mRNA. Treatment of rat islets for 24 h with IL-1 results in IRE1α activation, as evidenced by the processing of Xbp1 mRNA (accumulation of the spliced form; Fig. 1*D*). NO mediates IRE1α activation, as Xbp-1 processing is attenuated by NMMA. The N-linked glycosylation inhibitor tunicamycin, which stimulates CHOP expression, eIF2α phosphorylation, and Xbp1 splicing (Fig. 1*B*–*D*), was used as a positive control for UPR activation in these experiments.

Similar to rodent islets and RINm5F cells, NO also activates the UPR in human islets. Treatment of human islets for 3 h with the NO donor compound DEA-NO results in a three- to sixfold increase in the accumulation of the UPR-responsive mRNAs encoding CHOP, ATF-4, and gadd34 (Fig. 1*E*). In addition, DEA-NO also stimulates an ~sixfold increase in the accumulation of hsp 70 mRNA in human islets. Overall, these findings, consistent with previous reports by our laboratory and others (20,21,42), show that cytokine treatment stimulates UPR activation in a NO-dependent manner in rodent and human islets.

NO mediates IL-1–induced RINm5F cell death. Using a biochemical assay of membrane integrity, the effects of 6-, 12-, and 24-h exposures to IL-1 on RINm5F cell viability were examined. As shown in Fig. 2*A*, a 24-h incubation with IL-1 results in a 23% reduction in RINm5F cell viability. NO is the mediator of cytokine-induced β-cell death, as IL-1–induced nitrite production correlates temporally with cell death (Fig. 2*B*) and NMMA prevents IL-1–induced β-cell death. UPR activation following treatment with tunicamycin is associated with a loss of RINm5F cell viability (Fig. 2*A*), and this action is independent of β-cell production of NO. Taken together, these findings correlate cytokine-induced iNOS expression and NO production with activation of an ER stress response and the induction of a death cascade in β-cells.

Regulation of caspase-3 activity in response to cytokines and UPR activators. Because NO activates the UPR and prolonged UPR activation is associated with cell death, the role of UPR activation as a potential mediator of cytokine-induced β-cell death was further investigated by examining the effects of cytokines and UPR activators on caspase-3 activity in RINm5F cells and rat islets. This executioner caspase is activated by both the intrinsic and extrinsic apoptotic pathways (44,45). Following a 24-h incubation, tunicamycin enhances caspase-3 activity in RINm5F cells (2.4-fold) and rat islets (2.5-fold; Fig. 3*A* and *B*). The activation of caspase-3 following tunicamycin treatment correlates with the ability of this UPR activator to induce RINm5F cell death (Fig. 2). Using the MTT assay as a second methodology to examine cell death, a 24-h treatment with tunicamycin induces the death of 30.0 ± 7.3% of RINm5F cells. The caspase-3 inhibitor IV (100 μmol/l; CalBiochem, San Diego, CA), which attenuates caspase-3 activity in islets and RINm5F cells (19), reduces tunicamycin-induced RINm5F cell death to 11.8 ± 5.1%. These findings suggest that caspase-3 is activated and

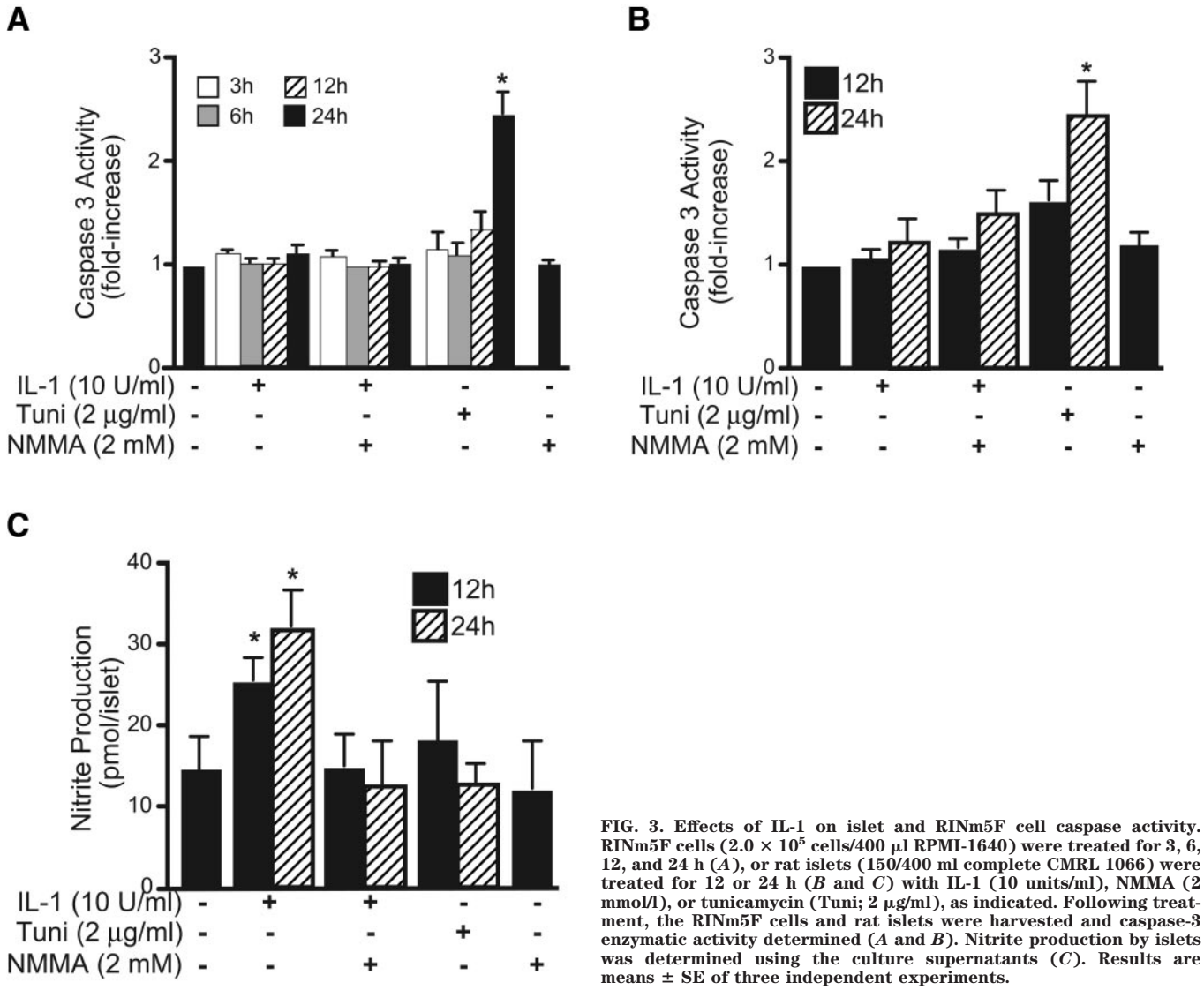

FIG. 3. Effects of IL-1 on islet and RINm5F cell caspase activity. RINm5F cells (2.0×10^5 cells/400 μl RPMI-1640) were treated for 3, 6, 12, and 24 h (*A*), or rat islets (150/400 ml complete CMRL 1066) were treated for 12 or 24 h (*B* and *C*) with IL-1 (10 units/ml), NMMA (2 mmol/l), or tunicamycin (Tuni; 2 μg/ml), as indicated. Following treatment, the RINm5F cells and rat islets were harvested and caspase-3 enzymatic activity determined (*A* and *B*). Nitrite production by islets was determined using the culture supernatants (*C*). Results are means ± SE of three independent experiments.

contributes to the loss of β-cell viability in response to ER stress activators such as tunicamycin.

In contrast to cell death induced by the UPR activator tunicamycin, cell death induced by IL-1 does not correlate with caspase-3 activation. IL-1 fails to activate caspase-3 in RINm5F cells (Fig. 3*A*) or rat islets (Fig. 3*B*) following exposures as long as 24 h. These observations are consistent with our previous studies showing that extended cultures up to 48 h with IL-1 also fail to stimulate caspase-3 activity in RINm5F cells (19). There is a slight 30–40% increase in caspase-3 activity in rat islets treated for 24 h with IL-1; however, this increase was variable and did not achieve statistical significance. While IL-1 failed to stimulate caspase-3 activation, it did stimulate NO production by RINm5F cells (Fig. 2*B*) and rat islets (Fig. 3*C*). These findings dissociate cytokine-induced NO production and UPR activation from the mechanisms by which cytokines mediate β-cell death. Importantly, known inducers of the UPR stimulate islet and RINm5F cell death by a process that is dependent on caspase-3 activation, while IL-1 fails to stimulate caspase-3 activation even though NO production in response to IL-1 is an effective activator of the UPR in islets. Since NO has been reported to inhibit caspase-3 activity (46–48), we also examined the effects of IL-1 and the NOS inhibitor NMMA on caspase activation. In the presence of NMMA, IL-1 still failed to activate caspase-3 (Fig. 3*A* and *B*). Although IL-1 is capable of activating the UPR in a NO-dependent manner, these findings suggest that the mechanisms responsible for β-cell death under conditions of prolonged UPR activation (tunicamycin) differ from cytokine-induced β-cell death, as IL-1 fails to stimulate caspase-3 activation or caspase-dependent β-cell death.

Regulation of caspase-3 cleavage in response to IL-1 and UPR activators. To further explore the relationship between cytokine-induced UPR activation and cytokine-induced β-cell death, we examined the effects of IL-1 on the processing of caspase-3 from the inactive procaspase to the active mature protease. As shown in Fig. 4*A*, a 24-h incubation of RINm5F cells with IL-1 fails to stimulate processing of caspase-3 to its active mature form. In contrast to the effects of IL-1, the peroxisome proliferator–activated receptor γ ligand PGJ_2 stimulates the processing of caspase-3 to the active mature protease. Importantly, we have recently shown that PGJ_2 activates the UPR and induces β-cell apoptosis in a caspase-3–dependent manner (33).

Cells undergoing necrosis are known to release HMGB1 (49–52). Indeed, we have recently shown that cytokines stimulate HMGB1 release from rat and human islets and

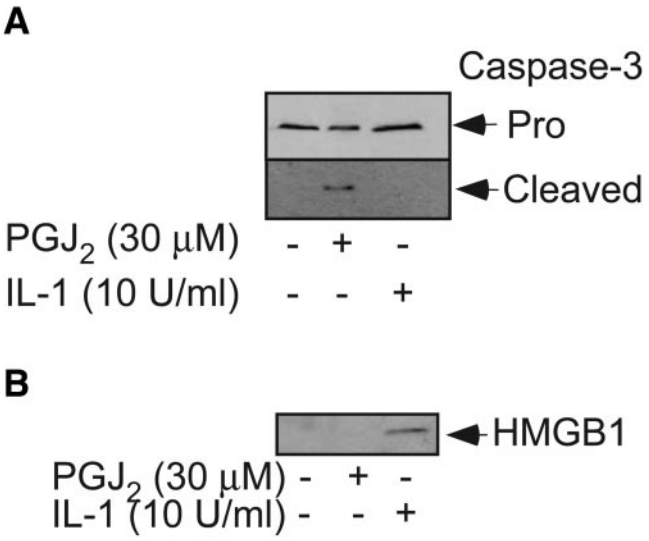

FIG. 4. IL-1 fails to stimulate caspase-3 cleavage but induces HMGB1 release from β-cells. RINm5F cells (*A*; 2.0 × 10^5 cells/400 μl RPMI-1640) or rat islets (*B*; 150 islets/400 μl RPMI-1640) were treated for 24 h with the indicated concentrations of IL-1 and the ER stress inducer PGJ_2. The cells were harvested and caspase-3 cleavage examined by Western blot analysis using an antibody specific for the cleaved active caspase and an antibody against procaspase-3 (*A*). The release of HMGB1 from rat islet cells was determined by Western blot analysis of the cell culture media following the 24-h incubation (*B*). HMGB1 release serves as a biomarker of necrosis. Results are representative of three independent experiments.

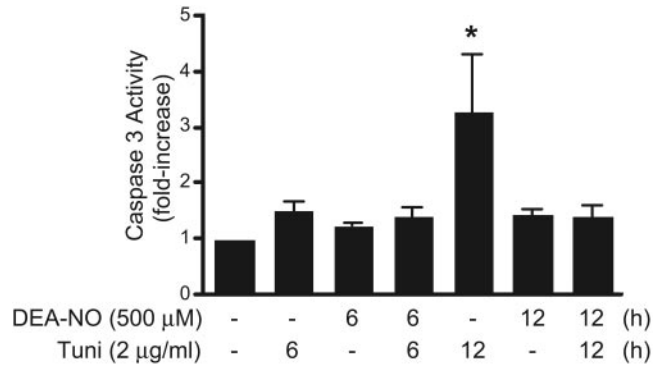

FIG. 5. NO inhibits tunicamycin-induced caspase-3 activity. RINm5F cells (2.0 × 10^5 cells/400 μl RPMI-1640) were treated with DEA-NO (500 μmol/l), tunicamycin (Tuni; 2 μg/ml), or DEA-NO plus tunicamycin for the indicated times. The cells were harvested and caspase-3 activity determined. Results are means ± SE of three independent experiments.

RINm5F cells in a NO-dependent manner (19). In contrast, inducers of apoptosis fail to stimulate HMGB1 release from rat and human islets and RINm5F cells (19,51). Consistent with a role for caspase-3 in β-cell death in response to UPR activators, PGJ_2 fails to stimulate HMGB1 release from rat islets, while IL-1 stimulates HMGB1 release from rat islets (Fig. 4*B*). These findings, using a second UPR activator, provide additional evidence dissociating the activation of the UPR from cytokine-mediated β-cell death.

Regulation of caspase-3 activity by NO. While cytokines stimulate UPR activation in islets and RINm5F cells in an NO-dependent fashion, experimental evidence presented in Figs. 2–4 indicates that the mechanisms of cytokine-induced β-cell death differ from the pathway responsible for UPR-mediated β-cell death. One potential explanation for these differences is in the ability of NO to inhibit caspase-3 activity. As shown in Fig. 5, tunicamycin stimulates a threefold increase in RINm5F cell caspase activity following a 12-h incubation. Importantly, tunicamycin-induced caspase-3 activation is prevented by the NO donor sodium DEA-NO, while DEA-NO alone fails to activate caspase-3 activity in RINm5F cells.

Effects of cytokines and UPR activators on the viability of cells deficient in UPR transducers. Consistent with a protective role of the UPR in prolonging survival in response to cellular stress, recent studies have shown that inhibition of UPR transducer activity increases the susceptibility of cells to ER stress (23). To further probe cell viability in response to cytokines and UPR activators, wild-type, $Perk^{-/-}$, A/A (expressing S51A eIF2α mutant), and $IRE1\alpha^{-/-}$ MEFs were treated for 24 h with IL-1 and IFN-γ or with tunicamycin. The combination of cytokines was required because IL-1 alone fails to stimulate NO production or the death of MEFs (data not shown). In combination, IL-1 and IFN-γ stimulate an ~10- to 15-fold increase in NO production (Fig. 6*A*) and the death of 17.3% of MEFs (Fig. 6*B*). Importantly, there is no statistically significant difference in the levels of cell death or the levels or NO production in response to IL-1 and IFN-γ when comparing wild-type, $Perk^{-/-}$, A/A, or $IRE1\alpha^{-/-}$ MEFs (Fig. 6). As expected, tunicamycin, which failed to stimulate NO production, reduced the viability of wild-type MEFs by 41.7%. Inhibition of the PERK signaling cascade, either by expression of a mutant eIF2α that cannot be phosphorylated (A/A) or via the absence of PERK, further enhanced the level of tunicamycin-induced cell death to 75–80% of the MEFs. We also examined the effects cytokines and tunicamycin on the viability of RINm5F cells or RINm5F cells expressing dnPERK mutant (K621M) or the S51A eIF2α mutant (Fig. 6*C*, data presented as percent increase in IL-1– and tunicamycin-induced death). Similar to MEFs, 19% of RINm5F cells are killed following a 24-h incubation with IL-1, and this level of death is not modified in cells expressing either the S51A eIF2α mutant or cells expressing dnPERK (K621M). In contrast to IL-1, tunicamycin induces the death of 26% of RINm5F cells following a 24-h incubation, and the level of death is enhanced by 77% in cells expressing the S51A eIF2α mutant. In RINm5F cells expressing dnPERK, the level of tunicamycin-induced death is enhanced approximately twofold (Fig. 6*C*). While the increase in tunicamycin-induced death of RINm5F cells expressing UPR transducer mutants may appear to be somewhat attenuated when compared with the increase in death in the corresponding MEFs, it is the expected increase in the level of RINm5F cell death when the 60% transfection efficiency of the UPR transducer mutants is taken into account in these assays. These findings, which support previous studies indicating that UPR-mediated cell death is enhanced in the absence of a functional PERK response (23,33), provide direct evidence that cytokine-induced death can occur independent of UPR activation, as deletion of either PERK or IRE1α or expression of the S51A eIF2α mutant does not alter the levels of death in response to cytokine treatment.

DISCUSSION

The purpose of this study was to evaluate the role of ER stress and the subsequent activation of the UPR as a mechanism by which cytokine treatment causes β-cell death. The UPR is a conserved cellular response designed to alleviate multiple forms of cellular stress due to oxida-

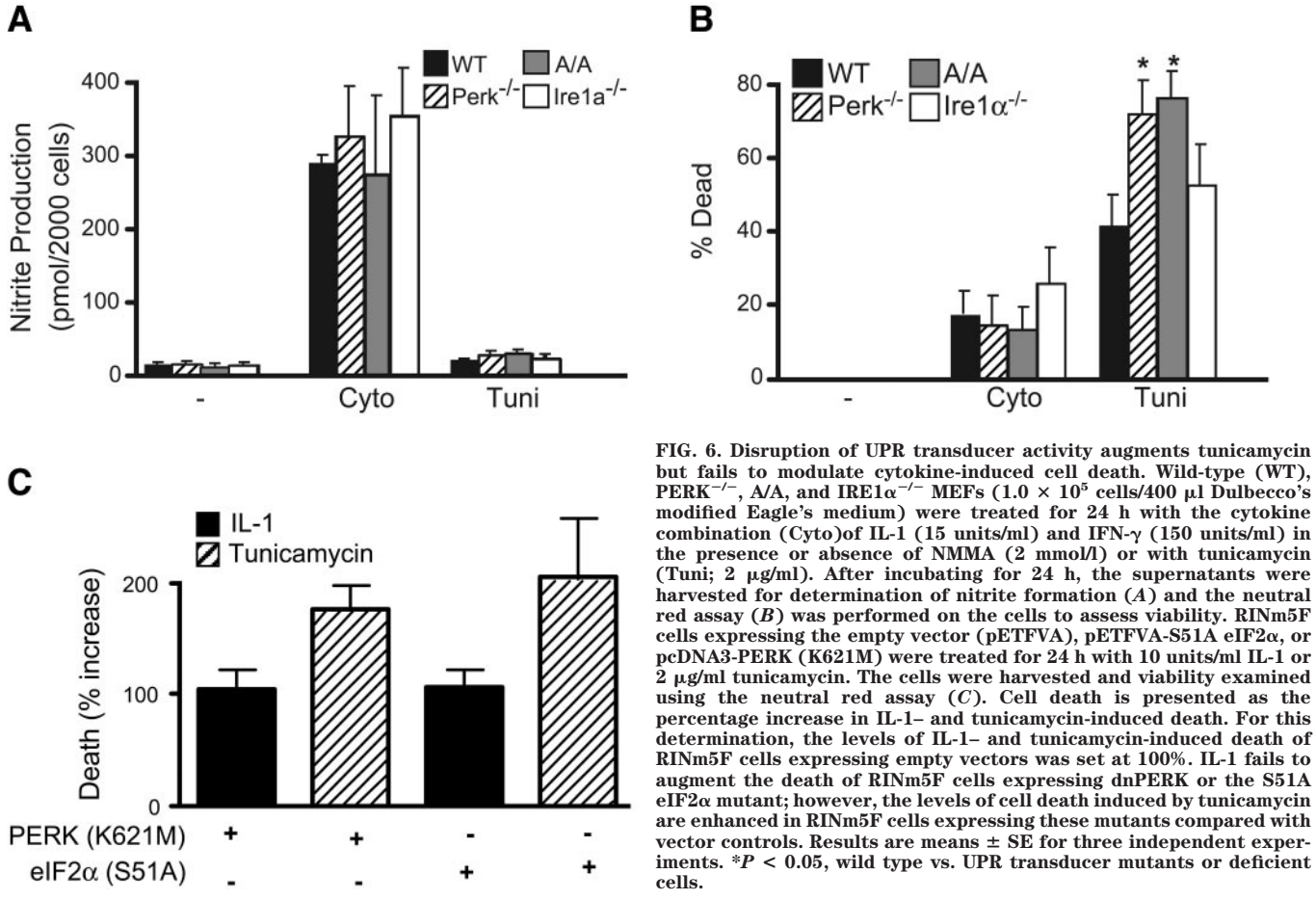

FIG. 6. Disruption of UPR transducer activity augments tunicamycin but fails to modulate cytokine-induced cell death. Wild-type (WT), PERK$^{-/-}$, A/A, and IRE1α$^{-/-}$ MEFs (1.0×10^5 cells/400 μl Dulbecco's modified Eagle's medium) were treated for 24 h with the cytokine combination (Cyto)of IL-1 (15 units/ml) and IFN-γ (150 units/ml) in the presence or absence of NMMA (2 mmol/l) or with tunicamycin (Tuni; 2 μg/ml). After incubating for 24 h, the supernatants were harvested for determination of nitrite formation (*A*) and the neutral red assay (*B*) was performed on the cells to assess viability. RINm5F cells expressing the empty vector (pETFVA), pETFVA-S51A eIF2α, or pcDNA3-PERK (K621M) were treated for 24 h with 10 units/ml IL-1 or 2 μg/ml tunicamycin. The cells were harvested and viability examined using the neutral red assay (*C*). Cell death is presented as the percentage increase in IL-1– and tunicamycin-induced death. For this determination, the levels of IL-1– and tunicamycin-induced death of RINm5F cells expressing empty vectors was set at 100%. IL-1 fails to augment the death of RINm5F cells expressing dnPERK or the S51A eIF2α mutant; however, the levels of cell death induced by tunicamycin are enhanced in RINm5F cells expressing these mutants compared with vector controls. Results are means ± SE for three independent experiments. *$P < 0.05$, wild type vs. UPR transducer mutants or deficient cells.

tive or nitrosative damage, aberrant accumulation of proteins in the ER, and stress associated with virus infection (12,22). If cellular stress is not eliminated or cells fails to recover from the stressful insult, apoptosis ensues (12,22,23). β-Cells are highly sensitive to perturbations in the response to ER stress. UPR activators, such as thapsigargin, have been shown to induce β-cell apoptosis (24). In addition, mice deficient in PERK develop diabetes that is due to the apoptotic loss of β-cells (26,53) and β-cell mass is reduced in mice expressing a S51A eIF2α mutant (23), which is not capable of being phosphorylated by PERK or other eIF2α kinases (23). NO, a known activator of the UPR (20,21,42), has been implicated as an inducer of ER stress in β-cells, and UPR activation in response to this ER stress has been reported to mediate β-cell apoptosis (20,21). In support of this hypothesis, cytokines stimulate eIF2α phosphorylation, IRE1α activation, and UPR gene expression in a NO-dependent fashion that correlates with cytokine-induced β-cell death (20,21).

In the current study, we have used a biochemical approach to investigate the role of UPR activation in cytokine-mediated β-cell death. Consistent with previous studies, we show that IL-1 stimulates the production of NO by islets and insulinoma cells in a time-dependent manner that correlates with the activation of the UPR. UPR activation is mediated by β-cell production of NO as IL-1–induced eIF2α phosphorylation, CHOP expression, and Xbp1 processing (IRE1α activation) are attenuated by the NOS inhibitor NMMA. While cytokine-induced NO production correlates with cytokine-induced UPR activation, the UPR does not appear to participate in cytokine-induced β-cell death. This conclusion is supported by three findings. *1*) β-Cell death in response to UPR activators is associated with a 2.5- to 3-fold increase in caspase-3 activity and is attenuated by the caspase-3 inhibitor IV. In contrast, IL-1 fails to activate caspase-3; however, this cytokine does induce the death of RINm5F cells and rat islets in an NO-dependent manner (Figs. 2 and 3 and reference 19). *2*) The peroxisome proliferator–activated receptor γ ligand PGJ_2 induces β-cell apoptosis by a pathway that is associated with UPR activation and that is dependent on the activation of caspase-3 (33). Consistent with these previous findings, PGJ_2 stimulates the proteolytic cleavage of caspase-3 to the active protease, whereas IL-1 fails to stimulate caspase-3 cleavage. We observed similar differences in the response of β-cells to apoptosis inducers, such as camptothecin and staurosporine (induce caspase-3–dependent β-cell apoptosis), as compared with cytokines that stimulate caspase-3–independent β-cell necrosis (19). *3*) NO is a known inhibitor of caspase-3 activity (via S-nitrosothiol formation at the active-site cysteine) (46–48), and we show that NO inhibits tunicamycin-induced caspase-3 activity in RINm5F cells (Fig. 5). Taken together, these findings dissociate the mechanisms associated with cytokine-induced β-cell death from cytokine-induced UPR activation. Cytokines appear to kill β-cells by NO-dependent necrosis that is caspase-independent and associated with the release of the necrosis marker HMGB1 (Fig. 4 and reference 19). In contrast, UPR activators stimulate β-cell apoptosis that is mediated in part by caspase-3.

If our conclusion that the UPR does not mediate cyto-

kine-induced β-cell death is correct, then inhibition of UPR transducer activity should not modulate the levels of cell death in response to cytokines. Specifically, this would support the hypothesis that NO is an activator of the UPR, and while UPR activation correlates temporally with cell death, it is not the mediator of cytokine-induced cell death. In contrast, if the UPR participates in cytokine-mediated damage, then inactivation of UPR transducer activity would be expected to enhance cell death. We have previously shown that inhibition of PERK enhances caspase-3 activation in response to PGJ_2 (33), and Scheuner et al. (23) demonstrated a 57% increase in tunicamycin-induced apoptosis of MEFs expressing a S51A mutation. Consistent with this hypothesis, there is no difference in the levels of cell death in wild-type, $PERK^{-/-}$, S51A (A/A), and $IRE1\alpha^{-/-}$ MEFs following a 24-h incubation with IL-1 and IFN-γ. In contrast, there is a 31% increase in the levels of tunicamycin-induced death in $PERK^{-/-}$ and a 35% increase in A/A MEFs compared with wild-type controls. Similar to MEFs, tunicamycin-induced death of RINm5F cells expressing dnPERK or eIF2α S51A mutant is enhanced compared with cells expressing the vector control, while the levels of NO-mediated IL-1–induced death are not modified. Importantly, these findings show that cell death mediated by NO is not affected by the absence of UPR transducer activity. In contrast, removal of the protective actions of UPR, specifically the PERK pathway (including A/A MEFs), renders cells more susceptible to ER stress–mediated cell death.

Our findings provide biochemical evidence that UPR activation correlates in a temporal fashion with the stimulatory actions of cytokines on NO production and that NO is an activator of the UPR in β-cells. Importantly, this is only a correlation, as UPR activation does not appear to contribute to cytokine-induced β-cell death. In view of these findings, new questions emerge regarding UPR activation and the role of this protective response in cytokine-mediated β-cell damage. In a series of studies, we have shown that β-cells have a unique ability to recover from the damaging actions of cytokines (54–56). The addition of a NOS inhibitor to islets treated for 24 h with IL-1 results in the time-dependent recovery of insulin secretion and mitochondrial oxidative metabolism that is complete in 8 h (56). This recovery response is activated by NO, is associated with enhanced expression of heat shock proteins and chaperones that assist in protein folding (data not shown), and requires de novo gene expression (54,55). We hypothesize that NO-induced UPR activation does not function as a mediator of β-cell death but may function to assist in the recovery of β-cells from NO-mediated damage. This is one potential mechanism we are currently exploring in our laboratory.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK52194 and DK068839 (to J.A.C.) and an American Heart Association predoctoral fellowship award (to K.T.C.).

We thank Colleen Kelly Bratcher for expert technical assistance.

REFERENCES

1. Gepts W: Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619–633, 1965
2. Mandrup-Poulsen T: The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39:1005–1029, 1996
3. Rabinovitch A, Suarez-Pinzon WL: Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol* 55:1139–1149, 1998
4. Southern C, Schulster D, Green IC: Inhibition of insulin secretion by interleukin-1 beta and tumour necrosis factor-alpha via an L-arginine-dependent nitric oxide generating mechanism. *FEBS Lett* 276:42–44, 1990
5. Eizirik DL, Bjorklund A, Welsh N: Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-fos induction and depends on gene transcription and protein synthesis. *FEBS Lett* 317:62–66, 1993
6. Corbett JA, Wang JL, Sweetland MA, Lancaster JR Jr, McDaniel ML: Interleukin 1 beta induces the formation of nitric oxide by beta-cells purified from rodent islets of Langerhans: evidence for the beta-cell as a source and site of action of nitric oxide. *J Clin Invest* 90:2384–2391, 1992
7. Corbett JA, Kwon G, Misko TP, Rodi CP, McDaniel ML: Tyrosine kinase involvement in IL-1 beta-induced expression of iNOS by beta-cells purified from islets of Langerhans. *Am J Physiol* 267:C48–C54, 1994
8. Corbett JA, McDaniel ML: Intraislet release of interleukin 1 inhibits beta cell function by inducing beta cell expression of inducible nitric oxide synthase. *J Exp Med* 181:559–568, 1995
9. Heitmeier MRH, Corbett JA: Cytotoxic role of nitric oxide in diabetes. In *Nitric Oxide Biology and Pathobiology*. San Diego, CA, Academic, 2000, p. 785–810
10. Thomas HE, Darwiche R, Corbett JA, Kay TW: Interleukin-1 plus γ-interferon–induced pancreatic β-cell dysfunction is mediated by β-cell nitric oxide production. *Diabetes* 51:311–316, 2002
11. Arnush M, Heitmeier MR, Scarim AL, Marino MH, Manning PT, Corbett JA: IL-1 produced and released endogenously within human islets inhibits beta cell function. *J Clin Invest* 102:516–526, 1998
12. Schroder M, Kaufman RJ: The mammalian unfolded protein response. *Annu Rev Biochem* 74:739–789, 2005
13. Eizirik DL, Flodstrom M, Karlsen AE, Welsh N: The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* 39:875–890, 1996
14. Koster JC, Marshall BA, Ensor N, Corbett JA, Nichols CG: Targeted overactivity of beta cell K(ATP) channels induces profound neonatal diabetes. *Cell* 100:645–654, 2000
15. Misler S, Barnett DW, Gillis KD, Pressel DM: Electrophysiology of stimulus-secretion coupling in human β-cells. *Diabetes* 41:1221–1228, 1992
16. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL: Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* 54 (Suppl. 2):S97–S107, 2005
17. Mandrup-Poulsen T: Apoptotic signal transduction pathways in diabetes. *Biochem Pharmacol* 66:1433–1440, 2003
18. Collier JJ, Fueger PT, Hohmeier HE, Newgard CB: Pro- and antiapoptotic proteins regulate apoptosis but do not protect against cytokine-mediated cytotoxicity in rat islets and β-cell lines. *Diabetes* 55:1398–1406, 2006
19. Steer SA, Scarim AL, Chambers KT, Corbett JA: Interleukin-1 stimulates beta-cell necrosis and release of the immunological adjuvant HMGB1. *PLoS Med* 3:e17, 2006
20. Cardozo AK, Ortis F, Storling J, Feng YM, Rasschaert J, Tonnesen M, Van Eylen F, Mandrup-Poulsen T, Herchuelz A, Eizirik DL: Cytokines downregulate the sarcoendoplasmic reticulum pump $Ca2^+$ ATPase 2b and deplete endoplasmic reticulum $Ca2^+$, leading to induction of endoplasmic reticulum stress in pancreatic β-cells. *Diabetes* 54:452–461, 2005
21. Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M: Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci U S A* 98:10845–10850, 2001
22. Harding HP, Calfon M, Urano F, Novoa I, Ron D: Transcriptional and translational control in the Mammalian unfolded protein response. *Annu Rev Cell Dev Biol* 18:575–599, 2002
23. Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ: Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* 7:1165–1176, 2001
24. Zhou YP, Teng D, Dralyuk F, Ostrega D, Roe MW, Philipson L, Polonsky KS: Apoptosis in insulin-secreting cells: evidence for the role of intracellular Ca2+ stores and arachidonic acid metabolism. *J Clin Invest* 101: 1623–1632, 1998
25. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR: The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 22:3864–3874, 2002
26. Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D: Diabetes mellitus and exocrine pancreatic dysfunction in perk-/-

mice reveals a role for translational control in secretory cell survival. *Mol Cell* 7:1153–1163, 2001
27. Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA, Urano F: Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab* 4:245–254, 2006
28. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M: Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 109:525–532, 2002
29. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664–666, 2000
30. Jiang HY, Wek SA, McGrath BC, Scheuner D, Kaufman RJ, Cavener DR, Wek RC: Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol Cell Biol* 23:5651–5663, 2003
31. Ma K, Vattem KM, Wek RC: Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress. *J Biol Chem* 277: 18728–18735, 2002
32. Kelly CB, Blair LA, Corbett JA, Scarim AL: Isolation of islets of Langerhans from rodent pancreas. *Methods Mol Med* 83:3–14, 2003
33. Chambers KT, Weber SM, Corbett JA: PGJ2 stimulated {beta}-cell apoptosis is associated with prolonged UPR activation. *Am J Physiol Endocrinol Metab* 292:E1052–E1061, 2006
34. Heitmeier MR, Scarim AL, Corbett JA: Interferon-gamma increases the sensitivity of islets of Langerhans for inducible nitric-oxide synthase expression induced by interleukin 1. *J Biol Chem* 272:13697–13704, 1997
35. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126:131–138, 1982
36. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D: IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415:92–96, 2002 [erratum in *Nature* 420:202, 2002].
37. Arnush M, Scarim AL, Heitmeier MR, Kelly CB, Corbett JA: Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. *J Immunol* 160:2684–2691, 1998
38. Borenfreund E, Shopsis C: Toxicity monitored with a correlated set of cell-culture assays. *Xenobiotica* 15:705–711, 1985
39. Mathews CE, Suarez-Pinzon WL, Baust JJ, Strynadka K, Leiter EH, Rabinovitch A: Mechanisms underlying resistance of pancreatic islets from ALR/Lt mice to cytokine-induced destruction. *J Immunol* 175:1248–1256, 2005
40. Saldeen J, Lee JC, Welsh N: Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. *Biochem Pharmacol* 61:1561–1569, 2001
41. Paschen W, Hotop S, Aufenberg C: Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress. *Cell Calcium* 33:83–89, 2003
42. Weber SM, Chambers KT, Bensch KG, Scarim AL, Corbett JA: PPARgamma ligands induce ER stress in pancreatic beta-cells: ER stress activation results in attenuation of cytokine signaling. *Am J Physiol Endocrinol Metab* 287:E1171–E1177, 2004
43. Corbett JA, Wang JL, Hughes JH, Wolf BA, Sweetland MA, Lancaster JR Jr, McDaniel ML: Nitric oxide and cyclic GMP formation induced by interleukin 1 beta in islets of Langerhans: evidence for an effector role of nitric oxide in islet dysfunction. *Biochem J* 287:229–235, 1992
44. Boatright KM, Salvesen GS: Mechanisms of caspase activation. *Curr Opin Cell Biol* 15:725–731, 2003
45. Wilson MR: Apoptotic signal transduction: emerging pathways. *Biochem Cell Biol* 76:573–582, 1998
46. Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, Dimmeler S: Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *J Biol Chem* 274:6823–6826, 1999
47. Li J, Billiar TR, Talanian RV, Kim YM: Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem Biophys Res Commun* 240:419–424, 1997
48. Kim YM, Talanian RV, Billiar TR: Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem* 272:31138–31148, 1997
49. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB: Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 18:1272–1282, 2004
50. Scaffidi P, Misteli T, Bianchi ME: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418:191–195, 2002
51. Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME: New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J* 20:4337–4340, 2001
52. Degryse B, Bonaldi T, Scaffidi P, Muller S, Resnati M, Sanvito F, Arrigoni G, Bianchi ME: The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J Cell Biol* 152:1197–1206, 2001
53. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D: Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 5:897–904, 2000
54. Scarim AL, Nishimoto SY, Weber SM, Corbett JA: Role for c-Jun N-terminal kinase in beta-cell recovery from nitric oxide-mediated damage. *Endocrinology* 144:3415–3422, 2003
55. Scarim AL, Heitmeier MR, Corbett JA: Heat shock inhibits cytokine-induced nitric oxide synthase expression by rat and human islets. *Endocrinology* 139:5050–5057, 1998
56. Scarim AL, Heitmeier MR, Corbett JA: Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1beta. *Endocrinology* 138:5301–5307, 1997